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Shock and Tissue Injury Induced by Recombinant Human Cachectin

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Cachectin (tumor necrosis factor), a protein produced in large quantities by endotoxin-activated macrophages, has been implicated as an important mediator of the lethal effect of endotoxin. Recombinant human cachectin was infused into rats in an effort to determine whether cachectin, by itself, can elicit the derangements of host physiology caused by administration of endotoxin. When administered in quantities similar to those produced endogenously in response to endotoxin, cachectin causes hypotension, metabolic acidosis, hemoconcentration, and death within minutes to hours, as a result of respiratory arrest. Hyperglycemia and hyperkalemia were also observed after infusion. At necropsy, diffuse pulmonary inflammation and hemorrhage were apparent on gross and histopathologic examination, along with ischemic and hemorrhagic lesions of the gastrointestinal tract, and acute renal tubular necrosis. Thus, it appears that a single protein mediator (cachectin) is capable of inducing many of the deleterious effects of endotoxin.

BACTERIAL ENDOTOXIN (LIPOPOLYSACCHARIDE, LPS) is highly toxic to most mammals. When administered intravenously, it evokes a "shock" state, characterized by fever, hypotension, and multi–organ system failure (*1–5*). Fatality is associated with injuries involving the lungs (shock lung syndrome), kidneys (acute tubular necrosis), and gastrointestinal tract (mesenteric ischemia) (*1, 2, 4, 6*). This syndrome frequently occurs in the course of invasive Gram-negative infections and is associated with a high mortality (*2, 5, 7*).

Recently, it has become clear that endotoxin does not injure host tissues directly, but does so through the action of an endogenous mediator or mediators. C3H/HeJ mice, which are highly resistant to the lethal effect of endotoxin as the result of a genetic lesion (the *lps*^{d} allele on chromosome 4) (*8*), are rendered endotoxin-sensitive if subjected to total body irradiation and transplanted with marrow obtained from mice of the closely related, endotoxin-sensitive strain C3H/HeN (*9*). Similarly, irradiated C3H/HeN mice reconstituted with C3H/HeJ marrow are endotoxin resistant (*9*). Thus, a host factor expressed by cells of hematopoietic origin appears to confer endotoxin sensitivity.

The macrophage appears to be the principal cell involved in mediating the effects of endotoxin. Infectious agents capable of stimulating reticuloendothelial hyperplasia render animals exquisitely sensitive to the effects of endotoxin (*10*). Moreover, macrophages activated by endotoxin in vitro produce a soluble factor that is capable of killing endotoxin-resistant animals (*11*).

Cachectin (*12, 13*) is a macrophage-derived polypeptide hormone known for its ability to modulate adipocyte metabolism (*12, 14*), lyse tumor cells in vitro (*15, 16*), and induce hemorrhagic necrosis of certain transplantable tumors in vivo (*15*). It constitutes between 1 and 2% of the total secretory protein produced by endotoxin-activated macrophages in vitro (*12*). Copious quantities of the protein are also produced in vivo in response to endotoxin. Approximately 5.5 mg of cachectin are present per liter of rabbit serum 1.5 hours after endotoxin administration (*17*). With consideration given to the half-life of the hormone in vivo (*18*), this would suggest the net production of milligram quantities of cachectin per kilogram of body mass.

Recently, Beutler *et al.* (*19*) suggested that cachectin itself might initiate the tissue injury elicited by endotoxin. This suggestion was based on the observation that animals passively immunized against cachectin were protected against the lethal effect of endotoxin (*19*). However, direct analysis of cachectin as a critical mediator of organ injury in sepsis required the isolation of large amounts of purified hormone, devoid of pharmacologically active quantities of endotoxin.

