1979

The Extrapulmonary pathogenic potential of Chrysosporium parvum and Chrysosporium parvum var. crescens

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EXTRAPULMONARY PATHOGENIC POTENTIAL OF CHRYSOSPORIUM PARVUM AND CHRYSOSPORIUM PARVUM VAR. CRESCENS

By

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B.A., Pacific Lutheran University, 1971

Presented in partial fulfillment of the requirements for the degree of

Master of Sciences

UNIVERSITY OF MONTANA

1979

Approved by:

Chairman, Board of Examiners

Dean, Graduate School

11-26-79

Date

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Jensen, J. Michael, M. S., August, 1979 Microbiology

Extrapulmonary Pathogenic Potential of Chrysosporium parvum and Chrysosporium parvum var. crescens

Director: John J. Taylor

Korean Hemorrhagic fever (KHF) is a disease of unknown etiology. There is however some evidence which suggests the adiaspore as a potential etiologic model for the disease, and a theory of etiology has been proposed (Jellison, 1971). Because the adiaspore can produce a pulmonary infection in both man and animals, the hypothesis to be considered is whether or not the vascular and renal syndromes of KHF are caused by a circulating toxin produced by the adiaspore or by one or more of its soluble products causing a hypersensitive reaction on the part of the host.

An in vitro model of human embryonic kidney cells (HEK) was used to investigate this hypothesis. The tissue culture experiments performed indicated that the adiaspores of Chrysosporium parvum and Chrysosporium parvum var. crescens were capable of producing certain toxic effects on the HEK cell cultures. Such toxic effects included granulation of the cytoplasm, loss of definition of the cell membranes, alterations of the shape of cells, and either a slowing or increase in the rate of growth of the cells. Both direct addition of adiaspores to HEK cell cultures and supernatant fractions from adiaspore cultures produced toxic effects on the HEK cells. The latter indicated the production of a soluble extracellular product by the adiaspore. The production of this soluble substance was dependent upon the age of the adiaspore and wide variations in toxicity were noted between 10 and 40 days of adiaspore age. The results of this study have given some support to the theory of the adiaspore's involvement in the etiology of KHF.

ACKNOWLEDGMENTS

Special regard should be payed to William L. Jellison whose pioneering efforts have formed the basis of this study. I would like to give my special thanks to Dr. John J. Taylor who has fostered my enthusiasm for this study with his expertise, patience, and enjoyable company. Also thanks should be extended to Dr. C.A. Speer, Dr. Gary L. Gustafson, and Dr. Richard N. Ushijima who have helped to mastermind this project.

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CHAPTER I

INTRODUCTION

Korean hemorrhagic fever (KHF) is a unique and baffling disease that does not appear to adhere to the concepts of most disease etiology. The etiologic agent has yet to be proven. The problem is still prevalant in Korea, especially among military personnel, and in a ten-year period from 1951 to 1971, 6,179 cases with a 5-7% death rate were reported (15,19).

Korean hemorrhagic fever is one of a group of diseases with the same clinical picture referred to as the hemorrhagic nephroso-nephritis group or hemorrhagic fever with renal syndrome (4,11,14). Other hemorrhagic fevers belonging to this group are Far Eastern (Manchurian), Yaroslavl, field fever of Finnish Lapland, nephropathia epidemica of Norway and Sweden, trench nephritis of France, and epidemic nephritis of Yugoslavia. A different group of Hemorrhagic fevers are presumably tick-borne and are caused by viruses (4). Examples of this group are Crimean hemorrhagic fever, Omsk hemorrhagic fever, and Kyasanur Forest disease of India. The hemorrhagic nephroso-nephritis group is presumably not tick-borne and an etiologic agent has not been isolated.

The predominant clinical feature of the hemorrhagic nephroso-nephritis (HNN) group is vascular injury

with a renal syndrome, and includes blood diathesis, severe intoxication, renal insufficiency and distinct changes in the blood and urine. The major pathologic mechanism is injury to the vascular system, mainly small vessels and capillaries. Vessel changes result in hemorrhage, leakage of protein-rich fluid into the tissues, proteinuria, hypotension and shock. Lesions are most severe in the kidneys, however a petechial rash as well as nasal and uterine bleeding may also occur. Renal pathology includes greatly dilated vessels full of blood, pinpoint hemorrhages in the pyramids, and foci of necrosis with severe swelling of fibrous tissue in Malphighian glomeruli. A fibrin-like substance may fill the lumina of the tubules, and cellular infiltration may occur. Gross changes in the kidneys include swelling, increase in weight, and a cloudy grey-yellow cortex strongly delineated from the dark red medullary zone. Also the renal capsule is easily peeled away. The pathology of the disease presents a broad spectrum of signs ranging from inapparent to fatal.

Past theories of etiology have included a chemical (Warfarin) which was used to control rodents in endemic areas, leptospirosis with acute epidemic nephritis, a rickettsial disease and a viral disease. Research has proven no connection between these theories and the disease (14).

A mycotic theory of etiology has been proposed but not tested through experimental means (14). This theory is that the fungal agent Chrysosporium parvum var. crescens (also known as Emmonsia crescens and Haplosporangium parvum) is inhaled as an aleuriospore and develops into an adiaspore (spherule form) in the lungs which would be the infective focus of the disease (adiaspiromycosis) (8). Previous studies of this organism (Jellison, 1969) indicate that no dissemination occurs but that the organism remains localized at the site of infection. Therefore renal and vascular syndromes of KHF would of necessity be caused by a circulating toxin or by a hypersensitive reaction on the part of the host.

There is substantial epidemiological evidence to suggest a relationship between C. parvum var. crescens and KHF (14). This organism has been isolated in rodents in endemic areas of KHF and was found to be widespread in these areas. In addition, the months of peak rodent populations are May and November, slightly before the maximum incidences of KHF (9). The incidence of KHF shows seasonal trends, reaching its peak in June (time of seeding, plowing, etc.) and in November (time of harvesting). Both of these months are dry seasons in Korea. With regard to the mycotic theory, dry conditions would aid the inhalation of conidia by those who are in close association with soil and dust (military personnel and agricultural workers are

predominantly the victims (15,19)). Also KHF has never been associated with insects, food, or water and the disease is not contagious. These observations suggest soil as a source of infection. Chrysosporium parvum var. crescens is a soil saprophyte, growing in a mycelial form and producing aleuriospores which can infect man and animals (5,18). Rodents could function as a reservoir of the fungus (7). When the animals die, adiaspores are returned to the soil which subsequently grow out in the mycelial state.

Comparison with other fungal diseases such as coccidioidomycosis shows enough similarity in symptomology to suggest a fungal etiology for KHF (14). Symptoms common to KHF and coccidiomycosis are fever, malaise, chills, anorexia, cough, headache, backache, and erythema. Other common entities are restricted endemic areas, sporadic cases, epidemics, non-transmissability, no effective specific treatment, seasonal occurance (dry), and rodent involvement. Other fungal agents, e.g. Fusarium sporotrichoides, are the source of toxic products which result in alimentary toxic aleukia (11). This disease mimics the symptomology of hemorrhagic fever, the severe hemorrhagic diathesis and petechical rash in particular. In regions where one of the hemorrhagic fevers and this mycotoxicosis both occur, their differentiation may be a diagnostic problem (Gajdusek, 1953)

The suspected agent of KHF, C. parvum var. crescens, is a dimorphic fungus existing in a mycelial form below 37 C and as an adiaspore at and above 37 C (13). When the fungus is incubated at 37, the aleuriospores enlarge to form thick-walled spherules (adiaspores). In the mycelial state, aleuriospores are formed on branched or single conidiophores. Inhaled aleuriospores and some hyphal cells increase in size in vivo from 2 um to approximately 700 um and develop into adiaspores in the pulmonary tree. These spherules do not proliferate to any great extent under usual in vivo conditions, but some budding has been observed (8). However the degree of infection depends on the initial inoculum.

Mycelia can be generated from germinating adiaspores and aleuriospores produced at temperatures below 37 C. Under certain conditions adiaspores may bud (8) and endosporulation has been observed in some (8,13). However exosporulation (budding) seems to be quantitatively more significant than endosporulation under experimental conditions (Hamacek et al. 1972). Budding forms may become disconnected from the original adiaspore and be transported to more distant sites by the circulatory system, but this occurrence is quantitatively insignificant. When the adiaspore has finally matured, the wall of the adiaspore often breaks, releasing the protoplast. Because of a cell-mediated response to the adiaspore in host tissues, the area

is walled off by monuclear cells, fibroblasts and giant cells. The infiltration of mononuclear cells is the basis for the formation of solid granulomatous mass which affects alveolar function (8,13). The adiaspore may in addition produce and release metabolic products which are toxic. Such products could circulate in the blood and cause KHF in humans.

