Estela Kaminagakura

Estudo imunohistoquímico das citoqueratinas, do índice de proliferação celular e da resposta inflamatória na Paracoccidioidomicose bucal.

Tese apresentada à Faculdade de Odontologia de Piracicaba, Universidade Estadual de Campinas, para obtenção do grau de Doutor em Estomatopatologia. Área de Estomatologia.

Piracicaba
2004

Estela Kaminagakura

Estudo imunohistoquímico das citoqueratinas, do índice de proliferação celular e da resposta inflamatória na Paracoccidioidomicose bucal.

Tese apresentada à Faculdade de Odontologia de Piracicaba, Universidade Estadual de Campinas, para obtenção do grau de Doutor em Estomatopatologia. Área de Estomatologia.

Orientador: Prof. Dr. Oslei Paes de Almeida

Banca examinadora:

Prof. Dr. Fábio Ramôa Pires
Prof. Dr. Jacks Jorge Júnior
Prof. Dr. José Roberto Pinto
Prof. Dr. Márcio Ajudarte Lopes
Prof. Dr. Oslei Paes de Almeida

Piracicaba
2004

Ficha Catalográfica

Kaminagakura, Estela.
K128e Estudo imunohistoquímico das citoqueratinas, do índice de proliferação celular e da resposta inflamatória na paracoccidioidomic bucal. / Estela Kaminagakura. -- Piracicaba, SP : [s.n.], 2004.
xiii, 70 : il.

Orientador : Prof. Dr. Oslei Paes de Almeida.
Tese (Doutorado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Fungos. 2. Micoses. I. Almeida, Oslei Paes de. II. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. III. Título.

Ficha catalográfica elaborada pela Bibliotecária Marilene Girello CRB/8–6159, da Biblioteca da Faculdade de Odontologia de Piracicaba - UNICAMP.

Aos meus pais, *Minoru e Masako*, pelo modo incondicional e infinito que me amam, por não medirem esforços para me proporcionar todas as oportunidades, por me ensinarem a valorizar a vida, o ser humano, o trabalho e a família. Porque nenhuma conquista seria possível sem vocês.

Aos meus irmãos *Ricardo e Walter* por estarem sempre torcendo por mim.

FACULDADE DE ODONTOLOGIA DE PIRACICABA

UNIVERSIDADE ESTADUAL DE CAMPINAS

A Comissão Julgadora dos trabalhos de Defesa de Tese de DOUTORADO, em sessão pública realizada em 27 de Fevereiro de 2004, considerou a candidata ESTELA KAMINAGAKURA aprovada.

1. Prof. Dr. OSLEI PAES DE ALMEIDA

2. Prof. Dr. FÁBIO RAMÔA PIRES

3. Prof. Dr. JOSÉ ROBERTO PINTO

4. Prof. Dr. MARCIO AJUDARTE LOPES

5. Prof. Dr. JACKS JORGE JUNIOR

Ao Prof. Dr. Oslei Paes de Almeida

por todos os bons exemplos que estou levando. Pela inquestionável competência e seriedade com que exerce a ciência e a docência, mas principalmente pela

simplicidade, humildade e honestidade

que o tornam ainda mais admirável.

Sinto-me privilegiada pela sua orientação.

Ao "tio", com carinho.

Ao Prof. Dr. *José Roberto Pinto*, por acompanhar e investir em cada etapa da minha formação, com a certeza de que cada sonho seria concretizado. A sua amizade tem um valor inestimável para mim.

Ao meu companheiro *Rubens Nísie Tango*

porque a cada dia você me confirma que "não haverá dificuldade que não possamos enfrentar, maus tempos que não sejam desafiados. Também não haverá tristezas que não superemos, doença que não nos ajudemos a suportar, pobreza que não combatamos juntos e riqueza que nos faça esquecer um do outro."

Riba LM

À Faculdade de Odontologia de Piracicaba/UNICAMP, na pessoa do seu diretor Prof. Dr. *Thales da Rocha Mattos Filho*.

Ao Prof. Dr. *Lourenço Correr Sobrinho*, coordenador dos cursos de pós-graduação da Faculdade de Odontologia de Piracicaba/UNICAMP.

Ao Prof. Dr. *Pablo Agustin Vargas*, coordenador do curso de pós-graduação em Estomatopatologia.

À Fundação de Amparo à pesquisa do Estado de São Paulo, *FAPESP*, pela concessão do auxílio financeiro que viabilizou a execução deste trabalho.

À Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, *CAPES*, pela concessão da bolsa de doutorado.

Aos professores das áreas de Patologia e Semiologia da Faculdade de Odontologia de Piracicaba/UNICAMP, *Edgar Graner, Jacks Jorge Júnior, Márcio Ajudarte Lopes, Osvaldo Di Hipólito Júnior, Pablo Agustin Vargas e Ricardo Della Coletta.*

Aos funcionários *Adriano Martins, Cristhiane Rizzo, João Carlos Silva, Rosa Fornasiare* pelo convívio e auxílio constantes. E de modo muito especial à *Ana Cristina do Amaral Godoy* pela colaboração fundamental na parte experimental deste trabalho.

À Universidade Norte do Paraná, na pessoa do coordenador do curso de odontologia Prof. *Fernão de Campos Leite Júnior*.

Às famílias dos tios *Ossamu* e *José Ascânio* e por estarem sempre ao lado da minha família e dispostos a nos ajudarem com desprendimento e alegria.

A *Silvana Pasetto*, *Mitsue Hayacibara*, *Mariliani Chicarelli da Silva* e *Cíntia Carvalhal* por tornarem a distância e a saudade de casa mais amena. Pela convivência harmônica, calorosa e feliz que tive nesses anos. Que os nossos sorrisos e palavras de incentivo nos acompanhem ao longo de nossas vidas. A todas vocês, com muito carinho!!!

Aos **estimados** amigos *Cláudio Maranhão Pereira*, *Danyel Perez* e *Paulo Rogério Ferreti Bonan* por irradiarem felicidade e entusiasmo, por serem acolhedores, companheiros e leais. Por acreditarem que o melhor sempre vai acontecer. Que bom que vocês existem!!!

A *Sandra Boucault Nakao* , *Ângela* e *María Cândida Almeida Lopes*, aos amigos de graduação, *Renato Bonacin Pires*, *Lia Ogawa* e *Josmar Antônio Moreira* porque mesmo distantes as nossas amizades continuam vibrantes.

Ao amigo *Bruno Antônio Giordani* e *Vera Lúcia Martins* pelo companheirismo em todos os momentos.

A toda a equipe do Orocentro: *Cláudio, Danyel, Matues Marchi, Mônica Alcure, Rogério Elias, Maria Aparecida Campion, Débora Gazolla, Katiane Silva, Rosa Scalco* e especialmente ao Prof. Dr. *Márcio Ajudarte Lopes* por nos propocionar um ambiente de trabalho agradável, nos conduzindo de um modo seguro, gentil e amigo que se refletiu em todo grupo. Sentirei muita saudade das terças-feiras.

Ao *Danyel* e *Fábio Ito* pelo auxílio na captura das imagens e ao *Paulo Bonan* pela co-autoria dos trabalhos.

Ao Prof. Dr. *Nelson Fonseca Júnior* pelo auxílio nas análises estatísticas. Ao *Danyllo Antunes* pela revisão dos textos redigidos em português.

A todos os demais colegas da pós-graduação em Estomatopatologia da Faculdade de Odontologia de Piracicaba/UNICAMP, *Ana Lúcia, Dawton, Fábio Ornellas, Juliana, Karina, Michele Pereira, Michelle Agostini, Paola, Roberto e Sabrina*, e de modo especial ao *Jorge Esquiche*, *Francisco Aguiar Júnior* e *Fábio Ito*. Aos amigos de outros departamentos e "**ESALQ**uianos" muito obrigada pela convivência fraterna e pelo incansável apoio que com certeza me encorajou nos momentos difícies.

A paracoccidioidomicose (Pmicose) é uma micose sistêmica, comum na América Latina, com apresentação clínica variável. Na sua forma crônica, freqüentemente envolve a mucosa bucal com lesões múltiplas de aspecto moriforme. Microscopicamente, caracteriza-se por hiperplasia pseudoepiteliomatosa (HPE) e resposta inflamatória granulomatosa, além de acúmulos de neutrófilos no epitélio e no conjuntivo. Os objetivos deste trabalho foram descrever, através da imunohistoquímica, a expressão de citoqueratinas (CKs) e a proliferação celular da HPE, assim como a distribuição das células inflamatórias nos tecidos epitelial e conjuntivo. Adicionalmente, foi descrito um caso clínico de Pmicose bucal. HPE foi evidente nos 28 espécimes de Pmicose bucal oriundas da mucosa jugal, lábio, gengiva e palato duro avaliados quanto à expressão de CKs (AE1/AE3, 34βE12, CK1, CK5, CK6, CK7, CK8, CK10, CK14, CK16, CK18 e CK19) e proliferação celular (Ki-67). Na camada basal, os resultados foram semelhantes para todas as CKs, nos casos de mucosa bucal normal (MBN) e HPEs. As diferenças observadas na camada suprabasal entre a MBN e a Pmicose estão descritas a seguir. Na Pmicose, CK1 e CK10 não foram expressas nas camadas espinhosa e superficial do lábio, gengiva e palato duro; CK14 foi expressa na camada suprabasal da mucosa jugal e lábio; CK6 foi mais freqüentemente expressa apenas na camada espinhosa do lábio, gengiva e palato duro, entretanto a expressão de CK16 foi menor nas camadas espinhosa e superficial da gengiva e palato duro. O índice de proliferação celular foi determinado utilizando-se a marcação imunohistoquímica para Ki-67 e quantificado com o auxílio do analisador de imagem Kontron 400. Os índices de proliferação foram de 7,7 (±3,6) e 28,2 (±9,8) para a MBN e HPE, respectivamente. Assim sendo, na HPE a proliferação celular mostrou-se aumentada, resultando no aumento da espessura e diminuição da queratinização. Para comparação, também foram incluídos 13 casos de displasia epitelial bucal de grau moderado, os quais revelaram índice médio de proliferação de 46,0 (±14,0). Quanto ao infiltrado inflamatório, nos granulomas organizados predominaram as células CD68+, com os linfócitos CD4+ distribuídos na periferia. Nas áreas não

granulomatosas, houve equilíbrio entre células CD4+ e CD8+. Linfócitos B (CD20+) estavam esparsamente distribuídos no tecido conjuntivo inflamado. Células dendríticas (S100+) foram observadas no epitélio, assim como no tecido conjuntivo subepitelial e na periferia dos granulomas organizados. Neutrófilos (CD15+) predominaram nos microabscessos intraepiteliais e nas ulcerações. Adicionalmente, descreve-se um caso clínico de Pmicose que apresentou uma lesão crônica, ulcerada e solitária, com aspecto clínico similar ao carcinoma espino celular em rebordo alveolar.

Palavras-chave: paracoccidioidomicose, citoqueratina, Ki-67, células inflamatórias.

Paracoccidioidomycosis (Pmycosis) is a common systemic mycosis in Latin America, with variable clinical presentation. The chronic form frequently involves the oral mucosa, showing multiple moriform like lesions. Microscopically, oral Pmycosis is characterized by pseudoepitheliomatous hyperplasia (PEH), and granulomatous inflammatory response, besides polimorphonuclear (PMN) accumulation in the epithelium and connective tissue. This work describes, by immunohistochemistry, cytokeratins (CKs) expression and cellular proliferation in PEH, as well as the distribution of inflammatory cells in the epithelial and connective tissues. Additionally, a clinical case of oral Pmycosis is described. PEHs were evident in all 28 oral Pmycosis specimens from the buccal mucosa, lip, gingiva and hard palate used to study CKs expression (AE1/AE3, 34βE12, CK1, CK5, CK6, CK7, CK8, CK10, CK14, CK16, CK18 and CK19) and cell proliferation (Ki-67). In the basal cell layer, the results were similar for all CKs, in normal oral mucosa (NOM) and PEHs. Differences found in the suprabasal layer of NOM and PEH are described below. In Pmycosis, CK1 and CK10 were not expressed in spinous and superficial layers of the lip, gingiva and hard palate. CK14 was positive in suprabasal layer of the buccal mucosa and lip. CK6 was more frequently expressed in spinous layer of the lip, gingiva and hard palate, nevertheless CK16 expression was decreased in the spinous and superficial layers of the gingiva and hard palate. Cellular proliferation index was determined by Ki-67 immunostaining and quantification with the aid of an image analyzer system (Kontron 400). The proliferation index was 7.7 (±3.6) and 28.2 (±9.8) for NOM and PEH, respectively. Therefore in PEH, epithelial proliferation was increased, resulting in a thicker and parakeratotic epithelium. Proliferative index of moderate oral dysplasia, included for comparison was 46.0 (±14.0). Organized granulomas showed a predominance of CD68+ cells, with CD4+ cells at the periphery. Similar number of CD4+ and CD8+ cells were found in non granulomatous areas. B lymphocytes (CD20+) were sparsely distributed throughout the connective tissue. Dendritic cells (S100+) were found in the epithelium, sub-epithelial connective tissue and periphery of organized granulomas. PMN (CD15+) predominated in areas of intraepithelial

microabscesses and ulcerations. Additionally, a clinical case of Pmycosis is described that showed a solitary chronic ulcerated lesion, similar to a spinous cell carcinoma of the alveolar ridge.

Key words: paracoccidioidomycosis, cytokeratin, Ki-67, inflammatory cells.

1.1 Considerações gerais

Lutz (1908) descreveu a paracoccidioidomicose (Pmicose), como uma micose sistêmica profunda, causada pelo fungo dimórfico *Paracoccidioides brasiliensis* (*Pb*), que cresce de forma micelial à temperatura ambiente e em forma de levedura entre 35-37°C (San-Blas, 1985). Essa infecção é endêmica na América Latina, com alta incidência no Brasil, Colômbia e Venezuela (Greer & Restrepo, 1977; Restrepo *et al.*, 2001). No Brasil, os estados que apresentam maior incidência são: São Paulo, Rio de Janeiro e Minas Gerais (Almeida *et al.*, 1991). Nas áreas endêmicas, estima-se que existam dez milhões de pessoas infectadas e que aproximadamente 2% dessa população possam desenvolver a doença (Januário *et al.*, 1999).

O habitat do *Pb* ainda não está totalmente elucidado, o que dificulta o esclarecimento da história natural da doença. No entanto, sugere-se que o fungo viva saprofiticamente na vegetação e nos solos úmidos, locais ricos em matéria orgânica e com mínimas alterações de temperatura (Brummer *et al.*, 1993; RESTREPO *et al.*, 2001). A infecção ocorre pela inalação dos conídios (McEwen *et al.*, 1987), que são estruturas uninucleadas de aproximadamente 4μm, originadas a partir dos micélios, sob condições de estresse como escassez de água e nutrientes (Restrepo, 1985). Esses conídios entram no organismo pelo trato aerodigestivo, instalam-se primariamente na porção distal dos pulmões, onde se transformam em leveduras e, posteriormente, crescem no parênquima pulmonar (McEwen *et al.*, 1987), de onde podem se disseminar por via hematogênica ou linfática para outras vísceras ou mucosas (Almeida *et al.*,1991; Scully & Paes de Almeida, 1992; Almeida *et al.*, 2003).