Accordingly, an artificial gene, encoding the amino acid sequence of cachectin, was constructed by means of an Applied Biosystems model 380A gene synthesizer. The gene was synthesized in 25 overlapping segments, which were annealed and ligated in a single reaction. Codons were chosen so as to optimize expression of the protein product in yeast. At the 5′ end, additional codons were added to specify the signal sequence of yeast α-factor, in order to obtain a secreted product (*20*). The synthetic

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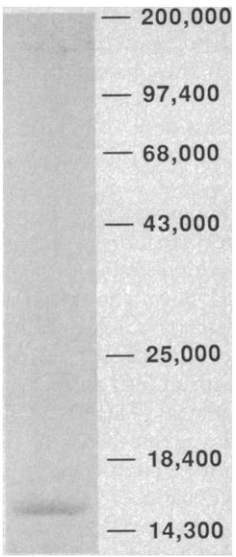

Fig. 1. Recombinant human cachectin, purified as described in the text, was subjected to electrophoresis in a 10 to 15% polyacrylamide gel under denaturing conditions and was stained with Coomassie blue. Molecular weights are indicated.

sequence was placed downstream from a fused alcohol dehydrogenase/glyceraldehyde-phosphate dehydrogenase (ADH2/GAPDH) promoter.

Cachectin biosynthesis and secretion was induced in cultures of transformed *Saccharomyces cerevisiae* by glucose deprivation. Approximately 30% of the secreted protein was cachectin. The sample was desalted by passage over a Sephadex G-25 column equilibrated with 5 m*M* tris-chloride buffer, *p*H 8.4. The protein-rich fraction was separated over a Mono-Q column, with a tris-chloride gradient, *p*H 8.4. Cachectin eluted as the major peak, at a concentration of 0.19*M* tris-chloride. The Mono-Q purified material (approximately 85% cachectin) was then mixed with an equal volume of 3.4*M* $(NH_4)_2SO_4$ and separated further on a phenyl Superose column. Proteins were eluted by means of a declining salt gradient [1.7*M* to 0*M* $(NH_4)_2SO_4$ buffered with 50 m*M* Na-PO_4, *p*H 7.0]. The endotoxin content of the sample was less than 0.4 μg per milligram of protein, as assessed by *Limulus* amebocyte lysate tests. The final product (Fig. 1) was electrophoretically and chromatographically homogeneous and behaved as a dimer on high-performance gel filtration analysis.

To establish the acute toxicity of cachectin, we infused the highly purified hormone into unanesthetized female Sprague-Dawley rats (225- to 250-g body weight) via the tail vein in various doses over 5 minutes (Table 1). The animals were observed for 12 hours after infusion. Those animals that succumbed to the effects of the infusion were necropsied immediately, and all survivors were killed for necropsy at the end of the experimental period. The animals receiving