Adiaspiromycosis in man is not common but chronic cases have been reported (5,18). The "less apparent" form of the disease may be related to the toxigenicity of or hypersensitivity to the fungus. Perhaps hypersensitivity can develop in some individuals in response to soluble antigens from the organism. In experimentally-induced infection, antibody response to this organism is prompt, persistent, and specific (3). A passive cutaneous anaphylaxis (PCA) test developed by Jellison has shown several positive reactions to C. parvum var. crescens from individuals associated with animals, animal habitats, farming, etc. However the PCA test has not been used in diagnosis of human infection nor has it resulted in the association of any disease syndrome with this organism. Renal pathology of KHF points to an acute toxic nephrosis (1), either by virtue of a nephrotoxin or of hypersensitive reactions.

Present studies were undertaken to shed some light on the question of the mycotic etiology of KHF and the disease potential of Chrysosporium parvum and Chrysosporium

parvum var crescens. An in vitro model of HEK cells was used to investigate the potential toxicity of the adiaspore to living tissues, and whether or not it produced a soluble metabolic product(s) that could relate it to the mycotic etiologic model described for KHF.

CHAPTER II

MATERIALS AND METHODS

Source of Fungi

Chrysosporium parvum University of Montana (UM) strain 636 and *Chrysosporium parvum* var. *crescens*, UM strain 753 were obtained from Dr. J. J. Taylor, University of Montana. The former strain was isolated from wild rodents and the latter was the type strain from W. J. Jellison.

Cultures

Mycobiotic agar cultures were inoculated with conidia of each strain and were incubated at 25 C to allow sporulation.

Harvesting of cells

Conidia of each strain were harvested by flooding sporulated mycelial cultures with phosphate-buffered saline (PBS) and by gently stroking the surface with a sterile wire. The resulting suspension of conidia were then centrifuged and resuspended in a smaller volume of diluent.

Viability of cells

Conidial suspensions were serially diluted (1:10) in PBS and plated out onto mycobiotic agar to determine conidial numbers and viability. Suspensions containing

desired conidial concentrations were then frozen and stored at -20 C and were used as needed.

In vitro model

The in vitro model used was the tissue culture of human embryonic kidney cells (HEK line). This cell line was selected because of its ease in handling and its apparent susceptibility to toxic products produced by the adiaspore. The sensitivity of the cells undergoing cytopathic changes could be used as an indices for the potency of the toxic products. These cells were maintained with Eagle's Minimum Essential Medium (MEM) containing 2% newborn calf serum. Tissue culture media were routinely drawn off and replaced with fresh media every two days throughout each phase of the tissue culture experiments. This procedure allowed normal cellular nutrition and the removal of metabolic waste products produced by the cells. Tissue culture flasks were 25 cm^2 in size (50 ml) and required 5 ml of tissue culture medium each time the medium was replaced.

Adiaspore cultures

Conidia harvested from bottle and plate cultures were introduced at concentrations of 10^5 to 10^6/ml into plastic tube cultures containing the same tissue culture medium used to maintain the HEK cell line. These tubes were gassed for several seconds with a 50% carbon dioxide in air mixture and sealed with air tight caps. These tubes

also contained 100 units of penicillin and 100 ug of streptomycin/ml to inhibit the growth of bacteria. These concentrations of antibiotics were not in any way toxic to HEK cells which were incubated at 37-38 C and monitored for approximately 50 days.

Tissue culture experiments

Exposure of tissue culture to varied numbers of conidia. Conidial inocula ranging from 10^1 to 10^5 conidia/flask were added to 2-day old cultures. Each concentration in tissue culture was prepared in triplicate. As controls, conidial inocula at the same concentrations were heat-inactivated in a boiling water bath for 15 minutes and were added to duplicate similar cultures. All tissue cultures were then observed for 3 weeks to detect any cytopathic damage.

Exposure of tissue culture to adiaspores of different ages. Adiaspores that were 2, 4, 6, 8, and 10 weeks old were added to 2-day old cultures. Each adiaspore age group was prepared in triplicate with an additional triplicate set of unexposed controls. In addition, adiaspores 2 weeks of age were heat-inactivated in a boiling water bath for 15 minutes and then were added to triplicate cultures. The concentration of adiaspores added to each tissue culture was approximately 10^5/flask. These cultures were then observed for a period of 3 weeks to detect any

cytopathic damage. This procedure was followed for both UM 636 and UM 753 strains of adiaspores.

Hematoxylin and eosin stains of tissue cultures. Experimental and control cultures were stained with hematoxylin and eosin. These cells were then examined using light microscopy.

Exposure of tissue culture to supernatant media in which adiaspores had been grown. Adiaspore cultures containing approximately $4x10^5$ adiaspores/ml were grown in the same kind of tissue culture medium (MEM with 2% serum) in which cells were maintained, and these adiaspores were incubated at 37-38 C. Replicate cultures were allowed to incubate for time periods of 2, 4, 6, 8, and 10 weeks. The supernatant media from these adiaspore cultures were decanted. Approximately 5 ml of supernatant medium from each adiaspore culture was added to duplicate tissue cultures along with 5 ml of normal medium. Controls received only 5 ml of normal medium. The frequency of medium changes for control and experimental tissue cultures was the same. At 2- or 3-day intervals the supernatant media were drawn off the tissue cultures and were replaced with 5 ml of fresh MEM plus 5 ml of supernatant adiaspore culture medium. These tissue cultures were observed for a period of 2 weeks to detect any cytopathic damage.

Exposure of tissue culture to supernatant media from concentrated adiaspore cultures at five-day increments of adiaspore age. Several adiaspore cultures containing 1-2 x 10^6 adiaspores/ml of tissue culture medium were incubated at 37 - 38 C. Supernatant media from these cultures were withdrawn at intervals of 5 days, and new tissue culture media were added to the adiaspore cultures. These 5-day supernatant fractions were then placed in a freezer at -20 C until fractions were obtained for up to 50 days of adiaspore age. Tissue culture experiments were then performed using these fractions. Five ml of each fraction were added to duplicate tissue cultures along with 5 ml of normal MEM. Controls were set up in triplicate. The tissue cultures were then observed for a period of 2 weeks to detect any cytopathic damage.

The employment of a more sensitive method in determining cytopathic effects. Trypsinized cells were exposed to various supernatant adiaspore culture fractions for a period of 6 hours. Cells were then washed and allowed 24 hours to settle and attach, then the total number of cells failing to attach was determined with a hemocytometer for each flask. The % of cells failing to attach was then determined for various supernatant culture fractions and control cultures.

Trypsinized cells were also exposed continuously

throughout passing to various supernatant adiaspore culture fractions to determine the number of cells failing to settle and attach. These cells were preincubated in the supernatant fractions for 3 and 6 hours.

Scanning electron micrographs of cytopathic effect. An attempt was made to determine the nature of the cytopathic effects using the techniques of scanning electron microscopy. The cells were grown on coverslips and adiaspores from each strain were added to the cell cultures. After 7 days the cells were fixed in 1/2% glutaraldehyde in Millonig's phosphate buffer, post-fixed in osmium tetroxide, treated with thiocarbohydrizide, dehydrated in a graded series of ethanol, critical point dried, coated with gold atoms, and observed with the scanning electron microscope.

CHAPTER III

RESULTS

Adiaspore growth

Conidia of Chrysosporium parvum and Chrysosporium parvum var. crescens grew successfully in cultures containing tissue culture media when incubated at 37 C. Occasionally contamination of some of these cultures with other fungi was noted and these were discarded. Successful cultures remained free from contamination, and samples from each were observed microscopically at intervals to check for such contamination. Occasionally conidia from sporulated cultures as old as 6 months did not develop into adiaspores, and at times there seemed to be a lag before any growth of C. parvum var. crescens into adiaspores was observed. Usually adiaspores of C. parvum reached sizes approaching 20 um after about 30 days in the incubating culture and then there was a cessation of spore enlargement. Adiaspores of C. parvum var. crescens were larger, reaching approximately 30 um in diameter after about 30 days of incubation in these cultures. Since conidia were approximately 3-5 um in diameter initially, the increase in volume of these adiaspores was a thousand fold. When conidia were added to tissue cultures, they developed into even larger adiaspores, perhaps because of some factor produced by the HEK cell line. Adiaspores of C. parvum

var. crescens reached 30-60 um in diameter under these conditions.

