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Latency and herpes simplex virus

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& Anamaris M. Colberg-Poley**

Introduction

Members of the herpesvirus group characteristically produce diseases with recurrent manifestations. Among these are varicella-zoster virus, Epstein-Barr virus, cytomegalovirus, and herpes simplex virus types 1 and 2 (HSV-1 and HSV-2). In an attempt to explain clinically recurrent herpetic lesions, the maintenance of a latent form of virus within the body was proposed (38, 92). Alternative explanations for causation of these clinically observed recurrences, not covered within the scope of the review, are persistent infection and exogenous reinfection (61).

This article reviews some of the literature pertaining to HSV latency but does not intend to be comprehensive for the vast number of publications within this field precludes such an attempt. Our intention is to present a clear summary of the pertinent information concerning HSV latency for readers not familiar with the work in this field. Citations already presented in other extensive reviews (18, 61, 93) have been included for the sake of clarity and continuity of this overview.

Table 1 — Recent reviews concerning HSV latency

Subject	References
Historical	Stevens, 1975 Stevens, 1978
Pathogenesis of recurrent lesions	Wheeler, 1975 Epstein, 1976
Comprehensive	Nahmias and Roizman, 1973 Docherty and Chopan, 1974 Roizman, 1974

HSV has been used as the prototype of herpesvirus latency. Yet, the concept of a latent virus, undetectable by standard isolation techniques and clinically inapparent, contrasts strongly with the usual rapidity and destructiveness of the HSV replicative cycle. These apparently conflicting observations (latency vs. lytic replication) could be reconciled if latency is viewed as an evolutionary mechanism enabling virus survival within the host's immune system (31). Productive replication of the virus with a concomitant increase in virus antigen expression early during the replication cycle could lead to the destruction of the infected cell by the host's immune system. Destruction of the infected cell prior to completion of virus replication would result in elimination of the virus within the body, thus excluding any opportunity for virus spread. One alternative would be blockage of virus replication prior to the synthesis of virus products expressed at the cell surface and maintenance of the virus in an inactive form within the cell until favorable conditions for replication are established. Subsequent replication of the virus under more favorable conditions (such as reduction in host immunity or upon hormonal stimulation) would permit survival and propagation of the virus. Furthermore, the ability of the virus to enter a latent state may correlate with its potential for malignant transformation of cells (79). Lytic replication, on the other hand, would destroy the transformed cell and preclude the development of cancer. It is therefore possible that latency, by blockage of lytic replication, could permit the expression of transforming ability.

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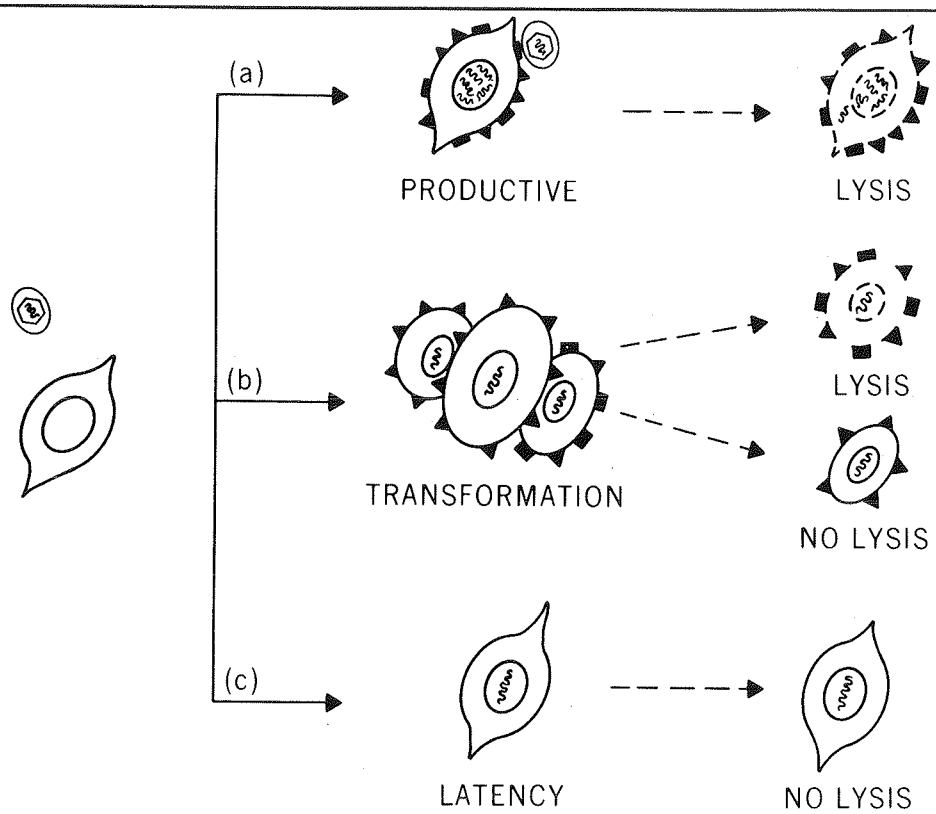
Figure 1 — Cellular-virus interactions following infection with herpes simplex virus



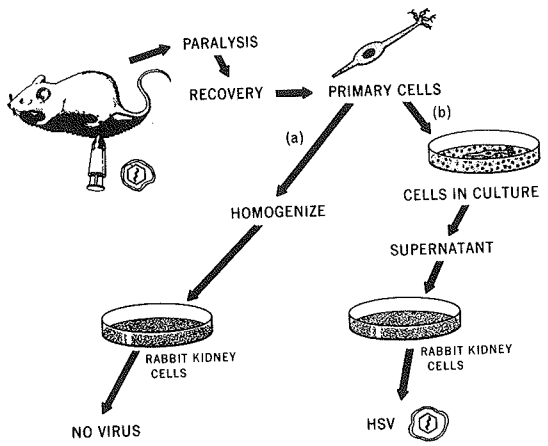
a. productive infection with concomitant increase of virus-specified proteins (■ and ▲) on cell surface; these may be recognized as foreign (■) by the host's immune system and result in lysis of the infected cell
b. Transformation may result after infection with HSV and the transformed cells may express virus antigens on its surface. This may lead to lysis (■) or not (▲), probably depending on the proteins present and the response of the host
c. Latency may represent a state in which virus proteins are not expressed on the cell surface but the virus genome is maintained; the infected cell escapes lysis and the survival of virus is possible

Evidence that HSV causes cancer — Strong evidence implicating HSV in cell transformation and cancer has developed from varied techniques utilized in numerous laboratories. Initial studies demonstrated the transforming potential of HSV by transformation of hamster embryo fibroblasts *in vitro* with ultraviolet (UV) — irradiated HSV (26, 27, 71, 72). Later, the presence of HSV DNA (34) and HSV antigens (75) in these cells was established. Alternately, the expression of HSV in neoplastic tissue has been demonstrated by immunofluorescent studies of exfoliated cells from a cervical carcinoma (81) and portions of HSV-2 DNA have also been found in cervical cancer tissue (35). More recent studies have examined biopsies from cervical carcinoma and premalignant tissue for the presence of HSV-2 mRNA using *in situ* hybridization. These studies have detected HSV-2 mRNA associated with the neoplasctic tissues (5). *In situ* hybridization has permitted even greater sensitivity for the detection of HSV sequences than previous techniques. It is perhaps for this reason that direct evidence establishing the presence of the HSV genome or portions of it within neoplastic tissue has been slow in emerging. Evidence for HSV involvement in cervical cancer and transformation has stemmed from studies using different techniques. HSV-2 has been induced from cervical carcinoma tissue by altering the pH of the culture medium in which the cells were grown *in vitro* (5). A correlation between the incidence of cervical cancer and genital herpes lesions has been noted (62) and strengthened by numerous epidemiological studies (3, 4, 60, 74, 82). These have been previously reviewed (61). The ubiquitous nature of HSV forces one to ask whether the association of HSV with cervical carcinoma represents, in effect, the causation of the malignancy or whether the association of virus with this cancer represents a superinfection of neo-

plastic tissue. This question raises an issue vital to the determination of cervical cancer etiology that has yet to be conclusively resolved.

The latent HSV — The historical development of the association between HSV latency, the nervous system, and recurrent lesions has been extensively reviewed (92, 93). Briefly, after initial observations that herpetic lesions were concurrent with inflammatory changes in the corresponding sensory ganglia (47, 48), it was noted that lesions developed after removal of contralateral ganglia (20). It soon became apparent that the lesions were infectious since it was possible to transmit them to rabbits (55). Ultimately, Goodpasture proposed that the herpesvirus remained latent in ganglionic neurons and reactivation would occur if these tissues were damaged (38). Direct evidence supporting Goodpasture's hypothesis was delayed for several decades until technology permitted the study and isolation of viruses from explanted tissues of animals and humans afflicted with herpetic lesions.

Figure 2 — Protocol for the isolation of HSV from the neuronal tissue of latently infected mice. The mice are injected in the footpad with HSV, and a paralysis ensues. After 2-3 weeks when recovery is complete, the spinal ganglia are excised and are maintained as organ cultures



a. Neither HSV nor virus-specified products are detectable at the time of excision.
b. The supernatant from these neurons, maintained in culture, is placed on permissive cultures (rabbit kidney cells) and production of virus is observed

HSV was isolated from the spinal ganglia of mice that recovered from paralysis induced by inoculation of HSV in the rear footpad (94). Further studies permitted the development of additional animal HSV latency models: rabbits inoculated in the cornea (97), mice inoculated in the ear (10, 46), and guinea pigs inoculated in the rear footpad or via the intravaginal route (87,89).

More recently, genetic and biochemical studies have been facilitated by the incorporation of HSV temperature-sensitive (ts) mutants into the mouse latency model. Several HSV ts mutants studied vary in their abilities to induce latency *in vivo* (54). Various HSV-1 ts mutants (Glasglow), which had been extensively characterized were selected and used to inoculate mice either intracerebrally or in the footpads. After the virus was undetectable, portions of the brain or spinal ganglia were explanted and observed for the reappearance of virus by cocultivation with baby hamster kidney (BHK) cells. It was found that of five ts mutants studied, tsD readily established latent infections, while tsG, tsS, tsJ were less efficient, and tsI was not recoverable. The authors suggest that tsI was not recoverable from neural tissue because of its inability to establish latent infection in the mice due to a defect in a virus function(s). This work has been extended to include other ts mutants, but thus far, the authors have not been able to establish a correlation between the inability of some ts mutants to establish latent infections in mice and defects within specific regions of the HSV genome.

These animal models have been established to permit studies on HSV latency not possible by experimentation with humans. Indeed, certain clinical observations have been reproduced using some of these animal models. Similar to human infections, spontaneous recurrences occur in guinea pigs (87, 88) rabbits (63), mice treated with prednisone (99), and mice inoculated in the ear (46). Nevertheless, this has not been the case in all systems studied. Mice inoculated in the footpads do not have spontaneous recurrences (95), and unfortunately, when recurrences do occur in experimental animals there is neuronal destruction. This destruction does not permit multiple recurrences commonly associated with humans afflicted with herpetic lesions (93). Both neuronal destruction and the lack of spontaneous recurrences in some animal models present direct conflicts with the human disease state these models attempt to simulate.

In the absence of apparent infection, it was proposed that the virus resided in the neuronal

tissue in a latent state. This was substantiated by the difficulty in establishing the presence of the virus in neural tissue. The presence of the virus was determined only after *in vitro* cultivation of explanted neural tissue for various periods after explantation. Neither virus nor virus-specific products could be detected, either by electron microscopy or immunofluorescence, at the time of explantation (93, 94). One noted exception is that virions were observed by electron microscopy in the trigeminal ganglia of a latently infected rabbit (8). However, spontaneous recurrences do occur in rabbits and the presence of virus detected in these studies may represent such an event.

Table 2 — State of HSV during latency

State	Presence[1]	Evidence	References
Active replication			
Virus particles	0	Electron microscopy	Stevens & Cook, 1971
	+	Electron microscopy	Baringer & Swoveland, 1974
Virus-specified proteins	0	Immunofluorescence	Stevens & Cook, 1971
Presence of Virus Genome			
Virus DNA	+	Hybridization	Cook & col., 1974 Puga & col., 1978
Expression of the Virus Genome			
Virus RNA	0	Hybridization	Puga & col., 1978
Thymidine Kinase	+	Enzyme assay	Yamamoto & col., 1977

1 + denotes the presence and 0 denotes the absence of HSV, its genome or its products as determined by each assay

Initial HSV latency studies utilized techniques that were relatively insensitive compared to more recent methodologies. Electron microscopy and immunofluorescence tests did not detect the presence of HSV virions and HSV antigens. More recent studies, however, have detected a herpesvirus-specified enzyme, thymidine kinase (TK) (22, 59, 64) in dorsal root ganglia of latently infected mice (107) and suggest that the HSV genome may be partially expressed. Hybridization studies have attempted to determine whether HSV is present in a replicating form during latency and have also examined the level of HSV-specific mRNA present in neural tissues of latently infected BALB/c mice. The presence of HSV-specific DNA in the trigeminal ganglia of acutely and latently infected mice was determined by this technique (70). Virus DNA sequences were detected in neurons during both the acute and latent (chronic) state, although an approximate 10-fold reduction in genome equivalents per cell was observed. In contrast, virus mRNA was detectable during the acute stage (0.1 to 0.2 RNA equivalents per cell) while the amount present during latent infection was below detection levels (70). The lack of detectable amounts of HSV mRNA during latent infection contrasts strongly with the presence of the HSV-specified TK found in the ganglia of latently infected mice (107). Nevertheless, it must be emphasized that this type of hybridization detect the average amount of mRNA in the total number of cells present. It is possible that transcription of the virus genome could occur in a small portion of the cells present in the tissue without being detected by this technique. Therefore, active transcription and translation of virus-specific sequences can not be excluded.

Latent HSV in humans — The major line of evidence supporting the presence of HSV in human neural tissue lies in the isolation of the virus from trigeminal ganglia (9, 77) and sacral ganglia (6, 7) obtained from human cadavers. The reactivation of HSV in humans occurs as a result of varied chemical and physical stimuli. Fever (14, 100), menstruation (85), excessive cold or sunlight (32), pesticides (98), exposure to UV light (78), and nerve root section (11, 12, 29) have been associated with recurrences (103). Stress of either a physiological (50) or emotional nature also induces virus reactivation. Most recently, reactivation of HSV has been found to occur as a result of decompression of the trigeminal nerve root (69). This new treatment for patients with trigeminal neuralgia resulted in the reactivation of HSV in a high percentage of patients.

The mechanisms involved in regulation of virus replication regulation in the body, virus disappearance, and subsequent reappearance as a result of various stimuli, are still controversial. Many authors feel that the immune system regulates the appearance of virus in the body. Roles have been proposed for serum antibody (37, 96), cell-mediated responses (61, 106), and interferon (13, 52). Others feel that dual action of the immune system is required for proper control of HSV replication (24, 53). Evidence supporting this proposal comes from *in vitro* work (30) suggesting that neither branch of the immune system is capable of limiting HSV replication alone but synergistic action of both cellular and humoral branches is required to control HSV replication. Yet not all the reactivations observed in humans seem to imply regulation by the immune response of the individual. Numerous stimuli, such as menstruation and pregnancy, seem to point to alterations in hormone activity. This has led to the suggestion that the control of virus reactivation may be influenced by hormone secretion, specifically prostaglandin E_2. Evidence sustaining this model includes the release of prostaglandins after exposure to UV radiation, and the presence of prostaglandin E_2 in mestrual fluid. This type of hormonal regulation may, in addition, reconcile otherwise inexplicable observations. Some recurrences in animal models have been found to result from the action of stimuli upon the skin and have led to the proposed "skin trigger" hypothesis (44). Recurrences have been induced by trauma as a result of repeated applications of cellophane tape (45) and plucking hair from the original site of infection (49). These stimuli do not alter the nervous system or immune status of the host, yet they reactivate the virus. Proponents of the skin trigger hypothesis attribute these recurrences to the action of prostaglandin E_2. Convincing experimental observations linking the release of prostaglandin E_2 with HSV recurrences have not been demonstrated. Experiments in which mice were injected with prostaglandin E_2 in saline or with saline alone showed recurrences in both groups at comparable levels (10).

