THE MORPHOLOGICAL MANIFESTATION OF THE ADIASPORES OF EMMONSIA CRESCENS IN VITRO AND IN VIVO

F. HAMÁČEK, J. DVOŘÁK, M. OTČENÁŠEK and V. SEMEČKY

Department of Epidemiology of the VŠVŠU, Hradec Králové and Institute of Parasiotology, Czechoslovak Academy of Sciences, Prague, Faculty of Pharmacy, Charles University, Hradec Králové

Dedicated to Professor B. Rosický, D.Sc., on the occasion of his 50th birthday.

Abstract. The results of studies on the spectrum of morphological manifestations of the adiaspore of *Emmonsia crescens* disclosed various modes of reproduction in vitro and in vivo. A description is given of the junction of cells of the parasitic phase, which has been observed recently. The authors present their concept of the life cycle of the adiaspore, emphasizing the necessity of revising various ideas on the pathogenesis of adiaspiromycosis and of re-evaluating its taxonomic position.

Recent reports on the first clinically manifested case of adiaspiromycosis (Hájek et al. 1970; Koňousek et al. 1970 a) increased, particularly in Czechoslovakia, the interest in this nosological entity and in its etiological agent. New knowledge was obtained from various fields: mycology (Dvořák et al. 1970; Hamáček et al. 1971 a, b; Hejtmánek et Koňousek 1971); histochemistry (Koňousek et al. 1971; Žďárská et Šlais 1971); ultrastructure of *E. crescens* (Havelková et al. 1971; Malinský et al. 1971); pathogenesis (Hamáček et al. 1971 a, d—g; Hamáček et Semeccký 1971); the clinic (Vojtek et al. 1970, 1971); treatment of adiaspiromycosis (Vojtek et al. 1971; Hamáček et al. 1970 a, b).

The study presents a synthesis of experimental results obtained by the authors in the morphophysiology of the fungus *Emmonsia crescens* under conditions of parasitism and in the experiment in vitro.

MATERIAL AND METHODS

White mice of both sexes (weight 18—20 g at the commencement of the experiment) were infected with a suspension of akeucocytes and fragments of the mycelium of *E. crescens*, using standard techniques (i.p., i.m. or s.c.). For this purpose we used a 20 day-old culture of the saprophytic phase of strains from Dvořák's collection (strain no. 1, 815, 2,499 and 2,570) (Otčenášek et al. 1965). The infective dose ranged from $10^3$—$10^4$ of viable elements. The mice were sacrificed at intervals from 1—24 weeks p.i. and we examined the liver, spleen, kidney, lungs and also the adiaspiromycoses.

a) Histological examination—after fixing the material with formalin or Bouin's solution and embedding it in paraffin or celloidin-paraffin, the sections were stained with either haematoxylin eosin, with trichrome or with our modification of Grocott and Gomori's impregnation method.

b) Cultivation—fresh adiaspores isolated from adiaspiromycotic foel of mice were cultivated on cover slips in the moist chamber either without nutritives or in microculture with Sabouraud's dextrose agar. Incubation at 37 °C lasted from 24 hrs to 42 days; the germination test of the endospores was performed at 25 °C.
RESULTS AND DISCUSSION

It has generally been accepted that the membrane of the adiaspore attains different sizes and is of a lamellar structure. In our material, we observed repeatedly the presence of capsules which, upon inspection in histological section, showed to contain a granular or basophilic structure placed inside an eosinophilic matrix. In these instances, the border of the membrane in its centrifugal or centripetal portion, which normally can be disclosed with standard methods, was not present. The outer surface of the adiaspore was uneven and the granules of the mentioned substance appeared, under certain conditions, to transform frequently into well-determinate extensions which, upon release, were responsible for the reproduction of the adiaspore.