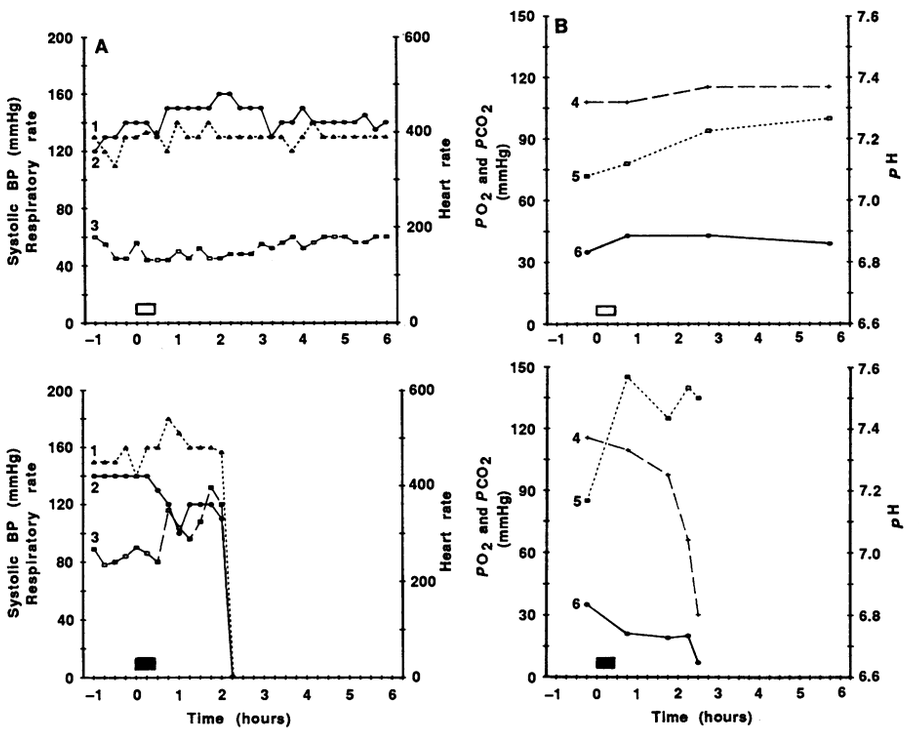

Fig. 2. Vital signs and arterial *p*H, PO_2, and PCO_2 monitored in individual rats. Animals were cannulated at $t = -1$ hour. Top (A and B), infusion of isotonic saline via the superior vena cava (open bars); bottom (A and B), infusion of 1.8 mg of cachectin per kilogram of body weight (solid bars). (A) (Line 1), heart rate (beats per minute); (line 2), systolic blood pressure (BP); (line 3), respiratory rate (respirations per minute). (B) (Line 4), *p*H; (line 5), PO_2; (line 6), PCO_2.

Fig. 3. Gross appearance of the gastrointestinal tract in an animal that died 4 hours after cachectin infusion (1.8 mg per kilogram of body weight).

doses in excess of 0.6 mg of cachectin per kilogram of body weight became lethargic within minutes after infusion. This was followed by piloerection without chills, bloody diarrhea, and tachypnea. The 12-hour mean lethal dose was estimated to be 0.7 mg of cachectin per kilogram of body weight by the method of Bliss and Litchfield (*21*).

We studied several physiological parameters to further characterize the response to cachectin administration. Pentobarbital-anesthetized rats were monitored via arterial cannula before, during, and after cachectin infusion into the superior vena cava. Arterial glucose, Na^+, K^+, and hematocrit were recorded in animals receiving various doses of cachectin (Table 2). Vital signs, as well as arterial *p*H, PO_2, and PCO_2, are compared graphically for experimental and control animals (Fig. 2).

Control animals (receiving no cachectin), maintained stable hemodynamic and vital functions over 6 hours of continuous monitoring. Similarly, no significant changes were observed in any of the other measured parameters except for hematocrit, which fell as a result of serial blood sampling with volume replacement (cubic centimeter for cubic centimeter) with lactated Ringer's solution. This decline in hematocrit induced no significant changes in hemodynamic or respiratory function throughout the 6-hour study period.

Rats infused with cachectin at low (0.2 to

0.6 mg/kg) doses exhibited mild to moderate tachypnea and hypotension followed by recovery. At higher doses, an overwhelming metabolic acidosis occurred, leading to a preterminal decline in arterial *p*H to 6.99 ± 0.11 (SD; $n = 10$). Plasma lactate levels were elevated by a factor of 3 to 5 when compared to concentrations before infusion. Death occurred after respiratory arrest. The physiological derangements that occurred in a typical animal infused with cachectin are detailed in graphic form in Fig. 2.

At necropsy, several gross abnormalities were readily apparent in cachectin-infused rats. Diffuse hyperemia and punctate areas of hemorrhage were evident in the lungs. Rats treated with cachectin in doses exceeding 0.6 mg/kg exhibited segmental ischemia of the bowel with regions of frank hemorrhage or necrosis (Fig. 3); the cecum appeared particularly sensitive to the hormone, and was invariably infarcted. The kidneys were uniformly distended and hyperemic. The pancreas was edematous and contained regions of focal hemorrhage. Adrenal hemorrhage was also observed in several animals.

Histopathologic examination was performed on tissues fixed with 10% formaldehyde in phosphate-buffered saline. Sections obtained from areas of the lung (Fig. 4A) that were hemorrhagic in appearance revealed occlusion of large arteries by thrombi composed primarily of polymorphonuclear leukocytes. An intense margination response was observed, with migration of polymorphonuclear cells through the walls of the pulmonary vessels. A severe interstitial and peribronchiolar pneumonitis was also apparent, with marked thickening of the alveolar membranes.

Histological sections taken through nonnecrotic regions of the intestinal tract (Fig. 4B) also showed inflammatory changes, with invasion of the submucosa and muscularis mucosa by polymorphonuclear leukocytes. The epithelium was denuded in a focal distribution throughout the bowel.