In vitro models of HSV latency — The cell-virus interactions which occur during latency and reactivation have not been extensively studied due to lack of appropriate *in vitro* models. Indeed, it seems that determination of these interactions and understanding of latency will require the use of *in vitro* models. Of course *in vitro* models, due to their oversimplified nature, do not permit the study of the complex array of factors that may be involved in HSV latency in humans. In addition, factors that may influence virus replication *in vitro* may not be of any consequence *in vivo*. Nevertheless, the attractiveness of *in vitro* models lies precisely in their simplicity. *In vitro* models can be manipulated easily, analyzed biochemically, and relatively simple conditions can be studied without the interplay of multiple factors.

In vitro HSV latency and persistence models commonly attempt to inhibit the lytic replication of HSV using conditions that are the least cytotoxic or detrimental to cell metabolism, i.e. HSV-specific antisera, cells that are semipermissive or nonpermissive for HSV replication, alteration of incubation temperature, and inhibitors of DNA synthesis.

By the early 1960s, it had been demonstrated that the addition of HSV-specific serum to the growth medium of HSV-infected HeLa cells produced a persistent infection (33, 102, 104). Virus persisted in the presence of antiserum (as determined by the formation of foci of infected cells), but spread of the infection resulting in total cell culture destruction was inhibited by the antiherpetic serum (33). Upon removal of the serum, foci became larger and the cell sheets were destroyed.

The lytic nature of HSV was discovered early by the extensive cytopathology observed in rabbit corneal cells (86) and HeLa cells infected with HSV (80). Later, it was demonstrated that certain cells were semipermissive for HSV replication. Following infection with HSV, Chinese hamster cells demonstrated persistent infection with cycles of cell destruction and regrowth (42). The mechanism of this persistent infection is thought to be dependent upon genetically determined cell-virus equilibrium (41). In addition, some neuronal cell lines are semipermissive for HSV replication. Persistent infection of a rat glioma (C143) cell line, characterized as having various neuronal properties, was possible upon selection of surviving cells after two successive infections with HSV-1. Three clones thus isolated lost the ability to produce HSV-1 after passage either in the presence or absence of antiherpetic serum and the expression of virus-specific antigens and structures persisted even in the absence of infectious virus (25). Another such cell line, a clone of rat glial primary (B103) cells, expressed HSV-1 thymidine

kinase, DNA polymerase, and some structural proteins although there was no detectable virus DNA synthesis (1). It appears that these cells do not permit virus replication as in other lytic systems. Stevens and his co-workers are attempting to utilize these neuronal cells in culture to establish an *in vitro* HSV latency model and are presently studying various clones of mouse C-1300 neuroblastoma cells infected with either wild-type HSV or ts mutants (93) to determine which virus products are required for establishment of latency.

Inhibition of HSV replication in permissive cells by alterations in incubation temperature necessitated drastic increases to 42°C (21) or decreases to 25°C (101, 105). Such drastic changes in temperature proved not only inhibitory to virus replication but also detrimental to the host cells. When corneal cell cultures were infected with HSV, (65), a marked inhibition of virus destruction of the culture was observed at 40°C compared to 30 or 36°C.

Inhibition of HSV replication, which appears naturally in some clones of neuronal cell lines and other semipermissive cells, can be experimentally and reproducibly induced by adding DNA synthesis inhibitors to lytically infected permissive cells. HSV replication has been found to be sensitive to a wide variety of nucleoside analogs. DNA synthesis inhibitors such as 1-β-arabinofuranosylcytosine (ara-C) (76), 5-iodo-2'-deoxyuridine (IUdR), and 5-trifluoromethyl-2-deoxyuridine (39) not only inhibit HSV replication but also inhibit cell replication. Other compounds as 9-β-D arabinofuranosyladenine (ara-A) (83), arabinofuranosylthymine (ara-T) (36, 58), 5-propyl-2-deoxyuridine (15, 23), 9-(2-hydroxyethoxymethyl) guanine (28, 84), 5'amino 2',5'-dideoxy-5-iodouridine (AIU) (16) have diminished detrimental effects upon the host cells and may be useful for clinical treatment of herpetic lesions. The mechanisms by which many of these drugs inhibit HSV replication have not been well established; yet information pertinent to the inhibition of virus replication has been obtained. Ara-C and ara-A inhibit the replication of DNA and RNA viruses which require a DNA intermediate as well as cell growth. Enzymes involved in the formation of the triphosphate forms of the nucleotides have been ruled out as the primary inhibitors *in vivo* (19). The triphosphorylated form ara-C is incorporated into DNA *in vivo* (17, 40, 91) but does not serve as a chain terminator (56). Ara-CTP and ara-ATP competitively inhibit the utilization of corresponding deoxyribonucleotide triphosphate in the DNA polymerase reaction (19, 73) and ara-T is phosphorylated by the viral pyrimidine kinase present in HSV-infected cells (2) to its active form which blocks DNA synthesis. In addition, 9-(2-hydroxyethoxymethyl) guanine is phosphorylated by virus-specified TK (28). It is this form that inhibits virus DNA polymerase activity and serves as a chain terminator upon incorporation into DNA.

Recently, O'Neill and co-workers (67) utilized the inhibition of HSV replication produced by ara-C to produce an HSV latency model. When HSV-infected human embryo cells were treated with ara-C, HSV replication was blocked and infectious virus was undetectable by plaque formation in permissive cells. The authors not only demonstrated the absence of detectable amounts of infectious virus in the presence of 25μg of ara-C/ml but also after the drug's removal from the culture medium. The reappearance of infectious virus occured only 5 to 11 days after reversal of drug treatment. Further-

Table 3 — Establishment of an *in vitro* model of latency by treatment with ara-C in presence and absence of alteration of incubation temperature

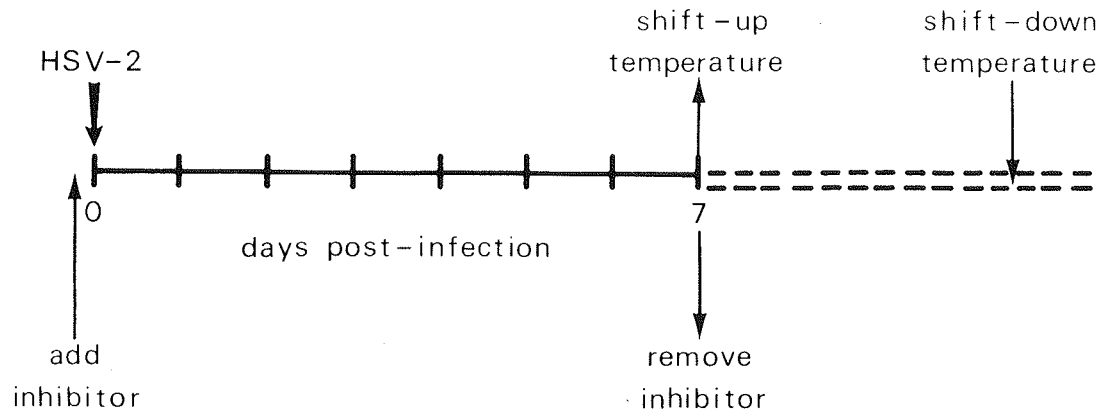
Conditions[1] and Incubation temperature (°C)	Presence of infectious virus[2]			
	2-7d[3]	7-9d	9-30d	>30d
Maintain at 37°	0	0	+	+
Shift from 37° to 40°	0	0	0	0
Shift from 37° to 40° followed by shift down to 37°	0	0	0	+

[1] Cultures were treated as schematically represented in Figure 3. In all cases, infected cells were treated daily with ara-C (25μg/ml) from 0 to 7 days after infection

[2] As detected by plaque formation in primary rabbit kidney cells, + denotes presence of infectious virus and 0 denotes undetectable levels of infectious virus

[3] Time after infection in days

Figure 3 — Protocol for the establishment of an *in vitro* model of HSV latency using ara-C. Confluent monolayers of human cells were pretreated with ara-C (25μg/ml), infected with HSV-2 at a low multiplicity of infection (0.025 to 0.1 plaque forming units/cell) and medium containing ara-C was replaced daily for seven days after infection. On the seventh day, cultures were washed twice with buffer and either maintained at 37°C or shifted to 40°C. At various intervals after shift to 40°C, cultures were once again shifted to 37°C. At various times after infection, cultures were harvested and titered by plaque formation in primary rabbit kidney cells. The results of these assays are presented in Table 3



more, this "latent" period has been extended up to 128 days post-infection by elevating the incubation temperature from 39.5 to 40°C at the time of reversal (66). It is also noteworthy that the concentration of ara-C (25μg/ml) utilized in this system proved to have only a mild effect upon confluent monolayers of human cells. This sytem permits the manipulation of virus replication such that the mechanism of its disappearance and reappearance may be studied.

These studies have been extended by use of another inhibitor of herpesvirus replication, phosphonoacetic acid (PAA) (68, 90). Low concentrations of PAA (20 to 40μg/ml) affect herpesvirus replication by inhibiting virus DNA polymerase (57) while not affecting host cellular function. A recent addition to this family of analogs, phosphonoformate (43) has stirred hope for clinical treatment of herpetic lesions because of its apparently low toxicity, high antiherpetic activity, and its therapeutic effect on herpetic lesions of guinea pigs.

Increased PAA concentrations have been used in this system to reproduce and extend the ara-C model. When PAA is added to HSV-infected human embryo cells, the reappearance of infectious virus is inhibited for a lengthened period of time after the removal of PAA. Similar to treatment with ara-C, increasing the incubation temperature to 40°C at the time of PAA removal also produced a period where infectious virus was undetectable.

In summary, a system involving HSV-infected human cells has been established where virus production is blocked early during replication and the effects of such a block persist after its removal. This model has produced a system where HSV, a normally lytic virus, does not reappear for extended periods of time but upon proper stimulation, such as shifts in temperature, virus reappearance is rapid and comparable to untreated controls.

Conclusions

What does the future hold? Perhaps the discovery of one of the most fascinating aspects of herpes virology, the mechanism of establishment and maintenance of latency. Our present understanding of HSV latency is quite limited. Indeed, the form in which the virus is maintained within the neuron during latency and the mechanism by which latency is established remain to be clarified. In theory, latency may represent a state induced by a virus-specified protein(s) or alternately, latency may result from the lack of some essential function for virus replication. Perhaps, latency represents a virus-cellular interaction induced by the unique metabolism of neurons which could signal the expression of a virus function necessary for latency or blockage of virus replication. As previously suggested, latency may represent an evolutionary adaptation enabling survival of a parasite under harsh conditions. The whole mechanism of latency remains to be explored. Further understanding of the regulation of virus replication will probably necessitate the use of *in*

vitro models which simplify the examination of the numerous factors involved in HSV latency. The resolution of this intriguing phenomenon would not only be academically satisfying but could open new insights into the regulation of virus replication and expression. This may, in turn, lead to alternate routes for treatment and prevention of recurrent herpetic lesions, and possibly even lead to better methods of cancer chemotherapy.

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Effect of mono and divalent cations on growth and sexual reproduction of Ceratocystis fimbriata, with emphasis on Ca^{+2} and Mg^{+2}*

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Summary

Ca^{+2} and Mg^{+2} stimulated vegetative growth and sexual reproduction on several isolates of *Ceratocystis fimbriata*. Those effects depended upon the particular isolate, concentration of Ca^{+2} and Mg^{+2} and N concentration in the culture media. Isolates that responded favorably to Ca^{+2} were not always stimulated by Mg^{+2} and vice versa. Addition of Ca^{+2} and/or Mg^{+2} reduced perithecial production of certain isolates depending on the N level of the medium. Except for the lack of response to Ca^{+2} and Mg^{+2} in vegetative growth by the isolates obtained from stone fruits there were no well defined correlations between response to Ca^{+2} and Mg^{+2} and host and geographic source of the isolates.

Resumo

Efeito de cátions mono e divalentes sobre crescimento vegetativo e reprodução sexual de Ceratocystis fimbriata, com ênfase em Ca^{+2} e Mg^{+2}

Estudou-se o efeito de vários cátions sobre o crescimento vegetativo e reprodução sexual de várias estirpes do fungo fitopatogênico *Ceratocystis fimbriata*. Dos cátions testados apenas Ca^{+2} e Mg^{+2} tiveram efeitos estimulatórios sobre o fungo, dependendo da estirpe considerada e também da concentração dos cátions e conteúdo de N do meio de cultura. Conforme o nível de N do meio, alguns dos isolados de *C. fimbriata* não foram afetados, outros foram prejudicados, no que diz respeito a crescimento e reprodução sexual, pela presença de Ca^{+2} e Mg^{+2} nas concentrações testadas. A resposta a Ca^{+2} nem sempre correspondeu em qualidade ou quantidade à resposta a Mg^{+2} e vice-versa. Em geral, não houve correlação entre as respostas dos diferentes isolados, planta hospedeira e sua distribuição geográfica. Entretanto, Ca^{+2} e Mg^{+2} não afetaram o crescimento vegetativo de nenhuma das quatro estirpes provenientes de plantas do gênero *Prunus*.

Introduction

Numerous studies have been made on the effect of minerals on the growth and sporulation of fungi (14). Two cations, Ca^{+2} and Mg^{+2}, have received special attention because of their effects singly or in combination with other minerals. For example, Mann (16) found that 20mM Mg^{+2} was an absolute requirement for growth of *Aspergillus niger* V. Tieghen and *Penicillium* spp.. Growth of *A. niger* increased in the presence of 100-800mM Na^{+1}, concentrations normally toxic to most organisms, while concentrations of Mg^{+2} above 200mM were inhibitory to this fungus. When Ca^{+2} was added to the culture medium, the inhibitory effects of high concentrations of Mg^{+2} were not reversed, but the stimulation by Na^{+1} or Zn^{+2} was decreased. Brian & Hemming (3) found that *Trichoderma viride* Pers. ex Fries sporulated more abundantly with $NaNO_3$ as N source when $MgSO_4 \cdot 7\,H_2O$

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was present in the media. Ca^{+2} (1 to 10mM) stimulated sporulation of three strains of *T. viride*. Steinberg (18) observed significantly reduced growth in both *Rhizoctonia solani* Khuen and *Sclerotium rolfsii* Sacc. when Ca^{+2} was not present in the growth media. Sr^{+2} but not Ba^{+2} partially replaced the effect of Ca^{+2} on growth of *R. solani*. Steinberg (18) concluded that Ca^{+2} functions as a micronutrient and that *R. solani* requires between 0.05 to 0.15mM Ca^{+2} with 0.6mM Mg^{+2} for optimum growth. Basu (1) studied 3 strains of *Chaetomium globosum* Kunze and reported that 0.25mM to 2.5mM Ca^{+2} stimulated perithecial production of 2 strains while the third produced the maximum number of perithecia at concentrations ranging from 0.025 to 0.25mM. Concentrations of 25mM Ca^{+2} reduced perithecial production. Basu (1) did not test Mg^{+2} as a replacement for Ca^{+2}, but found that Ba^{+2} and Sr^{+2} partially substituted for Ca^{+2}. Since Mg^{+2} was required for growth in addition to Ca^{+2}, Mg^{+2} was considered and absolute requirement for growth while Ca^{+2} had only a stimulatory effect on perithecial production. He also found that 10mM Ca^{+2} stimulated perithecial production and growth in several other species of *Chaetomium*. Concentrations of 0.01 to 0.125 mM Ca^{+2} promoted increase in the size of perithecia and the number of fertile perithecia of *Chaetomium brasiliensis* (2) while at 25mM fewer scattered fruit bodies occurred. Ca^{+2} concentrations between 0.01 to 25mM did not stimulate growth (mycelial dry weight). The effect of 0.25mM Sr^{+2} and 0.25 mM Ba^{+2} on fruiting of this species was equal to that of 0.25mM Ca^{+2} · Mn^{+2}, Fe^{+2}, Sn^{+2} and Pb^{+2} did not affect growth or fruiting while Co^{+2} and Ni^{+2} inhibited growth and sexual reproduction. When 0.25mM Ca^{+2} was added to colonies after they had reached maximum vegetative growth, ascospore production but not final dry weight was increased. Thus Basu (1, 2) concluded that Ca^{+2} stimulated fruiting but not vegetative growth.

