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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

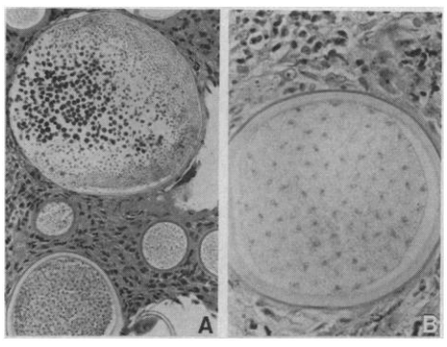

Fig. 1. (A) Histologic section from the nasal polyp contains developmental stages of *Rhinosporidium seeberi*. The largest structure is a sporangium filled with endospores. Endospores are more mature centrally and at one pole of the sporangium. (B) An intermediate stage in sporangial maturation of *R. 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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single organism was observed in every one or two fields (*9*), whereas by day 20 the average was more than ten organisms per field. Within 2 days after subculturing with spores 8 to 10 μm in diameter, organisms with diameters of up to 25 μm were observed, polyp-like proliferations were present, and mature sporangia were absent. Mature sporangia were first observed on day 4, when the average was fewer than one organism per field. By day 7 of subculture, two or three organisms were observed in each field, and nonadherent spores were removed and placed in four additional HRT-containing flasks to initiate the second subculture. Twenty-four hours later these flasks contained an average of ten adherent organisms, which then matured and released spores during the subsequent 43-day culture period. However, significant multiplication was not observed, and further subcultures were not successful. Analysis of the growth data indicates that during the three passages in culture we observed a 10- to 30-fold increase in the number of organisms. The sequence of development for organisms in all culture treatments consisted of adherence of spores to cells, increases in the diameter of the organism as well as the cell wall (with the interior containing amorphous contents), condensing of the contents with formation of spores, and expulsion of spores through a pore, leaving an empty sporangial case as previously described by other investigators for growth in vivo (*8*). The HRT-containing culture maintained at 27°C was treated as described above, and growth rates were similar, but HRT proliferation was either minimal or absent for all treatments. Various examples of *R. seeberi* propagated in vitro may be seen in Fig. 2.

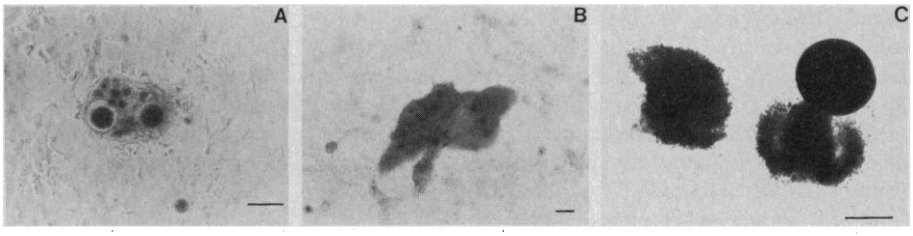

Fig. 2. *Rhinosporidium seeberi* propagated in vitro. (A) Polypoid proliferation of canine epithelial cells growing at 34°C surrounding growing *R. seeberi* of various sizes after 17 days of growth in vitro. (B) Advanced polypoid structure composed of *R. seeberi* and HRT cells grown at 34°C for 14 days. Dark circular structures within the polyp are *R. seeberi*. (C) Mature sporangium, after transfer to complete medium without cells, expelling spores still contained in a fibular matrix. The site of the pore through which the spores are extruded can easily be deduced. A similar mass of spores released by another sporangium can also be seen. Scale bar, approximately 200 μm.

Cultures were also initiated from homogenates of the canine polyp without additional HRT cells. Such cultures, when maintained at 34°C, also supported growth of *R. seeberi*. However, canine cells (*10*) adhered and proliferated only when they were adjacent to *R. seeberi* with polypoid growths during the first 10 days of culture. Because few organisms were isolated in this situation, subcultures were not attempted. Cultures at 27°C did not become established.