San-Blas & San-Blas (1977) relacionaram a virulência do *Pb* com a presença de α-1-3 glucana e pequena quantidade de galactomanana na parede celular da levedura, ao passo que fungos mutantes com α-manana perdem seu poder patogênico. Outro aspecto relevante à virulência é a produção de Gp43, uma proteinase de 43kDa que habilita o fungo a aderir aos tecidos, solubilizar a elastina e hidrolisar o colágeno (Stambuk *et al.*, 1988; Mendes-Giannini *et al.*, 1990).

Receptores para laminina na superfície das leveduras parecem estar relacionados com a invasão tecidual (Mendes-Giannini *et al.*, 1990). Experimentos *in vitro* e com camundongos mostraram que conídios e leveduras do *Pb* são capazes de sintetizar um pigmento semelhante à melanina, o qual especula-se exercer um papel importante na patogênese da Pmicose (Gómez *et al.*, 2001; Gómez & Nosanchuk, 2003).

Há uma predileção distinta para homens com relação de 30:1 (BICALHO *et al.*, 2001), embora o contato com o fungo ocorra em freqüência igual para ambos os sexos, visto que um número igual de homens e mulheres têm anticorpos contra ele (Brummer *et al.*, 1993). Loose *et al.* (1983) demonstraram que o *Pb* apresenta receptores específicos para hormônios esteróides, principalmente para o 17 β-estradiol. A presença desse hormônio em mulheres talvez impeça a transformação da forma micelial em levedura, justificando a reduzida freqüência da Pmicose nesse gênero (Loose *et al.* 1983).

A habilidade do linfócito Th-1 em induzir reação de hipersensibilidade tardia parece ser o principal mecanismo pelo qual o organismo se defende do *Pb* (Musatti *et al.*, 1994). A eficiência dessa resposta resulta na formação de granulomas bem organizados capazes de restringir a disseminação da levedura (Benard *et al.*, 2001; Oliveira *et al.*, 2002; Romano *et al.*, 2002), enquanto que a resposta tipo Th-2 é observada em pacientes com a forma severa da doença e parece estar associada com diminuição na função das células T, ativação policlonal de células B e altos níveis de produção de anticorpos (Mota *et al.*, 1988; Musatti *et al.*, 1994; Benard *et al.*, 2001).

A Pmicose pode ser classificada em duas formas polares principais, baseando-se na resposta imunológica do paciente. A forma polar hiperérgica é caracterizada por uma infecção localizada, o título de anticorpos específicos ao *Pb* é baixo ou ausente, a imunidade celular é preservada e as lesões granulomatosas são compactas e com poucos fungos (Franco *et al.*, 1993). Na forma polar anérgica, as lesões são generalizadas, há elevado título de anticorpos específicos

aos antígenos de *Pb,* imunidade celular enfraquecida e presença de reação granulomatosa não organizada contendo várias leveduras (Franco *et al.*, 1993).

A Pmicose pode se apresentar na forma de infecção sub-clínica ou doença clinicamente presente. Essa infecção é caracterizada pela ausência de sinais e sintomas clínicos, embora haja a evolução de uma resposta imune específica, evidenciada pelo teste intradérmico da paracoccidioidina (Lacaz *et al.*, 1956). São reconhecidas duas formas de doença: a aguda ou juvenil e a crônica ou adulta. A forma aguda representa apenas 3 a 5 % dos casos, possui curso rápido, envolvimento do sistema mononuclear fagocitário e resposta imune celular severamente deprimida. O quadro clínico é caracterizado pela hipertrofia dos órgãos do sistema mononuclear fagocitário e disfunção da medula óssea. Ao exame histopatológico, freqüentemente são observados muitos *Pb* em brotamento e não há a formação de granulomas (Franco *et al.*, 1987). A forma crônica tem duração prolongada, progride lentamente e as lesões podem permanecer localizadas (forma focal) ou envolver mais de um órgão ou sistema (forma multifocal) (Franco *et al.*, 1987). Em cerca de 90% dos casos, há envolvimento pulmonar radiograficamente detectável e as lesões nesses pacientes tendem a formar granulomas organizados (Londero *et al.*, 1978; Franco *et al.*, 1993).

A Pmicose foi considerada uma desordem incurável até 1940, quando as sulfonamidas foram introduzidas para seu tratamento (Brummer *et al.*, 1993). Essas drogas são eficazes e possuem baixo custo, porém, possuem a vida média sérica curta, dificultando sua posologia diária e, consequentemente, os episódios de recidivas são mais freqüentes (Brummer *et al.*, 1993; Shikanai-Yasuda *et al.*, 2002). Há também relatos da ocorrência de hematúria e obstrução renal em decorrência da formação de cristalúria (Shikanai-Yasuda *et al.*, 2002). Outros agentes antimicrobianos promovem a resolução das lesões sistêmicas e orais com muita eficácia, como, por exemplo, o cetoconazol, que também pode ser empregado em associação com as sulfonamidas (Almeida *et al.*, 1991). O cetoconazol requer um pH ácido para ser adequadamente absorvido, sendo, portanto, uma droga contra-indicada para pacientes que fazem uso de antiácidos e

β bloqueadores (Brummer *et al.*, 1993). Para pacientes com a forma severa, juvenil da doença, normalmente prescreve-se itraconazol, um anti-fúngico que pode ser administrado em dose única diária e não apresenta efeitos colaterais sérios (Shikanai-Yasuda *et al.*, 2002). A Pmicose também pode ser tratada com fluconazol oral ou miconazol por via intravenosa (Almeida *et al.*, 1991). A anfotericina B pode ser utilizada na forma grave e disseminada da doença. Entretanto, dificuldades na administração e efeitos colaterais tais como febre, calafrio, cefaléia, náusea, vômitos, flebite e toxicidade renal acabaram por limitar sua indicação (Lazow *et al.*, 1990; Brummer *et al.*, 1993). A ausência da superioridade de qualquer droga no tratamento da Pmicose faz necessária a avaliação dos efeitos colaterais produzidos pelas mesmas antes da sua indicação (Shikanai-Yasuda *et al.*, 2002). Uma das maiores complicações durante a fase de tratamento é o desenvolvimento de fibrose severa que, freqüentemente, causa dano funcional permanente do órgão afetado como, por exemplo, fibrose pulmonar e doença de Addison (Del Negro *et al.*, 1961; Afonso *et al.*, 1979; Shikanai-Yasuda *et al.*, 2002).

1.2 Paracoccidioidomicose bucal

Evidências indicam que a presença de lesões bucais sejam conseqüência da disseminação do fungo pelo organismo a partir do foco primário pulmonar (Almeida *et al.*, 2003). As lesões podem envolver principalmente as regiões de cabeça e pescoço, especialmente a pele da face, a mucosa nasal e bucal (Almeida *et al.*, 1991), justificando a importância do estudo das manifestações bucais da Pmicose. Cerca de 50% dos casos de Pmicose apresentam manifestações bucais como queixa principal.

Clinicamente, a Pmicose apresenta-se na mucosa bucal como áreas eritematosas, ulceradas, com pequenos pontos amarelados interpostos a áreas avermelhadas, dando um aspecto granular, denominado inicialmente de moriforme por Motta & Pupo (1936), posteriormente confirmado por diversos autores (Salman & Sheppard, 1962; Joseph *et al.*,1966; Limongelli *et al.*, 1978; Lazow *et al.*, 1990;

Almeida *et al.*, 1991; Sposto *et al.*, 1993; Almeida *et al.*, 2003). Na Pmicose juvenil, podem ocorrer alterações que mimetizam um quadro de doença periodontal com destruição progressiva e generalizada do osso alveolar, podendo causar recessão gengival, exposição das raízes, mobilidade e perda de elementos dentários (Migliarl *et al.*, 1998). Além disso, pode haver comprometimento dos linfonodos cervicais, com ulceração da pele que os recobre. Usualmente as lesões são múltiplas, envolvendo os lábios, gengiva, mucosa jugal, palato, língua e soalho de boca (Sposto *et al.*, 1994; Almeida *et al.*, 2003).

Na Pmicose bucal, a mucosa é revestida por epitélio estratificado escamoso que, freqüentemente, exibe microabcessos e hiperplasia pseudoepiteliomatosa, podendo simular a proliferação celular observada nos carcinomas espino celulares (Meneses-Garcia *et al.*, 2002; Almeida *et al.*, 2003). O tecido conjuntivo exibe reação inflamatória granulomatosa caracterizada por coleção de macrófagos, células epitelióides e células gigantes multinucleadas que, geralmente, ocupam o centro do granuloma com linfócitos, plasmócitos e eosinófilos freqüentemente ao redor desse aglomerado (Salman & Sheppard, 1962; Joseph *et al.*, 1966; Limongelli *et al.*, 1978; Moscardi-Bacchi *et al.*, 1989; Lazow *et al.*, 1990; Almeida *et al.*,1991; Villalba, 1998; Meneses-Garcia *et al.*, 2002; Almeida *et al.*, 2003). Nos granulomas amadurecidos, fibroblastos e fibras colágenas estão dispostos mais externamente (Coelho *et al.*, 1994). As células gigantes também são visualizadas fora do granuloma entre as células mononucleares, podendo ser do tipo Langhans ou células gigantes de corpo estranho. As leveduras podem ser ovais ou redondas, com diâmetro que varia de 5 a 30 μm, com dupla parede e múltiplos brotamentos e podem ser encontradas dentro e fora das células gigantes multinucleadas (Salman & Sheppard, 1962; Joseph *et al.*, 1966; Limongelli *et al.*, 1978; Lazow *et al.*, 1990; Almeida *et al.*,1991; Villalba, 1998; Meneses-Garcia *et al.*, 2002; Almeida *et al.*, 2003).

Os objetivos desse trabalho foram:

2.1. Analisar a expressão das citoqueratinas, AE1/AE3, 34βE12, CK1, CK5, CK6, CK7, CK8, CK10, CK14, CK16, CK18 e CK19 na hiperplasia pseudoepiteliomatosa presente na Pmicose bucal e compará-la com a mucosa bucal normal;

2.2. Comparar o índice de proliferação celular na hiperplasia pseudoepiteliomatosa, epitélio bucal normal e epitélio bucal com displasia moderada;

2.3. Descrever e quantificar as células inflamatórias presentes nas lesões bucais da paracoccidioidomicose;

2.4. Relatar um caso clínico de paracoccidioidomicose que mimetizava um carcinoma espino celular na região de rebordo alveolar inferior.

Cytokeratin expression in pseudoepitheliomatous hyperplasia in oral paracoccidioidomycosis

Kaminagakura E; Bonan PRF, Almeida OP*

Department of Oral Pathology, University of Campinas; Dental School, Piracicaba-SP, Brazil.

Short title: Cytokeratins in Paracoccidioidomycosis.

Supported by: FAPESP, Brazil.

*Corresponding author: Oslei Paes de Almeida, Department of Oral Pathology, University of Campinas; Dental School, Caixa Postal 52, CEP:13414-903. Piracicaba-SP, Brazil.

E-mail: oslei@fop.unicamp.br

Tel: +55-19-3412-5316.

Fax: +55-19-3412-5218.

Abstract

Expression of CKs in basal layer of the epithelium was similar in normal oral mucosa (NOM) and pseudoepitheliomatous hyperplasia of Pmycosis. Differences found in the spinous and superficial layers of NOM and pseudoepitheliomatous hyperplasia are described below. CK1 and CK10 were not expressed in the spinous and superficial layers of the lips, gingiva and hard palate of Pmycosis. Contrary to NOM, CK14 was positive in the spinous and superficial layers of the lip and buccal mucosa. In Pmycosis, CK6 was more frequently expressed in the spinous layer of the lip, gingiva and hard palate, nevertheless CK16 expression was decreased in the spinous and superficial layers of the gingiva and hard palate. The absence of CK1 and CK10 expression in Pmycosis can be related to the loss of keratinization in areas of pseudoepitheliomatous hyperplasia. Increased expression of CK14 can be due to effects of cytokines of inflammatory cells and/or decreased maturation of the epithelial cells in the spinous and superficial layers, maintaining some characteristics of the basal cells. We do not have a reasonable explanation for the apparently contradictory results of CK6 and CK16 but it can be a consequence of the higher cellular proliferative index of pseudoepitheliomatous hyperplasia. In summary, pseudoepitheliomatous hyperplasia of oral Pmycosis show a different pattern of CKs expression in relation to NOM, however further studies are necessary for a better understanding of the subject.

Key words: paracoccidioidomycosis, cytokeratins, oral mucosa, pseudoepitheliomatous hyperplasia.

Introduction

Paracoccidioidomycosis (Pmycosis) is one of the most common deep mycosis in many regions of Latin America (1,2). It is caused by *Paracoccidioides brasiliensis* (*Pb*) a dimorphous fungus, found in soil, although its precise habitat must yet to be determined (1,2). Humans are infected by inhalation of conidia, that in the lungs develops into yeast cells that can disseminate to lymph nodes, mucous membrane, skin, bone and adrenal (3,4). Clinically the disease has a wide spectrum of presentation, but a chronic form (adult type) is the most common, classically affecting the lungs of adult males, and in about half of the cases also involving the mouth (2,4-7). Oral mucosa lesions are commonly multiple, with a granular, mulberry like surface and microulcerations (5-10). Microscopically the granulomatous inflammatory reaction shows granulomas with giant cells, lymphocytes, PMN, eosinophils and intra and extracellular fungi cells, corresponding to *P. brasiliensis* (6-10).

The epithelium shows exocytosis, microabscesses and pseudoeptheliomatous hyperplasia that can mimic and be mistaken as neoplastic (9,10). Cytokeratins (CKs) are a group of intermediate filaments of epithelial cells, whose expression varies according to the epithelium type, differentiation and pathological process (11-15). The objective of this work was to assess by immunohistochemistry, the pattern of CKs expression in pseudoepitheliomatous hyperplasia of oral paracoccidioidomycosis (Pmycosis). This is the first report of CKs expression in pseudoepitheliomatous hyperplasia of oral Pmycosis.

Materials and Methods

Twenty eight specimens of normal oral mucosa (NOM) and 28 of oral Pmycosis were retrieved from the files of the Department of Oral Pathology, University of Campinas Dental School. The cases were divided in four groups of 14 cases each, corresponding to buccal mucosa, lip, gingiva and hard palate. Normal mucosa patients included 14 males and 14 females, age ranged from 18 to 59

years. All patients with Pmycosis were male, age ranged from 25 to 68 years. Microscopically, all 28 cases of Pmycosis showed typical pseudoepitheliomatous hyperplasia which was characterized by elongated rete pegs that extended into the underlying connective tissue. The stratified epithelium from NOM and Pmycosis was histologically divided into three compartments: basal, spinous and superficial.

Briefly, for immunohistochemical evaluation of CKs expression, 3μm paraffin sections were hydrated and treated with hydrogen peroxide for 30 min to inhibit endogenous peroxidase. Microwave antigen retrieval and overnight incubation with the primary antibodies were performed in all cases. A list of anti-CKs antibodies used is shown on Table 1. Secondary antibodies conjugated to streptavidin-biotin-peroxidase (StrepABC Complex/HRP Duet kit, Dako A/S, Denmark) were used, followed by diaminobenzidine (DAB, Sigma) as the chromogen. Slides were counterstained with Carazzi haematoxylin. Positive and negative controls were included in all reactions. The study protocol was approved by the Ethical Committee in Research (122/2001) of the University of Campinas Dental School.