Simonart & Chow (17) studied the growth of *Aspergillus oryzae* Whemer in a medium with 3% casamino acids (Difco) as the N source. Addition of Ca^{+2} to the culture medium increased the content of free amino acids of the mycelium. They suggested that Ca^{+2} favors the formation of basic amino acids and also the decarboxylation of glutamic acid with the production of amino butyric acid.

Several studies have been reported on the the mineral nutrition of *Phytophthora* spp.. Lopatecki & Newton (15) found that from Fe^{+2}, Zn^{+2}, Mn^{+2}, Cu^{+2} and $Mo04^{-2}$, only $Mo04^{-2}$ was not essential for the growth of these fungi. Ca^{+2} was not mentioned.

Davis (8) showed that 0.5 to 1.25mM Ca^{+2} was an absolute requirement for the growth (mycelial dry weight) of three isolates of *P. fragariae* Hickman on synthetic medium. Sr^{+2} could partially replace Ca^{+2} for two of those isolates while Ba^{+2} was toxic to all of them. The response to Ca^{+2} was independent of the C and N sources. However, Erwin & Katznelson (10) observed that requirement for 2.5mM Ca^{+2} by *P. cryptogea* Pethyb. and Laff. (now considered a variety of *P. megasperma* (9)), *P. drechsleri* Tucker, *P. parasitica* and *P. boehmeriae* Sawada was not absolute. Chee & Newhook (7) did not observe increases in radial growth of *P. cinnamomi* Rands when Ca^{+2} was added to a basal medium consisting of: anyone of twenty-three different amino acids, minerals, thiamine, agar and cholesterol. Change of the N source did not affect the lack of response to Ca^{+2} by this fungus. Erwin (9) compared the responses of two species of *Phytophthora* to Ca^{+2}. A concentration of 0.068mM of Ca^{+2} or Sr^{+2} stimulated the growth of *P. megasperma* and *P. cinnamomi*. The results for *P. cinnamomi* are contrary to those obtained by Chee & Newhook (7), perhaps because Erwin (9) measured dry weight instead of colony diameter and his glassware was washed with 1% EDTA and rinsed with deionized water, instead of a simple detergent washing. Furthermore, Erwin (9) carefully purified all chemicals to prevent the introduction of Ca^{+2} into his control medium. Hodson (13) found that 18mM Ca^{+2} did not affect the growth of 2 races of *P. infestans* in a synthetic medium. Hendrix & Guttman (12) observed that 0.68mM Ca^{+2} and cholesterol could stimulate growth (mycelial dry weight) of *P. parasitica* var. *nicotianae* in liquid medium when $NaNO_3$ or asparagine were used as N sources. When the N source was $NaNO_3$, there was an absolute growth requirement for either 0.68mM Ca^{+2} or cholesterol.

Yang & Mitchell (20) reported that Ca^{+2}, Mg^{+2} and K^{+1}, all at 10mM stimulated the production of sporangia by *Pythium* spp., however, only 0.1 to 10.0mM Ca^{+2} could promote the formation of oogonia by the species tested.

Campbell (4) reported that perithecial formation of one isolate of *Ceratocystis fimbriata* Ell.

and Halst. was greatest on a medium containing a mixture of $Ca(NO_3)_2$ plus asparagine and considered that nitrate-N might be utilized in the presence of asparagine. Further investigation (5, 6) however, showed that this isolate could not utilize nitrate and the increase on perithecial production was due to a lower asparagine-N level when $Ca(NO_3)_2$ was included as part of the total N. With another isolate there was an increase in perithecial production due to the addition of Ca^{+2} to the medium which was further enhanced by a higher N level (3.0g/l of asparagine) in the growth medium.

The present investigation was conceived to expand the knowledge of the effect of Ca^{+2} and other cations on sexual reproduction of *C. fimbriata* and its relationship to the asparagine content of the medium. Since previous studies did not consider the effect of Ca^{+2} on vegetative growth of *C. fimbriata*, this was also studied.

Material and Methods

Isolates As-1, Pe-4, and I-4 of *C. fimbriata* were included in all experiments. To study the effect of Ca^{+2} and Mg^{+2} on the species *C. fimbriata*, additional isolates were tested. Host and geographic origin of all isolates is presented in Table 1.

Table 1 — Designation and origin of *Ceratocystis fimbriata* isolates studied

Isolate	Origin	
	Hosts	Locations
A-2	Almond, *Prunus amygdalus* Batsch.	California, USA
Apr-1	Apricot, P. *armeniaca* L.	California, USA
As-1	Aspen, *Populus tremuloides* Michx.	Pennsylvania, USA
B-2	*Crotalaria* spp.	Campinas, Brazil
B-8	Cacao, *Theobroma cacao* L.	Campinas, Brazil
B-27	Mango, *Mangifera indica* L.	Campinas, Brazil
C-1	Cacao, *Theobroma cacao* L.	Turrialba, C. Rica
EC-1	Coffee, *Coffea arabica* L.	Turrialba, C. Rica
I-4	Oak, *Quercus ellipsoides* Hill.	Minnesota, USA
P-115	Prune, *Prunus domestica* L.	California, USA
Pe-4	Peach, *Prunus persica* Batsch.	California, USA
Pt-1	Plane-Tree, *Platanus* spp.	Mississipi, USA
Sp-196	Sweet potato, *pomoea batatas* Lam.	Louisiana, USA

Inoculum preparation and maintenance of cultures — To eliminate the possibility of transferring self-sterile and mycelial colonies that arise frequently in this species during ascospore formation (19) mycelial plugs (without perithecia) from monoascosporic, homothallic colonies of each isolate were transferred to plates of Difco's Nutrient Agar. That medium provided a sparse, starvation-type colony without mature perithecia. This method did not affect the future ability of the inoculum to grow and produce perithecia, as shown by maintaining 5 isolates at 18°C in culture tubes capped with parafilm. The subcultures showed no change in growth and sexual reproduction when transferred to malt agar, at intervals up to 11 months, except for slower growth during the first 5 days on the new medium. Stock cultures were genetically stable throughout the study. Inoculum for individual experiments was prepared by transferring a piece of mycelium from a stock colony into a 125ml Erlenmeyer flask containing the low N (solid) medium described below. After 8 to 15 days the endoconidia were removed, washed by low-speed centrifugation adjusted to 10^6 cells per ml. Plates of agar medium were inoculated with a 2mm nichrome loop of spore suspension. Plastic Petri dishes were used in all agar medium experiments. Liquid medium experiments were done in 125ml Erlenmeyer Pyrex flasks containing 40ml of medium and inoculated with 10^6 to 10^8 spores per flask. All glassware had been soaked in 1M HCl for at least 24 hours and then rinsed thoroughly in glass distilled water. The 2 basal media were modifications of the one used by Campbell (4). The first called low N basal medium contained 0.75g/l of asparagine whereas the second designated high N medium contained 3g/l of asparagine. The following reagent grade chemicals were included in both media:

$MgSO_4 \cdot 7\ H_2O$	0.083g
KH_2PO_4	1.361g
KCl	0.42g
$FeCl_3 \cdot 6\ H_2O$	0.24g
$ZnCl_2$	0.15g
H_3BO_3	0.06g
$CuCl_2 \cdot 2\ H_2O$	0.05g
$MnCl_2 \cdot 4\ H_2O$	0.04g
$Na_2MoO_4 \cdot 2\ H_2O$	0.03g
Maltose	20.0g

Glass distilled water to complete 1 liter.

The Ca^{+2} and Mg^{+2} salts were analyzed by X-ray diffraction and showed no significant traces of other ions. The cations: Ca^{+2}, Mg^{+2}, Sr^{+2}, Na^{+1}, K^{+1}, Mn^{+2}, Ba^{+2}, Co^{+2}, Ni^{+2}, Cu^{+2}, Sn^{+2} and Rb^{+1} were added to the basal media, in their chloride forms. Vitamins used in all media were: thiamine HCl (100μg/l), pyridoxine HCl (100μg/l), biotin (5μg/l), and i-inositol (5mg/l). They were prepared as stock solutions and kept frozen until used.

"Difco's Special Agar Nobel" was used in all solid media at 2% concentration. All media were adjusted to pH 5.5 and autoclaved separately. KH_2PO_4 was mixed with the other salts after sterilization and cooling of the media, to avoid precipitation of the phosphate. Between 25 to 30ml of medium was dispensed into each Petri dish.

Incubation procedure and measurements of growth and perithecial production — Growth was measured by comparing dry weights of mycelia obtained 12 to 15 days after inoculation of the flasks. To measure perithecial production, inoculated plates were incubated at 24 ± 1°C. Perithecia were counted 18 to 22 days after inoculation. The perithecial index was determined by counting mature perithecia with or without ascospore masses by the method described by Campbell (4). In this method perithecia were counted by following four radii from the center of the colonies with a dissecting microscope using a 15 X ocular and a 6 X objetive. The first radius was taken at random and the other three were symmetrically spaced around the colony. In each radial row, the first field was counted starting at the edge of a point made at the center of the colony using the tip of a needle. Then the plate was moved along the radius to the next field and the count started at the outer edge of the previous one. This was repeated until the margin of the colony was reached.

The data presented in all tables were analyzed statistically, via the Bourroughs 5500 computer employing a program for the Duncan's Multiple Range test at the 1% level. All values are means of three replicates. To simplify the statistical analysis and its presentation, the data for each isolate were analyzed separately. Thus, in the tables, for each isolate, data with one or more letters in common are not significantly different.

Results

Effect of mono and divalent cations on the sexual reproduction of Ceratocystis fimbriata at 2 N levels — Because the presence or absence of cations in media can affect the growth and sexual reproduction of fungi, several cations were tested to determine their effect on the production of perithecia by *C. fimbriata*. Two levels of N, 0.75g/l and 3.0g/l of asparagine, were included as basic treatments throughout the present work. Three isolates, I-4, Pe-4 and As-1 were included in all tests because in preliminary studies they responded differently when grown in the presence of Ca^{2+} · Pe-4 increased its perithecial production whenever 13.6mM Ca^{+2} was present at either low or high N, and I-4 was stimuled by this concentration of Ca^{+2} only in high N medium whereas As-1 did not respond to this cation at either N level.

1. *Effect of different cations on the sexual reproduction of C. fimbriata* — Experiments were set up with treatments consisting of low and high N basal media (controls) and the 2 basal media plus one of several cations at a concentration of 13.6mM. Ba^{+2}, Co^{+2}, Cu^{+2}, Mn^{+2}, Ni^{+2}, Rb^{+1}, Sn^{+2} and Zn^{+2}, showed extreme toxicity to *C. frimbriata* and were omitted from Table 2. Ca^{+2} and Mg^{+2} were the only cations to stimulate sexual reproduction of 2 out of the 3 isolates (I-4 and Pe-4) under some of the conditions tested (Table 2). I-4 was favorably affected by Ca^{+2} at high N but not at the low N level. Except for Ca^{+2}, other cations reduced perithecial production of I-4 at high N. I-4 was not affected by Ca^{+2} and K^{+1}, at low N but Na^{+1}, Mg^{+2} and Sr^{+2} reduced perithecial production. At either N level, Ca^{+2} and Mg^{+2} stimulated perithecial production of Pe-4 and Sr^{+2} inhibited. Perithecial production of Pe-4 was reduced by Na^{+1} and K^{+1} only at high N. Ca^{+2} did not affect sexual reproduction of As-1 but other cations tested re-

Table 2 — Effect of cations on perithecial production of 3 isolates of *Ceratocystis fimbriata*. The isolates were grown on low N (0.75g/l of asparagine and C/N = 54.6) and high N (3.0g/l of asparagine and C/N = 14.9) media, with 20.0g/l of maltose

Treatments		Perithecial indices (*)		
		Isolates		
Nitrogen Level	Cations (13.6 mM)	As-1	I-4	Pe-4
Low N				
	Control	188 c	284 d	345 d
	Ca^{+2}	191 c	249 cd	439 cd
	Mg^{+2}	130 b	209 c	619 e
	Sr^{+2}	7 a	68 a	87 a
	Na^{+1}	6 a	195 c	355 bc
	K^{+1}	22 a	262 cd	343 b
High N				
	Control	130 b	111 b	459 d
	Ca^{+2}	132 b	220 cd	771 f
	Mg^{+2}	15 a	89 a	884 c
	Sr^{+2}	2 a	10 a	79 a
	Na^{+1}	5 a	16 a	48 a
	K^{+1}	22 a	44 a	57 a

(*) The values in all tables are means of 3 replicates. The data were statistically analyzed using the Duncan's multiple range test, at the 1% level. The data for each isolate were analyzed separately. No comparisons can be made between isolates. For each isolate, means followed by the same letter are considered equal

duced it at either N level (Table 2). Further, As-1 and I-4 were favored by low N whereas Pe-4 responded positively to high N. The addition of the cations to the basal media sometimes changed this N response. The effect of Ca^{+2} on I-4 at high N can be interpreted either as a Ca^{+2} stimulation or as a reversion of an inhibition due to an excess of N.

In a further study, cation concentration was lowered from 13.6mM to 3.4mM. In this case, Ca^{+2}, Mg^{+2}, Sr^{+2}, Ba^{+2}, Na^{+1} and K^{+1} were tested and several changes occurred. At lower Ca^{+2} concentrations, the effect did not change qualitatively, but its intensity was reduced. Pe-4 and I-4, but not As-1, produced more perithecia on the Ba^{+2} medium when its concentrations was 3.4 instead of 13.6mM. Ba^{+2} was a stronger inhibitor of perithecia production than Sr^{+2} for As-1 in low N, as well as for I-4 and Pe-4 at either N level. In low N, As-1 was not affected by 3.4mM Mg^{+2}. In high N, Mg^{+2} reduced sexual reproduction of As-1 but less than at 13.6mM. At either N level, 3.4mM Mg^{+2} did not inhibit sexual reproduction of I-4. The Mg^{+2} stimulation of Pe-4 was still present at low and high N although at a lower level. Na^{+1} and K^{+1} at 3.4mM had no effect on sexual reproduction of all 3 isolates.

Thus the toxic effects of all cations diminished (Sr^{+2}, Ba^{+2} and Mg^{+2}) or even disappeared (Na^{+1} and K^{+1}) as their concentrations were lowered from 13.6mM to 3.4mM. The stimulatory effects, however, remained present although at a lower intensity.