The renal glomeruli appeared mildly hypercellular. In addition, acute tubular necrosis was evident, with disruption of both proximal and distal elements of the nephron (Fig. 4C). In longitudinal section, numerous red cell casts, as well as occasional inflammatory cells, were seen within the tubules. Histological examination of rats infused with phosphate-buffered saline (the solvent in which cachectin was administered) revealed none of the changes characteristic of cachectin-treated rats (Fig. 4, D to F).

The pathophysiological and histological findings reported here mimic changes evoked by administration of lethal doses of endotoxin (*1–4, 6*). The inflammatory pneumonitis, mesenteric ischemia, and acute tubular necrosis characteristic of rats treated with cachectin are also observed in endotoxin-treated animals. The cannulation and infusion of the rats in these studies may have led to the introduction of small quantities of bacterial endotoxin or other contaminants. However, no detectable physiological or histopathologic changes occurred with endotoxin infused in doses as high as 10 μg/kg. Moreover, preparations of cachectin that were boiled to destroy the biological activity of the hormone also failed to induce any changes.

We obtained further evidence implicating cachectin as the toxic agent by administering

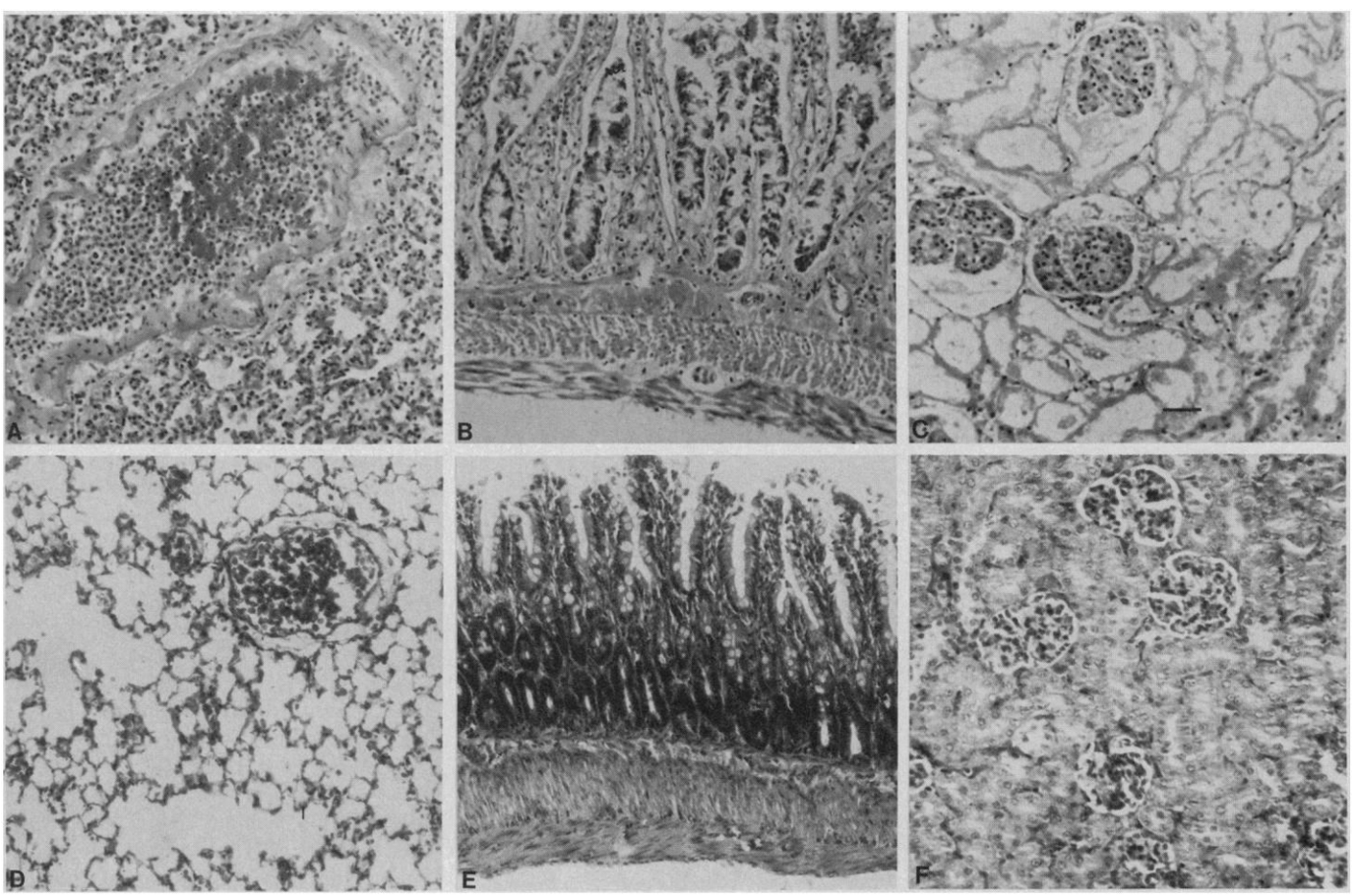

Fig. 4. Histological appearance of tissues obtained from a single rat treated with cachectin at a dose of 1.8 mg per kilogram of body weight. Sections were stained with hematoxylin and eosin. (A) Section taken through a region of confluent hemorrhage observed on gross examination of the lung. (B) Section of small bowel. (C) Sections of kidney cut perpendicular to the axis of the tubules. Control tissues: (D to F) lung, small bowel, and kidney sections (respectively) obtained from a rat infused with phosphate-buffered saline.

Table 1. Survival among Sprague-Dawley rats 12 hours after treatment with cachectin in various doses.

Cachectin dose (mg/kg body weight)	Survival		Mean survival time*
	No./group	%	
0	5/5	100	
0.2	5/5	100	
0.6	4/11	36	6.6 ± 2.5
1.8	4/11	36	4.9 ± 1.8
3.6	0/5	0	4.0 ± 1.2
3.6 (protected)†	5/5	100	

*Observation was continued for a period of 12 hours before the rats were killed, as described in the text. Animals receiving cachectin at a dose of 0.2 mg/kg exhibited segmental infarctions or perforation of the gastrointestinal tract, or both, particularly in the region of the cecum. Other animals treated with this dose exhibited acute tubular necrosis. These acute lesions would likely have decreased long-term survival. Means ± SD of those not surviving. †Rats in this group were given 4 mg of a neutralizing monoclonal antibody by an intravenous route 1 hour before cachectin infusion.