Results

Histopathology

The epithelium was acanthotic, infiltrated by inflammatory cells forming microabscesses (Fig 1). The connective tissue presented multinucleated giant cells in granulomas variably organized, and dense infiltration of lymphocytes, some plasma cells, PMN and eosinophils. *Pb* was found in all cases mainly in the connective tissue, either intra or extracellularly. NOM followed the characteristics of each site. Keratinized stratified epithelium was observed in the gingiva and hard palate and non-keratinized stratified epithelium was found in lip and buccal mucosa.

Immunohistochemical findings

NOM

All cases of NOM were strongly positive in all epithelial layers for AE1/AE3 and 34βE12. On the other hand, they did not express CK7, CK8, CK18 and CK19.

In buccal mucosa, which is not keratinized, CK1 and CK10 were negative In contrast, all cases of the hard palate were positive for CK1 (Fig 2A). In the gingiva, CK1 was observed in 85.7% and 71.4% of cases in spinous and superficial layers, respectively. In the lip, it was found in 57.1% in spinous and 42.8% in the superficial layers. CK10 was strongly expressed in the hard palate, gingiva and lip mainly in the superficial layer (Fig 3A and Table 2).

CK5 was found in all layers of all cases of NOM. CK14 was only expressed in the basal cells of the buccal mucosa (Fig 4A), however it was detected in all compartments of the hard palate and gingiva. On the lip, 100% and 28.5% of cases were positive for CK14 in the basal and spinous layers, respectively (Table 3).

CK6 was weakly positive in the spinous layer in all cases of the buccal mucosa; 42.8% of the hard palate, 57.1% of the lip and 71.4% of the gingiva. In the superficial compartment, CK6 was expressed in 14.2% of the cases of the buccal mucosa and lip and in 28.57% of the gingiva it was positive in basal and spinous layers. CK16 expression was observed in spinous layer of 14.2%, 57.1%, 85.7% and 100% of the buccal mucosa, lip, gingiva and hard palate respectively. In the superficial layer, CK16 was found in 71.4% of the gingiva and 85.7% of the hard palate (Table 4).

Pmycosis

As in NOM, AE1/AE3, 34βE12 and CK5 were expressed in all layers of Pmycosis, while CK7, CK8, CK18 and CK19 were negative. In the basal layer, the results were also similar for all CK in NOM and Pmycosis. Contrary to normal hard palate, gingiva and lip, CK1 and CK10 were negative in all cases of Pmycosis (Figs 2B and 3B; Table 2). CK14 was strongly positive in basal, spinous and superficial layers of all cases of the buccal mucosa and lip of Pmycosis (Fig 4B), however in

the adjacent areas of sub-epithelial inflammation it was only found in the basal layer. In the hard palate and gingiva, CK14 expression was similar to NOM (Table 3).

CK6 was seen in the spinous layer of all cases of pseudoepitheliomatous hyperplasia of the lip, gingiva and hard palate, where copious acanthotic areas were observed. In 71.4% of the buccal mucosa, CK6 was positive in the spinous layer. Superficial layers expressed CK6 in 14.2% and 28.5% of cases of the gingiva and hard palate respectively. CK16 expression was not evident as in NOM, except in buccal mucosa and lip. It was expressed in the spinous layer in 71.4% of cases of the buccal mucosa, 42.8% of the gingiva and 57.1% of the hard palate and lip. Contrary to NOM, CK16 was negative in the superficial layer of the hard palate and gingiva (Table 4).

Discussion

As in NOM, AE1/AE3, 34βE12 and CK5 were expressed in all cases of Pmycosis in the basal, spinous and superficial layers. AE1 recognizes CK1 to CK8, AE3 recognizes CK9 to CK20 and 34βE12 identifies CKs of 66 to 55kDa (CK1, 5, 10 and 14) (11,12). CK7, CK8, CK18 and CK19 were not found in NOM and Pmycosis as expected, because these keratins are typically expressed in simple epithelia (11).

CK1 and CK10, differentiation keratins pair, were present in spinous and superficial layers of normal hard palate and gingiva, confirming previous studies (11,16). They are markers of keratinization (11) and their expression by masticatory mucosa and epidermis appears to be associated with epithelial toughness and rigidity (17) and may be decreased with inflammation and in hyperproliferative lesions (18). Expression of CK1 was stronger and more frequent than CK10 in normal hard palate and gingiva. It is not clear why there is such dominance of CK1 over CK10 in NOM and in keratotic lesions (19). Different from NOM, CK1 and CK10 were absent in pseudoepitheliomatous hyperplasia. It can be speculated that lack of these keratins may be associated with inflammation and

might contribute to increase epithelial fragility (17), facilitating ulcerations common in oral Pmycosis.

CK5 and CK14 are the major keratins in the basal layer (20). They are considered important resisting shear forces, thus large amounts of these filaments are present in keratinized epithelium (21). Our results showed similar CK5 expression in all compartments of all specimens of NOM and Pmycosis. CK14 showed similar results, except in buccal mucosa and lip from Pmycosis. It is interesting that in areas of intense sub-epithelial inflammation in the buccal mucosa and lip of Pmycosis, CK14 was expressed in the three compartments, suggesting that cytokines from inflammatory cells can alter its expression in the suprabasal layer (18).

CK6 and CK16, high turnover keratins (22,23), are positive in palmar and plantar epidermis, buccal mucosa, gingiva, palate (16,24), hyperproliferative skin disorders (22) and wound healing (23). Their expression is important to create a cytoskeleton more compatible with cell division and to enable a greater number of divisions before keratinocyte differentiation (18). According to previous studies, CK6 and CK16 expression in hyperproliferating epidermal cells, might inhibit expression of CK1 and CK10 (18,25,26). CK16 could act on retinoblastoma protein hyperphosphorylation promoting keratinocyte proliferation while CK10 plays an opposite role (18,25). Discordance between CK6 and CK16 expression has been reported (27) and our cases showed that CK6 had a tendency to increase in the spinous layer of pseudoepitheliomatous hyperplasia, however CK16 seemed to be decreased. They can be related to cell proliferation but the results found in our work are not sufficient for a reasonable explanation.

Histologically, pseudoepitheliomatous hyperplasia present characteristic similar to well-differentiated carcinoma, however without tendency to keratinization. It also shows a more intense intraepithelial inflammatory infiltration, frequently forming microabscesses. Nevertheless differences of CKs expression have been described (13,15,28). Well-differentiated oral squamous cell carcinoma expresses variable combinations of CK1, CK10, CK4 and CK13, whereas poorly differentiated

tumors predominantly expresses CK8, CK18 and CK19 (15,27). Chu et al (29) studied squamous cell carcinoma from different origin and degree of differentiation and reported that over 90% of cases expressed CK14. On the other hand, CKs expression in benign hyperplastic lesions of oral cavity, do not differ significantly from the corresponding NOM. The main difference is a higher suprabasal expression of CK14 (30,31), also found in pseudoepitheliomatous hyperplasia in non-keratinized mucosa.

In summary, alterations of CKs pattern expression were observed in oral Pmycosis, mainly CK1, CK10 and CK14 comparing with NOM. Absence of CK1 and CK10 expression in pseudoepitheliomatous hyperplasia might contribute for decreasing epithelium resistance, facilitating ulceration and cell proliferation. The mechanisms that control CK14 expression in non-keratinized epithelium might be altered in Pmycosis. Factors involved in the development of pseudoepitheliomatous hyperplasia are not yet known, but cytokines of inflammatory cells either in the epithelium and sub-epithelial connective tissue possibly play a major role.

Acknowledgements

The authors thank Ana Cristina do Amaral Godoy (University of Campinas Dental School) for the technical assistance with the immunohistochemical procedures.

References

1. Restrepo A, McEwen JG, Castañeda E. The habitat of *Paracoccidioides brasiliensis*: how far from solving the riddle? Review article. *Med Mycol* 2001;**39**:233-41.
2. Brummer E, Castaneda E, Restrepo A. Paracoccidioidomycosis: An update. *Clinical Microbiol Reviews* 1993;**6**:89-117.

3. McEwen JG, Bedoya V, Patiño MM, Salazar ME, Restrepo A. Experimental murine paracodioidomycosis induced by the inhalation of conidia. *J Med Vet Mycol* 1987;**25**:165-75.
4. Borges-Walmsley MI, Chen D, Shu X, Walmsley AR. The Pathobiology of *Paracoccidioides brasiliensis. TRENDS in Microbiol* 2002;**10**:80-7.
5. Sposto MR, Scully C, Almeida OP, Jorge J, Graner E, Bozzo L. Oral paracoccidioidomycosis. A study of 36 South American patients. *Oral Surg Oral Med Oral Pathol* 1993;**75**:461-5.
6. Almeida OP, Jorge Jr J, Scully C, Bozzo L. Oral manifestation of paracoccidioidomycosis (South American Blastomycosis). *Oral Surg Oral Med Oral Pathol* 1991;**72**:430-5.
7. Scully C, Paes de Almeida O. Orofacial manifestations of the systemic mycoses. *J Oral Pathol Med* 1992;**21**:289-94.
8. Bacchi MM, Soares A, Mendes R, Marques S, Franco M. In situ localization of T lymphocyte subsets in human paracoccidioidomycosis. *J Med Vet Mycol* 1989;**27**:149-58.
9 Meneses-Garcia A, Mosqueda-Taylor A, Luz RM, Rivera LMRG. Paracoccidioidomycosis: Report of 2 cases mimicking squamous cell-carcinoma. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2002;**94**:609-13.
10. Almeida OP, Jorge Jr J, Scully C. Paracoccidioidomycosis of the mouth: An emerging deep mycosis. *Crit Rev Oral Biol Med* 2003;**14**:377-83.
11. Eicher R, Bonitz P, Sun TT. Classification of epidermal keratins according to their immunoreactivity, isoelectric point, and mode of expression. *J Cell Biol* 1984;**98**:1388-96.
12. Cooper D, Schermer A, Sun TT. Classification of human epithelia and their neoplasms using monoclonal antibodies to keratins: strategies, applications, and limitations. *Lab Invest* 1985;**52**:243-56.

13. Odgen GR, Chisholm DM, Adi M, Lane EB. Cytokeratin expression in oral cancer and its relationship to tumor differentiation. *J Oral Pathol Med* 1993;**22**:82-6.
14. Su L, Morgan PR, Lane EB. Keratin 14 and 19 expression in normal, dysplastic and malignant oral epithelia. A study using *in situ* hybridization and immunohistochemistry. *J Oral Pathol Med* 1996;**25**:293-01.
15. Heyden A, Huitfeldt HS, Koppang HS, Thrane PS, Bryne M, Brandtzaeg P. Cytokeratins as epithelial differentiation markers in premalignant and malignant oral lesions. *J Oral Pathol Med* 1992;**21**:7-11.
16. Clausen H, Moe D, Buschard K, Dabelsteen E. Keratin proteins in human oral mucosa. *J Oral Pathol Med* 1986;**15**:36-42.
17. Mackenzie IC, Rittman G, Gao Z, Leigh I, Lane EB. Patterns of cytokeratin expression in human gingival epithelia. *J Periodont Res* 1991;**26**:468-78.
18. Stoler A, Kopan R, Duvic M, Fuchs E. Use of monospecific antisera and cRNA probes to localize the major changes in keratins expression during normal and abnormal epidermal differentiation. *J Cell Biol* 1988;**107**:427-46.
19. Bloor BK, Su L, Shirlaw PJ, Morgan PR. Gene expression of differentiation-specific keratins (K4, K13, K1 and K10) in normal human buccal mucosa. *Lab Invest* 1998;**78**:787-95.
20. Purkis PE, Steel JB, Mackenzie IC, Nathrath WBJ, Leigh IM, Lane EB. Antibody markers of basal cells in complex epithelia. *J Cell Sci* 1990;**97**:39-50.
21. Fuchs E. The cytoskeleton and disease: Genetic disorders of intermediate filaments. *Annu Rev Genet* 1996;**30**:197-31.
22. Weiss RA, Eichner R, Sun TT. Monoclonal antibody analysis of keratin expression in epidermal diseases: a 48-and 56-kdalton keratin as molecular markers for hyperproliferative keratinocytes. *J Cell Biol* 1984;**98**:1397-406.
23. Mansbridge JN, Knapp AM. Changes in keratinocyte maturation during wound healing. *J Invest Dermatol* 1987;**89**:253-62.

24. Moll R, Franke WW, Schiller DL, Geiger B, Krepler R. The catalog of human cytokeratins: Patterns of expression in normal epithelia, tumors and cultured cells. *Cell* 1982;**31**:11-24.
25. Paramio JM, Casanova ML, Segrelles C, Mittnacht S, Lane EB, Jorcano JL. Modulation of cell proliferation by cytokeratins K10 and K16. *Mol Cell Biol* 1999;**19**:3086-94.
26. Paramio JM, Jorcano JL. Beyond structure: do intermediate filaments modulate cell signalling? *BioEssays* 2002;**24**:836-44.
27. Hansson A, Bloor BK, Haig Y, Morgan PR, Ekstrand J, Grafström RC. Expression of keratins in normal, immortalized and malignant oral epithelia in organotypic culture. *Oral Oncol* 2001;**37**:419-30.
28. Morgan PR, Shirlaw PJ, Johnson NW, Leigh IM, Lane EB. Potential applications of anti-keratin antibodies in oral diagnosis. *J Oral Pathol* 1987;**16**:212-22.
29. Chu PG, Lyda MH, Weiss LM. Cytokeratin 14 expression in the epithelial neoplasms: a survey of 435 cases with emphasis on its value in differentiating squamous cell carcinomas from other epithelial tumours. *Histopathology* 2001;**39**:9-16.
30. Schulz J, Ermich T, Kasper M, Raabe G, Schumann D. Cytokeratin pattern of clinically intact and pathologically changed oral mucosa. *Int J Oral Maxillofac Surg* 1992;**21**:35-9.
31. van der Velden LA, Manni JJ, Ramaekers FCS, Kuijpers W. Expression of intermediate filament proteins in benign lesions of the oral mucosa. *Eur Arch Otorhinolaryngol* 1999;**256**:514-9.

Table 1. List of primary antibodies

Keratin	Dilutions	Clone	Supplier
1	1:200	34βB4	Novocastra Lab. Ltda, Newcastle,UK
5	1:400	XM 26	Novocastra Lab. Ltda, Newcastle, UK
6	1:200	LHK6B	Novocastra Lab. Ltda, Newcastle, UK
7	1:400	OV-TL 12/30	Dako Corp. Carpenteria, CA, USA
8	1:200	35βH11	Dako Corp. Carpenteria, CA, USA
10	1:200	DE-K10	Dako Corp. Carpenteria, CA, USA
14	1:200	LL002	Novocastra Lab. Ltda, Newcastle, UK
16	1:200	LL025	Novocastra Lab. Ltda, Newcastle, UK
18	1:400	DC10	Dako Corp. Carpenteria, CA, USA
19	1:400	RCK108	Dako Corp. Carpenteria, CA, USA
Pan-keratin	1:200	34βE12	Dako Corp. Carpenteria, CA, USA
Pan-keratin	1:500	AE1/AE3	Dako Corp. Carpenteria, CA, USA

Table 2. Percentage of CK1 and CK10 expression in normal oral mucosa (NOM) and in pseudoepitheliomatous hyperplasia of Pmycosis. Each group of buccal mucosa, lip, gingiva and hard palate is formed by 14 cases.