Adiaspores remained viable in incubating cultures for about 50 days. Much variety in internal organization was observed throughout this time period. Young adiaspores (20 days) of C. parvum had a well-defined nucleus and C. parvum var. crescens was multinucleated. A well-defined nucleus could not be observed in older (40 days) adiaspores. The walls were thicker and irregular, and the well-defined internal organization was replaced by random granulation. Lysis and extrusion of the protoplast was occasionally observed in the older cultures (40-50 days) as well as an occasional budding of mature adiaspores.

Tissue culture experiments

Exposure of tissue culture to varied inocula of conidia of C. parvum. Of the varied concentrations of conidia added directly to the HEK cell line, only the highest concentration (10^5 adiaspores/flask) produced observable changes in the HEK cell line. However conidia of C. parvum developed into adiaspores in all the tissue cultures to which they were added. The viable conidia settled onto the surface of the HEK cell monolayer and became anchored, making changes of media possible without displacement of the adiaspores. Also many conidia were phagocytosed by the cells. Definite enlargement of viable conidia

was observed after 4 days in tissue cultures. No enlargement of non-viable (heat-inactivated) conidia was observed at any time. New HEK cells continued to grow in all tissue cultures which after a time pushed many adiaspores into groups or aggregates of adiaspores. The tissue cultures with 10^5 viable conidia also showed that adiaspores were causing some changes in the HEK cells. After 2 weeks, the cells became granular and there were no distinct cell membranes. In addition, many cells were observed with adiaspores growing inside them which eventually resulted in the breaking up of the cell. Other than this latter case, no cell destruction was observed. Also the tissue cultures with 10^5 viable conidia lasted much longer than the tissue cultures with 10^4 viable conidia or less. The former remained viable for a month and the cells simply remained in their granular state while the latter (including control tissue cultures) detached after about 2 weeks. Perhaps this resulted from a slowing of the HEK cellular metabolism.

Conidia of C. parvum var. crescens were also added to HEK cell cultures at concentrations of 10^5 conidia/flask. These conidia, however, did not develop into adiaspores in the cell culture, and no HEK cell changes were observed. Viability of conidia was later determined to be only 10^3 viable conidia/ml in a 10^6/ml suspension.

Exposure of tissue culture to adiaspores of

different ages. Cytopathic effects in tissue cultures containing conidia of C. parvum (Table I) were first observed after about 10 days. However, when adiaspores of C. parvum, which had matured for two weeks in a separate culture, were added to tissue cultures, the cellular changes occurred much more rapidly. The HEK cells changed shape, becoming more elongated after these older adiaspores had been in tissue culture only 3 days. By the 5th day, the cells had definitely become granular, and cell membranes were not distinct (Figure 3 and 4). These changes in the cells persisted throughout the period of observation, and the monolayer remained attached to the growing surface. The media however continued to become acidic after each medium change, indicating continuing metabolic activity in the HEK cell cultures despite alteration in cellular appearance. When 4-week old adiaspores of C. parvum were added to tissue cultures, the same effects on the cells were observed after 10 days, indicating some toxicity to the cells, but of a lesser degree than the 2-week old adiaspores were capable of producing. When 6-week old adiaspores were added to tissue culture, a rather pronounced cell granulation and unclear cell membranes were observed after 12 days. Neither the 8-week old or 10-week old adiaspores produced changes in the HEK cells that were different from the control group. These adiaspores were simply pushed into aggregates of adiaspores as new cell growth pushed old cells with their attached

Table 1

Sensitivity of HEK cell line exposed to C. parvum adiaspores of various ages

	Age of Adiaspore:					
	Viable conidia	2 Week	4 Week	6 Week	8 Week	10 Week
Nature of CPE[1]	unclear membrane, granulation of cytoplasm	same	same	same	none	none
Age of Tissue Culture when CPE appears (days)	9	5	10	12		

[1] CPE=Cytopathic Effect

Figure 1

Normal HEK Cell Line for 10 days

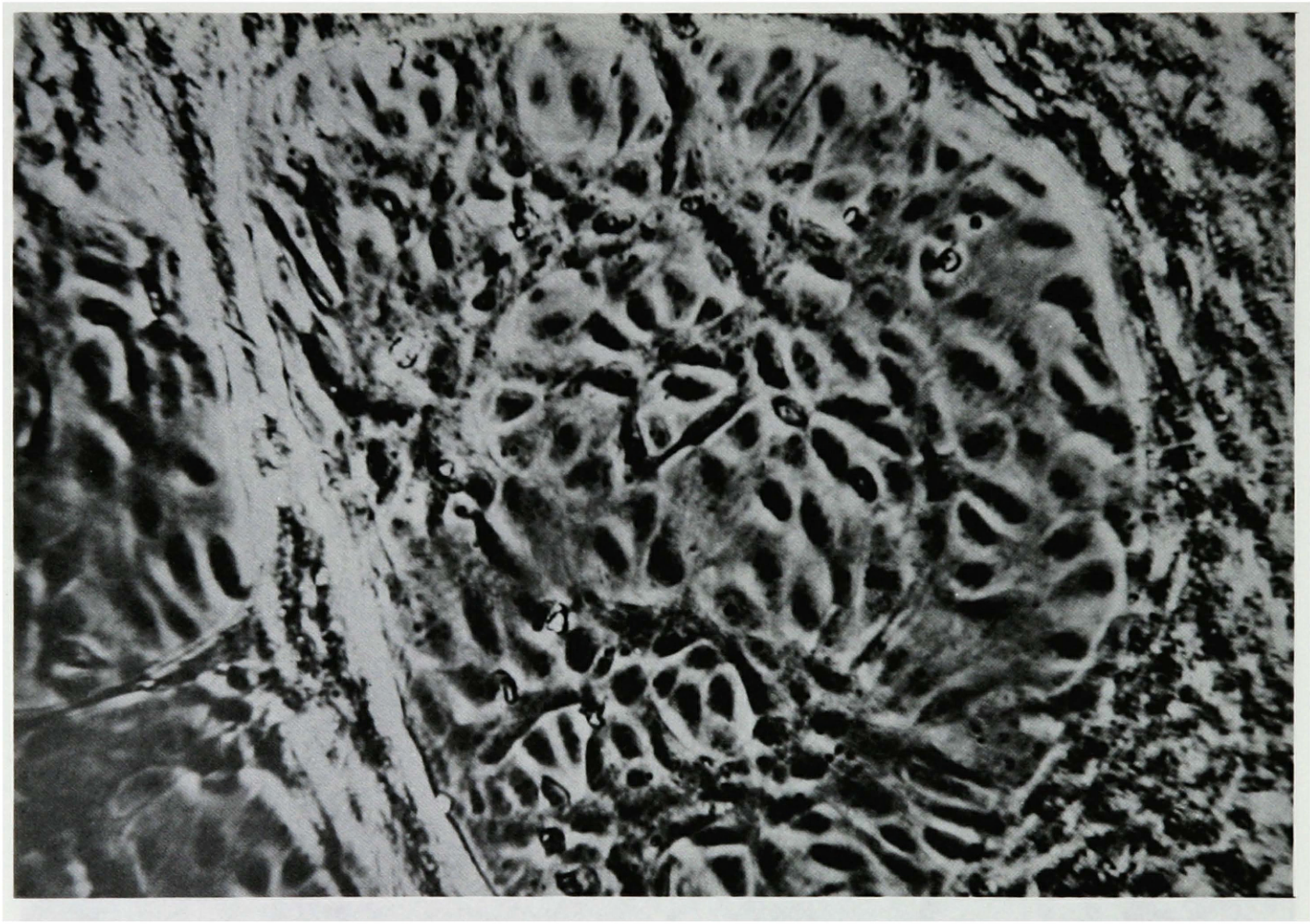
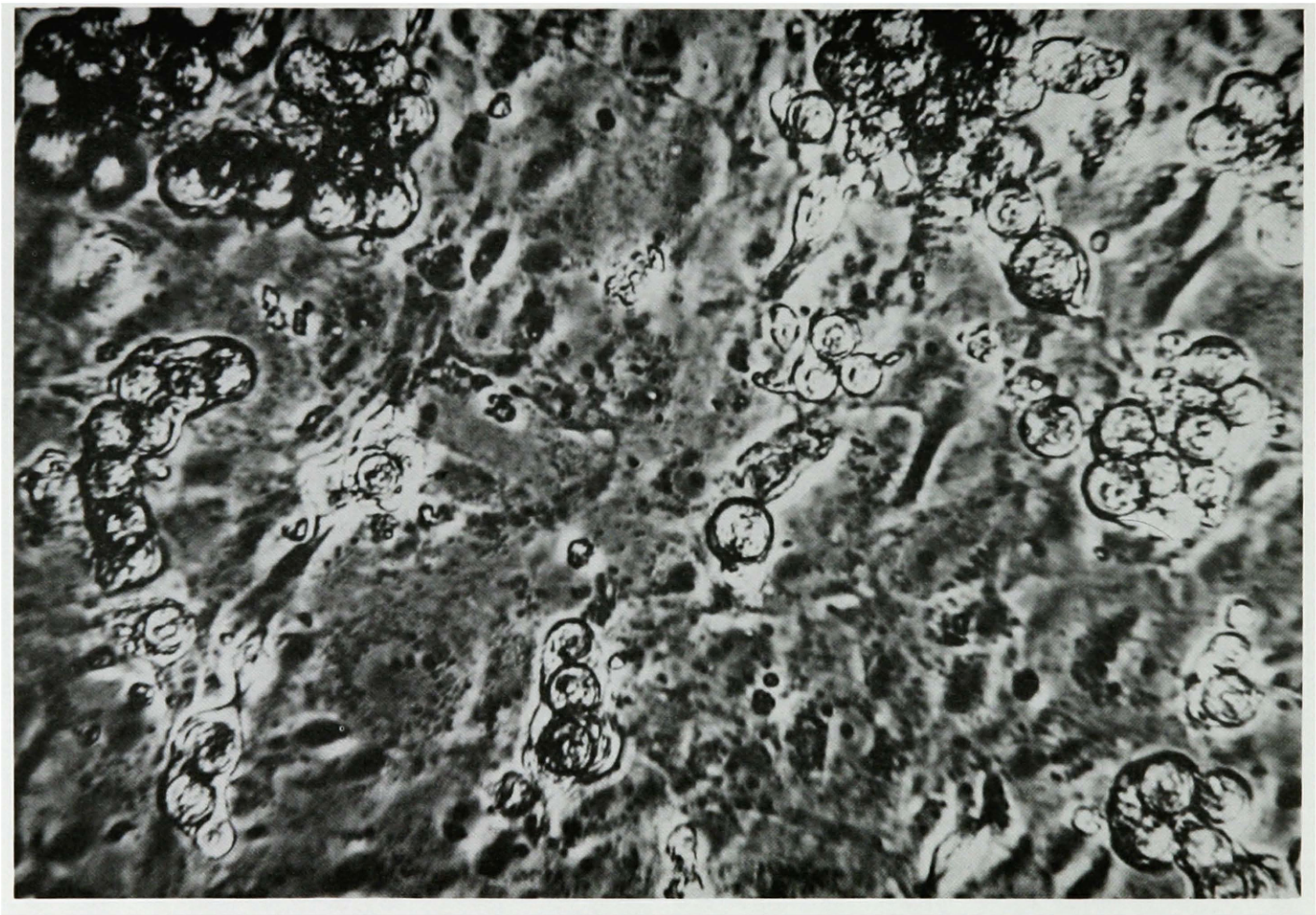