The granular structure of a membrane without a morphologically identifiable bordering layer towards the centre of the adiaspore enters continuously the submembraneous reticulum. This was pointed out by Kodousek et al. (1970 b) and we shall describe it in detail later in the text. As regards the membranes of the adiaspore, we observed several adiaspores in infected host tissue, lying close to one another. They were surrounded by a sheath of varying thickness (up to several tens of μm), but it was impossible to determine whether these adiaspores were of different age (Plate I*), Fig. 1. This situation having been observed also in unsectioned and fresh material excludes any confusion of a subtangential with an equatorial section (Plate I, Fig. 2).

We observed nipple-like prominences at the outer periphery of several membranes visible in both selectively and standardly stained sections (Plate I, Fig. 3) and in vitro (Plate I, Fig. 4). These formations suggest the phenomenon of budding, and their release from the mother adiaspore has been observed in several cases (Plate I, Fig. 5). The formations appear to be capable of further independent existence. Perhaps a decrease of external temperature may have been responsible for the formation of microgerms transforming into daughter adiaspores after a reversion to the status quo ante. In our opinion, however, this explanation appears to be doubtful, because

— during cultivation of adiaspores in artificial nutritive media, budding occurs normally and independently on changes of incubation temperatures (a temporary decrease) (Plate I, Fig. 6);

— germination and budding was observed to occur simultaneously in the same adiaspore at 37 °C (Plate II, Fig. 1), throwing some doubt on the hypothesis of a dominant thermodependence of the conversion of the phases of B. crescens;

— the presence of elements of the mycelial phase of the fungus could not be observed even in experimentally infected hibernated mice (Sekecký et al. 1971). Post-mortem examination of experimentally infected mice kept for 48 hrs in the refrigerator, disclosed germinating forms of adiaspores (Kodousek et Hejtmánek 1971). This suggests that certain processes typical of the viable macroorganism, are factors inhibiting the phenomenon of germination and that also under conditions which, theoretically, appear to be suitable for the occurrence of this phenomenon.

It has been impossible to estimate the degree of dependence of the process of exophytic growth on the temperature of the environment or on other factors as well as the amount of energy supplied by the element of the parasite or by tissue activity of the macroorganism to this process.

The formation of a submembraneous reticulum appears to depend on certain components of the sheath. This consists of a system of chambers bordered by a basophilic reticulum and filled with fat droplets (Plate II, Fig. 2). The course of the fibres of the reticulum is accompanied by basophilic granules of doubtful function. Towards the

*) The Plates I—IV will be found at the end of this issue.
centre of the adiaspore, this regular arrangement is disturbed, because the minute fat droplets merge and form larger droplets until, in the later stages, they occupy most of the inner part of the adiaspore compressing even the submembranous reticulum. This adiaspore if inspected in an unfrozen section not stained for fat, gives the impression of being empty.

No description has been available of the endospores inside the adiaspore of *E. crescens*. In our material we observed an occasional minute formation (2—5 μm in diameter) (Plate II, Fig. 3) sometimes accompanied by a larger formation which, judging from its size, may be its more advanced stage. The endospores are generally arranged in chains interconnected with plasmic bridges; the complete formation gives the impression of a fragmented pseudomycelium. The origin of this formation may be associated with the germination of the original element; it seems more probable, however, that they originate from the developing submembranous reticulum during a certain developmental stage. The endospores or batches of endospores have no special arrangement and are dispersed throughout the content of the adiaspore and take on the function of a sporangium. We observed similar formations in the content of elements cultivated in vitro (Plate II, Fig. 4). In view of the fact that endospores are encountered only occasionally in smears of fresh adiaspores, it is difficult to perform the germination test. We were fortunate enough to perform this test and to confirm the reproductive character of the endospore (Plate II, Fig. 5). The microphotograph shows a disintegrated membrane (due to prolonged cultivation in a medium without nutritives at 25 °C) containing a group of endospores at a more advanced stage of development, which germinated multipolar. In our opinion, however, exosporelation (budding) appears to be quantitatively more important than endosporelation.