Group/epithelial layer	CK1						CK10					
	Basal layer		Spinous layer		Superficial layer		Basal layer		Spinous layer		Superficial layer	
	NOM	PCM	NOM	PCM	NOM	PCM	NOM	PCM	NOM	PCM	NOM	PCM
Buccal mucosa	0	0	0	0	0	0	0	0	0	0	0	0
Lip	0	0	57.1	0	42.8	0	0	0	42.8	0	57.1	0
Gingiva	0	0	85.7	0	71.4	0	0	0	42.8	0	85.7	0
Hard Palate	0	0	100	0	100	0	0	0	85.7	0	100	0

Table 3. Percentage of Ck14 expression in normal oral mucosa (NOM) and in pseudoepitheliomatous hyperplasia of Pmycosis. Each group of buccal mucosa, lip, gingiva and hard palate is formed by 14 cases.

Group/epithelial layer	Basal layer		Spinous layer		Superficial layer	
	NOM	PCM	NOM	PCM	NOM	PCM
Buccal mucosa	100	100	0	100	0	100
Lip	100	100	28.5	100	0	100
Gingiva	100	100	100	100	100	85.7
Hard palate	100	100	100	100	100	100

Table 4. Percentage of CK6 and CK16 expression in normal oral mucosa (NOM) and in pseudoepitheliomatous hyperplasia of Pmycosis. Each group of buccal mucosa, lip, gingiva and hard palate is formed by 14 cases.

Group/epithelial layer	CK6						CK16					
	Basal layer		Spinous layer		Superficial layer		Basal layer		Spinous layer		Superficial layer	
	NOM	PCM	NOM	PCM	NOM	PCM	NOM	PCM	NOM	PCM	NOM	PCM
Buccal mucosa	0	0	100	71.4	14.2	0	0	0	14.2	71.4	0	14.2
Lip	0	28.5	57.1	100	14.2	0	0	0	57.1	57.1	0	0
Gingiva	28.5	0	71.4	100	28.5	14.2	0	0	85.7	42.8	71.4	0
Hard palate	0	0	42.8	100	0	28.5	0	0	100	57.1	85.7	0

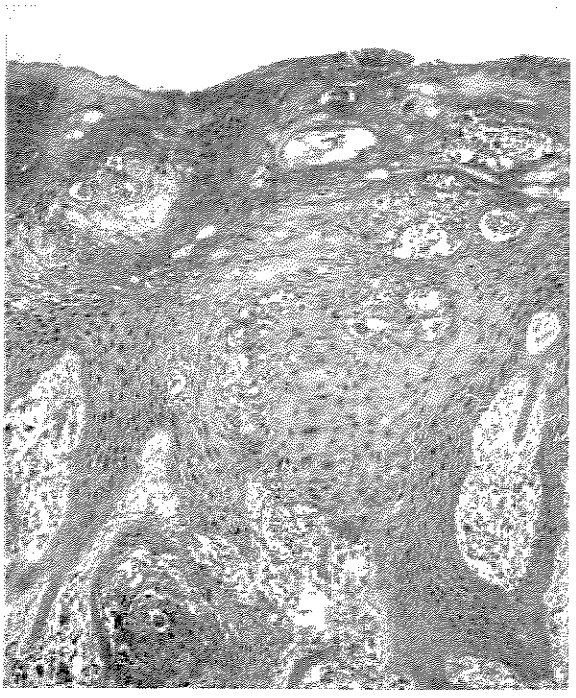


Fig 1. Pmycosis of the gingiva showing typical pseudoepitheliomatous hyperplasia (H&E, x50).

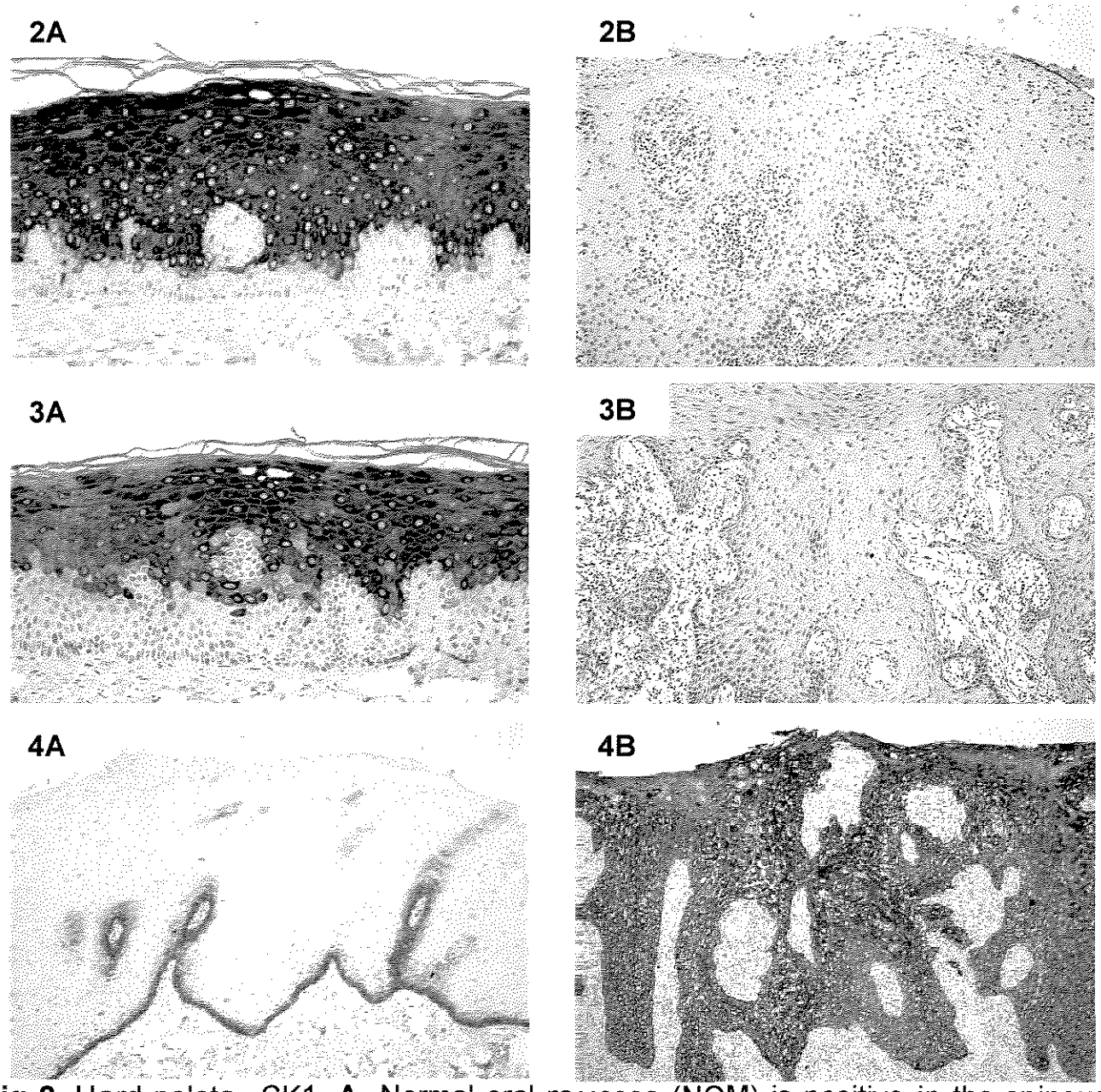


Fig 2. Hard palate, CK1. **A**- Normal oral mucosa (NOM) is positive in the spinous and superficial layers. **B**- Paracoccidioidomycosis (Pmycosis) is negative. (x200).
Fig 3. Hard palate, CK10. **A**- NOM is positive in the spinous and superficial layers. **B**- Pmycosis is negative. (x200).
Fig 4. Buccal mucosa, CK14. **A**- NOM is positive in the basal cells. **B**- Strongly positive in the whole epithelium in Pmycosis. (x100).

Cell proliferation in pseudoepitheliomatous hyperplasia of oral paracoccidioidomycosis

Kaminagakura E; Bonan PRF, Almeida OP*

Department of Oral Pathology, University of Campinas; Dental School, Piracicaba-SP, Brazil

Short title: Paracoccidioidomycosis.

Supported by: FAPESP, Brazil.

*Corresponding author: Dr. Oslei Paes de Almeida, Department of Oral Pathology, University of Campinas; Dental School, Caixa Postal 52, CEP:13414-903. Piracicaba-SP, Brazil.

E-mail: oslei@fop.unicamp.br

Tel: +55-19-3412-5316.

Fax: +55-19-3412-5218.

Abstract

Paracoccidioidomycosis (Pmycosis) is one of the most prevalent deep systemic mycosis in Latin America. It is characterized by granulomatous inflammation and pseudoepitheliomatous hyperplasia (PEH). This work describes the proliferation index in PEH, using Ki-67 immunostaining, comparing with normal oral mucosa (NOM) and moderate oral epithelial dysplasia (ED). Ki-67 positive cells were present in the basal and parabasal layers in NOM and PEH. In ED, it was observed in the basal, parabasal and spinous epithelial layers. Percentage of positive cells were 7.7 (±3.6), 28.2 (±9.8) and 46.0 (±14.0) in NOM, PEH and ED respectively, this three values were statistically different. Although histologically PEH mimics well-differentiated squamous cell carcinoma, the proliferative pattern is more similar to NOM than to dysplasia, i.e., restricted to the basal and parabasal layers, helping to understand the benign characteristics of PEH.

Key words: paracoccidioidmycosis, pseudoepitheliomatous hyperplasia, Ki-67, MIB-1.

Introduction

Paracoccidioidomycosis (Pmycosis) is one of the most prevalent deep systemic mycosis in Latin America. It is caused by the thermally dimorphic fungus, *Paracoccidioides brasiliensis (Pb)*, which is found in soil and vegetation (1). The fungus induces the infection by respiratory route (2) and oropharyngeal lesions are secondary to pulmonary infection (3,4). Clinically, the oral lesions classically show an erythematous finely granular ulceration, speckled with pinpoint hemorrhages, with a mulberry-like surface (4-7). They are usually multiple, affecting gingiva, palate, lip, buccal mucosa, tongue and floor of the mouth (4-7). Microscopically, the lesions show epithelioid granulomas, intra-epithelial microabscess and pseudoepitheliomatous hyperplasia (PEH) (4,8).

PEH is characterized by hyperplasia of the epithelium with irregular expansion and downgrowth of rete pegs into the connective tissue that may mimic a well-differentiated squamous cell carcinoma (9-11). However the basement membrane is intact and there are no evident dysplasia (10). PEH may occur in many oral lesions (11). In some cases, it can play a role in healing process, by containing the infection within the epithelial barrier and then extruding the necrotic material (9). The aim of this work was to investigate epithelial cell proliferation in PEH of oral Pmycosis and compare the results with those of normal oral mucosa (NOM) and oral epithelial dysplasia (ED).

Material and Methods

A total of 49 cases, including 18 NOM, 18 oral Pmycosis and 13 cases of oral ED were retrieved from the files of the Department of Oral Pathology, University of Campinas Dental School. Microscopically, all cases of Pmycosis showed evident PEH, and cases of epithelial dysplasia were classified as moderate. The epithelia from NOM, PEH and ED were divided into three compartments: basal, parabasal and spinous layers. Cells of the basal layer were in contact with basement membrane; parabasal layer was considered as the two next rows of cells above

the basal, and spinous layer was composed by all cells towards the surface from the parabasal layer.

Briefly, for immunohistochemical reactions, 3μm paraffin sections were hydrated and treated with hydrogen peroxide for 30 min to inhibit endogenous peroxidase. Microwave antigen retrieval and overnight incubation with the primary antibody against Ki-67 (clone MIB-1; dilution 1:200; Dako, Denmark) were performed in all cases. Secondary antibodies conjugated to streptavidin-biotin-peroxidase (StrepABC Complex/HRP Duet kit, Dako A/S, Denmark) were used, followed by diaminobenzidine (DAB, Sigma) as the chromogen. Slides were counter stained with Carazzi haematoxylin. Positive and negative controls were included in all reactions.

The proliferative index of the epithelium was determined by counting the number of positive nuclei per 1000 cells, and the values were expressed in percentage. The counting of immunopositive nuclei was performed with a x10 ocular and x40 objetive using KONTRON 400 image analyzer system (Zeiss, Germany). The fields were selected at random across each section. Statistical analysis of data was performed by ANOVA and Mann Whitney test and $p \leq 0.01$ was considered significant. The study protocol was approved by the Ethical Committee in Research (122/2001) of the University of Campinas Dental School.

Results

NOM patients included 9 male and 9 female; all patients with Pmycosis were male and oral dysplasia included 10 male and three female. The mean age of NOM, Pmycosis and ED was 24.3; 44.3 and 56.9 years, respectively. All cases of Pmycosis showed typical PEH which was characterized by elongated rete pegs that extended into the underlying connective tissue. Cases of moderate dysplasia showed acanthosis, and pleomorphic cells with hypercromatism.

In NOM, most of the labeled cells were in the parabasal layer (Fig 1A) while in PEH positive cells were in basal and parabasal layers (Fig 1B). In ED, some cells of the spinous layer were also positive (Fig 1C). Percentual of Ki-67 positive cells

layers as in NOM. This suggests that PEH is a reactive process, without oncogenic characteristics as seen in ED.

Acknowledgements

The authors thank Ana Cristina do Amaral Godoy (University of Campinas Dental School) for the technical assistance with the immunohistochemical staining.

References

1. Restrepo A, McEwen JG, Castañeda E. The habitat of *Paracoccidioides brasiliensis*: how far from solving the riddle? Review article *Med Mycol* 2001;**39**:233-41.
2. McEwen JG, Bedoya V, Patiño MM, Salazar ME, Restrepo A. Experimental murine paracodioidomycosis induced by the inhalation of conidia. *J Med Vet Mycol* 1987;**25**:165-75.
3. Brummer E, Castaneda E, Restrepo AM. Paracoccidioidomycosis: An update. *Clinical Microbiology Reviews* 1993;**6**:89-117.
4. Almeida OP, Jorge Jr J, Scully C. Paracoccidioidomycosis of the mouth: An emerging deep mycosis. *Crit Rev Oral Biol Med* 2003;**14**:377-83.
5. Sposto MR, Scully C, Almeida OP, Jorge J, Graner E, Bozzo L. Oral paracoccidioidomycosis. A study of 36 South American patients. *Oral Surg Oral Med Oral Pathol* 1993;**75**:461-5.
6. Almeida OP, Jorge J, Scully C, Bozzo L. Oral manifestation of paracoccidioidomycosis (South American Blastomycosis). *Oral Surg Oral Med Oral Pathol* 1991;**72**:430-5.
7. Scully C, Paes de Almeida O. Orofacial manifestations of the systemic mycoses. *J Oral Pathol Med* 1992;**21**:289-94.
8. Meneses-Garcia A, Mosqueda-Taylor A, Luz RM, Rivera LMRG. Paracoccidioidomycosis: Report of 2 cases mimicking squamous cell carcinoma. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2002;**94**:609-13.