Occasionally, maturing sporangia became detached from the HRT substrate and, when these were transferred to flasks containing medium only, would mature and release spores. After spores were expelled through a distinct pore (Fig. 2), only a collapsed shell remained. In one experiment, several hundred maturing sporangia were recovered from the first subculture at 34°C and spores accumulated at 23°C, a temperature that supported maturation but prevented establishment of contaminating HRT cells. When these spores were placed onto HRT monolayers at 34°C 2 weeks later, adherence and subsequent development did not occur. One hundred and one days after isolation of the spores in flasks containing only medium at 23°C (the media were replaced at approximately weekly intervals), adherence and development occurred when spores were placed onto HRT monolayers at 34°C (Table 1). These organisms elicited a proliferative response from the HRT cells similar to that described earlier. This finding indicates that during the time the spores were maintained in culture medium they acquired the ability to adhere to cultured epithelial cells. This change appears to be a prerequisite to subsequent development. Once the organisms began to enlarge they appeared to be committed to complete development and sporulation. However, a substantial percentage of adherent spores did not grow during the observation period and failed to induce significant cellular changes in the adjacent HRT monolayer. Inoculation of nude mice with these aged spores did not result in infection (*11*), indicating that conditions for establishment in vivo differ from requirements in vitro.

The complete development of *R. seeberi* in vitro appears to require interaction with mammalian cells. Perhaps this will explain the failure to propagate this organism in

Table 1. Growth of *Rhinosporidium seeberi* on HRT cells from aged spores. The spores were obtained from sporangia that had been placed in complete medium without cells and that had sporulated their contents into the medium. Spores obtained in this manner were not infectious to HRT cells when tested 2 weeks after production. Spores for this experiment were allowed to incubate at 23°C in complete medium, with medium changed every 1 or 2 weeks for 101 days. A suspension of spores was placed into each of six 35-mm-diameter petri plates containing a recently confluent monolayer of HRT cells. The spores were allowed to incubate at 34°C for 24 hours, after which nonadherent organisms were removed. Measurements are of 25 organisms from each of three cultures at each time. The nonadherent organisms were returned to complete medium without cells and were incubated at 34°C. No subsequent growth of these spores was observed. Three noninfected cultures of HRT cells were handled in an identical manner and served as negative controls for development of polypoid structures. Organisms were classified as small or large according to diameter. Small organisms (spores and early developmental forms) had diameters of ≤15.9 μm and large organisms (growing forms) had diameters of >16 μm. Mean diameters are given with standard deviations in parentheses. Mature sporangia containing spores were observed 11 days after inoculation and were present throughout the remainder of the experiment. Sporangia that had expelled spores were not measured.

Day post-inoculation	Type	*N*	Diameter (μm)		Percentage of large forms
			Mean	Range	
1	Small	75	8.8 (1.2)	6.7–12.5	
1	Large	0			0
4	Small	75	8.9 (1.2)	6.3–11.9	
4	Large	0			0
11	Small	70	9.7 (1.4)	7.2–14.4	
11	Large	5	31.8 (10.6)	24.1–43.6	6.7
15	Small	71	10.9 (1.6)	5.8–15.8	
15	Large	4	35.8 (12.6)	23.8–61.6	5.3
19	Small	69	10.4 (1.3)	7.5–14.5	
19	Large	6	35.8 (12.6)	27.0–60.3	8.0
23	Small	68	10.4 (1.8)	7.5–15.6	
23	Large	7	36.0 (18.6)	16.1–69.0	9.3
30	Small	57	10.0 (1.5)	7.7–15.5	
30	Large	17	48.7 (22.6)	16.5–88.5	22.7

standard microbiological media. Failure of the organism to induce proliferation of HRT cells at 27°C was probably due to the quiescent nature of HRT cells at this temperature. Interestingly, fungal growth was not markedly inhibited and was independent of HRT proliferation. The ability of *R. seeberi* to induce cellular proliferation appears to require close contact with actively growing organisms and associated mammalian cells, as empty sporangial shells or organisms that attached but failed to grow did not induce this response. When cryopreserved *R. seeberi* were placed in culture, both fungal development and HRT proliferation were delayed by several days in comparison with freshly collected organisms (*12*). This delay appeared to be associated with the lag in development that followed recovery from −70°C storage conditions. Results from Table 1 suggest that a period of development or aging (or both) might be required under some circumstances before spores are capable of further development. This finding may help explain why transmission does not occur directly between hosts (*1*).

Utilization of an in vitro method that supports complete development of *R. seeberi* may facilitate study of its life cycle and assist in development of effective pharmacologic agents for treatment of this infection. The mechanism by which this organism induces cellular proliferation may now be investigated.