Figure 2

HEK Cell Line exposed to 2 week old Adiaspores
of C. parvum for 10 days



adiaspores aside. This phenomenon did not occur when 2-, 4-, and 6-week old adiaspores were introduced into cell culture, presumably because little or no new HEK cell growth occurred and the monolayer simply became granular. A control culture containing 2-week old adiaspores which had been heat-inactivated in a boiling water bath also demonstrated no effects on the HEK cells, whereas the viable 2-week old adiaspores were cytopathogenic. Again, the inactivated adiaspores were pushed into aggregates by new HEK cell growth.

The adiaspores of C. parvum var. crescens produced a different kind of effect on the cells (Table 2). Conidia which finally developed into adiaspores in tissue culture were able to produce an effect on the cells after 13 days. In this case, a marked elongation of the cell occurred, and cell membranes seemed to acquire distinct though rough appearing margins. Two-week old adiaspores of the UM 753 strain produced the same type of effect on the cells, the first appearance of this change in shape being observed after 6 days in tissue culture. This change in shape of the cell persisted throughout the observation period. Often in the tissue cultures with the UM 753 strain, the cells would not aggregate as in the control cultures indicating a reduced rate of new HEK cell growth. Four-week old adiaspores of the UM 753 strain produced the same change in shape of the cells slightly earlier. The

Table 2

Sensitivity of HEK Cell Line exposed to C. parvum var. crescens adiaspores of various ages

	Age of Adiaspore:			
	Viable conidia	2 Week	4 Week	6 Week
Nature of CPE[1]	distinct rough cell membrane, elongation	same	same	none
Age of Tissue Culture when CPE appears (days)	13	6	4	

[1] CPE=Cytopathic Effect

cells started to become elongated with rough margins by the fourth day after the adiaspores were added. Again this effect persisted for 3 weeks or the extent of the observation period. Six-week old adiaspores added to tissue cultures produced nothing differing from the control group.

Hematoxylin and eosin stains of tissue cultures. HEK cell cultures, to which adiaspores of C. parvum had been added, were prepared with hematoxylin and eosin staining for observation using light microscopy. Numerous cytoplasmic bridges could be seen between the cells and adiaspores. The cytoplasm of the cells seemed to be highly vacuolated, giving it a foamy appearance. It was also spotted with acidophilic granulation, and no definite cell membranes could be observed. Adiaspores in lysis could occasionally be seen, and a pocket of dark granules analogous to the materials seen to be expelled from lysing adiaspores could occasionally be observed in the cytoplasm of HEK cells. The nuclei of HEK cells did not seem to have any abnormal qualities, but appeared normal or were in some mitotic stage.

Exposure of tissue culture to supernatant culture media in which adiaspores had been grown. Because adiaspores added directly to cell cultures were capable of causing a cytopathic effect in the cells, the question arose whether this was caused directly by the interaction

of the adiaspore with the cell culture, or whether the adiaspore produced a soluble product(s) in the media that was affecting the cells. Supernatant medium from a 2-week old adiaspore culture of C. parvum (Table 3) was added with each medium change in the first experimental group. After 12 days the cells began to change shape and an elongation of the cell occurred. This initial elongation of the cells also occurred when 2-week old adiaspores of C. parvum were added to tissue culture. Older adiaspores or adiaspore supernatants of C. parvum did not produce this elongation of the HEK cells. Supernatant medium from a 4-week old culture of C. parvum produced the more notable type of cellular alteration. The cell membranes became less clear and the cells became granular. In addition, there was no aggregation of the cells as in controls, indicating a lack of normal cell division. This alteration was first observed on the fifth day of continuous exposure to the supernatant media and lasted throughout the period of observation (2 weeks). Supernatant media from a 6-week old adiaspore culture produced a result nearly identical with the 4-week supernatant. The cell membranes were unclear, and the cells were very granular by the sixth day of incubation. Again this cellular alteration remained throughout the period of observation. No changes different from controls were observed with supernatants from either 8- or 10-week old adiaspore cultures.