9. Hayman JA, Smith IM, Flood P. Pseudoepitheliomatous hyperplasia in *Mycobacterium ulcerans* infection. *Pathology* 1996;**28**:131-4.
10. Grauwin MY, Mane I, Cartel JL. Pseudoepitheliomatous hyperplasia in trophic ulcers in leprosy patients. *Lepr Rev* 1996;**67**:203-7.
11. Dorji T, Cavazza A, Nappi O, Rosai J. Spitz nevus of the tongue with pseudoepitheliomatous hyperplasia. Report of three cases of a pseudomalignant condition. *Am J Surg Pathol* 2002;**26**:774-7.
12. Verheijen R, Kuijpers HJH, Schlingemann RO et al. Ki-67 detects a nuclear matrix-associated proliferation-related antigen. I. intracellular localization during interphase. *J Cell Sci* 1989;**92**:123-30.
13. Verheijen R, Kuijpers HJH, Driel R et al. Ki-67 detects a nuclear matrix-associated proliferation-related antigen. II. Localization in mitotic cells and association with chromossomes. *J Cell Sci* 1989;**92**:531-40.
14. Gerdes J, Schwab U, Lemke H, Stein H. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. *Int J Cancer* 1983;**31**:13-20.
15. Liu SC, Sauter ER, Clapper ML et al. Markers of cell proliferation in normal epithelia and dysplastic leukoplakias of the oral cavity. *Cancer Epidemiol Biomakers Prev* 1998;**7**:597-603.
16. Liu SC, Klein-Szanto AJP. Markers of proliferation in normal and leukoplakic oral epithelia. Review. *Oral Oncol* 2000;**36**:145-51.
17. van Oijen MGCT, Gilsing MMA, Rijksen G, Hordijk GJ, Slootweg PJ. Increased number of proliferating cells in oral epithelium from smokers and ex-smokers. *Oral Oncol* 1998;**34**:297-03.
18. Macluskey M, Odgen GR, Green M, Chisholm DM, Schor SL, Schor AM. The association between epithelial proliferation and disease progression in the oral mucosa. *Oral Oncol* 1999;**35**:409-14.
19. Loose DS, Stover EP, Restrepo A, Stevens DA, Feldman D. Estradiol binds to a receptor-like cytosol binding protein and initiates a biological response in *Paracoccidioides brasiliensis*. *Proc Natl Acad Sci* 1983;**80**:7659-63.

20. Krutchkoff DJ, Eisenberg E, Anderson C. Dysplasia of oral mucosa: a unified approch to proper evaluation. *Mod Pathol* 1991;**4**:113-9.
21. Li LL, Crotty KA, McCarthy SW, Palmer AA, Kril JL. A zonal comparison of MIB1-Ki67 immunoreactivity in benign and malignant melanocytic lesions. *Am J Dermatopathol* 2000;**6**:489-95.
22. Gonzales-Moles MA, Ruiz-Avila I, Rodriguez-Archilla A, Martinez-Lara I. Suprabasal expression of Ki-67 antigen as a marker for the presence and severity of oral epithelial dysplasia. *Head Neck* 2000;**22**:658-61.
23. Girod SC, Krueger G, Pape H-D. p53 and Ki-67 expression in preneoplastic and neoplastic lesions of the oral mucosa. *Int J Oral Maxillofac Surg* 1993;**22**:285-8.
24. Kushner J, Bradley G, Jordan RCK. Patterns of p53 and Ki-67 protein expression in epithelial dysplasia from the floor of the mouth. *J Pathol* 1997;**183**:418-23.
25. Oliver RJ, MacDonald DG, Felix DH. Aspects of cell proliferation in oral epithelial dysplastic lesions. *J Oral Pathol Med* 2000;**29**:49-55.
26. Zidar N, Gale N, Cör A, Kambic V. Expression of Ki-67 antigen and proliferative cell nuclear antigen in benign and malignant epithelial lesions of the larynx. *J Laryngol Otol* 1996;**110**:440-5.
27. Piatelli A, Rubini C, Fioroni M, Iezzi G, Santinelli A .Prevalence of p53, bcl-2, and Ki-67 immunoreactivity and of apoptosis in normal oral epithelium and in premalignant and malignant lesions of the oral cavity. *J Oral Maxillofac Surg* 2002;**60**:532-40.
28. Bjarnason GA, Jordan RCK, Sothern RB. Circadian variation in the expression of cell-cycle proteins in human oral epithelium. *Am J Pathol* 1999;**154**:613-22.

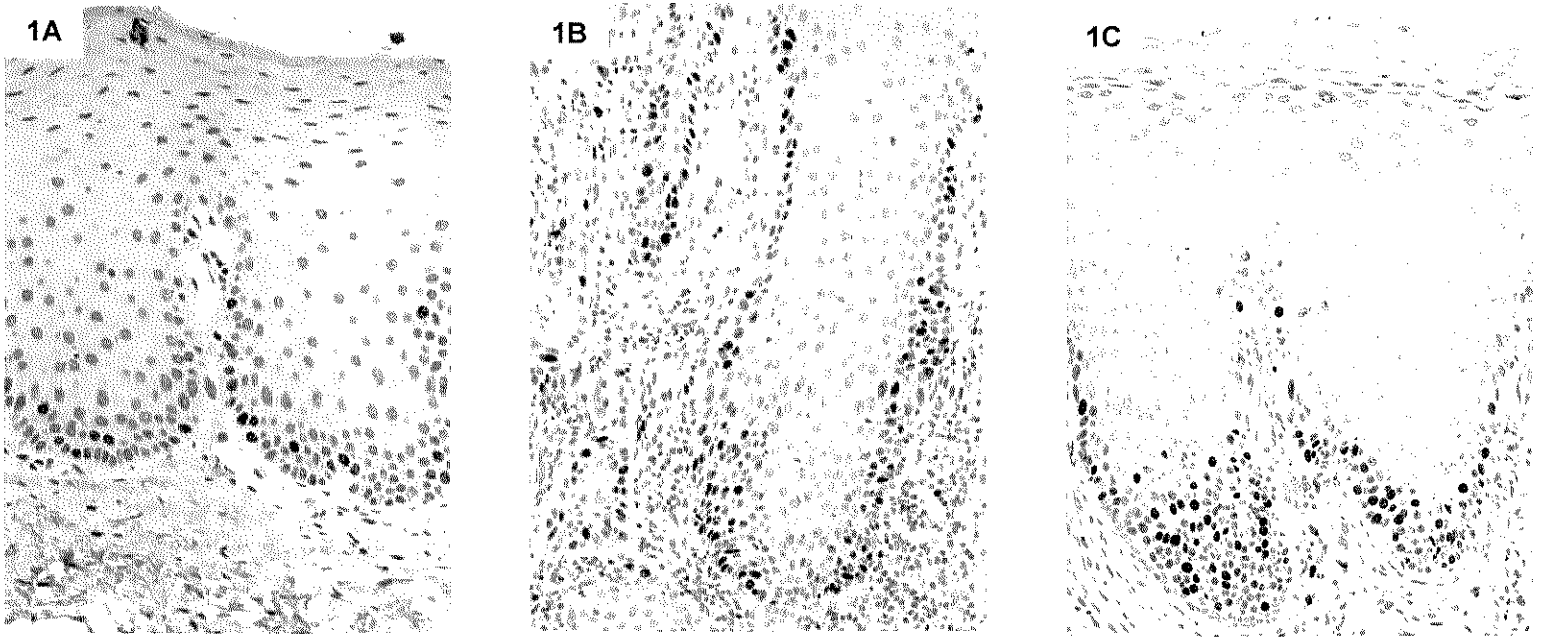


Fig 1. Ki-67 expression. **A** NOM basal and parabasal positive cells (x200); **B** Pmycosis many basal and parabasal epithelial positive cells (x200); **C** ED basal, parabasal and some spinous epithelial positive cells (x200).

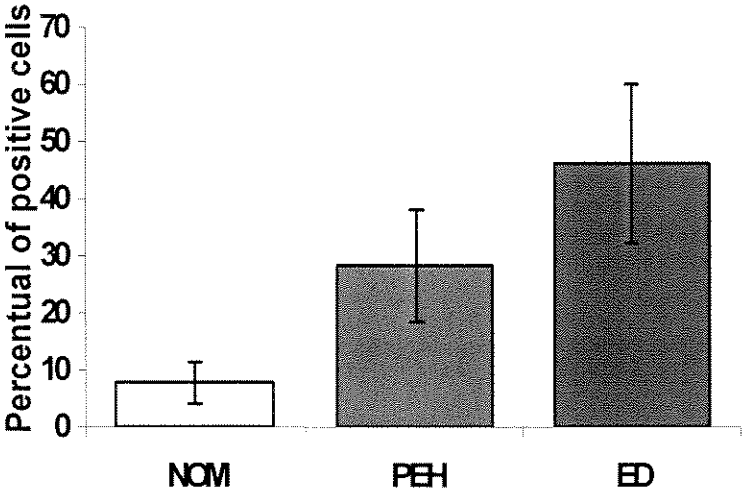


Fig 2. Percentual of Ki-67 positive cells in normal oral mucosa (NOM), pseudoepitheliomatous hyperplasia (PEH) and moderate epithelial dysplasia (ED).The three values were statistically different, $p \leq 0,01$.

Characterization of inflammatory cells in oral paracoccidioidomycosis

Kaminagakura E, Bonan PRF, Almeida OP* .

Department of Oral Pathology, University of Campinas; Dental School, Piracicaba-SP, Brazil.

Short title: Oral paracoccidioidomycosis.

Supported by: FAPESP, Brazil.

Corresponding author: Dr. Oslei Paes de Almeida, Department of Oral Pathology, University of Campinas; Dental School, Caixa Postal 52, CEP:13414-903. Piracicaba-SP, Brazil.

E-mail: oslei@fop.unicamp.br

Tel: +55-19-3412-5316.

Fax: +55-19-3412-5218.

Abstract

Paracoccidioidomycosis (Pmycosis) is one of most common deep mycosis in many regions of Latin America. Microscopically, it shows granulomatous inflammatory reaction with giant cells, lymphocytes, plasma cells, PMN and eosinophils. We assessed inflammatory cells, in 15 cases of oral Pmycosis by immunohistochemistry. Macrophages and T cells were the predominant cells in well-organized and non granulomatous areas. CD4 phenotype was predominant in well-organized granulomas and a balance between CD4+ and CD8+ expressing cells was observed in non granulomatous areas. Dendritic, S100+ cells were found mainly in the epithelium, in subepithelial connective tissue and at periphery of organized granulomas. CD15+ cells were accumulated mainly in areas of intraepithelial microabscess and ulceration.

Key words: paracoccidioidomycosis, inflammatory cells, immunohistochemistry.

Introduction

Paracoccidioides brasiliensis (Pb) causes one of the most prevalent systemic mycosis in Latin America, the paracoccidioidomycosis (Pmycosis). It is a thermally dimorphic fungus, which secretes a 43kDa glycoproteic protease (Gp43)(1) and it has been isolated from soil, but its precise environmental niche remains undefined (2). Humans are infected by inhalation of conidia, that inside of the lungs develops into pathogenic yeast cells (3).

Clinically the disease has a wide spectrum of clinical presentation, two main forms are described. Acute or subacute form (juvenile) is severe, develops in weeks to months, involving the mononuclear phagocytic system, showing depressed cellular immunity, increased antibody level (4,5) and loose granulomatous inflammation with numerous fungal cells (4). The chronic form (adult type) is the most common, classically affecting adult male, initially involving the lungs, disseminating to lymph nodes, mucous membrane, skin, bone and adrenal (4). The mouth is involved in about half of the cases (6). In this form, the host immune response induces dense and well-organized granulomas that are associated with low numbers of *Pb* yeast cells (4).

Oral lesions are microscopically characterized by formation of epithelioid granulomas with giant cells interspersed by lymphocytes, PMN and plasma cells (6-9). The microscopical aspects of Pmycosis are well known but the literature is scarce in relation to the description and quantification of the involved cells in oral Pmycosis. The aim of this study was to characterize and describe the inflammatory cells of oral Pmycosis.

Material and methods

Fifteen cases of oral Pmycosis were retrieved from files of the Department of Oral Pathology, University of Campinas Dental School. All patients were male, age range from 29 to 62 years old (mean 48 $\pm$ 8.97 years). Biopsies were taken from buccal mucosa (n=7), tongue (n=3), lip (n=2), palate (n=1), gingiva (n=1) and alveolar process (n=1). Each specimen was classified according to the

predominant tissue response as showing well-organized granulomas, non granulomatous areas (10) and microabscesses.

Briefly, for immunohistochemical reactions, 3 μm paraffin sections were hydrated and treated with hydrogen peroxide for 30 min to inhibit endogenous peroxidase. Microwave antigen retrieval and overnight incubation with the primary antibodies were performed against CD68, CD45, CD8, CD4, CD20, CD15 and S100 in all cases (Table 1). Secondary antibodies conjugated to streptavidin-biotin-peroxidase (StrepABC Complex/HRP Duet kit, Dako A/S, Denmark) were used, followed by diaminobenzidine (DAB, Sigma) as the chromogen. Slides were counterstained with Carazzi`s haematoxylin. Positive and negative controls were included in all reactions.

Cells were quantified in 3 randomized high power fields (HPF) in well-organized and non granulomatous areas and microabscess. At least 9 areas were analyzed in each case. The counting of immunopositive cells was performed with a x10 ocular and x40 objetive with a square grid of 21208,57μm^2 using KONTRON 400 image analyzer system (Zeiss, Germany). Statistical analysis was performed by ANOVA and comparison between means by Tuckey`s test using SAS Institute program, considering $p \leq 0.05$ as significant.

The study protocol was approved by the Ethical Committee in Research of the University of Campinas Dental School (122/2001).

Results

All cases of oral Pmycosis showed stratified squamous epithelium with areas of pseudoepitheliomatous hyperplasia and microabscesses. In four cases predominated well-organized granulomas, formed by macrophages, giant and epithelioid cells in the center of the granuloma, surrounded by lymphocytes and plasma cells. Fibroblasts were present at the periphery (Fig.1A). In other four cases predominated non granulomatous areas with diffuse foci of lymphocytes, macrophages, giant, epithelioid, eosinophils, mast and plasma cells in the

connective tissue (Fig.1B). In seven cases, both types of inflammation reaction were found in similar proportion.

Paracoccidioides brasiliensis (Pb) were easily seen in all cases by haematoxylin-eosin stain (H&E), although Schiff periodic acid stain was also used. The fungus were observed inside and sometimes outside multinucleated giant cells. They appeared as round and birefringent structures, often surrounded by daughter spores.

Immunohistochemical analysis

The center of well-organized granulomas were mostly composed by CD68+ cells, corresponding to macrophages, epithelioid cells and multinucleated giant cells (Fig.2A). On the periphery predominated CD45 lymphocytes (Fig.2B), mainly with CD4 phenotype (OPD-4), which was also found between CD68+ cells in the center of granulomas (Fig.2C). Small number of CD8+ and S100+ cells were also observed among CD4+ cells (Fig.2D and 2E) and few CD20+ cells were seen in only four cases (Fig.2F).