4 mg of a neutralizing mouse monoclonal antibody directed against human cachectin to rats 1 hour before infusion of the hormone. Animals pretreated with the monoclonal antibody were fully protected from the effects of otherwise lethal doses of cachectin (Table 1). After cachectin infusion, the immunized animals were active and did not exhibit piloerection, diarrhea, or tachypnea. Histopathologic examination of tissue specimens obtained from these passively immunized animals after they were killed showed none of the lesions typically induced by cachectin.

Several investigators have reported that cachectin [tumor necrosis factor (TNF)] activates human neutrophils, stimulating their adherence to endothelial cell surfaces and increasing their phagocytic potential (*22*). Cachectin also initiates skeletal muscle depolarization (*23*) and may play a role in the sequestration of water and electrolytes in shock. Moreover, cachectin stimulates endothelial cell secretion of a procoagulant factor, and decreases endothelial cell expression of thrombomodulin (*24*); hence, the hormone may initiate inappropriate hemostasis, as occurs in septic shock.

Cachectin interacts with specific receptors present on a wide variety of tissue targets (*12, 18*), suggesting that the hormone may elicit diverse physiological effects. Cachectin-induced hemoconcentration may reflect plasma volume contraction resulting from transudation of fluid. Hyperkalemia presumably occurs as a result of renal failure, widespread tissue injury, and metabolic acidosis. Transient hyperglycemia, which is often observed in endotoxemic states (*25*), may result from mobilization of glycogen stores in the setting of a generalized adrenergic response. Purified cachectin, administered in quantities similar to those evoked by lethal doses of endotoxin, can produce a potentially lethal syndrome of physiological decompensation and tissue injury. To our knowledge, no single endogenous mediator has previously been shown to initiate this spectrum of host responses.

Cachectin has been shown to induce the release of interleukin-1 (IL-1) in vitro (*26, 27*) and in vivo (*26*). The IL-1, in turn, might trigger the production of interleukin-2, leading to further fluid sequestration, and a net decline in plasma volume (*28*). Moreover, several other molecules, including leukotrienes (*29*) and platelet-activating factor (*30*) have also been implicated in the pathogenesis of inflammation and cardiovascular collapse as they occur in the setting of endotoxemia. It would seem likely that these effectors are involved in the processes that we have described, and that cachectin may act to initiate their production.