Table 3

Sensitivity of HEK cell line exposed to supernatant media from *C. parvum* adiaspore cultures of various ages

	Supernatant Media from Adiaspore Cultures of Age:				
	2 Weeks	4 Weeks	6 Weeks	8 Weeks	10 Weeks
Nature of CPE[1]	elongation of cell	unclear membrane, granulation of cytoplasm	same	none	none
Age of Tissue Culture when CPE appears (days)	12	6	6		

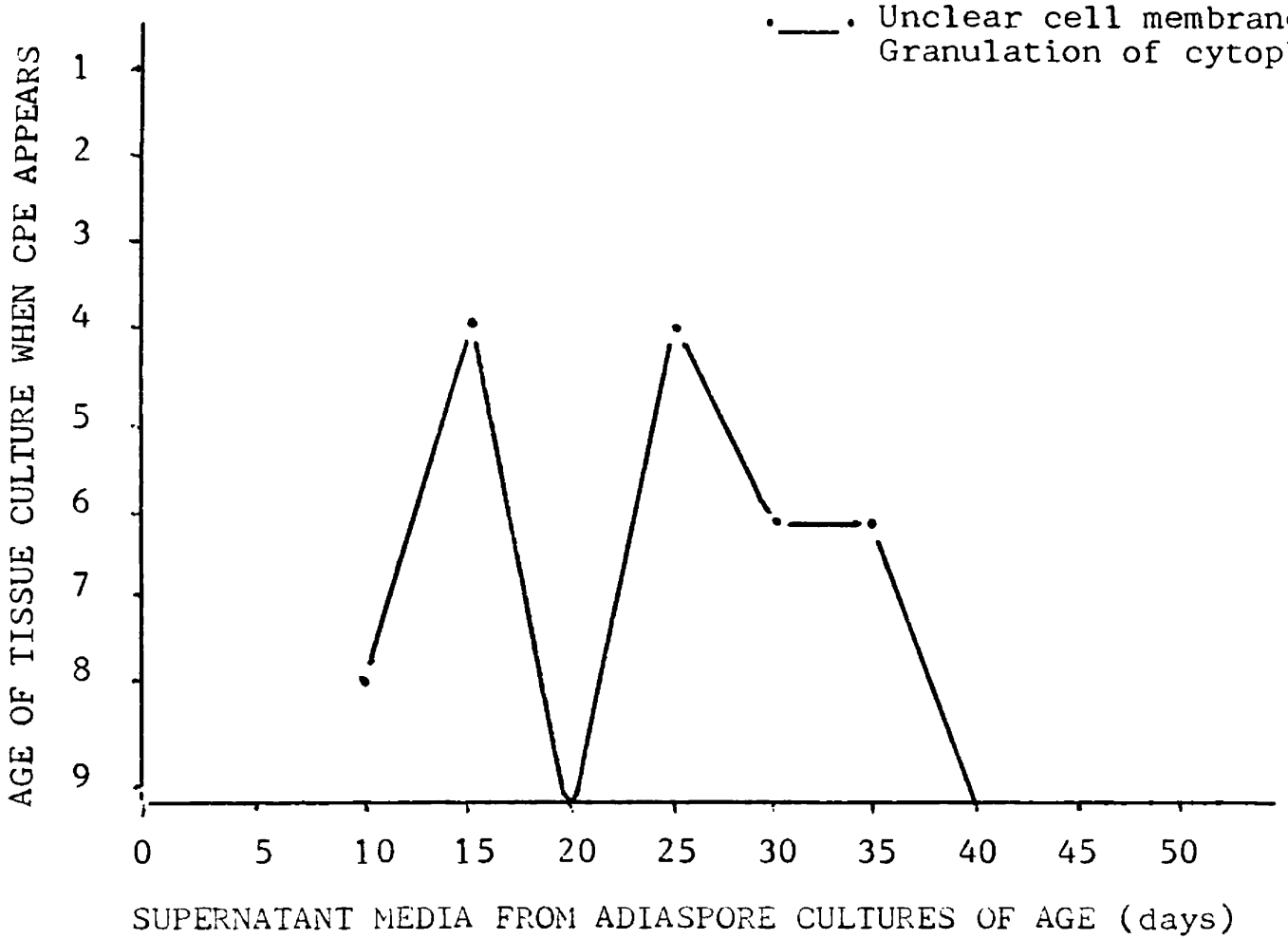
[1] CPE=Cytopathic Effect

Exposure of tissue cultures to supernatant media from concentrated adiaspore cultures at 5-day increments of adiaspore age. The adiaspore cultures of C. parvum and C. parvum var. crescens used in this phase of research contained higher concentrations of adiaspores (10^6 adiaspores/ml). Supernatant media was pipetted off every 5 days so that any possible adiaspore products were not allowed to accumulate over a wide range of time. This approach gave a more detailed picture of the activity of the adiaspore.

Supernatant from a 10-day old culture of C. parvum (Figure 1) produced the usual cellular changes (granular cytoplasm and unclear cell membranes) by the eighth day of observation. The supernatant medium from a 15-day old culture seemed to be more cytopathogenic, producing the same effect after 4 days in tissue culture. The supernatant medium from the 20-day old adiaspore culture did not show any effect on the cells except a slight granulation by the ninth day. During this period the adiaspores did not seem to be cytopathogenic. However the supernatant taken from a 25-day old culture demonstrated the recurrence of cytopathogenicity. The same cytopathic effect was noted after the same 4-day period of observation as had occurred prior to absence of CPE observed at 20 days. Both the supernatant media taken from the 30-and 35-day old adiaspore culture produced the same cytopathic effect slightly later in tissue culture. On the sixth day of

Figure 3

Sensitivity of HEK cell line exposed to supernatant media from concentrated Adiaspore cultures of C. parvum taken at 5-day intervals

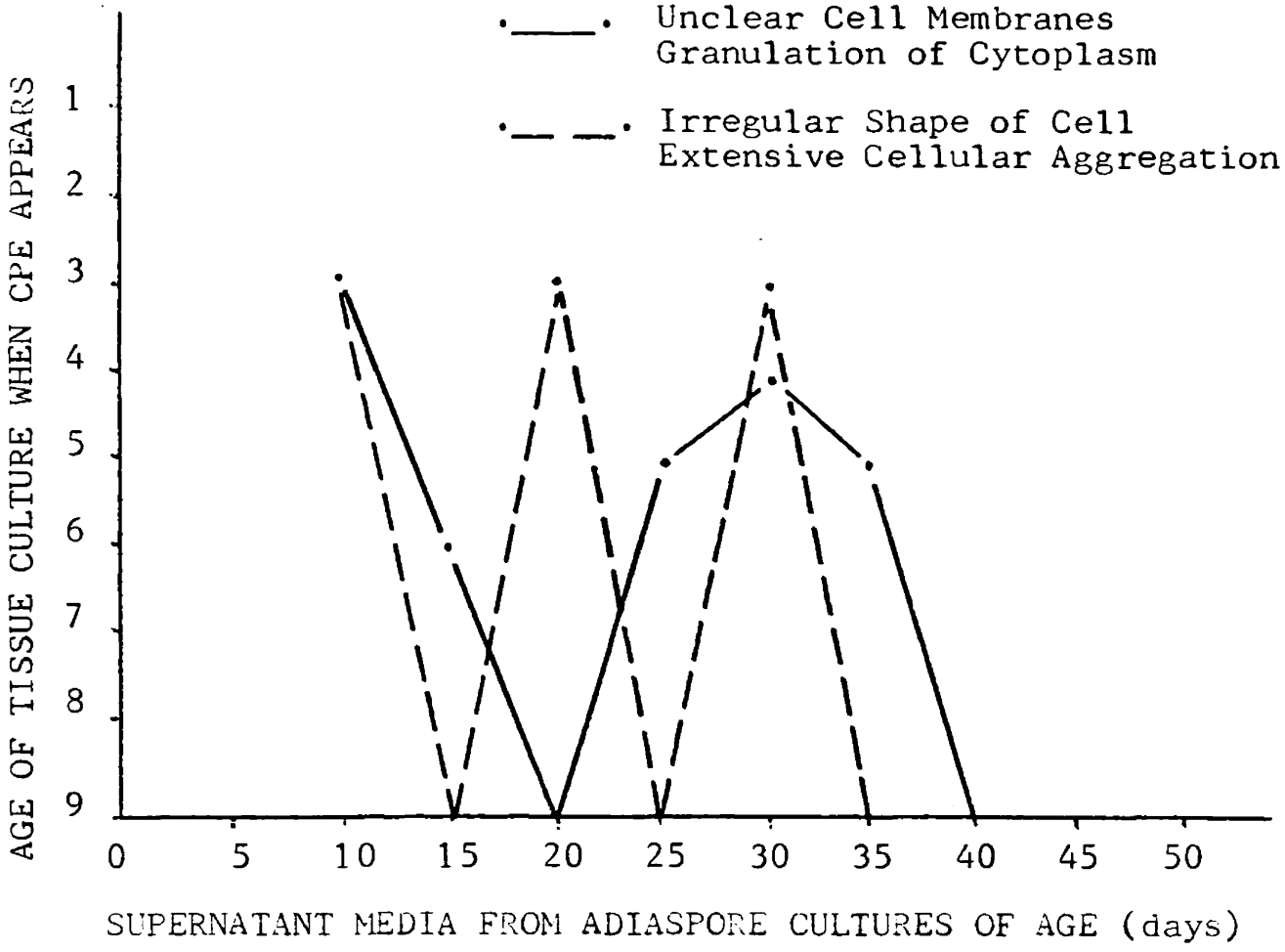


observation both unclear HEK membranes and granular cytoplasm appeared in tissue culture, indicating a slightly lower degree of cytopathogenicity. Both the supernatant media taken from 40-day and 50-day old adiaspore cultures produced no changes in HEK tissue cultures different from controls. This would seem to indicate cessation of adiaspore cytopathogenicity after 40 days. This correlates well with the appearance of senescent changes which were observed in adiaspores of this age.