In non granulomatous areas CD45+ cells, corresponding to CD4+ and CD8+ cells, predominated and they were interspersed with CD68+ cells (Fig.3A; 3B; 3C and 3D). Multinucleated giant cells were less frequent, but particles of the fungus were more easily found. Isolated macrophages, multinucleated giant cells and S100+ cells were common in the connective tissue close to the epithelium (Fig.3E). CD20+ and CD15+ were more frequent when compared with well-organized granulomas (Fig. 3F).

Areas of ulceration and microabscesses were formed almost exclusively by CD15+ (Fig.4A) with some CD68+ (Fig.4B), although eventual CD4+, CD8+, S100+ and CD20+ cells were also present. Table 2 summarizes the quantitative results.

Discussion

Granulomatous inflammation is an important component of immune response to intracellular pathogens and some non-degradable antigens. These responses are initiated by CD4+ T lymphocytes (11) with accumulation of macrophages, and other effector cells in response to T cell cytokines (11).

Macrophages form the major cell defense against *Pb.* These cells and their products are involved in the development of granulomatous inflammation, promoting antigen recognition and processing (12). They are also of central importance in the induction of antigen-specific T lymphocyte activation (12). The interaction of macrophages with *Pb* yeast cell and their structural constituents may activate the microbicidal system by nitric oxide (NO), however it might promote suppression of Ia antigen (product of MHC II), consequently inhibiting antigen presentation by them (12). These events might contribute in different degrees to immunosupression and to clinical pathologic diversity of this infection (12). Pmycosis shows organized granulomas and non granulomatous areas, and this probably is related to the host response to the fungus. CD4+ cells predominate at the periphery of well-organized granulomas, and this confirms the data of Moscardi-Bacchi et al. (13). On other hand, CD8 and other inflammatory cells are also interspersed with macrophages and multinucleated giant cells in non granulomatous areas. It was also common to find isolated multinucleated giant cells in oral Pmycosis. These characteristics have also been described in other infectious granulomatous disease, including the presence of helper and supressor T cells among macrophages in poorly organized granulomas (14). It is speculated that in this situation, inhibition of antigen presentation occurs, as well as maturation of macrophages to epithelioid cells (14). Interactions among these cells are complex, and at the moment it is difficult to conclude that in oral mucosa the response is in someway different from other tissues.

The humoral system responds to antigenic stimulation by producing specific antibodies against *Pb* through B-cell activation (5). High levels of IgG, IgM and IgE

anti-Gp43 are more frequent in patients with acute than chronic disease and it could be related to the dissemination of the yeast resulting from the inability of host defenses to control fungal replication in the phagocytic mononuclear system (5,15). It has been reported that increased levels of these antibodies are correlated with severity of the clinical manifestations (15). CD20+ cells and plasma cells were uncommon in oral Pmycosis suggesting that humoral imune response do not play an important role in this form of the disease. In fact they were more frequent in non granulomatous areas which usually contain more fungi.

S100+ cells were found around the well-organized granulomas, as previously described in the skin (16,17). Studies reported a reduced number of epidermal Langerhans cells (LCs) with short and irregular dendrites when compared with normal skin (17,18). In addition, Sandoval et al (19) did not detect *Pb* antigens in the cytoplasm of LCs, suggesting that they would not be capable of presenting fungal antigen efficiently. Conversely, the yeast has been described inside FXIIIa+ dermal dendrocytes, another group of dendritic cells with similar characteristics to Langerhans cells (16). In oral Pmycosis, S100+ cells were observed in the epithelium, in the subepithelial connective tissue and also at the periphery of well-organized granulomas, suggesting that these cells act as antigen presenting cells.

Oral mucosa lesions commonly show an erythematous finely granular hyperplasia, speckled with multiple pinpoint hemorrhages and a mulberry-like surface. Areas of ulceration are frequent in oral Pmycosis (6-9). Areas of microabscesses were composed almost exclusively by CD15+ cells. PMN phagocytes (20-22), but fail to digest the *Pb* yeast cell unless the cell membrane has been altered by amphotericin B (22). Their fungistatic effect on the *Pb* yeast cell is enhanced by IFN-γ and granulocyte-macrophage colony-stimulating factor (21). *Pb* yeast cell were also observed in areas of microabscess, frequently surrounded by PMN.

Villalba (23) studied 64 cases of patients with oral Pmycosis and reported that in 92.18% of cases eosinophils were observed, mainly in non granulomatous

areas. Natural killer, plasma and mast cells have been described in granulomatous inflammation (23-25), however they were not quantified in this study.

In summary, well-organized granulomas were formed mainly by macrophages and CD4+ cells, while in non granulomatous areas CD8+ cells were also found in quantities similar to CD4+ cells. CD15+ cells were seen mainly in areas of intraepithelial microabscesses. S100+ cells were observed in the epithelium, but also in the subepithelial connective tissue, and at the periphery of well-organized granulomas. Eosinophils, CD20+, plasma and mast cells were found dispersed among other inflammatory cells in non granulomatous areas. Cases with predominance of well-organized granulomas tend to have fewer yeast particles, indicating a better host response. Nevertheless there are not yet studies considering the microscopical characteristics of oral Pmycosis with clinical response to treatment, or severity or systemic involvement of the disease.

Acknowledgements

The authors thank Ana Cristina do Amaral Godoy (FOP-UNICAMP) for the technical assistance with the immunohistochemical procedures.

References

1. Stambuk BU, Puccia R, Almeida MLC, Travassos LR, Schenkman S. Secretion of the 43 kDa glycoprotein antigen by *Paracoccidioides brasiliensis*. *J Med Vet Mycol* 1988;**26**:367-73.
2. Restrepo A, McEwen JG, Castañeda E. The habitat of *Paracoccidioides brasiliensis*: how far from solving the riddle? Review article. *Med Mycol* 2001;**39**:233-41.
3. McEwen JG, Bedoya V, Patiño MM, Salazar ME, Restrepo AM. Experimental murine paracodioidomycosis induced by the inhalation of conidia. *J Med Vet Mycol* 1987;**25**:165-75.

4. Franco M, Montenegro MR, Mendes RP, Marques AR, Dillon NL, Mota NG. Paracoccidioidomycosis: a recently proposed classification of its clinical forms. *Rev Soc Bras Med Trop* 1987;**20**:129-32.
5. Bueno JP, Mendes-Gianini MJS, Del Negro GMB, Assis CM, Takiguti CK, Shikanai-Yasuda MA. IgG, IgM, IgA antibody response for the diagnosis and follow-up of paracoccidioidomycosis: comparison of counterimmunoelectrophoresis and complement fixation. *J Med Vet Mycol* 1997;**35**:213-7.
6. Almeida OP, Jorge Jr J, Scully C. Paracoccidioidomycosis of the mouth: An emerging deep mycosis. *Crit Rev Oral Biol Med* 2003;**14**:377-83.
7. Almeida OP, Jorge J, Scully C, Bozzo L. Oral manifestation of paracoccidioidomycosis (South American Blastomycosis). *Oral Surg Oral Med Oral Pathol* 1991;**72**:430-5.
8. Scully C, Paes de Almeida. Orofacial manifestations of the systemic mycoses. *J Oral Pathol Med* 1992;**21**:289-94.
9. Sposto MR, Scully C, Almeida OP, Jorge J, Graner E, Bozzo L. Oral paracoccidiodomycosis. A study of 36 South American patients. *Oral Surg Oral Med Oral Pathol* 1993;**75**:461-5.
10. Brito T, Franco MF. Granulomatous inflammation. *Rev Inst Med Trop São Paulo* 1994;**36**:185-92.
11. Hogan LH, Weinstock JV, Sandor M. TCR specificity in infection induced granulomas. *Immunol Lett* 1999;**68**:115-20.
12. Bocca AL, Silva M, Silva CL, Cunha FQ, Figueiredo F. Macrophage expression of class II major histocompatibility complex gene products in *Paracoccidioides brasiliensis*-infected mice. *Am J Trop Med Hyg* 1999;**61**:280-7.
13. Moscardi-Bacchi M, Soares A, Mendes R, Marques S, Franco M. *In situ* localization of T lymphocyte subsets in human paracoccidioidomycosis. *J Med Vet Mycol* 1989;**27**:149-58.

14. Moldlin RL, Hofman FM, Meyer PR, Sharma OP, Taylor CR, Rea TH. *In situ* demonstration of T lymphocyte subsets in granulomatous inflammation: leprosy, rhinoescleroma and sarcoidosis. *Clin and Exp Immunol* 1983;**51**:430-8.
15. Calich VLG, Singer-Vermes LM, Russo M, Vaz CAC, Burger E. Immunogenetics in paracoccidioidomycosis. *In:* Franco M, Lacaz CS, Restrepo-Moreno A, Del Negro G. *Paracoccidioidomycosis* CRC Press, Inc. Cap.10, 1993.
16. Pagliari C, Sotto MN. Correlation of factor XIIIa+ dermal dendrocytes with paracoccidioidomycosis skin lesions. *Med Mycol* 2002;**40**:407-10.
17. Pagliari C; Sotto MN. Dendritic cells and pattern of cytokines in paracoccidioidomycosis skin lesions. *Am J Dermatopathol* 2003;**25**:107-12.
18. Gimenez MF, Tausk F, Gimenez MM, Gigli I. Langerhan`s cells in paracoccidioidomicosis. *Arch Dermatol* 1987;**123**:479-81.
19. Sandoval MP, Brito T, Sotto MN et al. Antigen distribuition in mucocutaneous biopsies of human paracoccidioidomycosis. *Int J Surg Pathol* 1996;**3**:181-8.
20. Goihman-Yahr M, Essenfeld-Yahr E, de Albornoz MC et al. Defect of in vitro digestive ability of polymorphonuclear leukocytes in paracoccidioidomycosis. *Infect and Immun* 1980;**28**:557-66.
21. Goihman-Yahr M, Rothenberg A, Bretaña A al. Digestion of killed *Paracoccidioides brasiliensis* by neutrophils. *Mycopathol* 1989;**106**;53-8.
22. Kurita N, Oarada M, Miyaji M, Ito E. Effects of cytokines on antifungical activity of human polymorphonuclear leucocytes against yeast cells of *Paracoccidioides brasiliensis. Med Mycol* 2000;**38**:177-82.
23. Villalba H. Características microscópicas da paracoccidioidomicose bucal. (dissertation) Piracicaba-SP, Brazil. Univ. of Campinas.
24. Peçaroli MTS, Soares AMV, Mendes RP et al. Studies of natural killer in paracoccidioidomycosis. *J Med Vet Mycol* 1991;**29**:373-80.
25. Mahaisavariya P, Jiamton S, Manonukul J, Klemnger S. Mast cells in leprosy and leprosy reaction. *Int J Dermatol* 2000;**39**:274-7.

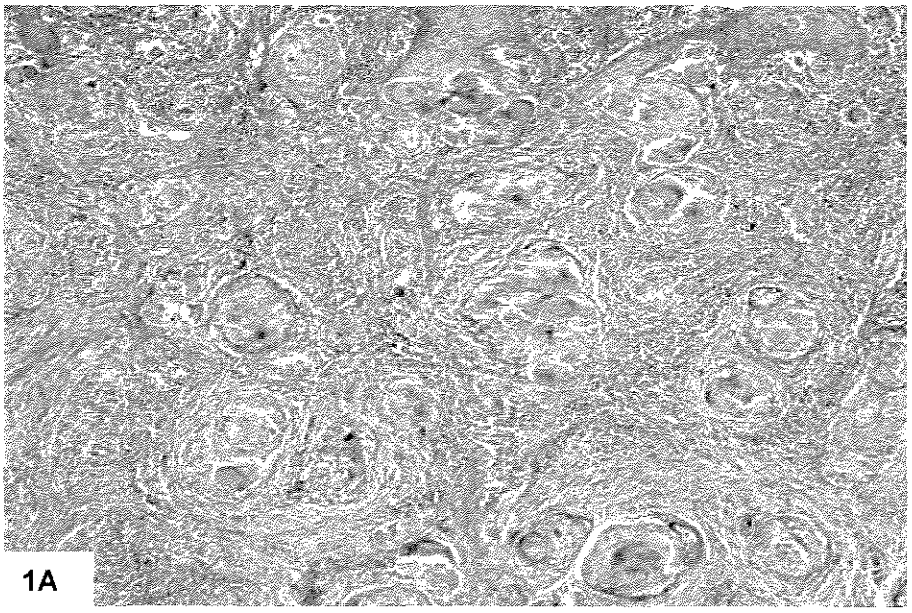
Table1. List of primary antibodies

Antigen	Dilution	Clone	Supplier
CD68	1:400	PG-M1	Dako Corp. Carpenteria, CA, USA
CD45	1:200	UCHL-1	Dako Corp. Carpenteria, CA, USA
CD8	1:100	C8/144B	Dako Corp. Carpenteria, CA, USA
CD4	1:200	OPD4	Dako Corp. Carpenteria, CA, USA
CD20	1:10000	L26	Dako Corp. Carpenteria, CA, USA
CD15	1:200	C3D-1	Dako Corp. Carpenteria, CA, USA
S100	1:10000	policlonal	Sigma Aldrich Inc, Saint Louis, USA

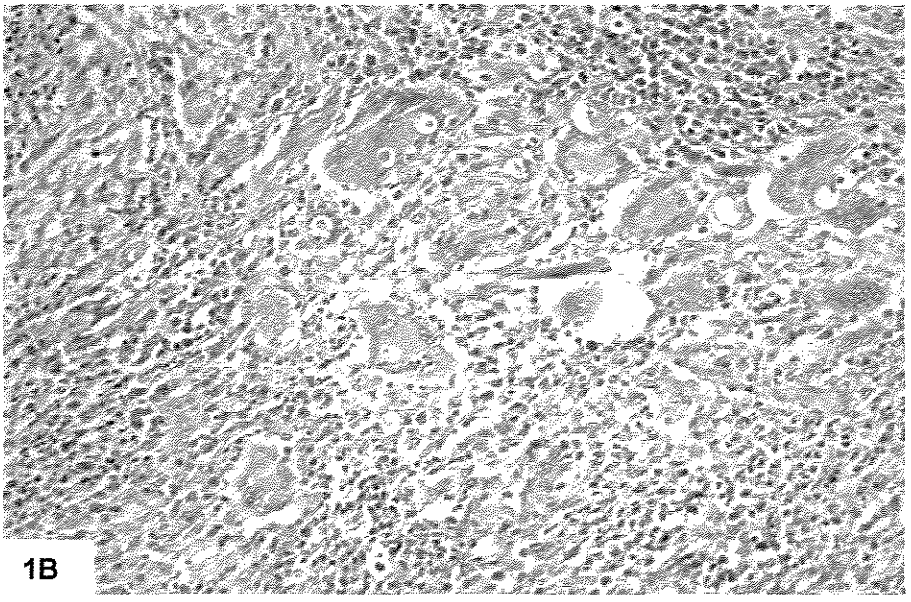
Table 2. Percentage of cells in well-organized granulomas, non granulomatous areas and microabscesses.

	well-organized granulomas	non granulomatous areas	microabscesses
CD68	A 34.2 a	A 30.4 a	B 12.2 b
CD45	A 29.8 b	A 45.9 a	B 11.3 c
CD8	C 1.4 b	BC 12.5 a	C 1.4 b
CD4	B 8.0 a	B 14.3 a	C 0.7 b
CD20	C 0.1 b	BC 9.4 a	C 0.2 b
CD15	C 0.8 c	C 5.7 b	A 70.7 a
S100	BC 4.0 a	C 5.4 a	C 0.6 b

the same capital letters in the vertical line and small letters in the horizontal line does not differ between itself. (Tuckey`s test, $p \leq 0,05$).