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5. Complete medium with single strength antibiotics consists of 20 ml of heat-inactivated (56°C for 30 minutes) fetal bovine serum, 80 ml of minimum essential medium (Eagle) (Gibco), 1.5 ml of 4.2% $NaHCO_3$, and streptomycin sulfate (100 μg/ml). Each 25-cm^2 flask received 5 ml of medium, which was changed daily during the first week and every 2 or 3 days thereafter. The *p*H was adjusted daily to 7.2 to 7.4 with 7.2% sodium bicarbonate.
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7. The initial inoculum consisted primarily of 8- to 10-μm spores released by the tissue grinder. Intact organisms up to 50 μm were also observed but there were fewer than five such organisms per flask at culture initiation. Larger organisms were disrupted. All measurements were made with an American Optical ×10 eyepiece micrometer.
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9. A microscope field was 100.4 mm^2.
10. A mixture of cell types, but primarily epithelial cells, were released from the *R. seeberi*–containing tumor (polyp). Although not fully characterized, the cells that did become established appeared by gross observation to be epithelial.
11. After spores were incubated for 101 days at 23°C, with medium changed approximately at weekly intervals, a suspension of several hundred rhinosporidium organisms (average diameter of 15.9 ± 6.5 μm, $n = 25$) per milliliter of medium was made in protein free Dulbecco's modified Eagle medium. No sporangia were identified in this suspension. The nasal mucosa of three male nude mice was scarified and then 0.2 ml of the protein-free rhinosporidium suspension was injected intranasally. Two-tenths of the rhinosporidium suspension was also injected in the right flank of each of these male mice and 0.2 ml was injected intraperitoneally into three female nude mice. Sixty and ninety days after inoculation one male and one female mouse were killed by cervical dislocation. There were no significant macroscopic or microscopic lesions in the mice. The remaining mice were examined periodically for 1 year, and no macroscopic lesions were observed.
12. M. G. Levy and N. Russel-Henry, unpublished data.

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Human Monoclonals from Antigen-Specific Selection of B Lymphocytes and Transformation by EBV

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Hybridoma technology has made it possible to prepare monoclonal antibodies with the use of murine lymphocytes. Attempts to extend this technology to the human level, however, have met with difficulties. A method has been developed for making human monoclonal antibodies of predetermined specificity. Biotinylated antigens (human thyroglobulin or tetanus toxoid) were incubated with human B lymphocytes from peripheral blood. The lymphocytes to which the antigens bound were selected by fluorescence-activated cell sorting. Positively selected (high fluorescence) and negatively selected (low fluorescence) cells were then transformed with Epstein-Barr virus (EBV) and grown in microculture wells. All wells from the positively selected fraction produced antigen-specific antibody (95 to 1800 nanogram-equivalents per milliliter), whereas fewer than 6% of the wells from negatively selected fraction made any detectable antibody (less than 10 nanogram-equivalents per milliliter). When the positively selected EBV-transformed cells were cultured in limiting dilution, clones were obtained that made antigen-specific monoclonal antibodies. By this method, monoclonal antibodies to both foreign antigens and autoantigens can be prepared from the normal human B-cell repertoire.

In 1975 KÖHLER AND MILSTEIN described a method for making antibodies in vitro by fusing spleen cells from immunized mice with mouse myeloma cells (*1*). The resulting hybridomas produced large quantities of antigen-specific monoclonal antibodies. This technique has revolutionized immunology and is widely used. Attempts to extend this approach to the production of human monoclonal antibodies, however, have met with difficulty for several reasons (*2–5*): (i) the lack of good human cells for fusion partners (that is, myeloma cells), (ii) the low frequency of fusion events (approximately 10^{-5}), and (iii) the relative paucity of circulating B cells with given specificity in human subjects who cannot be actively immunized with certain antigens. These factors reduce the probability of obtaining cell lines secreting antibodies of predetermined specificity.

We now describe an alternative approach for making human monoclonal antibodies. It takes advantage of the fact that receptors (that is, immunoglobulin molecules) for antigens are expressed on the surface of B lymphocytes in the normal B-cell repertoire, and, thus, B cells capable of making antibody with a desired specificity can be separated from irrelevant B cells by using the antigen in question as a probe. In our system, purified antigens are biotinylated and incubated with B lymphocytes from peripheral blood. The B lymphocytes to which the antigens bind are selected by fluorescence-activated cell sorting (FACS). The sorted cells are then immortalized by infection with Epstein-Barr virus (EBV) and propagated in culture. Under these conditions B cells from normal individuals can be used to generate clones secreting human monoclonal antibodies of selected antigen specificity.

Experiment 1 was designed to investigate the binding of biotinylated ligands to B

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