The supernatant taken from concentrated adiaspore cultures of C. parvum var. crescens showed some diversity of cellular effects but with a striking similarity of pattern when compared with C. parvum (Figure 2). The supernatant medium taken from a 10-day old adiaspore culture of C. parvum var. crescens produced an irregular shape in the cells, and elongation of cells after a 3-day exposure in tissue culture. In these cultures the cells also became granular, and excessive aggregation of HEK cells was noticed, indicating that cell division in these cultures was more rapid than in control cultures. Supernatant media from a 15-day old adiaspore culture produced only one distinct change in the cells, i.e. granulation, which also occurred with the 10-day supernatant. The granulation was more severe with the 15-day supernatant and was first noticed after the sixth day of exposure. The supernatant medium from the 20-day old adiaspore culture

Figure 4

Sensitivity of HEK cell line exposed to supernatant media from concentrated adiaspore cultures of C. parvum var. crescens taken at 5-day intervals



produced cellular changes in the same fashion as the 10-day supernatant but with only slight granulation. Again many of the cells became elongated, and there was excessive aggregation of the HEK cells after 3 days incubation. The supernatant from the 25-day old adiaspore culture produced the previously observed effect on the cells, i.e. the cells became granular with unclear cell membranes. These effects were noticed by the fifth day of exposure in tissue culture and continued throughout the 9-day observation period. The supernatant from the 30-day old adiaspore culture produced a composite of all effects previously noted. The cells became elongated, and excessive aggregation and overcrowding of the cells occurred by the third day of exposure in tissue culture. On the fourth day of exposure granulation of the cells occurred, and cell membranes became unclear. By the seventh day of exposure the cells had detached from the growing surface. The supernatant from the 35-day old culture again produced limited effects; the cells became granular and cell membranes became unclear. These changes were observed by the fifth day of exposure in tissue culture. Again there was detachment of the cells by the seventh day of exposure. The supernatant from the 40-day old culture produced only a slight granulation observed at the fifth day in tissue culture and the supernatant from the fifty day old culture produced no effect on the cells, indicating cessation of adiaspore activity.

The employment of a more sensitive method in determining cytopathic effect. All previous work with the supernatants had been done with continuous exposure to the supernatant media throughout the period of observation. In this experiment cells were suspended in the supernatant media for a period of 6 hours. Any period of exposure less than this did not noticeably affect the cells. The cells were, however, more sensitive to toxic products when in these suspension and had a greater amount of surface area exposed to the supernatant media.

The percentage of cells failing to settle and attach in the control group after 24 hours was 7.5% of the total inoculum of cells being transferred (Table 4). (These cells had no exposure to supernatant media in the interim.) Supernatant media from 10-, 30-, and 50-day old adiaspore cultures of C. parvum produced a higher percentage of cells failing to settle and attach in the flask - 21%, 17% and 11% of the total inoculum respectively. Only the 10- and 30-day supernatants had significantly greater cytopathogenicity than controls with the 6-hour exposure. (Cells exposed for 3 hours in the same manner did not differ from controls.)

Scanning electron micrographs of cytopathic effect. Adiaspores anchored to HEK cell surfaces could be observed in greater detail, but nothing of a cytopathic nature could be discerned. The monolayers prepared for SEM were

extremely fragile and were not very stable under electron bombardment. However, budding of adiaspores could be observed and a "foamy looking" substance was observed to be in association with adiaspore walls.

Table 4

Sensitivity of HEK cell suspension exposed to supernatant media from C. parvum adiaspore cultures of various ages[1]

	Supernatant Media from Adiaspore Cultures of Age:			
	10 Days	30 Days	50 Days	Normal media
% of cells failing to attach and grow after 24 hours	21%	17%	11%	7.5%

[1]HEK cells were exposed 6 hours.

Figure 5

Scanning electron micrograph of C. parvum var. crescens adiaspore attached to HEK cell surface (2300x)

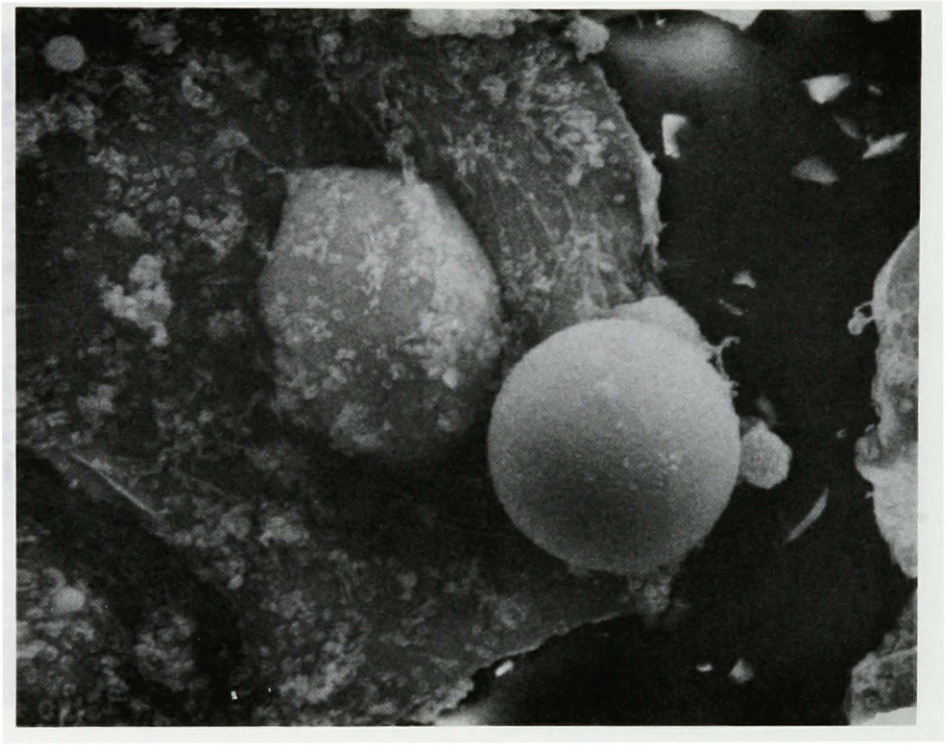
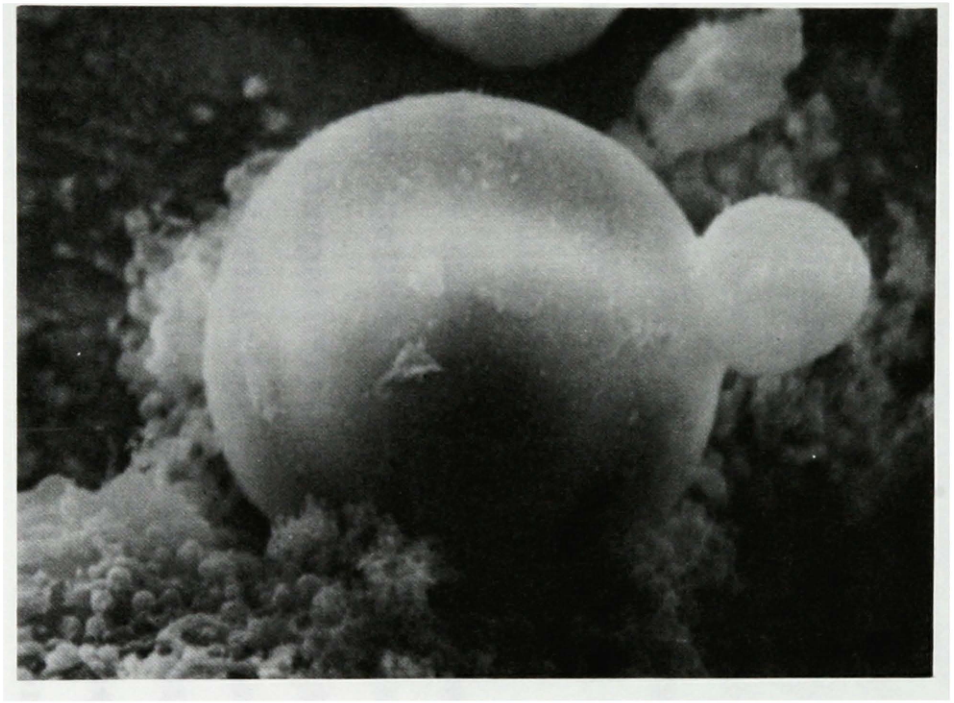