The inflammatory response may serve to protect the host from invasion, or may injure the host by causing extensive destruction of normal tissues. The protective effects of cachectin, which have led to its preservation throughout mammalian evolution, remain unknown. The effects of localized or low-level production of cachectin have not yet been determined. However, our data suggest that systemic release of cachectin, as occurs in endotoxemic states, can initiate shock and tissue injury.

It would appear that cachectin occupies a proximal position in the pathogenesis of endotoxin-induced tissue injury. Further studies directed at interruption of cachectin production or inhibition of its action might provide new therapeutic directives.

Table 2. Acute alterations of hematocrit (HCT) and arterial glucose, Na^+, and K^+, after cachectin infusion into Sprague-Dawley rats. Means ± SD. ND, data not obtained.

Cachectin dose (mg/kg body weight)	*n*	*t**	HCT	Glucose (mg/dl)	Na^+ (meq/liter)	K^+ (meq/liter)
0	3	0	42 ± 1.7	137 ± 23	146 ± 5	3.9 ± 0.4
		I	38 ± 2.5	130 ± 11	144 ± 6	4.1 ± 0.1
		II	37 ± 2.0	146 ± 42	146 ± 4	4.0 ± 0.3
		III	34 ± 0.7	159 ± 23	145 ± 5	4.5 ± 0.2
0.067	3	0	40 ± 0.0	128 ± 9	144 ± 5	4.0 ± 0.2
		I	37 ± 0.6	122 ± 0	142 ± 3	3.4 ± 0.0
		II	34 ± 2.3	140 ± 4	140 ± 3	3.6 ± 0.5
		III	28 ± 1.7	133 ± 6	141 ± 1	3.3 ± 0.1
0.2	3	0	37 ± 2.1	130 ± 12	146 ± 1	3.9 ± 0.3
		I	44 ± 2.0	262 ± 135	144 ± 4	4.7 ± 1.4
		II	ND	ND	ND	ND
		III	38 ± 3.0	150 ± 56	143 ± 2	5.3 ± 2.5
0.6	4	0	43 ± 2.3	146 ± 13	140 ± 1.4	3.6 ± 0.1
		I	51 ± 1.0	188 ± 27	141 ± 1.2	3.8 ± 0.1
		II	52 ± 1.5	240 ± 36	142 ± 4.2	4.1 ± 0.2
		III	53 ± 1.4	89 ± 72	145 ± 6.0	5.6 ± 1.4
1.8	3	0	40 ± 2.6	113 ± 16	151 ± 3.1	4.1 ± 0.4
		I	48 ± 6.8	273 ± 110	145 ± 7.0	4.8 ± 1.0
		II	55 ± 1.4	204 ± 58	148 ± 4.0	4.8 ± 1.5
		III	57 ± 2.8	83 ± 8	150 ± 0.0	8.8 ± 0.4

*Cachectin was infused into the superior vena cava at a constant rate over a 20-minute interval. Control animals were identically cannulated, but received no active hormone. Rats succumbed to the effects of cachectin at different times; hence, precisely timed comparisons among members of each group were often impossible. To compare the physiological response of animals within each group, we sampled blood before infusion (0) and at several time points after infusion was complete. Data labeled I represent samples collected within 15 minutes after infusion; data labeled II, samples collected 1 to 1.5 hours after completion of infusion; data labeled III, samples collected 3 hours after the samples labeled II or immediately before death.

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Cultivation of *Rhinosporidium seeberi* in Vitro: Interaction with Epithelial Cells

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***Rhinosporidium seeberi*, a fungus that is associated with polyp-like tumors in animals and man, was successfully cultivated. This organism stimulated proliferation of epithelial cells in vitro, producing polyp-like structures. Spores produced in culture required a period of aging or development, or both, before they were capable of reinitiating the growth cycle.**

RHINOSPORIDIUM SEEBERI, A FUNgus of uncertain classification, is associated with tumor-like growths of epithelial tissues in various mammalian hosts including man (*1*). Although infection with this organism is most commonly reported from south Asia, infection is known to occur within the United States (*2*). Despite numerous attempts, experimental infection of animals or complete development in vitro has not been achieved (*1, 3*). Therefore detailed information about the life history of *R. seeberi* or the mechanism by which it induces tumor-like growths is unavailable. We now report successful cultivation of this organism and describe the ability of *R. seeberi* to induce proliferation of epithelial cells in vitro.