As all divalent cations tested at 13.6mM (except for Ca^{+2} and Mg^{+2}) reduced the sexual reproduction of the 3 isolates of *C. fimbriata*, they could not be used in further experimentation to equilibrate the ionic strength of the control media to that of the treatments containing Ca^{+2} or Mg^{+2}. Thus throughout this investigation the control media, having neither Ca^{+2} nor Mg^{+2}, had always a lower ionic strength than that of the treatments containing Ca^{+2} or Mg^{+2}. Further, to better characterize the effects of Ca^{+2} and Mg^{+2} a concentration of 27.2mM was used, based on the 25mM Ca^{+2} level used by Basu (2) for *C. brasiliensis.*

2) *The response to Ca^{+2} and Mg^{+2} in varying combinations* — Mixtures of various proportions of Ca^{+2}, plus Mg^{+2}, adding up to a final concentration of 27.2mM, differed in their effects on perithecial production of 3 isolates of *C. fimbriata* (Table 3). Pe-4 produced more perithecia whenever the proportion of Mg^{+2} increase in the mixture of Ca^{+2} plus Mg^{+2}, although there was also a Ca^{+2} stimulation. At either N level As-1 had a lower perithecial index with all Ca^{+2} - Mg^{+2} combinations, except for

Table 3 — Response of 3 isolates of *Ceratocystis fimbriata* to mixtures of Ca^{+2} and Mg^{+2} at a total concentration of Ca^{+2} plus Mg^{+2} of 27.2mM, as expressed by changes in perithecial indices, at 2 N levels: low N : 0.75g/l and high N : 3.0g/l of asparagine. Carbon source: maltose 20.0g/l

Treatments			Perithecial Indices (*)		
	Cations (mM)		Isolates		
N level	Ca^{+2}	Mg^{+2}	As-1	I-4	Pe-4
Low					
	0.0	0.0	120 d	138 c	259 a
	27.2	0.0	187 d	132 c	442 b
	20.4	6.8	86 c	88 b	459 b
	13.6	13.6	92 c	85 b	514 b
	6.8	20.4	69 b	74 b	616 c
	0.0	27.2	56 a	75 b	634 c
High					
	0.0	0.0	91 c	84 b	475 b
	27.2	0.0	13 a	148 c	1047 d
	20.4	6.8	1 a	120 c	1210 e
	13.6	13.6	16 a	65 a	1351 f
	6.8	20.4	15 a	24 a	1371 f
	0.0	27.2	6 a	32 a	1391 f

(*) See Table 2 for an explanation of the statistical analysis

the treatment with 27.2 mM Ca^{+2} that did not affect perithecial production. At high N, 27.2mM Ca^{+2} and 20.4mM Ca^{+2} plus 6.8mM Mg^{+2} stimulated I-4. As the Mg^{+2} concentration was increased the treatments gradually became more inhibitory to I-4 at either N level. I-4 was not stimulated by any of the treatments in low N. These results confirm those of Table 2 and they show that for As-1, 27.2mM Ca^{+2} in high N has an inhibitory effect not found with 13.6mM Ca^{+2}.

Effects of Ca^{+2} (27.2mM) and Mg^{+2} (27.2mM) on growth and sexual reproduction of the species C. fimbriata represented by isolates from 11 geographical regions and 12 different hosts — 1) *Effect of Ca^{+2} on sexual reproduction* — The results indicate that isolates of *C. fimbriata* differ in their response to Ca^{+2} and to the N content of the medium. Because the species *C. fimbriata* constitutes many strains with a wide-host range and geographical distribution, a comparison of additional isolates was done to determine if correlations between Ca^{+2} response and origin of the isolates existed. Both low and high N media were used. Thirteen isolates were used and they were grouped as follows:

a) Isolates from temperate areas.
   1. Isolated from stone-fruit trees: A-2, Apr-1, P-115 and Pe-4.
   2. Isolated from other hosts: As-1, I-4, Pt-1 and Sp-196.

b) Isolates from the tropics: B-2, B-8, B-27, C-1, EC-1.

Five of the 13 isolates (Table 4) responded by an increase in perithecial production when Ca^{+2} was added to the medium at both N levels. These 5 isolates were obtained from 3 different areas, namely: 2 from California (one from Almond — A-2 — and the other from peach — Pe-4); 2 from Brazil (isolated from mango — B-27 — and *Crotalaria* spp. — B-2) and the other isolate from Mississippi isolated from plane tree (Pt-1). Only 3 isolates responded favorably to Ca^{+2} exclusively at low N: the B-8 strain isolated from cacao in Brazil, EC-1 from Costa Rica isolated from coffee and P-115 isolated from prune in California. Isolates Apr-1; C-1 and I-4 (respectively from: apricot-California; cacao — Costa Rica; oak — Minnesota) were stimulated by Ca^{+2} only in high N whereas Sp-196 was not affected by Ca^{+2}. As-1 (in high N) and C-1 (in low N) had, however, their sexual reproduction significantly reduced by Ca^{+2}.

The isolates from the 3 groups (isolated from: stonefruit trees, other hosts from temperate areas and tropical plants) varied in their responses to Ca^{+2} and there was no correlation between the origin (location and hosts) of the isolates and their response to Ca^{+2} at the 2 N le-

Table 4 — Effect of 27.2mM Ca^{+2} on the sexual reproduction of 13 isolates of *Ceratocystis fimbriata* at 2N level (low N: 0.75g/l and high N: 3.0g/l of asparagine)

Isolates	Perithecial Indices (*)			
	Low N		High N	
	Control	Ca^{+2}	Control	Ca^{+2}
A-2	166 a	251 b	176 a	210 b
B-2	1379 a	2091 b	674 a	2886 b
B-27	121 a	306 b	156 a	523 c
Pe-4	236 a	302 b	341 b	495 c
Pt-1	631 b	714 c	243 a	807 d
B-8	1302 a	1877 b	1819 ab	1671 ab
EC-1	239 a	322 b	154 a	114 a
P-115	128 a	186 b	159 ab	199 b
Apr-1	450 b	452 b	71 a	450 b
C-1	332 d	177 c	26 a	119 b
I-4	354 b	268 b	77 a	351 b
As-1	193 c	197 c	124 b	22 a
Sp-196	562 a	1056 a	746 a	692 a

(*) See Table 2 for an explanation of the statistical analysis

vels, as expressed in perithecial indices. All isolates, except for As-1 and Sp-196, favorably responded to Ca^{+2} at both or at one of the N levels tested.

2) *Effect of 27.2mM Ca^{+2} on the growth of C. fimbriata as it relates to its effect on sexual reproduction* — Nine isolates of *C. fimbriata* were used in a investigation of the influence of 27.2mM Ca^{+2} on the vegetative growth of *C. fimbriata* in liquid media (Table 5).

Of the 5 isolates that produced more perithecia in response to Ca^{+2} at either N level, 2 (A-2 and Pe-4) were not affected in vegetative growth by the addition of Ca^{+2} and 3 (B-2, B-27 and Pt-1) had a greater dry weight of mycelium whenever Ca^{+2} was added to the medium, but only in high N. As far as vegetative growth is concerned, B-8 was stimulated by Ca^{+2} in high N while this cation increased perithecial production of this isolate only in low N.

Isolates Apr-1 and I-4 were not stimulated by the addition of Ca^{+2} at either N level although the perithecial formation by both of them was stimulated by Ca^{+2}. Finally, isolate As-1 which was not affected by Ca^{+2} in low N but had its sexual reproduction reduced upon addition of this cation in high N, was stimulated by Ca^{+2} in its vegetative growth at either N level.

Table 5 — Effect of 27.2mM Ca^{+2} on vegetative growth of 9 isolates of *Ceratocystis fimbriata* in liquid media at 2N levels (low N: 0.75g/l and high N: 3.0g/l of asparagine)

Isolates	Mycelial Dry Weight (mg) (*)			
	Low N		High N	
	Control	Ca^{+2}	Control	Ca^{+2}
A-2	96 a	96 a	136 b	141 b
B-2	101 a	109 a	93 a	166 b
B-27	94 a	93 a	105 a	165 b
Pe-4	127 a	119 a	161 b	162 b
Pt-1	116 a	123 a	100 a	168 b
B-8	118 a	117 a	127 a	163 b
Apr-1	78 a	86 a	104 b	110 b
I-4	73 a	82 a	104 b	99 b
As-1	105 a	142 b	150 b	173 c

(*) See Table 2 for an explanation of the statistical analysis

Some correlation existed, however, between the Ca^{+2} effect on growth (mycelial dry weight) and the origin of some of the isolates. For example, all isolates from California (from stone fruits) did not increase in vegetative growth when Ca^{+2} was added, although these isolates had a higher perithecial production in the presence of Ca^{+2} in solid medium at one or both N levels, depending on the isolates considered. Furthermore, all the isolates from Brazil (B-2, B-8 and B-27) increased vegetative growth with the addition of Ca^{+2} to the high N medium, although the perithecial formation by two of the Brazilian isolates (B-2 and B-27) was increased upon adition of Ca^{+2} at either N level, the other isolate (B-8) was not affect by Ca^{+2} in high N but, on the other hand, was stimulated by this cation at low N. As far as vegetative growth and perithecial production are concerned, the isolates from temperate areas did not respond as a group.

3) *The effect of 27.2mM Mg^{+2} on perithecial production and vegetative growth of C. fimbriata and its relationship to the 27.2mM Ca^{+2} response* — To study the effect of Mg^{+2} on the sexual reproduction of *C. fimbriata* and its relation to the Ca^{+2} response further experiments were done using the same isolates used for the Ca^{+2} study. The isolates (A-2, B-2, B-27, Pe-4 and Pt-1), that responded to the addition of Ca^{+2} with an increase in perithecial production at either N level, had different responses to Mg^{+2} except for B-27 and Pe-4 which responded equally to Ca^{+2} and Mg^{+2} (Table 6). Isolates A-2 and B-27 were not affected by Mg^{+2} at any of the N levels, whereas, Pt-1 responded favorably to Mg^{+2} only in high N. B-8 increased its sexual reproduction upon addition of Ca^{+2} to the low N medium but was not affected by Mg^{+2}. Apr-1 and I-4 that were stimulated by Ca^{+2} only at high N were never favorably affected by Mg^{+2}. I-4 had a lower sexual reproduction at either N level when Mg^{+2} was present, while Apr-1 did not respond to Mg^{+2} at either N level. As-1 was negatively affected by Mg^{+2} at low and high N although Ca^{+2} did not affect its sexual reproduction in low N and inhibited it in high N.

Of the isolates from Brazil, only B-2 responded to Mg^{+2} (in high N) and the other isolates were not affected by this cation. This was different from the effect of Ca^{+2} that was beneficial to the sexual reproduction of all 3 tropical isolates (B-2 or B-27 at either N level and B-8 only at low N). Of the isolates from California (from stone-fruit trees) only Pe-4 responded favorably to either Ca^{+2} or Mg^{+2}, while the other two isolates responded to Ca^{+2} (A-2 at either N level and Apr-1 at high N) but not to Mg^{+2}.

Each of the isolates from temperate areas and hosts other than stone-fruit trees presented a peculiar set of responses to the 2 cations at the N levels. Pt-1 which favorably responded to Ca^{+2} at both N levels was stimulated by Mg^{+2} at the high N level, although this cation had no effect on Pt-1 in the low N medium. As-1 whose sexual reproduction was not affected by Ca^{+2}, at low N, but was reduced by that cation in high N was negatively affected by Mg^{+2} at either N level. Mg^{+2} had the same effect on isolate I-4 which was stimulated by Ca^{+2} in high N and not affected by this cation in low N.

Table 6 — Effect of 27.2mM Mg^{+2} on perithecial production of 9 isolates of *Ceratocystis fimbriata* at 2N levels (low N: 0.75g/l and high N: 3.0g/l of asparagine)

Isolates	Perithecial Indices (*)			
	Low N		High N	
	Control	Mg^{+2}	Control	Mg^{+2}
A-2	204 ab	303 b	110 a	130 a
B-2	954 a	897 a	1040 a	2857 b
B-27	120 a	94 a	131 a	180 a
Pe-4	338 a	964 c	587 b	1175 d
Pt-1	1180 ab	1208 b	808 a	1304 b
B-8	1131 a	1430 a	1679 a	1181 a
Apr-1	445 b	442 b	228 a	216 a
I-4	261 c	132 b	134 b	91 a
As-1	150 c	119 b	98 b	41 a

(*) See Table 2 for an explanation of the statistical analysis

Table 7 — Effect of 27.2mM Mg^{+2} on vegetative growth of 9 isolates of *Ceratocystis fimbriata* in liquid medium at 2N levels (low N: 0.75g/l and high N: 3.0g/l of asparagine)

Isolates	Mycelial Dry Weight (mg) (*)			
	Low N		High N	
	Control	Mg^{+2}	Control	Mg^{+2}
A-2	74 a	77 a	123 b	143 b
B-2	74 a	83 b	80 ab	133 c
B-27	187 a	189 a	187 a	226 b
Pe-4	94 a	103 a	152 b	151 b
Pt-1	94 a	147 b	109 ab	215 c
B-8	123 a	131 a	137 a	187 b
Apr-1	99 a	96 a	124 b	129 b
I-4	65 a	75 a	93 b	119 b
As-1	95 a	96 a	117 b	113 b

(*) See Table 2 for an explanation of the statistical analysis

Table 8 — Summary of the data on the effect of 27.2mM Ca^{+2} and 27.2mM Mg^{+2} upon the vegetative growth and sexual reproduction of 9 isolates of *Ceratocystis fimbriata* at 2N levels (low N: 0.75g/l and high N: 3.0g/l of asparagine) (*)

Isolates	Change in growth (mycelial dry weight)				Change in sexual reproduction (perithecial index)			
	Low N		High N		Low N		High N	
	Ca^{+2}	Mg^{+2}	Ca^{+2}	Mg^{+2}	Ca^{+2}	Mg^{+2}	Ca^{+2}	Mg^{+2}
A-2	0	0	0	0	+	0	+	0
B-2	0	+	+	+	+	0	+	+
B-27	0	0	+	+	+	0	+	0
Pe-4	0	0	0	0	+	+	+	+
Pt-1	0	+	+	+	+	0	+	+
B-8	0	0	+	+	+	0	0	0
Apr-1	0	0	0	0	0	0	+	0
I-4	0	0	0	0	0	–	+	–
As-1	+	0	+	0	0	–	–	–

(*) 0 means no effect; – means reduction and + means increase

Effect of Mg^{+2} on vegetative growth — A study of the effect of Mg^{+2} on the growth of the fungus in liquid medium was done in the same manner as with Ca^{+2} (Table 7). Two isolates (B-8 and B-27) grew better when Mg^{+2} was added to the high N medium and 2 others (B-2 and Pt-1) had more growth upon addition of Mg^{+2} to either low or high N medium. The other isolates did not respond to Mg^{+2}.

Table 8 contains a summary of the effects of Ca^{+2} and Mg^{+2} on the vegetative growth and sexual reproduction of the 9 isolates of *C. fimbriata*. It shows that 5 out of the 9 isolates responded to Ca^{+2} with an increase in vegetative growth at high N and only 1 out of 9 at low N. Four of 9 isolates were significantly stimulated by Mg^{+2} in high N, with all 4 responding to Ca^{+2} at that N level. In low N only 2 isolates responded favorably to Mg^{+2} in growth as compared to 1 out of 9 of the same isolates when Ca^{+2} was the cation added to the medium. In solid medium at low N, Mg^{+2} depressed the sexual reproduction of two isolates and stimulated the perithecial production by another one. Ca^{+2} was more effective than Mg^{+2} in stimulating sexual reproduction of *C. fimbriata* at both N levels. The addition of Ca^{+2} to the low N medium enhanced perithecial indices in 6 of the 9 isolates tested. Five of these also favorably responded to Ca^{+2} in high N medium. Two additional isolates were stimulated by Ca^{+2} only at high N.

Discussion

In the present study, several divalent and monovalent cations were tested for their effect on sexual reproduction and growth of *C. fimbriata*. Of those tested, only Ca^{+2} and Mg^{+2} stimulated the production of perithecia and vegetative growth. The quantitative responses observed depended not only on the concentration of the cations but also on the isolate and N levels. Sr^{+2} and Ba^{+2} have been successfully used as total (2) or partial (1) replacements for Ca^{+2} in other fungi but not in *C. fimbriata*.

The relationship between the effect of Ca^{+2} and Mg^{+2} to N concentration on sexual reproduction and growth of *C. fimbriata* cannot be explained on the basis of the results presented. Although this relationship exists, critical work on the uptake and metabolism of Ca^{+2}, Mg^{+2}, amino acids and sugars is needed before a conclusion can be reached as to the reasons for the interdependence among nutrients.

The response of *C. fimbriata* to Ca^{+2}, differs from that reported by Basu (1, 2) in several species of *Chaetomium*. In *C. fimbriata* Ca^{+2}, besides increasing perithecial production, stimulated vegetative growth in several isolates. However, in some *Chaetomium* spp., the effect seems to be exclusively related to fruiting and ascospore formation since vegetative growth was not affected even by Ca^{+2} concentrations of 25.0mM. No further comparison with the results of Basu (1, 2) can be made because of differences in experimental approaches. This is the first time that a detailed study has been done on the response of a wide range of isolates of one species of the genus *Ceratocystis*.