Figure 6

Scanning electron micrograph of C. parvum var. crescens adiaspore showing evidence of budding (4800x)



CHAPTER IV

DISCUSSION

Cytopathology in tissue culture

In tissue culture, signs of cellular intoxication include a decreased or total arrest cell growth, changes in the shape of the cell,loss of membrane definition, swelling of the cell, the accumulation in the cytoplasm of substances that are normally visible as granulation, absent, or present only in small amounts (granular cytoplasm), and degenerative nuclear changes such as nuclear pyknosis karyorrhexis, and karyolysis (22).

The kinds of cytopathic changes produced by adiaspores of C. parvum included a decreased rate of cell growth, an initial elongation of the HEK cells, and eventual loss of membrane definition with granulation of the cytoplasm. These changes produced by the adiaspores indicated a diffuse general intoxication of the cell culture. It is interesting that some of the changes produced by the adiaspores could be duplicated by adding a toxic amount of tetracycline (100 ug/ml) which could slow cell growth and produce loss of membrane definition with extreme granulation of cytoplasm. Also bacterial toxins often produce similar effects on cells in culture (22). For example, diphtheria toxin will cause inhibition of cellular multiplication followed by vacuolization of the cytoplasm

characterized by an increase in lipids (lipid phanerosis or lipid degeneration). Also with this toxin the nucleus will become pyknotic. Such changes occur after 24-48 hours. Staphylococcus toxin will also cause swelling and vacuolization of the cytoplasm, but in this case vacuoli represent vacuolar degeneration and not fatty degeneration. The cells have a "foamy" appearance due to vacuolization which seemed to be similar to observations made when adiaspores of C. parvum were added to HEK cell cultures. Again the nucleus becomes pyknotic, and the cells die. This latter effect was not observed when adiaspores or adiaspore products were added to HEK cell cultures. When staphylococcus toxin is added to tissue culture, these cytopathic effects occur after 1 hour. The cytopathic effects produced by adiaspores or their products never occurred earlier than 4 days after addition to tissue culture. Other toxins producing similar cytopathic effects are Shigella toxin which produced granulation of the cytoplasm around a pyknotic nucleus and the poison from all snakes which produces pyknosis of the nucleus and vacuolization of the cytoplasm. These toxins are very specific with regard to the pathogenic effect they produce on cells in culture.

Some compounds toxic to cells can act on resting cells and others on cells in division (22). Compounds that are toxic to resting cells act by inducing changes in the

protoplasm. They penetrate the cells and change the colloidal status and permiability of the cells. This results in slowing and arrest of cell growth, changes in shape of the cells, and lipid degeneration. Many drugs when added at toxic levels to tissue culture produce these cytopathic effects. Protoplasmic alterations were also the main feature of the cytopathic effects produced by adiaspores and their products. Nuclear pyknosis may or may not occur when cells are exposed to toxic compounds, and cells can resume normal activity if transferred to normal medium only if the nucleus remains normal. Otherwise they die. HEK cells retained some viability when cytopathic effects were present, and often their condition improved when supernatant media was withdrawn and normal media was added to cell cultures suggesting that there were no adverse nuclear alterations caused by the adiaspores or adiaspore products. The results would imply that adiaspores probably do not produce a toxin which usually kills the tissue culture cells rapidly but some compound of less toxicity.

The adiaspores and adiaspore products of C. parvum var. crescens also produced many of the same cytopathic effects that were produced by C. parvum. However, additional cellular alterations were observed. Loss of membrane definition with granulation of the cytoplasm was observed in common with C. parvum, but an increased rate of HEK cell growth was also observed at times along with

changes in cell density resulting in an elongation and flattening of the cells and these cytopathic effects occurred only when adiaspores or adiaspore products of C. parvum var. crescens were added to HEK cell culture. Bacterial toxins added to tissue culture can sometimes produce retraction of the cytoplasm resulting in cells that are triangular, quadrangular, or polygonal (22). Vacuolar degeneration can also cause cells to become elongated and stretched. Such an elongation of cells occurred when adiaspores of C. parvum var. crescens were added directly to HEK cell culture.

The increased rate of cell growth observed with supernatants from C. parvum var. crescens is a phenomenon which can occur in many mycotic infections in vivo. For example, Rhinosporidium seeberi (another spherule form) produces an epithelial hyperplasia resulting in vascular polyps, tumors, and hyperplasic papillomas (23). Some mycotic agents such as Rhinocladiella, Phialophora, and Cladosporium also produce hyperplastic responses in tissues resulting in verrucoid, warty, cutaneous nodules on the skin (verrucous dermatitis). Often such hyperplastic responses are mistaken for epidermoid tumors or carcinomas but are rather considered to be an extreme acanthosis (proliferation of cells in prickle layer of skin). Candida albicans when present in large numbers in the skin can also cause a hyperkeratosis (proliferation of cornified

cells) and acanthosis, and this is caused by toxic injury from fungal cellular components (23). Also Blastomyces dermatitidis can produce a pseudoepitheliomatous hyperplasia and extensive acanthosis when the chronic cutaneous form of the disease caused by this organism occurs. A very extensive cellular proliferation occurs which can be mistaken for a basal cell carcinoma, but cells remain well-differentiated in contrast to the latter. The role of metabolic products such as ethylene and lipids from this yeast have been postulated to have a rule in pathogenesis, but no experimental evidence has been established (23). The spherules of Coccidioides immitis, an organism similar to the adiaspore, can also produce pseudoepitheliomatous hyperplasia which is very similar to that of cutaneous blastomycosis. These organisms are possibly causing an in vivo response related to the in vitro proliferation of cells caused by the supernatant media from adiaspore cultures of C. parvum var. crescens. The involvement of a soluble product in producing such tissue responses might by analogy further be supported by the work of Gale et al where culture filtrates of C. immitis were demonstrated to be toxic for mice (12).

Other factors were observed to have an influence on the condition of the HEK cells. The age of the tissue culture itself was observed to have such an effect. Older cultures would often become overcrowded, and cells would

either strip off or cell growth would stabilize. Cells would eventually become a bit granular with no clear membranes (after about 3 weeks). The changes produced by the adiaspore, however, occurred much earlier in tissue culture and were quite distinct from controls. Failure to change the media also resulted in cellular intoxication, but cells simply stripped off in this case. Contamination from other fungal or bacterial agents also resulted in rapid stripping off of cells. No changes were observed which occur with viruses such as rounding up of cells, syncitium formation, inclusion bodies, or nuclear changes (21).

While a number of cytopathic changes were observed in HEK cells, it should be noted that cultured cells are more sensitive to toxic substances than are the same tissues in a living animal. Therefore the substance(s) produced by the adiaspore which is toxic to HEK cells *in vitro* may not be toxic to renal tissues *in vivo*.

Effective concentrations of adiaspores in relation to HEK cell numbers.

The results of this experiment have indicated that a high number of adiaspores in relation to the number of HEK cells is required before any discernable cytopathic effects occur. A 10^5 inocula of adiaspores was required to produce a discernable cytopathic effect on a growing HEK cell culture containing approximately 10^6 cells. A more pronounced effect occurred when increased numbers of

adiaspores were added, and concentrations of adiaspores greater than 10^6/flask tended to obscure observations in the tissue cultures.