1A



1B

Fig 1. Patterns of tissue reaction in Pmycosis. **(A)** Well-organized granulomas characterized by compact clusters of giant cells and macrophages in the center of the granulomas and lymphocytes and plasma cells at periphery (H&E, x50). **(B)** Non granulomatous areas showing diffuse foci of giant and epithelioid cells, macrophages interspersed with lymphocytes, neutrophils, plasma cells and some eosinophlis (H&E, x200).

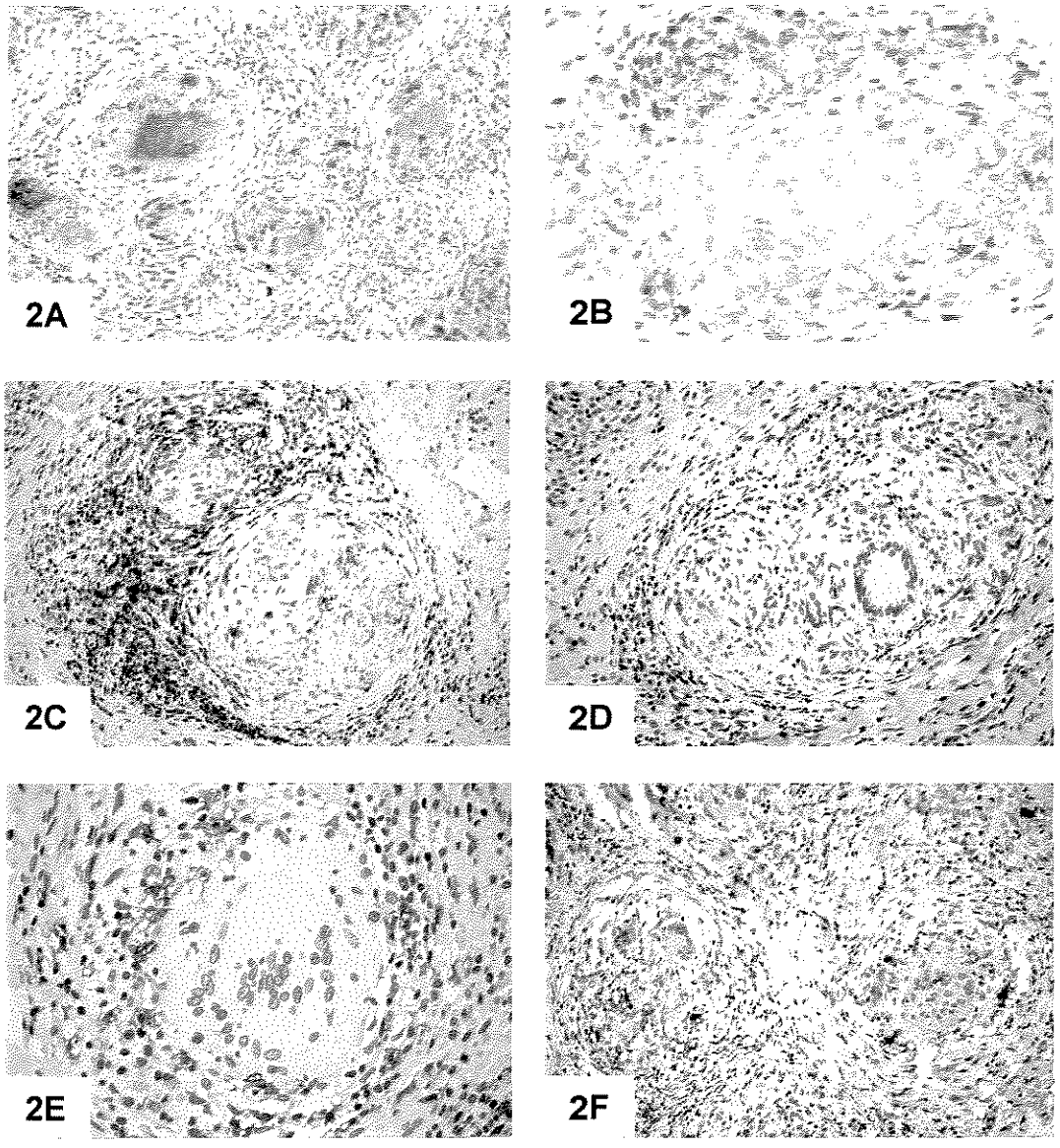


Fig 2. Well-organized granulomas- **(A)** CD68 positive cells, were mainly found in the center of the granuloma (x200); **(B)** CD45 positive cells were found on the granuloma periphery (x40O); **(C)** CD4 expressing cells were around macrophages and giant cells (x200); **(D)** a few CD8 expressing cells were observed on granuloma periphery (x200); **(E)** CD20 positive cells were also observed on granuloma periphery (x400); **(F)** some S100 expressing cells were observed on granuloma periphery (x200). Immunoperoxidase.

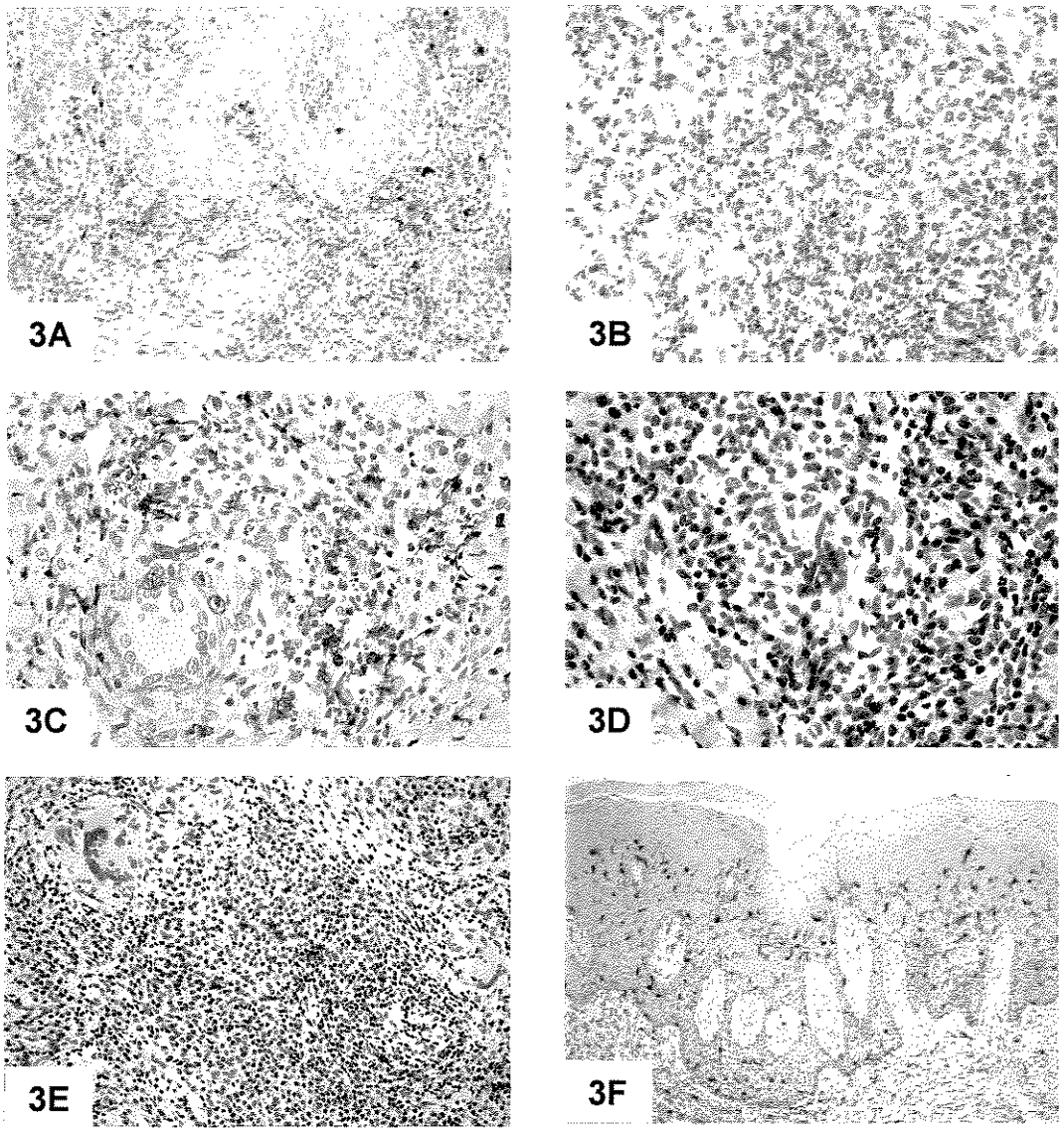
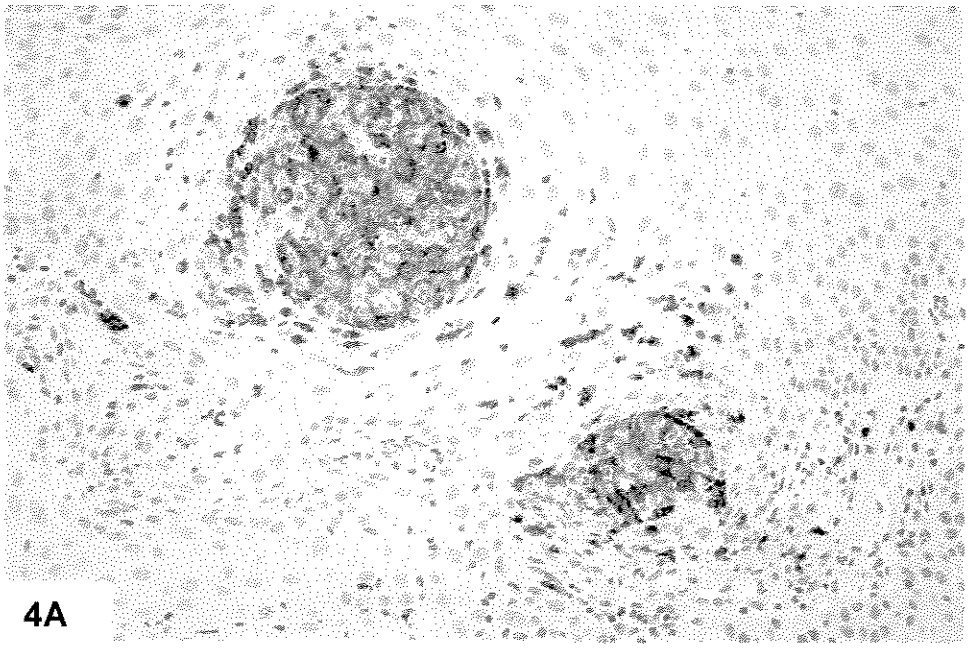
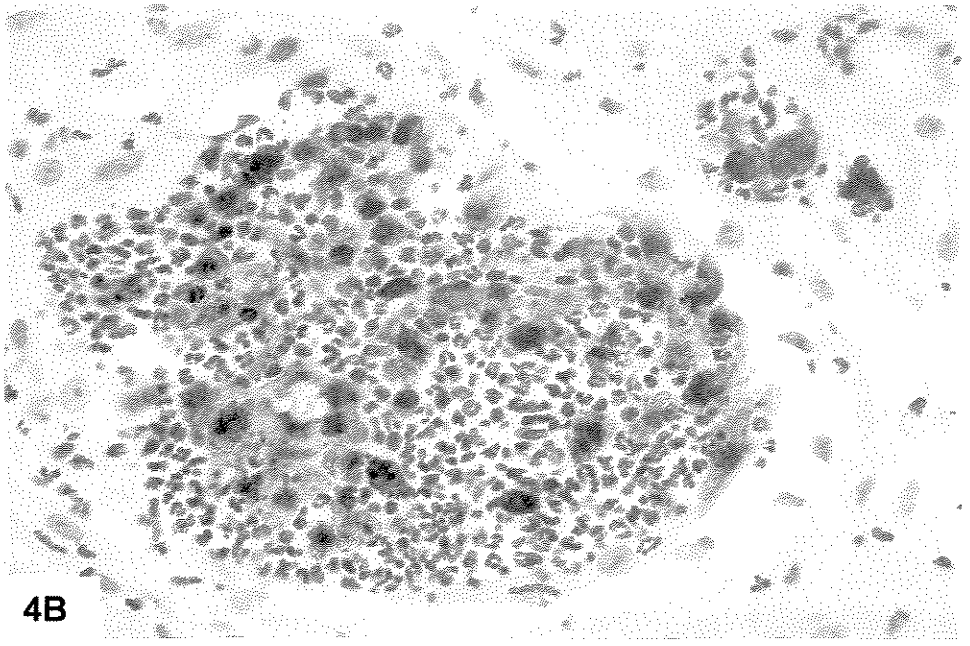


Fig 3. Non granulomatous areas- **(A)** CD68 positive cells were accumulated close to the epithelium (x100); **(B)** CD45 immunopositive cells were seen in whole connective tissue (x200); **(C)** CD4 expressing cells were numerous and close to giant cells (x400); **(D)** CD8 expressing cells were distributed throughouth the connective tissue (x200); **(E)** CD20 expressing cells (x200); **(F)** S100 immunopositive cells were observed in the epithelium and in the connective tissue close to the epithelium, they showed numerous dendrites (x100). Immunoperoxidase.



4A



4B

Fig 4. Areas of microabscess- **(A)** CD15 positive cells were almost exclusive in areas of microabsecess (x200); **(B)** CD68 expressing cells were also found close to CD15 positive cells (x400). Immunoperoxidase.

Oral paracoccidioidomycosis or squamous cell carcinoma?

Kaminagakura E, Graner E, Almeida OP, Di Hipólito Jr O, Lopes MA*

Department of Oral Pathology, University of Campinas; Dental School, Piracicaba-SP, Brazil.

Short title: Oral paracoccidioidomycosis.

Supported by: CAPES, Brazil.

Corresponding author: Márcio Ajudarte Lopes, Department of Oral Pathology, University of Campinas; Dental School, Caixa Postal 52, CEP:13414-903. Piracicaba-SP, Brazil.

E-mail: malopes@fop.unicamp.br

Tel: +55-19-3412-5319.

Fax: +55-19-3412-5218.

Abstract

Paracoccidioidomycosis is a deep, systemic and progressive mycosis caused by *Paracoccidioides brasiliensis.* Oral lesions normally are multiples with a mulberry-like appearance. This article reviews an unusual case involving a chronic, solitary, and ulcerated lesion whose clinical aspects were similar to squamous cell carcinoma. Viewed microscopically, the lesion showed pseudoepitheliomatous hyperplasia and non-necrotizing granulomas. The patient was treated with systemic Ketoconazole. After 11 years of follow-up examinations no recurrence was observed.