The studies with fungi other than Ascomycetes are mainly on the effect of small amounts of Ca^{+2}. An indispensable step for the success of such studies was the use of highly purified chemicals and the use of carefully washed glassware. The work of Erwin (9) with *Phytophthora* spp. is an example of successful methodology in dealing with low Ca^{+2} media.

The method used by Basu (1, 2), to quantatize the Ca^{+2} effect on perithecial production by *Chaetomium* spp., was different from that of Campbell (4) with *C. fimbriata*. Campbell (4) used the concept of "perithecial index" as a measure of sexual reproduction in *C. fimbriata*. This concept provides a practical approach to the quantitative evaluation of fruiting in many fungi. His method with some minor changes, was used throughout the present study. As Campbell (5, 6) did not wash his glassware either with a chelating agent, such as EDTA, or a strong acid, he ran a risk of working with a control with a high Ca^{+2} content. He also used a lower phosphate concentration than in the present investigation. The differences may account for the lack of response of isolate I-4 to Ca^{+2} in his N treatment. There is also the possibility that some of the physiological characteristics of I-4 have changed since the time of Campbell's work, because of a long period of storage in culture.

Although there is a great variability in the response to Ca^{+2} between isolates of *C. fimbriata* the isolate L-35 (from almond), used by Campbell (5, 6), responded the same way as another almond isolate (A-2) used here. In the present study the fertility of the inoculum was maintained as described in the Methods section, avoiding the problem encountered by Campbell (4) of mutations to self-sterile or mycelial type colonies (19).

Throughout the present investigation the effects of Ca^{+2} and Mg^{+2} on growth and fruiting of *C. fimbriata*, as well as their relationship to the asparagine concentrations were repeatedly dependent on the isolate considered. This variability among the isolates tested was verified when a large number of them was studied. Further, it was observed that the response of the various strains of *C. fimbriata* to Ca^{+2} and Mg^{+2} had no relation to their origin, i. e., geographical locations and hosts. Some isolates showed an identical response to either Ca^{+2} or Mg^{+2}. Depending on the asparagine content in the medium however, other isolates responded to either one of those cations or to neither of them. Thus, the fact that a strain of *C. fimbriata* responded to Ca^{+2} does not necessarily mean that it will also respond to Mg^{+2}, and vice versa.

Due to the low concentration of minerals at either basal medium it seems unlikely that Ca^{+2} or Mg^{+2} were protecting several strains of *C. fimbriata* against a mineral toxicity. There was, however, an inhibition of sexual reproduction in the case of isolates Apr-1, As-1, I-4 and Pt-1 when the asparagine content of the medium was raised from 0.75 to 3.0g/l. With all these isolates, except for As-1, 27.2mM Ca^{+2} could completely reverse this inhibition. Mg^{+2} counteracted the high N effect only with isolate Pt-1. Thus Ca^{+2} was more effective than Mg^{+2}, in reversing the inhibitory effect of N for a larger number of isolates. The fact that some of the isolates responded to Ca^{+2} and/or Mg^{+2} at low

and high N and others only at one of the N levels, diminishes the likelihood of the possibility that Ca^{+2} and/or Mg^{+2} may always be protecting the fungus against the toxic effects of a N excess.

The variable response to Ca^{+2} and Mg^{+2} might be due to differences in the ability of the various isolates of *C. fimbriata* to take up Ca^{+2} and Mg^{+2}. If, however, Ca^{+2} and Mg^{+2} are taken up at the same rates and amounts by the different isolates, it might be that Ca^{+2} and Mg^{+2} are involved in different aspects of the fungal cell metabolism. Then, the answer may only be found studying the role of high concentrations of Ca^{+2} and Mg^{+2} on metabolic pathways of the fungal cell. Mg^{+2} is considered as a general requirement by all fungi due to its role as an activator of enzymes (11, 14). Further work is, however, needed on the nature of the stimulus of *C. fimbriata* by high concentrations of Mg^{+2}, such as 27.2mM. The same is true for the interactions between cations and several of the main nutrients required by *C. fimbriata*.

Until more information is accumulated on ion uptake and metabolism, conclusions regarding the roles of Ca^{+2} or Mg^{+2} in the process of sexual reproduction and growth of *C. fimbriata* cannot be reached.

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Metabolismo oxidativo de formas de cultura de Fonsecaea pedrosoi*

Maria Aparecida de Resende**
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Resumo

Com amostra de *Fonsecaea pedrosoi*, isolada de paciente de cromomicose, foram realizados: curva de crescimento, usando método de peso seco e experimentos de respiração, através do uso do método manométrico, utilizando-se diversos substratos como fonte energética. Inibidores metabólicos foram testados em presença de glicose. Na respiração de carboidratos, frutose apresentou maior efeito estimulante sobre a respiração, enquanto que trealose e lactose apresentaram estímulo menor. Sorbitol, glicose, manose, reafinose, arabinose e galactose tiveram efeito estimulante menos acentuado, enquanto que glicerol, sacarose e xilose não apresentaram qualquer estímulo. Há grande variabilidade na resposta energética à adição de carboidratos. Dos ácidos orgânicos testados, somente oxalacetato produz efeito estimulante em pH 5,0. Succinato, piruvato, oxalacetato, malato e α-cetoglutarato produziram fraco efeito estimulante em pH 7,0. Citrato não produziu efeito em nenhum dos valores de pH testados. De todos os L-aminoácidos testados, cisteína apresentou maior efeito estimulante sobre a respiração, enquanto que isoleucina, metionina e lisina tiveram seus efeitos menos acentuados. Prolina, alanina, fenilalanina, glutamina e asparagina tiveram pequeno efeito estimulante. Não houve nenhum efeito estimulante em presença dos seguintes aminoácidos: hidroxiprolina, leucina, tirosina, arginina, ácido aspártico, ácido glutâmico, triptofano, glicina, histidina, serina, treonina, valina e cistina. Todos os inibidores metabólicos testados inibiram a respiração da glicose. Foram eles: pirofosfato de sódio, arsenito de sódio, cianeto de potássio, antimicina A, iodo-acetato de sódio e rotenona.

Summary

Oxidative metabolism in culture forms of Fonsecaea pedrosoi

With a sample of *Fonsecaea pedrosoi*, isolated from a chromomycosis patient, a growth curve was determined by the dry weigth method, and the respiration rates manometrically determined in a Warburg apparatus, with the use of several substrates as energy sources. Metabolic inhibitors were used with glucose. In respiration experiments with carbohydrates fructose caused greater stimulation, while trehalose and lactose showed weaker effects. Sorbitol, glucose, mannose, raffinose, arabinose, and galactose had still smaller effects, while glycerol, sucrose, and xylose had no effect at all. Great variability occurs in energetic response to carbohydrate addition. Of all organic acids tested, only oxalacetate stimulated respiration at pH 5.0. Succinate, pyruvate, oxalacetate, malate, and α-ketoglutarate showed weaker effects at pH 7.0, while citrate had no effect at either pH. Of all L-aminoacids used, cysteine showed the greatest stimulating effect in respiration, while isoleucyne, methionine, and lysine showed a lesser effect. Proline, alanine, phenylalanine, glutamine, and asparagine had little stimulating effect. The following aminoacids caused no stimulatory effects: hydroxyproline, leucine, tyrosine, arginine, aspartic acid, glutamic acid, tryptophan, glycine, hystidine, serine, threonine, valine, and cystine. All metabolic inhibitors used inhibited glucose respiration ant these included: natrium pirophosphate, natrium arsenite, potassium cyanate, antimycin A, rotenone, and iodoacetate.

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Introdução

O fungo *Fonsecaea pedrosoi* tem sido isolado predominantemente como agente da cromomicose em nosso meio (15).

Cromomicose inclui um grupo de entidades clínicas causadas por vários fungos dematiáceos (pigmentados).

Rippon (17) agrupou a doença em quatro tipos clínicos: 1) forma verrucosa ou cromoblastomicose clássica; 2) síndrome de abscesso cerebral (Cladosporiose); 3) cromomicose de cistos simples ou múltiplos (feoesporotricose); e 4) lesões mais ou menos sistêmicas da patologia variada.

O tratamento da cromomicose é difícil e as tentativas de tratamento têm sido numerosas. Atualmente, tem-se tentado associar anfotericina B com 5-fluorocitosina (11). Anfotericina B é um antibiótico poliênico, altamente tóxica, enquanto que 5-fluorocitosina induz resistência em casos de lesões muito extensas e antigas (10).

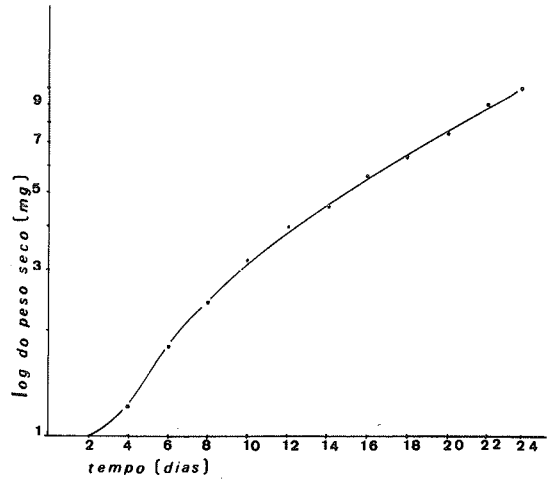
Pouco se conhece a respeito dos aspectos biológicos e bioquímicos de *Fonsecaea pedrosoi*, com exceção do estudo da utilização de alguns carboidratos e compostos nitrogenados (14, 18). O estudo mais detalhado dos organismos responsáveis por uma infecção, nos seus aspectos biológicos e bioquímicos, tem, como uma das finalidades, fornecer subsídios para a busca de novas drogas para o tratamento da doença, baseando-se no encontro de novos alvos específicos a serem atingidos.

Material e Métodos

Amostra — Foi utilizada amostra 5VPL de *Fonsecaea pedrosoi*, isolada de paciente de cromomicose, na Santa Casa de Belo Horizonte. A amostra foi identificada por Oliveira & col. (15) e confirmada por Borelli, mantida em nosso Laboratório em meio de ágar Sabouraud-dextrose.

Inóculo — Para todos os experimentos, o inóculo foi obtido a partir de uma cultura de sete dias de crescimento em meio sintético de asparagina (19). O micélio era colhido e homogeneizado em solução isotônica, em homogeneizador manual de vidro. A suspensão de micélio era então inoculada em 50ml do mesmo meio, em frascos Erlenmeyer de 250ml e incubada a 37°C, em repouso. Para os experimentos de

Gráfico 1 — Curva de crescimento de *Fonsecaea pedrosoi*



crescimento foram adicionados três gotas de homogeneizado de micélio, em tubo contendo o substrato a ser utilizado, em meio de cultura, num volume final de 5ml. Os tubos eram incubados a 37°C, em repouso.

Obtenção da massa de células — Após os sete dias de cultivo, o micélio era lavado três vezes, em tampão fosfato pH 7,0, 0,2M por centrifugação, a 4°C, a 18.000g. Após, era homogeneizado em homogeneizador manual e ressuspenso na mesma solução, até se obter o valor de turvação de 350 unidades Klett (fotocolorímetro Klett Summerson, filtro verde). Foram feitas dosagens de proteína, pelo método de Lowry & col. (12), no sobrenadante da suspensão micelial, antes e após homogeneização. Não se constatou extravasamento de proteína do protoplasma, após a homogeneização.

Medida de crescimento — Na curva de crescimento, a medida foi determinada em função do peso seco de micélio.

Método manométrico — As medidas de consumo de oxigênio foram tomadas no respirômetro de Warburg (Gilson Medical Eletronics Inc. — Modelo RWB-3), a 37°C, segundo as regras do "Manometric Techniques" (21). Cada frasco do aparelho continha o volume final de 3,2ml. As leituras foram tomadas com intervalos de 60 minutos. Os valores foram calculados em microlitros de O_2/hora/unidade de densidade ótica e os resultados foram expressos, subtraindo-se o consumo de O_2 do endógeno. O efeito dos inibidores metabólicos foi testado em presença de glicose.

Sistemas montados

Cuba central..... 0,2ml de KOH a 20%
Cuba de reação.... 2,6ml da suspensão celular + 0,2ml de água (ou inibidor metabólico)
Tubo lateral..... 0,2ml de substrato (100μ moles)

Os aminoácidos usados (todos da série L-), carboidratos, poliálcoois, ácidos orgânicos e inibidores metabólicos foram obtidos da Sigma Chemical Co., J.T. Baker, Carlo Erba, Difco, Dyne Produtos Químicos, E. Merck Ag. Darmstad e Hippo Farma.

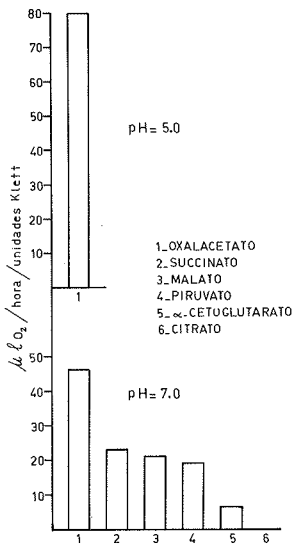
Resultados

Amostra de *F. pedrosoi* apresentou uma fase "lag" de dois dias, após a qual cresceu até esgotar os nutrientes do meio.

Experimentos de respiração — De todos os resultados de experimentos de respiração, foram descontados os valores da respiração endógena (Gráficos II, II, IV e V).

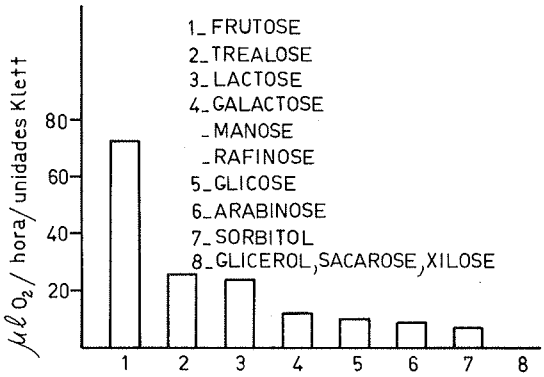
Carboidratos — A frutose, de todos os carboidratos testados, foi a que apresentou maior efeito estimulante da respiração (Gráfico II). Trealose e lactose demonstraram um estímulo razoável, enquanto que sorbitol, glicose, manose, rafinose, arabinose e galactose tiveram seu efeito menos acentuado. Glicerol, sacarose e xilose não apresentaram nenhum efeito estimulante.

Gráfico II — Atividade respiratória da *Fonsecaea pedrosoi* frente a diversos carboidratos



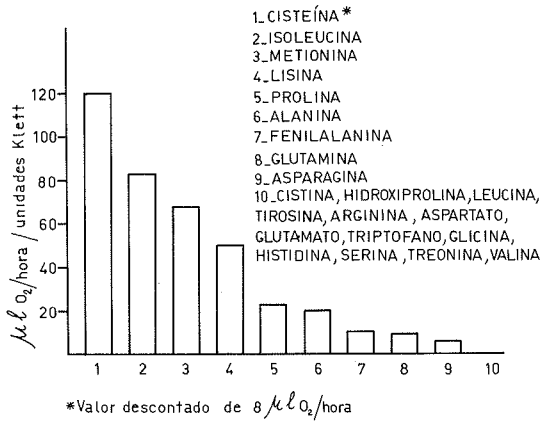
Aminoácidos — Cisteína foi que apresentou maior efeito sobre a respiração (Gráfico III), seguida de isoleucina, metionina e lisina. Outros aminoácidos apresentaram um pequeno estímulo: prolina, alanina, fenilalanina, glutamina e asparagina. Os seguintes aminoácidos não apresentaram nenhum efeito estimulante: hidroxiprolina, leucina, tirosina, arginina, ácido aspártico, ácido glutâmico, triptofano, glicina, histidina, serina, treonina, valina e cistina.