The internal bodies were described in detail by Šlais et al. (1970). We should like to emphasize only that a daughter adiaspore may originate inside the mother adiaspore from an occasional internal body (Plate II, Fig. 6). We consider this phenomenon to be endosporelation sensu lato, because it seems most unlikely that any of the mentioned endospores sensu stricto should be capable of developing into an adiaspore of typical structure. The origin and importance of the remaining types of internal bodies remains obscure.

The semilunar forms of adiaspores observed in adiaspiromycotic lesions (Plate III, Fig. 4) have mostly been considered to be artifacts. We observed in fresh smear preparations that this semilunar shape occurs only in daughter adiaspores which originated from budding and only in connection with changes of environmental humidity, while the mother adiaspores do not change morphologically under these conditions. (Plate III, Fig. 1.) In view of the fact that these factors have no noticeable influence on the parasitic stages, the formation of semilunar forms appears to be a reaction of the young adiaspore to unfavourable conditions of the external environment. This suggests that the presence of semilunar forms in the tissue may be an indirect proof of the budding of the adiaspore in vivo.

Attention should be given to an element occurring closely before the commencement of the parasitic phase. Its structure is similar to that of the brightly staining activated content of an adiaspore, which contains granular, filamentous and reticular structures, but has no morphologically confirmable membrane (Plate III, Fig. 5). It appears to be an analogue of the uncapsulated formations observed during the cultivation of the fungus in several liquid media at 37 °C under the paraffin layer (Oťenášek 1969). In view of the marked antigenic properties of the spherule sheath of several other fungi (and evidently also that of the adiaspore of *E. crescens*) it appears unlikely that the humoral mechanism of the host would attack an uncapsulated organism. It is of interest that uncapsulated formations of the parasitic phase were observed also in the culture of adiaspores isolated from infected organs (Plate III, Figs. 2, 3; Plate IV, Fig. 2); their
presence, however, could not be confirmed in standard culture of aleuriospores and mycelial fragments.

Evidently, there is variation in the mode of destruction of the mother adiaspore. Its lysis and replacement by connective tissue should, under certain conditions, have a sterilizing effect, but may favour also the dissemination of the parasite's cells. This occurs, if elements capable of reproduction, are present in the content of the adiaspore at the time of rupture of the membrane. Upon release, these elements gain more space for further development.

The number of pyriform adiaspores in the tissue is remarkably high. According to the original concept, the pear-shaped prominence may represent the locus minoris resistentiae originating at the site of attachment of the aleuriospore to the aleuriophore. It may possibly be the site which is attacked by the defence mechanisms of the host, or in which the prolate of the inner content of the adiaspore occurs. In each case, this leads to the formation of a pore (Plate IV, Fig. 4) through which the macrophages and fibroblasts enter the original adiaspore. Its content and, later, its membrane are resorbed and a connective tissue scar is formed. Sometimes, reproductive particles escape from the inside of the dying adiaspore along the same route.

Basing mainly on our experiences we are presenting our concept on the developmental cycle of the adiaspore of E. crescents (Fig. 1). We are aware that the scheme is far from being complete and it may even be incorrect in some details. It appeared, however, important to revise several older and incorrect ideas on the character of the fungus and of the disease caused by it.

Under natural conditions of a primoinfection of the host, the adiaspore originates generally from the aleuriospore, the intercalar or terminal chlamydsopore. Thereby, it is evident that not every adiaspore originates from these formation (endosporulation, exosporulation, germination etc.). In addition, it is theoretically possible, that adiaspironmycosis is a "terminal type" infection. It has been confirmed in experiments that the viable elements of the fungus can be disseminated along circulation routes; this depends on the strength of the barriers encountered (Hamáček et al. 1970 c-f; Hamáček et al. 1971 c). By contrast, it seems that an aleuriospore or chlamydsopore produces a single adiaspore only.

The later fate of a parasitizing adiaspore may show considerable variation. Metabolic activity and its degenerative character is responsible for larger or lesser concentrations of lipid substances. It is uncertain as yet, whether this process is the result of the the-sauring function of the adiaspore or, whether its life cycle is interrupted by it at a certain stage.