Rhinosporidium seeberi was recovered from a dog with a nasal tumor (*4*). Examination of a thin section of this tissue revealed various developmental stages of the organism (Fig. 1). The polypoid mass containing the organisms was removed, and a piece weighing approximately 1.5 g was placed in several volumes of complete tissue culture medium (*5*) containing antibiotics (double the described concentration) and maintained at 4°C for 4 hours. The tissue was then rinsed several times with sterile medium containing antibiotics, minced, and placed in a chilled (4°C) tissue grinder (Ten Brock) with 2 ml of medium. The homogenate was resuspended in 50 ml of medium and sedimented at 1500*g* for 10 minutes at 4°C. The pellet was suspended in 5 ml of complete medium, and 1-ml portions of the suspension were put into 25-cm^2 plastic tissue culture flasks containing recently confluent monolayers of an epitheloid human rectal tumor (HRT) cell line (*6*) or into control flasks without cells. Pairs of these flasks, one with and one without cells, were placed in environments of 5% CO_2 in humidified air at either 27° or 34°C. Within minutes, organisms of various sizes (*7*) were observed to be strongly adherent to the HRT monolayers. Few organisms adhered to the flasks that did not contain cells. Twenty-four hours after the culture was initiated, organisms were strongly attached to the HRT monolayers, and phase-contrast microscopy at ×250 revealed numerous cells in the immediate vicinity of organisms in the 34°C culture. Although rhinosporidia were strongly adherent, cellular changes were not observed in the 27°C culture. Cultures without HRT cells contained many free rhinosporidia and degenerating canine cells. A few organisms and canine cells adhered to the control flasks at 34°C, but none were adherent at 27°C. At 34°C and 2 days after inoculation of the HRT-containing culture, polyp-like structures were observed surrounding groups of two to ten organisms. These polypoid structures consisted of HRT cells that were proliferating immediately next to organisms. Polyps were not observed in areas of the flask not containing adherent organisms. By day 4, numerous polyps were present in the culture, and mature sporangia were observed developing and releasing spores into the medium. The spores were initially confined close to the sporangia and were contained within a fibular matrix as described earlier (*8*), but most eventually became free in the medium. A small percentage, estimated at less than 1% of the total number of spores, adhered to the HRT monolayer and reinitiated the growth cycle. Development of these spores into mature sporangia was confirmed by daily monitoring. After sporulation, an empty cyst-like structure was left and HRT proliferation ceased, whereas HRT cells adjacent to growing organisms continued to proliferate. On day 6, during a routine medium change, nonadherent spores were removed and placed in other flasks containing recently confluent HRT cells to initiate the first subculture.

The original culture was maintained for 63 days during which the organisms continued to grow and induce polyp-like proliferations. On day 1 of the original culture, a

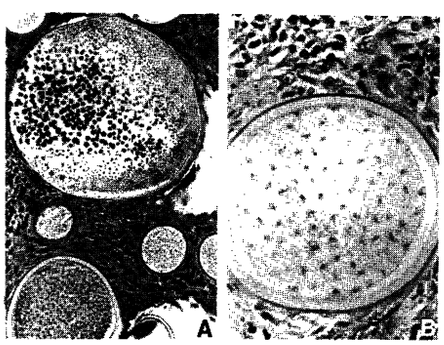

Fig. 1. (A) Histologic section from the nasal polyp contains developmental stages of *Rhinosporidium seeberi*. The largest structure is a sporangium filled with endospores. Endospores are more mature centrally and at one pole of the sporangium. (B) An intermediate stage in sporangial maturation of *R. seeberi*. At this stage the capsule is thick; it consists of two layers, and the central cavity contains a mass of developing endospores. Original magnifications: (A) ×200; (B) ×400. Stained with hematoxylin and eosin.

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