Gráfico III — Atividade respiratória da *Fonsecaea pedrosoi* frente a diversos aminoácidos



Ácidos orgânicos em pH 5,0 e 7,0 — Somente oxalacetato estimulou a respiração em pH 5,0. Succinato, piruvato, oxalacetato, malato e α-ceto-glutarato estimularam fracamente a respiração a pH 7,0. Citrato não foi utilizado em nenhum dos valores de pH testados (Gráfico IV).

Gráfico IV — Atividade respiratória da *Fonsecaea pedrosoi* frente ácidos orgânicos em pH 5.0 e 7.0

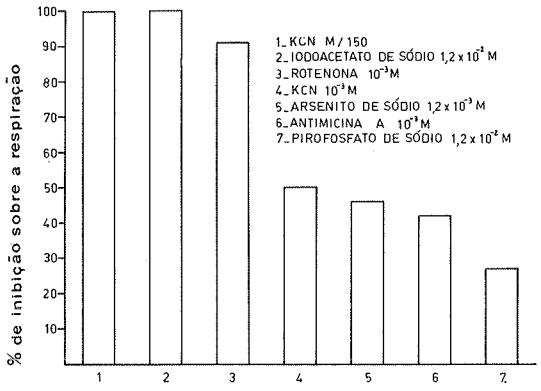


Inibidores metabólicos — A respiração de glicose foi inibida por todos os inibidores testados: pirofosfato de sódio, arsenito de sódio, iodoacetato de sódio, cianeto de potássio, antimicina A e rotenona (Gráfico V).

De todos os resultados obtidos desses experimentos foram descontados os valores da respiração do endógeno.

Não foram testados os efeitos dos inibidores sem a adição de glicose.

Gráfico V — Efeito de inibidores metabólicos em *Fonsecaea pedrosoi*



Discussão

A amostra de *F. pedrosoi*, após uma fase "lag" de dois dias, apresentou uma fase logarítmica menos acentuada que a observada em *Cladosporium carrionii*, nas mesmas condições (16).

A respiração estimulada por frutose foi demonstrada ser intensa: nossos resultados concordam com o obtido por West (22), em *Phialophora verrucosa* e por Silva (18), em *F. pedrosoi*. A utilização de glicose foi baixa, resultado que entrou em desacordo com os experimentos nutricionais de Silva (18), que admitiu ser esse carboidrato tão eficiente quanto a frutose no crescimento de *F. pedrosoi*. *P. verrucosa*, no entanto, apresenta utilização de glicose em níveis semelhantes ao de *F. pedrosoi* (22). Níveis superiores a 100% de estímulo da respiração endógena foram obtidos pelo mesmo método em *Cladosporium carrionii* (16), com a frutose. Baixo estímulo como substrato energético foi observado também com galactose, manose e rafinose. No caso da galactose, nossos resultados foram diferentes dos de Montemayor (14), com *F. pedrosoi* e os obtidos com *C. carrionii* (16). A utilização de lactose sugere a presença de β-galactosidase no parasita. Esta hipótese é reforçada pela resposta positiva, frente à galactose. Inclusive, o nível de estímulo produzido pela lactose correspondente aproximadamente à soma dos estímulos produzidos por glicose e galactose. A não utilização de outros oligossacárides testados poderia ser devida à ausência de enzimas hidrolíticas específicas, no fungo. A rafinose, por exemplo, também pouco utilizada por *P. verrucosa* (22), deveria produzir estímulo da ordem de 30$\mu\ell$ O_2/hora, no caso do microrganismo possuir as enzimas. A ausência de β-frutofuranosidase é também evidente (Gráfico II). Ao contrário, níveis evidentes de enzimas implicadas na hidrólise de sacarose e rafinose parecem estar presentes em *C. carrionii* (16). Baixo estímulo foi produzido por arabinose em *F. pedrosoi*, o mesmo ocorrendo com *C. carrionii* (16). Entretanto, o glicerol, não utilizado por *F. pedrosoi*, alcançou níveis de estímulo próximos a 100% em *C. carrionii* (16). A não utilização de glicerol poderia ser conseqüência de uma ausência do sistema quinase, implicado na sua transformação em α-glicerofosfato (1) ou ausência do sistema α-glicerofosfato desidrogenase. Sorbitol apresentou baixos níveis de estímulo, enquanto que xilose não foi utilizada por *F. pedrosoi*. Em *P. verrucosa*, xilose foi pouco utilizada (22). Os resultados apresentados no Gráfico II demonstram uma grande variabilidade na resposta energética à adição de carboidratos, conforme preconiza Hawker (5). *C. carrionii* e *F. pedrosoi* apresentam grande variabilidade nutricional entre si, havendo maior concordância dos resultados entre *F. pedrosoi* e *P. verrucosa*.

O único ácido orgânico capaz de estimular a respiração em pH 5,0 foi o oxalacetato. Os restantes, com exceção do citrato (Gráfico IV), foram utilizados apenas em pH 7,0. Tal fato entra em desacordo com os dados observados na maioria dos trabalhos na literatura (2, 4, 20, 23), onde a maior permeabilidade dos ácidos orgânicos é observada em sua forma aniônica. Esse fato, entretanto, é observado da mesma maneira em *C. carrionii* (16). A não utilização de citrato, por ambos os microrganismos, poderia estar associada a algum bloqueio ou inexistência de um sistema de permease, ou, ainda, ausência de enzimas implicadas em sua metabolização. Esses resultados, assim como a ação do

arsenito e pirofosfato de sódio (Gráfico V), inibidores respectivamente dos complexos α-cetoglutarato desidrogenase e desidrogenase succínica, sugerem a existência de um ciclo dos ácidos tricarboxílidos funcional, com finalidade energética ou pelo menos biossintética.

Dos aminoácidos testados, a cisteína forneceu maior estímulo sobre a respiração. Nos dados do Gráfico III foram descontados os $\mu\ell$ de O_2 consumidos em função da oxidação espontânea da substância. Com a não utilização de cistina, é provável haver bloqueio ou inexistência de sistemas de permeação para este último, ou, ainda, inexistência de cistina redutase, uma vez que o restante da via de degradação de cistina é usualmente idêntica à de cisteína (3).

Metionina e isoleucina foram utilizadas em proporções semelhantes. Ambas vão levar ao mesmo produto final: succinil-CoA. A utilização de metionina era esperada, uma vez que sua degradação forma, como uma das possibilidades, cisteína. Lisina, em menores proporções que metionina, foi estimulante da respiração.

A utilização de prolina provavelmente envolve o sistema prolina oxidase, proposto para *Leishmania donovani* (9) e para *T. cruzi* (4).

A utilização da alanina, nas mesmas proporções que piruvato, sugere a participação de um sistema aminotransferase, transformando alanina em piruvato. Fenilalanina mostrou-se fraco estimulante da respiração. O catabolismo de fenil-alanina não seria mediado pela fenilalanina hidroxilase, com formação de tirosina, uma vez que essa não é utilizada. É provável que haja participação de uma transaminase, com formação de ácido fenilpirúvico (7).

Com a finalidade de se obter dados para a compreensão do sistema de transporte de elétrons na forma micelial do microrganismo, foram utilizados inibidores metabólicos que agem nos sítios abaixo relacionados (8, 13):

Rotenona: inibe o complexo I da cadeia respiratória, impedindo a oxidação do $NADH_2$

Antimicina A: citocromo b_2 —— citocromo C_1
Cianeto de potássio: citocromo a + a_3

A succínico desidrogenase de *F. pedrosoi* foi evidenciada pela inibição promovida pelo pirofosfato de sódio. A inibição provocada por antimicina A sugere a existência de citocromo b e o mesmo efeito, observado em presença de cianeto de potássio, revela a possibilidade de um citocromo a + a_3 funcional. A diferença percentual de inibição, provocada por diferentes concentrações de cianeto, sugere como uma das possibilidades a dicotomização na cadeia de transporte de elétrons a partir do citocromo b. A concentração M/1.000, de cianeto, inibe 50% da respiração, enquanto que M/150 inibe 100%, ou seja, apenas altas concentrações de cianeto inibiriam ambos os ramos da cadeia de transporte de elétrons propostos por Hill (6). Por outro lado, o cianeto não inibe certas reações que consomem oxigênio, como as reações que envolvem o íon superóxido e a água oxigenada.

Torna-se necessário um estudo mais amplo dos componentes enzimáticos do microrganismo, assim como de outros agentes da cromomicose. O melhor conhecimento da fisiologia de *F. pedrosoi* virá também esclarecer problemas relacionados com os mecanismos da doença, permitindo ainda o desenvolvimento de um meio quimicamente definido, com balanceamento fisiológico adequado, possibilitando, em conseqüência, a identificação, isolamento e caracterização de substâncias imunogênicas. O presente estudo abre ainda, perspectivas de trabalho, como, por exemplo, permitir o uso do método respirométrico, para o "screening" de drogas anti-fúngicas, com grande facilidade.

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Micobactérias isoladas de suínos do Estado de Minas Gerais *

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Resumo

Exame de linfonodos submaxilares em 490 suínos de abate, colhidos ao acaso, procedente de nove municípios do Estado de Minas Gerais. Em 22 (4,4%), foram encontradas lesões macroscópicas semelhantes às da tuberculose. O exame bacteriológico destes órgãos e de mais 216 linfonodos aparentemente normais, permitiu isolar 63 culturas de micobactérias, sendo uma de *Mycobacterium bovis*, 58 do complexo *M. avium-intracellulare-scrofulaceum* e quatro de *M. gordonae*. A sorotipagem das micobactérias do complexo MAIS permitiu identificar representantes de 10 sorotipos, destacando-se a prevalência dos sorotipos 7 e 15. Não foi encontrado nenhum dos três sorotipos de *M. avium*.

Summary

Mycobacteria isolated from pigs of Minas Gerais, Brazil

Twenty two (4.4%) out of 490 lymphonodes from slaughtered pigs showed tuberculous-like lesions. The bacteriological examination of these and other 216 lymphonodes without macroscopic alterations revealed 63 mycobacteria: one strain identified as *Mycobacterium bovis,* 58 belonging to the *M. avian-intracellulare-scrofulaceum* complex and four classified as *M. gordonae*. The serological characterization of the MAIS complex revealed members from 10 serotypes. The most prevalent serotypes were 7 and 15. No *M. avium* serotypes were found.

Introdução

O suíno pode ser considerado excelente sentinela epidemiológico da tuberculose e das micobacterioses do homem e animais, no meio rural, pois é susceptível à infecção por *Mycobacterium tuberculosis, M. bovis,* vários sorotipos do complexo *M. avium-intracellulare-scrofulaceum* e, ainda esporadicamente, outras micobactérias oportunistas. Os linfonodos submaxilares e os mesentéricos constituem filtros biológicos, a partir dos quais são isoladas, mais freqüentemente, as micobactérias.

O exame de amostragem representativa de suínos de uma região, em que a maioria dos fazendeiros cria ou mantém porcos, permite avaliar a situação epidemiológica da tuberculose e das micobactérias da população humana e das espécies animais. Além disso, orienta os inspetores de carne sobre a etiologia das lesões tuberculóides e alerta o veterinário sanitarista para o problema das reações inespecíficas, que podem ser encontradas na tuberculinização de bovinos.

Visando estes objetivos, foram feitas pesquisas em suínos do interior do Paraná (8), do lito-

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ral e do agreste de Pernambuco (9), da região de Brasília (10), do Rio de Janeiro (11) e do Paraná e São Paulo (5), examinando linfonodos lesados e aparentemente normais de suínos de abate.

Neste trabalho, são relatados os resultados do exame de 490 materiais de suínos, oriundos de nove municípios do Estado de Minas Gerais, em cujas fazendas se cria habitualmente bonivos de leite, suínos e aves.

Material e Métodos

Coleta de material — No matadouro, foram colhidos, ao acaso, os linfonodos submaxilares de 490 suínos recém abatidos, procedentes dos seguintes municípios.

Municípios	N.º de materiais
Alvinopólis	49
Vespaciano	111
Jequitibá	42
Pitangui	58
Montes Claros	55
Matosinho	57
Betim	30
Igarapé	53
Pedro Leopoldo	35

A maioria dos suínos abatidos era de mestiços de várias raças, predominando a raça nacional Piau. São animais rústicos, com desenvolvimento mais lento, pois atingem o ponto de abate (± 100kg de peso vivo) entre 1 e 2 anos de idade. A maioria dos suínos foi criada em currais coletivos, sendo confinados para ceva três a quatro meses antes do abate.

Cada material foi colocado separadamente em pequeno saco plástico, por ocasião da coleta, transportado, em gelo, até o laboratório e conservado à temperatura de −20°C, até a hora do exame, realizado dentro de oito a 10 dias. Foram submetidos ao exame bacteriológico 22 linfonodos submaxilares portadores de lesões macroscópicas semelhantes às da tuberculose e 216 aparentemente normais.

Exame bacteriológico — No laboratório, cada material era despido do tecido adiposo envolvente, mergulhado sobre papel esterilizado. O linfonodo era inspecionado, enquanto era seccionado em fatias de dois a três mm de espessura. Para cada material era utilizado novo papel e instrumental recém-esterilizado. Dos órgãos lesados, transferia-se, para o gral, a parte alterada, com apenas pouco tecido normal e de cada material, fazia-se semeadura em agar-sangue, antes da descontaminação. Dos linfonodos, aparentemente normais, retirava-se, de várias partes do órgão, fragmento com cerca de duas gramas que era triturado em gral e areia estéreis. A descontaminação era feita com solução de ácido sulfúrico a 6%, na proporção de 1:6 do material, durante 30 minutos, incluindo-se a centrifugação de 15 minutos a 2.000rpm. O depósito era lavado duas vezes em 10ml de soro fisiológico e, depois, semeado no meio de Löwenstein-Jensen com glicerina, em tubos de ensaio com dispositivo que permite a aeração da cultura. O mesmo material também foi semeado no meio de Löwenstein-Jensen sem glicerina, em tubos de ensaio que não permite entrada de ar. As culturas eram incubadas em estufa bacteriológica, a 37°C, durante 2 meses, fazendo-se controles bi-semanais para o registro do crescimento, forma e coloração de colônias suspeitas. Quando estas atingiam desenvolvimento adequado, eram feitos controles microscópicos, em esfregaços e subculturas, para o processo de identificação.

Comportamento bioquímico — Com um único tubo de cultura com crescimento abundante foram feitos os testes de niacina, de catalase à temperatura ambiente e a 68°C e de redução de nitratos, o que permite diferenciar *M. tuberculosis* e *M. bovis* entre si e de outras micobactérias (2, 3).

As micobactérias, ditas atípicas, além do controle da velocidade do crescimento e da produção de pigmento no escuro e na luz, foram submetidas às provas de arilsulfatase (rápida e lenta), hidrólise do Tween (aos cinco e 21 dias), redução de telurito (aos três dias), através das quais era possível reconhecer as micobactérias do complexo *M. avium-intracellulare-scrofulaceum*. As culturas restantes ainda foram submetidas aos testes da urease, da pirazinamidase e da nicotinamidase.

Identificação sorológica — As culturas, que bioquimicamente apresentaram características do complexo *M. avium-intracellulare-scrofulaceum*, foram estudadas sorologicamente (18). Soros e culturas padrões, procedentes do National Institute of Health, Bethesda, Maryland, USA, serviram de referência para os soros pro-

duzidos em nosso laboratório, compreendendo os sorotipos: 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 41, 42 e 43.

Quando necessário, a especificidade da prova de soro-aglutinação foi testada pela absorção de aglutininas (18).

Resultados

A inspeção macroscópica dos dois linfonodos submaxilares em 490 suínos de abate permitiu evidenciar 22 (4,4%) órgãos portadores de lesões semelhantes às da tuberculose. Na maioria dos casos, as lesões apresentam-se sob forma nodular contendo, massa caseosa, ora mais ou menos seca, de cor branca-amarelada, medindo entre um e sete mm de diâmetro. Freqüentemente, havia dois ou mais nódulos no mesmo órgão. Não foi possível diferenciar, pela inspeção macroscópica, as lesões tuberculosas das tuberculóides.