Under different circumstances, the adiaspore loses its morphologically confirmable membrane. We assume that, during later development,
— the membrane may be renewed;
— it may be resorbed and replaced by connective tissue;
— at a certain stage occurring simultaneously with the resorption, particles capable of reproduction may be released and, at the same time, a nest of secondary adiaspores may originate in a single granuloma.

By the rupture of the membrane of the adiaspore, elements of endosporic nature may be transported from the inside of the adiaspore to different sites and develop there. It has also been confirmed that the nipple-like prominences of the membrane are, in fact, the budding of the adiaspore and this results in the formation of daughter cells.

After having obtained definitive experimental confirmation of the reproduction of adiaspores in vivo (Hamáček et Semecký 1971) we are fully consistent with the nomenclatoric changes suggested by Hejtmánek et Kodoušek (1971). In view of recent
Fig. 1. Schematic illustration of the morphological forms of the cells of *Emmonsia crescens*.  
-- = confirmed transformation, --- = hypothetical transformation.
knowledge, the term “adiaspore” used for the incomplete formation of the parasitic phase, should be replaced by the term “trophocyte” (a thin-walled formation of 6—20 μm in diameter). The mature element should be called “spherule” as long as it does not contain reproductive particles. A cell containing endospores sensu lato is nothing else but a “sporangium”. Exosporation is effectuated by budding and, hence, there is no need for a special name for a budding spherule. The disease itself, i. e., the infection of the macroorganism with cells of a fungus of the genus Emmonsia should be called “emmonsiosis” (Dvořák 1970).

In spite of the fact that the present hypotheses on the pathogenesis of adiaspiromycosis needs to be revised, it is evident from experimental results that only a weakened organism is susceptible to spontaneous infection (Hamáček et al. 1971 d-f). In spite of considerable research work on adiaspiromycosis (emmonsiosis) there remain numerous problems which will have to be solved.

Acknowledgements. The authors wish to express their gratitude for perfect technical assistance to Mrs. M. Navrátilová.

МОРФОЛОГИЧЕСКОЕ ПРОЯВЛЕНИЕ АДИАСПОР ГРИБКА
EMMONSIA CRESCENS IN VITRO И IN VIVO

Ф. Гамачек, Я. Дворжак, М. Отченашек и В. Семечки

Резюме. Результаты изучения морфологического проявления адиспор грибка Emmonsia crescens обнаружили разные способы размножения in vitro и in vivo. Данные описания соединения клеток подтверждает наблюдаемой параситической стадии грибка. Авторами представлена концепция жизненного цикла адиспор, причем подчеркнута необходимость пересмотра разных взглядов относительно патогенеза адиспоромикоза и перепонки его таксономического положения.

REFERENCES


THE „PARASITOLOGICAL DAYS“ IN BRATISLAVA

The „Parasitological Days“ in Bratislava were held from September 16 to 17, 1971. This meeting was organized by the Czechoslovak Parasitological Society in collaboration with the Slovak Academy of Sciences and the Medical School, Komenský University, Bratislava. The 69 contributions delivered at this meeting were concerned with human parasitology.

The problems discussed at the meeting can be divided into several thematic groups. Group 1 concerned papers on the occurrence, diagnosis and serology of protozoan infection of man (particularly toxoplasmosis, amoebiasis, lambliasis), including several theoretical problems such as the antigenic analysis of parasite antigens, culture methods and therapeutic experiences.

Group 2 included papers on helminthiasis of man, particularly on the incidence of strongyloidiasis, enterobiasis and taeniarhynchosis in Czechoslovakia, and the treatment of these infections.

Papers on arachnoentomology dealt mainly with questions on the incidence and diagnosis of scabies, a current infection in recent years. Several papers discussed the influence of certain insects on arthropods and helminths.