Supernatant fluids from adiaspore cultures of 10^5/ml produced a barely discernable effect when 3 ml of supernatant fluid was added to tissue culture. When adiaspore cultures were at concentrations of 10^6/ml, the effects were more obvious. It is not known how these concentrations compare with infection densities in tissues *in vivo*.

Direct addition of adiaspores to tissue culture and supernatant effects.

Adiaspores between 2 and 6 weeks of age when added to tissue culture resulted in the observation of a variety of cytopathic effects on the cells. The nature of this interaction with the cells was not clear, however. Was the adiaspore competing with the cell for available nutrients, could cell changes be attributed to direct contact with the adiaspore, or was the adiaspore producing a toxic substance which diffused through the media?

The initial studies with the adiaspore culture supernatants indicated that the adiaspore was producing one or more metabolic products that were capable of producing cellular changes identical to those of intact adiaspores though not perhaps to the same degree. This would preserve the adiaspore as an etiologic model for Korean hemorrhagic

fever and related diseases. A diffusible product produced by the adiaspore in the lung might circulate through the body causing the symptoms of the disease. This pattern of pathogenicity occurs with any of the fungi that produce a toxin though in these cases the preformed toxins are ingested, leaving the idea of a pulmonary origin of the toxin in a unique position as far as fungi are concerned. Some examples of fungi that produce mycotoxicoses are Aspergillus flavus which produces aflatoxin that causes vascular necrosis and Fusarium sporotrichoides which causes symptoms nearly identical to those of Korean hemorrhagic fever (23). There are many other mycotoxicoses.

Pattern of adiaspore activity

The more detailed study of the adiaspore supernatant media (5-day incubation intervals) permitted a better indication of what the adiaspore was doing at any one time. Initial studies of supernatants involved an accumulation of metabolic products over an extended period of time.

Both C. parvum and C. parvum var. crescens had similar cytopathic activity with regard to the production of cytoplasmic granulation and unclear cell membranes. Both strains produced these effects when adiaspores were 10-and 15-days old but not when 20-days old. Then both strains regained cytopathogenicity when they were 25-to

proteins (28). It was noticed that a decrease in the factor producing granulation in HEK cell cultures seemed to coincide with a rapid increase in growth of the adiaspore and a leveling off of growth with an increase of this factor.

An elongation of the HEK cells was observed with increased cell growth when adiaspores were added directly to tissue cultures. Oddly, no other effect was seen when adiaspores of C. parvum var. crescens were added directly to the tissue cultures. An initial elongation of the cell also occurred when 2-week old adiaspores of C. parvum were added directly to tissue culture, and this effect was also observed when the supernatant from the adiaspore culture of this age was added to tissue culture. However elongation was never observed with old adiaspores or culture supernatants of this strain, and it was quickly masked by an extreme granulation of the cytoplasm when adiaspores were directly added to tissue culture.

A number of adiaspore products might be produced by each strain of adiaspore which could be common to both, and these could be regulated in the same way. However certain strain specific metabolites may also exist. Although species specific metabolites are common in the production of antibiotics (e.g. Penicillium sp.) and often the same metabolite is produced by different species, no examples of strain specific metabolites are known.

The adiaspore and Korean hemorrhagic fever.

The toxigenicity of the adiaspore is still an unresolved question. Whether the adiaspore causes the pathology and symptomology associated with the hemorrhagic nephroso-nephritis group of diseases is still uncertain. It does seem evident, however, that the adiaspore has the capacity to produce a diffusible substance that via the blood circulation could reach more distant sites in the body from its usually well-restricted locus in the lung of the host. This possibility has been supported by the results of this experiment. It is not known, however, whether the adiaspore would produce cytotoxic products under in vivo conditions. For example, the production of many mycotoxins is substrate dependent or depends on environmental factors e.g. the production of mycotoxins from Aspergillus flavus and Fusarium moniliforme. Also the morphogenesis of other spherule forms like C. immitis depends on several environmental factors besides temperature, and culture spherules may be quite different from tissue spherules (2).

The nature of the adiaspore's soluble products is yet another unresolved question. Does the adiaspore produce one or more cytotoxins or are the products essentially nontoxic? In vivo responses to such substances could help determine the answer to this question if pathological changes occurred in experimentally induced infection.

Even if the adiaspore were producing a diffusible substance that was not itself cytotoxic, this substance being foreign to the body could be responsible for producing a disease state involving the immune system such as an immune complex disease. The antigenic substance from the adiaspore could localize in small blood vessels (systemic and renal) or immune complexes might circulate and localize in the same locations. The antigenic substances would then be phagocytosed by polymorphonuclear leucocytes, and the proteolytic enzymes released could result in damage to blood vessel walls and kidneys. When a soluble antigen is directed into the circulatory system of a host and a hypersensitive state exists from previous or chronic exposure to the antigen, extensive vascular damage is the result. The intravascular immune complex type of disease occurs most often in persistant microbial infections in which microbial antigens are continuously released into the blood but antibody responses are minimal or of poor quality. Then immune complexes are deposited in kidney glomeruli and blood vessels over the course of weeks, months, or even years (20).

Many cases of glomerulonephritis are due to complexes of this nature (24). Examples of diseases that can result in such a syndrome are a nephrotic syndrome associated with malaria, chronic viral diseases such as that caused by the lymphocytic choriomeningitis virus,

nephritogenic streptococci infections, glomerulonephritis associated with systemic lupus erythematosus, and some hemorrhagic arthropod-borne viral diseases such as dengue hemorrhagic fever.

Perhaps this disease mechanism also produces the similar clinical picture of Korean hemorrhagic fever of which vasculitis and a renal syndrome are the main features. Respiratory symptoms rarely occur in this disease, but radiologic evidence of pulmonary lesions which may involve adiaspores is commonly seen (4). No hemorrhagic lesions or renal syndrome has ever been noted in reported cases of human adiaspiromycosis (29,30). There is, however, a prompt and specific antibody response to the adiaspore in experimental infection of rabbits (3) indicating the presence of diffusable antigenic substances from adiaspores in the lungs. Positive skin test responses to various antigenic preparations from both C. parvum and C. parvum var. crescens indicate this. Some serological testing has also been done in Sweden (C. Kindmark) on 18 cases of proven Nephropathia epidemica in which 72% of the cases were found to have a positive seroreaction to antigen from C. parvum var. crescens whereas only 2% of a control group were positive. Further study of the adiaspore's products would be needed before such proposals could be proved or disproved.

CHAPTER V

SUMMARY

The potential of the adiaspore as the etiologic agent of Korean hemorrhagic fever has been investigated by using human embryonic kidney cells in tissue culture as an *in vitro* model for the disease. These cells would likely be susceptible to toxic products produced by the adiaspore.

The tissue culture experiments performed indicated that the adiaspores of *Chrysosporium parvum* and *Chrysosporium parvum* var. *crescens* were capable of producing certain toxic effects on the cell line. Both direct addition of adiaspores and supernatant fractions from adiaspore cultures produced toxic effects on the cells. Toxic effects produced by supernatant fractions added to tissue culture indicated the presence of one or more soluble products produced by the adiaspore. Such toxic effects included granulation of the cytoplasm, loss of definition of cell membranes, slowing of cell growth, and failure to settle and attach in the tissue culture flask when suspended cells received a limited exposure to adiaspores or their soluble extracellular products. Additional effects noted were an elongation of the cell, and an increased rate of HEK cell growth which was noted when several supernatant fractions of *C. parvum* var. *crescens*

were added to tissue culture. It is not known how other cell lines would react to the adiaspore, although cells of renal origin are generally more sensitive to toxic products (22).

The addition of adiaspores of various ages to tissue cultures indicated that both qualitative and quantitative aspects of cytopathology could be correlated with adiaspore age. These effects occurred when the adiaspores were between 10 and 40 days old.

A certain pattern of adiaspore activity was indicated by the study of supernatant culture media taken from adiaspore cultures at 5-day intervals of adiaspore age. Both strains produced cytoplasmic granulation and loss of cell membrane definition when adiaspores were 10- and 15-days old but not when 20-days old. Then both strains regained cytopathogenicity when they were 25- to 35-days old, but were without effect when older than 5 weeks. An increased rate of HEK cell growth was produced by supernatant media from 10-, 20-, and 30-day old adiaspore cultures of C. parvum var. crescens.

Further research on the connection of the adiaspore to Korean hemorrhagic fever should proceed in the direction of characterization of the adiaspore's products and further in vivo responses to such substances.

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