O exame bacteriológico dos 22 materiais permitiu isolar apenas uma cultura de *Mycobacterium bovis*, duas de *M. intracellulare* sorotipo 8, nove de *Corynebacterium equi* e uma de *C. pyogenes.*

O exame bacteriológico de 216 linfonodos, aparentemente normais, permitiu o isolamento de 60 culturas de micobactérias, das quais 56 apresentavam as características culturas e bioquímicas do complexo MAIS e quatro comportavam-se como *M. gordonae*. Foram também isoladas 17 culturas de *Corynebacterium equi*.

A caracterização sorológica das micobactérias do complexo MAIS revelou que 46 amostras pertenciam a 10 sorotipos de *M. intracellulare*, quatro não foram tipáveis e oito se filiaram aos três sorotipos de *M. scrofulaceum*. A Tabela 1 resume as características culturais, bioquímicas e a prevalência dos sorotipos, destacando-se os sorotipos 7 e 15 dos demais, pela alta freqüência.

Tabela 1 — Características culturais, bioquímicas e sorológicas das micobactérias isoladas

N.º de amostras	Vel de Crescim.	Pigmento	Niacina	Catalase T.A. 68°C		Nitratase	Arilsulfase 3 - 14 dias		Tween 5 - 21 dias		Telurito 3 dias	Sorotipos	Espécies
1	Lenta	–	–	+	–	–							M. bovis*
4	"	–	–	+	+	–	–	+	–	–	+	4	M. intracellulare
3	"	–	–	+	+	–	–	+	–	–	+	6	" "
15	"	–	–	+	+	–	–	+	–	–	+	7	" "
6	"	–	–	+	+	–	–	+	–	–	+	8	" "
1	"	–	–	+	+	–	–	+	–	–	+	9	" "
1	"	–	–	+	+	–	–	+	–	–	+	13	" "
12	"	–	–	+	+	–	–	+	–	–	+	15	" "
1	"	–	–	+	+	–	–	+	–	–	+	16	" "
2	"	–	–	+	+	–	–	+	–	–	+	17	" "
1	"	–	–	+	+	–	–	+	–	–	+	18	" "
4	"	–	–	+	+	–	–	+	–	–	+	**	" "
2	"	+	–	+	+	–	(±)	+	–	+	–	41	M. scrofulaceum
4	"	+	–	+	+	–	(±)	+	–	+	–	42	" "
2	"	+	–	+	+	–	–	+	–	+	–	43	" "
4	"	+	–	+	+	–	(±)	+	+	+	–	***	M. gordonae

* Confirmado por inoculação em cobaio
** não tipáveis sorologicamente
*** não identicadas sorologicamente

Discussão

Comparando-se o resultado do exame bacteriológico dos 22 linfonodos portadores de lesões macroscópicas e dos 216 órgãos aparentemente normais verificou-se, com surpresa, maior proporção de isolamento de culturas de micobactérias dos linfonodos macroscopicamente não lesados, respectivamente 13,6% e 25,9%.

O exame bacteriológico dos 22 linfonodos lesados demonstrou que, em apenas três casos (13,6%), foi possível isolar micobactérias; em 10 (45,4%), *Corynebacterium* sp.; e, em nove casos (40,9%) não puderam ser isolados agentes causais. Este achado difere muito ao do en-

contrado no Paraná (8), em que, de 62 linfonodos lesados, foram isoladas 41 (66,1%) culturas de micobactérias. Mas assemelha-se ao de Pernambuco (9), no que se refere à alta incidência de *Corynebacterium equi*.

Por outro lado, comparando-se os resultados observados aos obtidos com a pesquisa realizada com suínos de Brasília (10), verifica-se que, entre estes, há em comum, o alto percentual de lesões das quais não puderam ser isolados agentes etiológicos. A presença de lesões, aparentemente estéreis, poderia ser justificada com as observações feitas por Brooks (4) e Loveday (12), que admitem ocorrer autocuras e resoluções de lesões, em suínos mais velhos. Isto se aplicaria a nosso caso, pois, tratando-se de suínos de raça mista e de desenvolvimento mais lento, estes, ao invés de atingirem o ponto de abate aos seis meses, o alcançam entre a idade de um a dois anos.

O exame bacteriológico de 216 linfonodos submaxilares, aparentemente normais, permitindo o isolamento de 60 micobactérias (23,9% dos órgãos), demonstra que os linfonodos submaxilares também agem como eficiente filtro biológico, para micobactérias com reduzida ação patogênica e assegura, ao suíno, o papel de bom sentinela epidemiológico da tuberculose e das micobacterioses. Castro & col. (5), obtiveram resultado semelhante, partindo de 500 linfonodos mesentéricos, dos quais isolaram 95 culturas de micobactérias (19,0% dos órgãos).

Comparando-se os resultados da tipagem sorológica, verificou-se que, dentre os sorotipos do complexo MAIS, identificados por Castro & col. (5), prevalecem os sorotipos 8, 6 e 7, por ordem de freqüência, enquanto, em nosso trabalho, destacaram-se os sorotipos 7 e 15. Esta divergência está possivelmente relacionada com a procedência dos suínos, aqueles oriundos dos Estados do Paraná e São Paulo e estes do Estado de Minas Gerais, regiões em que diferem as condições ecológicas e o manejo dos animais.

Confrontando-se os achados da tipagem sorológica de micobactérias do complexo MAIS, em nosso trabalho e os de Castro & col. (5), com similares de vários países do hemisfério norte, chama atenção a alta freqüência com que são encontrados os sorotipos 1, 2 e 3 de *M. avium*, por exemplo, Alemanha (16, 21, 23), França (15), Checoslovaquia (6), Hungria (22) e Suécia (19). No Brasil, a tuberculose aviária é rara, pois foram descrito apenas alguns focos, em aves nos Estados de São Paulo (13, 14, 17), Minas Gerais (7), Rio Grande do Sul (20) e em Santa Catarina (1), o que justifica a ausência de *M. avium* em nossos achados.

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Aglutininas anti Yersinia enterocolitica e anti Yersinia pseudotuberculosis em soros humanos*

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Resumo

Pesquisa de anticorpos anti *Y. enterocolitica* dos sorotipos O3, O8 e O9 e anti *Y. pseudotuberculosis* dos grupos OI e OII em soros de 1.609 pacientes. A técnica empregada foi a de aglutinação em lâmina e em tubo, sendo considerados positivos os soros com títulos igual ou superior a 1:80. Os soros com aglutininas anti *Y. enterocolitica* do sorotipo O9 foram absorvidos com antígenos preparados com *Brucella abortus* e aqueles com aglutininas anti *Y. pseudotuberculosis* do grupo OII, com antígenos preparados com *Salmonella schleissheim.* Cerca de 1% dos soros examinados revelaram-se positivos para *Yersinia enterocolitica* (*Y. enterocolitica* O3 - 0,62% e para *Y. enterocolitica* O9 - 0,37%). Observou-se reação cruzada entre *Y. pseudotuberculosis* OII e *Salmonella* do grupo B. Conclui-se pela baixa freqüência desses anticorpos na população examinada.

Summary

Anti Yersinia enterocolitica and anti Y. pseudotuberculosis agglutinins in human sera

A study of the antibodies anti *Yersinia enterocolitica* of serotypes 03, 08 and 09 and anti *Y. pseudotuberculosis* of groups OI and OII, in sera of 1,609 patients. The procedure used was the agglutination on slides and in tubes. The sera considered positive were those with titles equal or superior to 1:80. Sera with agglutinins anti *Y. enterocolitica* of serotype O9 were absorbed by antigens prepared with *Brucella abortus* while those with agglutinins ant *Y. pseudotuberculosis* of groups OII, were absorbed using antigen prepared with *Salmonella schleissheim.* About 1% of the sera examined were positive to *Y. enterocolitica* (0,62% for the serotype O3 and 0,37 for the serotype O9). A cross reaction was observed between *Y. pseudotuberculosis* OII and *Salmonella* of group B. It was thefore established that there was a low incidence of these antibodies among the population examined.

Introdução

As espécies *Y. enterocolitica* e *Y. pseudotuberculosis* fazem parte da família *Enterobacteriaceae* (4).

Segundo Mair (15, 16) e Mollaret (18), a principal entidade clínica devida a *Y. pseudotuberculosis* é a linfoadenite mesentérica aguda, ocorrendo principalmente entre jovens do sexo masculino, na faixa etária compreendida entre 5 e 15 anos. O quadro clínico é de apendicite aguda ou sub-aguda. Na maioria dos casos, a laparatomia revela apêndice normal ou ligeiramente inflamado. os linfonodos mesentéricos, especialmente aqueles do ângulo íleo-cecal, estão inflamados e o mesentérico, freqüentemente, apresenta vermelhidão difusa ou limitada à região dos nódulos linfáticos afetados. Algumas vezes, o íleo e o ceco terminal apresentam edema hiperêmico grosseiro, o qual pode ser confundido com tumor malígno ou Doença de Chron. Ao lado da forma apendicular, aparecem, menos freqüentemente, enterite aguda, intussepção aguda ou eritema nodoso, os quais ocorrem durante o curso de uma linfoadenite mesentérica aguda. Outras formas clínicas, de-

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vidas a *Y. pseudotuberculosis*, são a septicemia e púrpura reumatóide, entre outras menos freqüentes.

Estudos sobre *Y. enterocolitica* são bem mais recentes que os sobre *Y. pseudotuberculosis*, pois somente a partir de 1964 é que a primeira espécie passou a constituir uma entidade bacteriana (6).

De acordo com Mollaret (17), dois terços dos casos de infecção por *Y. enterocolitica* aparecem sob a forma de enterocolite, afetando principalmente crianças de até sete anos. O principal sintoma é a diarréia, que pode ser o único, ou que pode ser acompanhado por dores abdominais difusas. A temperatura varia de normal a 39°C ou mais e há variação das condições gerais. O quadro clínico é muito semelhante ao produzido por *Salmonella, Shigella* ou *E. coli* enteropatogênica. As outras formas clínicas de infecção por *Y. enterocolitica* podem ser: linfoadenite mesentérica, ileíte terminal aguda, eritema nodoso, sendo estas entidades clinicamente indistinguíveis das causadas por *Y. pseudotuberculosis*. Aparece também sob a forma septicêmica e artrítica.

Foram descritas reações cruzadas entre *Y. enterocolitica* do sorotipo O9 com *Brucella* (6) e entre os sorotipos O8, O14, O16, O17, O18 e O21 com diferentes fatores somáticos de *Salmonella* (7).

Vários autores têm demonstrado relações antigênicas entre *Y. pseudotuberculosis* dos grupos OII, OIV e OVI com fatores somáticos de *Salmonella*, de *E. coli* e de *Enterobacter cloacae*, conforme relatado por Falcão & col. (7).

Os sorotipos de *Y. enterocolitica*, que predominam entre aqueles causadores de infecções humanas, em países europeus, Canadá, Japão e África do Sul, são o O3 e o O9 (6). Nos Estados Unidos, há predominância do sorotipo O8, seguido do O5 (13, 27).

No que diz respeito a *Y. pseudotuberculosis*, há grande predominância do sorogrupo OI, seguido do sorogrupo OII (12, 16).

O diagnóstico laboratorial de infecções por *Y. enterocolitica* e *Y. pseudotuberculosis* pode ser bacteriológico, sorológico ou anátomo-patológico.

Essas duas espécies têm sido estudadas em todo o mundo, relatando-se número cada vez maior de isolamentos e de formas clínicas, sob as quais são caracterizadas. Isto não tem ocorrido na América Latina, onde são raras as referências sobre isolamento dessas bactérias, há apenas o relato de isolamento de *Y. pseudotuberculosis*, de caso humano na Argentina (10). Quanto a *Y. enterocolitica*, a espécie isolada a partir de abscessos hepáticos, em macacos, em São Paulo (11, 22); a partir de fezes de porcos, em Botucatu, São Paulo (comunicação pessoal); a partir de fezes de crianças, em Araraquara, São Paulo (24); em São Paulo (8) e no Rio de Janeiro (25).

Em vista da escassez de dados, sobre o isolamento dessas bactérias, em nosso continente, propusemos-nos a investigar se são raramente isoladas entre nós, devido à quase inexistência ou por falta de melhor conhecimento de suas características.

O presente trabalho relata a ocorrência de infecções devidas a *Y. enterocolitica* e *Y. pseudotuberculosis*, em nosso meio, através da pesquisa de aglutininas contra os sorotipos mais freqüentes das duas espécies bacterianas, em soros humanos, procurando detectar contatos anteriores, de nossa população, com as bactérias.

Material e Métodos

1 — *Antígenos testados* — Foram preparados antígenos com amostras de *Y. enterocolitica* dos sorotipos O3, O8 e O9 e com amostras dos grupos OI e OII de *Y. pseudotuberculosis*.

2 — *Preparo dos antígenos* — As amostras foram cultivadas em "Soy Broth", durante 36 horas, a 22°C. Após, foram semeadas em placas de "Blood Agar Base", incubadas durante 36 horas a 22°C e o crescimento retirado com salina formolinizada, a 0,5%. A suspensão, após uma hora de repouso, era centrifugada, a baixa temperatura, durante cinco minutos. A seguir, a massa de células era novamente ressuspensa, em salina formolinizada e centrifugada.

O sedimento, a seguir, era diluído adequadamente, para constituir o antígeno das reações de aglutinação, em lâmina e em tubo.

3 — *Soros examinados* — Foram pesquisados anticorpos contra *Y. enterocolitica* e *Y. pseudotuberculosis*, em 1.609 soros humanos, assim distribuídos:

a — 854 soros a serem submetidos a exames sorológicos de rotina

b — 191 soros de indivíduos com quadro clínico de apendicite aguda, a serem submetidos a cirurgia

c — 559 soros a serem submetidos a provas reumáticas

d — 5 soros de indivíduos com eritema nodoso.

Os soros de indivíduos com eritema nodoso foram colhidos pelo pessoal do Departamento de Dermatologia da Faculdade de Medicina de Ribeirão Preto. São em pequeno número porque poucos foram os pacientes, com esse quadro clínico, que procuraram aquele Departamento, no período em que os estudos foram realizados.

4 — *Pesquisa de anticorpos* — Cada soro foi testado com os cinco antígenos, em aglutinação em lâmina. Para a realização deste teste, os soros eram diluídos a 1:10 e os antígenos usados em solução concentrada padronizada. Este teste serviu como triagem. Todos os soros que aglutinavam, com um ou mais antígenos, eram submetidos a teste de aglutinação em tubo.

Para a realização da aglutinação em tubo, os soros foram diluídos e adicionados dos antígenos, dando a diluição inicial de 1:20. Usou-se antígenos diluídos à concentração semelhante à do tubo 3, da Escala de MacFarland. A incubação foi realizada em banho-maria a 48-50°C, durante 24 horas. Foram consideradas reações positivas aquelas com título superior a 1:80 (29, 30).

Na fase inicial do trabalho, todos os soros eram submetidos, ao mesmo tempo, à reação de aglutinação em tubo e aglutinação em lâmina. Após o estudo de 200 amostras, observou-se perfeita correlação dos resultados das reações positivas para os dois testes. Passou-se então a realizar a triagem, com teste de aglutinação em lâmina e a realizar aglutinação em tubo, apenas dos soros positivos nos testes em lâmina.

5 — *Absorção dos soros* — Os soros positivos, quanto à presença de anticorpos contra *Y. enterocolitica* sorotipo O9 e *Y. pseudotuberculosis* do grupo OII, foram absorvidos, respectivamente, com *Brucella abortus* e *Salmonella* do grupo B (*S. schleissheim* — Fator O27)

a — *Soros anti Y. enterocolitica* O9 (i) — *Preparo do antígeno de Brucella abortus* — Os microrganismos eram semeados em "Trypticase Soy Broth" e incubados a 35-37°C, por 24 horas, em atmosfera de 10% de CO_2 e, posteriormente, semeados em placas de "Tripticase Soy Agar", as quais eram incubadas a 35-37°C, por 24 horas, em atmosfera de CO_2. O crescimento era retirado com salina fenicada a 0,5% e a mistura aquecida em banho-maria fervente, durante uma hora, com agitação constante e, após resfriada, filtrada em papel.