Problems of the diagnosis and therapy of tropical and subtropical parasites were another subject of discussion among parasitologists who had encountered these in persons returning from these countries.

The „Parasitological Days“ in Bratislava enabled an interesting confrontation of the results obtained by the various institutions of Czechoslovakia. They offered new stimulating lines of research upon which the future work of the participants could be conducted.

Dr. Ž. Černá CSc.
Fig. 1. Adiaspores with an uniformly thick membrane. Subcutaneous granuloma in a mouse on day 21 p.i. Stained with the modified Grocott-Gomori method (x 120).

Fig. 2. Adiaspores with an uniformly thick membrane cultivated in vitro. Sabouraud’s dextrose agar, age of culture 21 days. HE (x 120).

Fig. 3. Adiaspore with nipple-like promincences. Intraperitoneal granuloma in a mouse on day 21 p.i. HE (x 120).

Fig. 4. Adiaspore with nipple-like promincences cultivated in vitro. Sabouraud’s dextrose agar; on day 21 of cultivation. Fresh preparation (x 270).

Fig. 5. Exosporulation caused by the release of the nipple-like promincences in the membrane of the adiaspore from a mouse lung. Intrapulmonary infection, on day 21 p.i. Stained with the modified Grocott-Gomori method (x 120).

Fig. 6. Budding adiaspore. Cultivation on Sabouraud’s dextrose agar at 37 °C. Age of culture 28 days. Fresh preparation (x 600).
Fig. 1. Concomitant budding and germination of adiaspores on Sabouraud's dextrose agar at 37 °C. Age of culture 32 days. Fresh preparation. (× 120).

Fig. 2. Subtangential section through submembranous reticulum showing the structure of the sheath. Intraperitoneal granuloma of a mouse, 28 days p.i. HE (× 270).

Fig. 3. Endospores inside the adiaspore. Subcutaneous granuloma of a mouse; on day 21 p.i. HE (× 270).

Fig. 4. Endospores inside the adiaspore cultivated on Sabouraud's dextrose agar at 37 °C for 32 days. Fresh preparation (× 600).

Fig. 5. Endospore germination test. Original adiaspore with desintegrated membrane after 12 days of cultivation on blood agar at 25 °C. Fresh preparation. (× 600).

Fig. 6. Adiaspore inside adiaspore. Subcutaneous granuloma from a mouse on day 35 p.i. HE (× 270).
Fig. 1. Semilunar forms cultured on Sabouraud's dextrose agar at 37 °C for 21 days. Fresh preparation (×600).

Figs. 2, 3. Uncapsulated forms of *E. crescens* cells cultured on Sabouraud's dextrose agar at 37 °C for 21 days. Fresh preparation (×120).

Fig. 4. Bottom right — semilunar form of an adiaspore with, apparently, a budding adiaspore in the centre. Mouse lungs, day 35 p.i. Stained with the modified Grocott-Gomori method (×120).

Fig. 5. Uncapsulated form of *E. crescens* cell in the intraperitoneal granuloma of a mouse on day 28 p.i. HE (×270).
Fig. 1. Star-like extensions of the adiasporic membrane pointing towards another adiaspore in the culture. Sabouraud's dextrose agar; duration of cultivation 12 days; temperature 37 °C. Fresh preparation (× 600).

Fig. 2. Germinating unencapsulated form of E. crescens. Originally cultivated for 16 days on Sabouraud's dextrose agar at 37 °C then at 25 °C for 48 hrs. Fresh preparation. (× 120).

Fig. 3. Junction of adiaspores in a granuloma from a mouse. Intraperitoneal granuloma on day 35 p.i. Trichrome.

Fig. 4. Proliferation of granulation tissue through the pore in the sheath into the inside of the adiaspore. Mouse lungs, 28 days p.i. HE (× 270).

Fig. 5. Junction of adiaspores with the help of star-like bridges. Cultivation on Sabouraud's dextrose agar at 37 °C for 35 days. Fresh preparation (× 120). Focused upon the bridges.