A densidade da suspensão de antígeno foi medida com um espectofotômetro "Colleman Junior II" para dar uma absorbância de 0.30 a um comprimento de onda de 540 mμ (cubeta de 10mm); (ii) — *Absorção* — Misturava-se 2ml de soro diluído a 1:20, com o depósito de antígeno centrifugado (correspondente a 80ml da suspensão do antígeno), homogeneizava-se bem e incubava-se a 37°C, por 2 horas. A seguir, a mistura era centrifugada e o soro testado (3). Realizava-se testes com soro não absorvido e antígeno de *Brucella*, pela técnica do "Test Card" e, com soro absorvido, com antígeno de *Brucella* e de *Y. enterocolitica* O9, em reações de aglutinação em lâmina e em tubo.

b — *Soros anti Y. pseudotuberculosis* OII — (i) — *Preparo do antígeno* — Os antígenos de *Salmonella*, quer para reações de aglutinação, quer para absorção, foram preparados segundo Edwards & Ewing (5); (ii) — *Absorção* — A massa de células de *Salmonella* (provenientes do crescimento em 16 placas de 10mm de diâmetro) era misturada a 0,5ml de soro a ser absorvido e 0,75ml de salina fenolada a 0,5% e incubada em banho-maria a 48°C, por duas horas, após o que a mistura era centrifugada. O soro não absorvido era testado com antígeno de *Salmonella* do grupo B; o soro absorvido com antígenos, quer de *Salmonella* grupo B, quer de *Y. enterocolitica* O9, era testado em reações de aglutinação em lâmina e em tubo.

Resultados e Discussão

Segundo Wauters (26), o sorodiagnóstico constitui precioso mecanismo para estabelecimento da etiologia de infecções por *Y. enterocolitica* e *Y. pseudotuberculosis*. No que diz respeito a *Y. enterocolitica*, a pesquisa de aglutininas é método diagnóstico muito específico, para o sorotipo O3, sendo que, para o sorotipo O9, há necessidade de realizar a absorção, para eliminar o antígeno que possui, em comum com *Brucella abortus*.

Mair (16) diz que anticorpos contra *Y. pseudotuberculosis* podem ser detectados no sangue de pacientes, na fase aguda da doença, sendo que o teste é específico somente para os

sorogrupos I, III e V. A especificidade dos tipos II e IV é afetada pela relação antigênica existente entre esses sorogrupos, com diferentes fatores somáticos de *Salmonella*, havendo, nesse caso, necessidade de realizar a absorção.

Outros autores têm caracterizado as infecções por *Y. enterocolitica* e *Y. pseudotuberculosis*, empregando o sorodiagnóstico (1, 2, 9, 14, 19, 20, 21, 23, 28, 31).

Ao testar 1609 soros, com antígenos de *Y. enterocolitica* (O3, O8 e O9) e de *Y. pseudotuberculosis* (OI e OII), em reações de aglutinação, observou-se que apenas 16 apresentaram anticorpos em títulos significativos, isto é, 1:80 ou maior (0,99%). Essas reações ocorreram somente com os antígenos O3 e O9, de *Y. enterocolitica* e OII, de *Y. pseudotuberculosis*. Não foram observadas aglutininas anti *Y. enterocolitica* O8, que é o sorotipo caracterizado com maior freqüência nos Estados Unidos, nem contra *Y. pseudotuberculosis* do grupo OI, que constitui o sorogrupo responsável pelo maior número de infecções humanas, causadas por esses microrganismos.

A Tabela 1 mostra os resultados de aglutinação em tubo, de 854 amostras de soros do grupo "Sorologia Geral", com os cinco antígenos testados. A Tabela 2 apresenta os resultados de aglutinação em tubo, de 559 amostras de soros do grupo "Provas Reumáticas", com os cinco antígenos testados. A Tabela 3 apresenta os resultados de aglutinação em tubo, de 191 amostras de soros do grupo "Suspeita de Apendicite", com os cinco antígenos testados. Os soros

Tabela 1 — Aglutinação positiva em tubo de 854 amostras de soros não absorvidos do grupo de "Sorologia Geral" com os cinco antígenos testados

Antígenos	Título do Soro								
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
Ye O_3	7	3	5	—	—	—	—	—	—
Ye O_8	—	—	—	—	—	—	—	—	—
Ye O_9	2	3	2	—	—	—	—	—	—
Yp OI	2	—	—	—	—	—	—	—	—
Yp OII	49	28	17	7	2	—	—	—	—

Ye = *Y. enterocolitica* Yp = *Y. pseudotuberculosis*

Tabela 2 — Aglutinação positiva em tubo de 559 amostras de soros não absorvidos do grupo de "Provas Reumáticas" com os cinco antígenos testados

Antígenos	Título do Soro								
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
Ye O_3	6	3	2	1	—	—	—	—	—
Ye O_8	—	—	—	—	—	—	—	—	—
Ye O_9	3	4	1	1	—	—	—	—	—
Yp OI	1	—	—	—	—	—	—	—	—
Yp OII	32	33	13	2	1	—	—	—	—

Ye = *Y. enterocolitica* Yp = *Y. pseudotuberculosis*

Tabela 3 — Aglutinação positiva em tubo de 191 amostras de soros não absorvidos do grupo de "Indivíduos Suspeitos de Apendicite" com os cinco antígenos testados

Antígenos	Título do Soro								
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
Ye O_3	4	3	1	1	—	—	—	—	—
Ye O_8	—	—	—	—	—	—	—	—	—
Ye O_9	1	—	2	—	2	—	—	—	—
Yp OI	—	—	—	—	—	—	—	—	—
Yp OII	8	3	3	3	—	—	—	—	—

Ye = *Y. enterocolitica* Yp = *Y. Yersinia pseudotuberculosis*

dos indivíduos com eritema nodoso apresentaram resultados negativos, quanto à presença de anticorpos contra os cinco antígenos testados. Note-se que esses resultados referem-se a soros não absorvidos.

Os resultados das Tabelas 1, 2 e 3, revelam que 3% do total dos soros examinados apresentaram reações de aglutinação positivas para *Y. pseudotuberculosis* do grupo OII, porcentagem esta bastante superior à apresentada por sorotipos de *Y. enterocolitica*.

No entanto, na totalidade das amostras de soros positivos, as aglutininas anti *Y. pseudotuberculosis* OII desapareceram após absorção com *Salmonella schleissheim*, evidenciando a ocorrência de reações cruzadas, entre o antígeno OII de *Y. pseudotuberculosis* e o fator O27 de *Salmonella*, mostrando assim a inexistência de anticorpos anti *Y. pseudotuberculosis*, dos grupos somáticos I e II, na população examinada.

Os soros com títulos significativos de anticorpos anti *Y. enterocolitica* do sorotipo O9, foram testados também com antígeno de *Brucella*, quando se observou que dois deles (um do grupo "Sorologia Geral" e outro do grupo "Suspeita de Apendicite") também apresentaram reação positiva com esse antígeno, mostrando reação cruzada entre o sorotipo O9 de *Y. enterocolitica* com *Brucella*, confirmando, nos soros examinados, achados de outros autores, conforme descrito em trabalho anterior (7).

Apesar de se ter evidenciada reação cruzada em apenas duas amostras de soros, todos aqueles positivos, para o sorotipo O9 de *Y. enterocolitica*, foram absorvidos com antígeno de *Brucella*. Após a absorção, verificou-se que apenas aqueles dois soros, que também haviam aglutinado com *Brucella*, foram absorvidos. Os outros soros continuaram apresentando reação de aglutinação com antígeno de *Y. enterocolitica* O9, em título significativo.

A Tabela 4 mostra os resultados das reações positivas de aglutinação das 1609 amostras de soros examinados antes e após as absorções. Através desses resultados, observa-se que, após absorção, a porcentagem de reações positivas, para *Y. enterocolitica* O3, foi cerca de duas vezes superior à de *Y. enterocolitica* O9, confirmando, desse modo, dados de autores europeus e canadenses, de que os sorotipos de *Y. enterocolitica*, causadores com maior freqüência de doenças na população humana, é o O3, seguido do O9 (6).

Tabela 4 — Número e percentual de aglutinações positivas dos 1609 soros, antes e após as absorções necessárias, segundo a diluição 1:80 ou maior e segundo os antígenos

Antígeno	Antes		Após	
	Número	%	Número	%
y. *enterocolitica* O_3	10	0,62	10	0,62
y. *enterocolitica* O_8	—	—	—	—
y. *enterocolitica* O_9	8	0,49	6	0,37
y. *pseudotuberculosis* OI	—	—	—	—
y. *pseudotuberculosis* OII	48	2,98	—	—

Tabela 5 — Porcentagens de reações de aglutinação positivas para y. *enterocolitica* O_3 e O_9 nos três grupos de soros estudados

Grupo Sorológico	Antígeno	
	Ye O_3	Ye O_9
Sorologia Geral	0,58%	0,11%
Provas Reumáticas	0,53%	0,35%
Suspeita Apendicite	1,0 %	1,5 %

A Tabela 5 apresenta as porcentagens de reações de aglutinação positivas para *Y. enterocolitica* O3 e O9, nos grupos de "Sorologia Geral", "Provas Reumáticas" e "Suspeitos de Apendicite". Esses resultados mostram que, na população estudada, o grupo com maior porcentagem de reações positivas, para os dois sorotipos de *Y. enterocolitica*, foi aquele de "Suspeita de Apendicite". Como não utilizamos, nesse estudo, soros de indivíduos com quadro clínico anterior de diarréia, não podemos concluir se a síndrome de gastroenterite é a que é mais freqüentemente devida a *Y. enterocolitica*, em nosso meio.

Winblad & col. (31), examinando soros de pacientes submetidos a apencicectomia, com ileíte regional crônica, com diarréia de várias origens e de doadores de sangue e aqueles a serem submetidos a sorologia de rotina (grupo controle), quanto à presença de anticorpos anti

Y. enterocolitica O3, verificaram que as aglutininas eram muito mais freqüentes em pacientes com sintomas de apendicite aguda que em qualquer outro, sendo que, nos pacientes com diarréia, pouco excedia à freqüência do grupo controle. Esses resultados assemelham-se aos apresentados por nós.

Os resultados da presente pesquisa mostram, portanto, a inexistência de anticorpos anti *Y. pseudotuberculosis* dos sorogrupos OI e OII e anti *Y. enterocolitica* do sorotipo O8, na população examinada. Mostra também porcentagens relativamente baixas de anticorpos anti *Y. enterocolitica* dos sorotipos O3 e O9, indicando que, possivelmente, esses microrganismos causam doença em faixa reduzida de nossa população.

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Concentrações inibitórias de cefoxitina sódica para amostras de enterobactérias e Staphylococcus aureus isoladas de infecções humanas no Rio de Janeiro*

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Resumo

Foram determinadas as concentrações inibitórias de cefoxitina sódica para 85 amostras de enterobactérias e 15 de *Staphylococcus aureus,* recentemente isoladas de infecções humanas, ocorridas no Rio de Janeiro. O crescimento de todas as amostras foi inibido na concentração de 25μg/ml, mas quatro amostras cresceram na presença de 12,5μg/ml deste antibiótico.

Summary

Inhibitory concentrations of sodium cefoxitin for Enterobacteriaceae and Staphylococcus aureus

Inhibitory concentrations of sodium cefoxitin for 85 strains of *Enterobacteriaceae* and 15 strains of *Staphylococcus aureus* recently isolated in Rio de Janeiro from human infections were determined. All strains were inhibited at the concentration of 25μg/ml, but 4 strains showed growth in broth containing 12,5μg/ml of the antimicrobial agent.

Introdução

A cefoxitina é um antibiótico beta-lactâmico, semi-sintético, derivado da cefamicina C. Entre suas principais características, destacam-se a resistência à ação das beta-lactamases, produzidas por enterobactérias, como também a excelente atividade sobre *Staphylococcus aureus* e sobre bactérias anaeróbias (2,5,6).

Em 1974, Wallick & Hendlin (7) estudaram a atividade da cefoxitina sobre enterobactérias, verificando que este antibiótico era mais ativo que a cefalotina para amostras de *Escherichia coli Proteus mirabilis* e *Klebsiella* sp.

Com a finalidade de investigar a aplicação da cefoxitina na terapia de infecções humanas, foram desenvolvidos diversos trabalhos em países da Europa e nos Estados Unidos da América do Norte (9). Os resultados obtidos na clínica confirmaram a eficácia do antibiótico, demonstrada inicialmente *in vitro*. No Brasil, o interesse repetiu-se, porque diversos grupos de médicos e microbiologistas estão desenvolvendo investigações voltadas para o mesmo objetivo.

Nosso trabalho relata as concentrações de cefoxitina sódica que inibem enterobactérias e *Staphylococcus aureus*, isolados de infecções humanas no Rio de Janeiro.

Material e Métodos

Foram testadas 100 amostras de bactérias, recentemente isoladas de espécimes provenientes de diversos processos infecciosos hu-

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manos: *Escherichia coli* (15 amostras), *Klebsiella* (15), *Proteus mirabilis* (13), *Proteus* indol-positivo (12), *Salmonella* (15), *Shigella* (15) e *Staphylococcus aureus* (15).

A diluição seriada da cefoxitina sódica (concentrações variando de 0,39 a 100μg/ml) e a determinação das concentrações inibitórias do antibiótico foram realizadas de acordo com Bailey & Scott (1).

Resultados e Discussão

A freqüência acumulada da inibição de bactérias, nas diferentes concentrações do antibiótico, está apresentada na Tabela 1.

Na concentração de 0,39μg/ml não houve inibição do crescimento dos microrganismos testados; entretanto, 25μg de cefoxitina sódica por mililitro inibiram o crescimento de todas as amostras testadas. A concentração de 12,5μg/ml inibiu 96% das amostras testadas; apenas quatro amostras — *E. coli* (1), *P. mirabilis* (1), *Proteus* indol-positivo (1) e *Salmonella* (1) — não foram inibidas nesta concentração.

Parece significativo que as amostras testadas determinem uma freqüência acumulada de 96% de sensibilidade à cefoxitina sódica, na concentração de 12,5μg/ml, considerando-se que Brumfitt & col. (3) e Sommerville & col. (4) observaram que concentrações mais elevadas do antibiótico podem ser obtidas nos fluídos do organismo, após a administração de doses moderadas do antibiótico.

Tabela 1 — Freqüência acumulada das amostras inibidas nas diferentes concentrações de cefoxitina sódica

Microrganismos	N.º de Amostras Testadas	N.º de Amostras Inibidas nas Diferentes Concentrações de Cefoxitina Sódica (μg/ml)								
		0,39	0,78	1,56	3,12	6,25	12,5	25	50	100
Escherichia coli	15	0	12	14	15					
Klebsiella	15	0	4	9	11	12	14	15		
Proteus mirabilis	13	0	2	11	11	12	12	13		
Proteus indol pos.	12	0	2	7	9	11	11	12		
Salmonella	15	0	4	13	14	14	14	15		
Shigella	15	0	11	15						
Staphylococcus aureus	15	0	8	12	13	14	15			
Total	100	0	43	81	88	93	96	100		

Analisando-se o comportamento de diversos gêneros bacterianos testados, é importante ressaltar que todas as amostras de *Shigella* e de *Escherichia* foram inibidas nas concentrações 1,56μg/ml e 3,12μg/ml, respectivamente, demonstrando portanto que, mesmo em concentrações bastante reduzidas, a cefoxitina sódica ainda exerce atividade sobre estes microrganismos.

Em relação às amostras de *Pseudomonas aeruginosa* e *Enterobacter*, isoladas de infecções humanas, Washington (8) caracterizou a ineficácia da cefoxitina, quando investigou as possíveis concentrações inibitórias do antibiótico.

Os resultados obtidos neste trabalho são semelhantes aos descritos por Birnbaum & col. (2), que estabeleceram a concentração de 12,5μg/ml como limite crítico para análise da sensibilidade das bactérias à cefoxitina sódica, favorecendo assim o emprego deste antimicrobiano na maioria das infecções bacterianas humanas.

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