Received: May 5, 2003
Last revisions: August 15, 2003
Accepted: August 18, 2003

Introduction

Paracoccidioidomycosis (Pmycosis) is a deep mycosis common to many regions of Latin America, diagnosed frequently in Brazil, Venezuela, and Colombia.[1,2] It is caused by *Paracoccidioides brasiliensis*, which is found more commonly in soil and on vegetables; however, its precise habitat has not yet been determined.[1,2] *P. brasiliensis* is a thermally dimorphic fungus. Humans contract the infection by inhaling small conidia 4.0 μm in diameter. When the conidia reaches the distal portion of the pulmonary parenchyma, it becomes a pathogenic yeast cell that either may be confined locally or may propagate and disseminate to lymph nodes, mucous membrane, skin, bone, and adrenal glands.[3,4] The disease manifests most commonly in a chronic form. It affects adult males more frequently and occurs primarily in the lungs; approximately 50% of the cases appear as oral manifestations.[2,5] Oral mucosa lesions have a granular, mulberry-like surface and usually appear in multiple numbers, although microulcerations or deep ulcers also may appear eventually.[5,6] Isolated lesions resemble neoplastic disease, which may confuse dentists.[7] Viewed microscopically, the chronic inflammatory reaction shows granulomas to be rich in giant cells, lymphocytes, neutrophils, eosinophils, plasma cells and intra- and extracellular fungi cells, corresponding to *P. brasiliensis*.[7] The epithelium displays exocytosis, microabscesses, and pseudoeptheliomatous hyperplasia that resemble squamous cell carcinoma so closely that it is possible to mistake one for the other.[7] This study presents a case of oral Pmycosis that mimics squamous cell carcinoma.

Case report

A 61-year-old white man was referred for evaluation of an ulcerated lesion that had been evident for approximately four months. The patient reported that he had smoked 20 cigarettes per day for about 40 years. His previous medical history revealed an allergy to sulfonamide.

Extraoral analysis displayed a tender, mobile, and enlarged right submandibular lymph node, as well as a scar on the mandibular lip resulting from surgical excision of a squamous cell carcinoma.

Intraoral examination revealed an ulcerated lesion with central necrosis involving the inferior alveolar ridge (Fig. 1). The maximum dimensions of this lesion were approximately 2.0 x 1.0 cm. A granulomatous disease or squamous cell carcinoma were suspected and an incisional biopsy was performed. The lesion showed stratified squamous epithelium as well as pseudoepitheliomatous hyperplasia, acanthosis, spongiosis and microabscesses (Fig. 2). In the connective tissue, organized tubercule-like granulomas were noted, as were macrophages, giant cells, and epithelioid cells surrounded by lymphocytes. Use of periodic acid-Schiff stain revealed *P. brasiliensis* and the patient was diagnosed with Paracoccidioidomycosis brasiliensis (Fig. 3). The patient was referred for evaluation and treatment. A radiograph of the patient's chest showed bilateral interstitial infiltrates with hilar adenopathy (Fig. 4). Blood tests revealed discreet anisocytosis, leukocytosis, eosinophilia, lymphocytosis, and neutrophils with toxic granulations that were larger and darker than those seen in segmented neutrophils.

The patient was given Ketoconazole (400 mg/day for 14 months and 200 mg/day for 8 more months) under medical supervision. Two months after beginning Ketoconazole treatment, the oral lesion was healed completely (Fig. 5); after 11 years of follow-up examinations, no recurrence was observed.

Discussion

Before making a differential diagnosis of Pmycosis, the following conditions must be considered: squamous cell carcinoma, Wegner's granulomatosis, Crohn's disease, sarcoidosis, lupus erythematosus, north American blastomycosis, histoplasmosis, coccidioidomycosis, tuberculosis, syphilis, actinomycosis, leishmaniasis, and leprosy.[5,6,8-10] Traditionally, Pmycosis displays multiple oral

lesions that are mulberry-like in appearance, with pinpoint hemorrhages that affect the gingiva and alveolar mucosa primarily.[5,6,9] Different aspects may appear over time; the patient in this report displayed an atypical oral Pmycosis, a chronic, solitary, ulcerated lesion whose properties were similar to those of squamous cell carcinoma.

A biopsy usually is sufficient for establishing a definitive diagnosis. However, Pmycosis and other lesions, such as leprosy, syphilis and *Mycobacterium ulcerans* infection may exhibit pseudoepitheliomatous hyperplasia. These conditions may be confused with squamous cell carcinoma; as a result, small specimens may lead to erroneous interpretation.[7,11,12] Pmycosis is characterized by non-necrotizing granuloma with giant cells. The fungus blastospores appear double-contoured and approximately 30 μm in diameter and often are surrounded by daughter spores.[10]

North American blastomycosis includes granulomas and yeast forms with single buds.[10] A diagnosis of histoplasmosis is confirmed by the presence of granulomas and periodic acid-Schiff positive spores, which is characterized by a narrow halo of about 2 to 5 μm of dimension in the macrophages.[10] A diagnosis of actinomycosis depends on the culture, the histopathological examination of the biopsy specimen, and the presence of sulphur granules in exudate.[13] Warthin-Starry and Ziehl-Nielsen are special stains that are utilized to rule out syphillis and tuberculosis respectively.[14] A sarcoidosis diagnosis may depend on the presence of non-necrotizing granulomas with laminated concretions composed of calcium and proteins; these concretions are known as *Schaumann bodies.* One also may observe stellate inclusions enclosed within giant cells; these are known as *asteroid bodies.*[15] Gastrointestinal involvement, represented by chronic ulcers and granulomatous inflammation, may be helpful to establish the diagnosis of Crohn's disease, inasmuch as other orofacial granulomatosis do not present gastrointestinal manifestation. [16] Wegner's granulomatosis is a granulomatous vasculitis.[17] In this case, organized granulomas with macrophages, giant cells, epithelioid and lymphocytes were found. Periodic acid-Schiff staining was

performed to detect the fungus and confirm the diagnosis.

Pmycosis treatment usually is prolonged; many patients receive therapy for one to two years. It is important to emphasize that treating Pmycosis without drug therapy usually is fatal.[4] Sulfonamides have several advantages over other drugs used for treating Pmycosis, including their low cost and relatively low toxicity; however, sulfonamides also require long periods of treatment, which could result in a resistance to the fungus.[2]

Ketoconazole can be given intraorally and resultant toxicity is minor, although it requires an acid pH to be absorbed properly; as a result, antiacids and beta-blockers are contraindicated during therapy.[2,10,18] In addition, because various antimycotics inhibit peroxidase and catalase activities, the erythrocyte metabolism of patients undergoing sulfadoxin or ketoconazole therapy might be investigated.[2] Ketoconazole sometimes is used in association with sulfonamide.[10] Fluconazole and itraconazole normally are prescribed last for patients with the severe juvenile form of Pmycosis.[2] Intravenous miconazole is another treatment option.[10] Amphotericin B is a major tool for treating patients with severe disseminated disease. Because it is administered by intravenous route, normally depends on physician supervision in hospital environment. The drug may have some side effects including fever, chills, headache, nausea, vomiting, phlebitis and renal toxicity.[2,18] The patient in this case was treated with Ketoconazole for 22 months. No signs of blood test alterations or recurrence were observed over 11 years.

Summary

Due to their similar clinical and histopathological aspects, Pmycosis should be considered when performing a differential diagnosis of squamous cell carcinoma. This is particularly important in endemic regions of Latin America, because Pmycosis is rare in other parts of the world.

References

1. Restrepo A, McEwen JG, Castaneda E. The habitat of *Paracoccidioides brasiliensis*: How far from solving the riddle? Med Mycol 2001;39:233-241.
2. Brummer E, Castaneda E, Restrepo A. Paracoccidioidomycosis: An update. Clin Microbiol Rev 1993;6:89-117.
3. McEwen JG, Bedoya V, Patino MM, Salazar ME, Restrepo A. Experimental murine paracoccidiodomycosis induced by the inhalation of conidia. J Med Vet Mycol 1987;25:165-175.
4. Borges-Walmsley MI, Chen D, Shu X, Walmsley AR. The pathobiology of *Paracoccidioides brasiliensis.* Trends Microbiol 2002;10:80-87.
5. Sposto MR, Scully C, de Almeida OP, Jorge J, Graner E, Bozzo L. Oral paracoccidioidomycosis. A study of 36 South American patients. Oral Surg Oral Med Oral Pathol 1993;75:461-465.
6. de Almeida OP, Jorge J, Scully C, Bozzo L. Oral manifestations of paracoccidioidomycosis (South American blastomycosis). Oral Surg Oral Med Oral Pathol 1991;72:430-435.
7. Meneses-Garcia A, Mosqueda-Taylor A, Morales-de la Luz R, Rivera LM. Paracoccidioidomycosis: Report of 2 cases mimicking squamous cell carcinoma. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2002;94:609-613.
8. Limongelli WA, Rothstein SS, Smith LG, Clark MS. Disseminated South American blastomycosis (paracoccidioidomycosis): Report of case. J Oral Surg 1978;36:625-630.
9. Sposto MR, Mendes-Giannini MJ, Moraes RA, Branco FC, Scully C. Paracoccidioidomycosis manifesting as oral lesions: Clinical, cytological and serological investigation. J Oral Pathol Med 1994;23:85-87.
10. Scully C, de Almeida OP. Orofacial manifestations of the systemic mycoses. J Oral Pathol Med 1992;21:289-294.

11 Hayman JA, Smith IM, Flood P. Pseudoepitheliomatous hyperplasia in Mycobacterium ulcerans infection. Pathology 1996;28:131-134.

12. Grauwin MY, Mane I, Cartel JL. Pseudoepitheliomatous hyperplasia in trophic ulcers in leprosy patients. A 28-case study. Lepr Rev 1996;67:203-207.

13. Nagler R, Peled M, Laufer D. Cervicofacial actinomycosis. A diagnostic challenge. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1997;83:652-656.

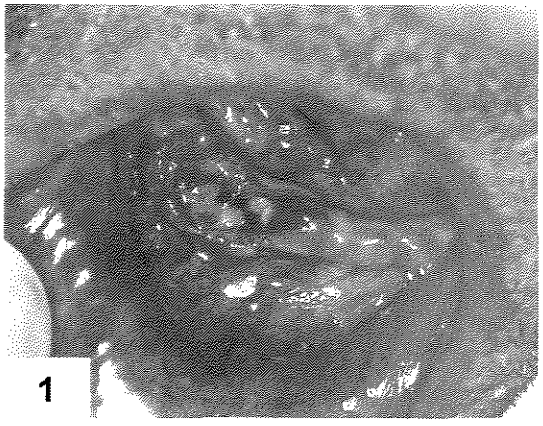
14. Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Jr Winn WC. Color atlas and textbook of diagnostic microbiology, ed. 5. Philadelphia: Lippincott Williams & Wilkins;1997:91-93.

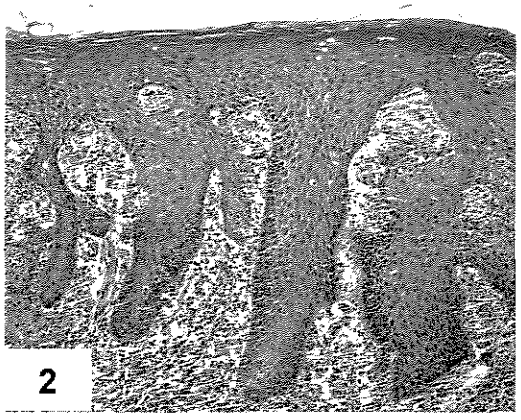
15. Cotran RS, Kumar V, Robbins SL. Robbins patologia estrutural e funcional, ed. 6. Rio de Janeiro, Brazil: Guanabara Koogan;2000:660.

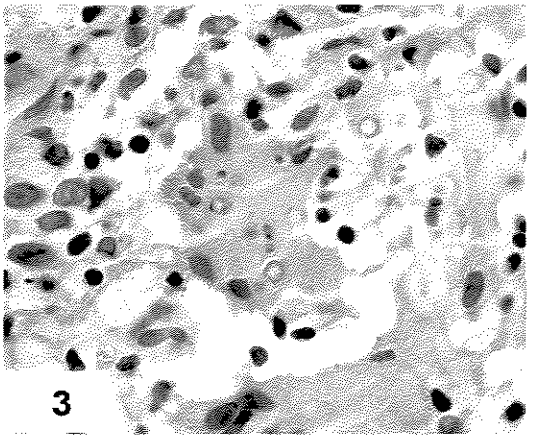
16. Manganaro AM, Holmes SM. Persistent lip swelling. J Oral Maxillofac Surg 1997;55:842-846.

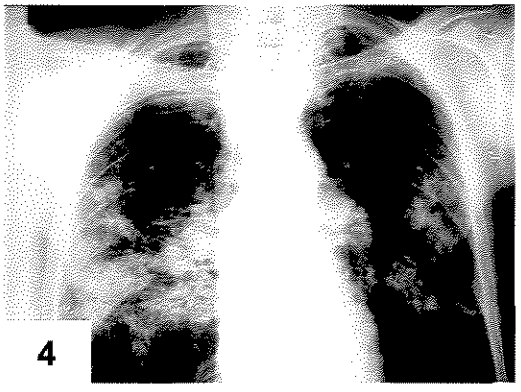
17. Shiboski CH, Regezi JA, Sanchez HC, Silverman S Jr. Oral lesions as the first clinical sign of microscopic polyangiitis: A case report. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2002;94:707-711.

18. Lazow SK, Seldin RD, Solomon MP. South American blastomycosis of the maxilla: Report of a case. J Oral Maxillofac Surg 1990;48:68-71.









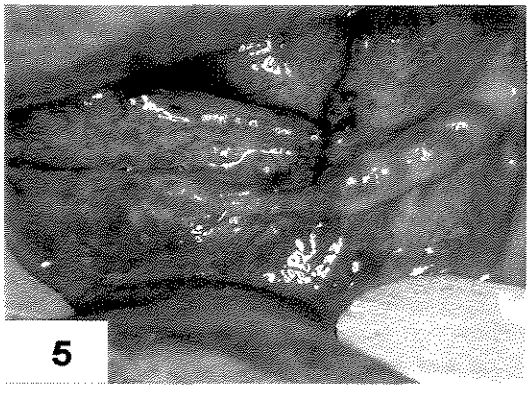


Fig 1. Ulcerative lesion on the inferior alveolar ridge. **Fig 2**. Photomicrography of oral lesion shows pseudoepitheliomatous hyperplasia, microabscesses on the epithelial surface (H&E, 50x). **Fig 3**. *P. brasiliensis* inside giant cells (PAS, 1000x). **Fig 4**. A radiograph showing pulmonary involvement. **Fig 5**. The oral lesion is resolved after two months of treatment.

7.1. Houve diferença no padrão de expressão das citoqueratinas na Pmicose bucal em relação ao epitélio normal. Na Pmicose:

7.1.a. as CK1 e CK10 não foram expressas na gengiva e palato duro;

7.1.b. a CK14 foi expressa nas camadas basal, espinhosa e superficial da mucosa jugal e lábio e,

7.1.c. a CK6 foi mais expressa na camada superficial do lábio, gengiva e palato duro, enquanto a CK16 foi aparentemente menos expressa nas camadas espinhosa e superficial da gengiva e lábio.

7.2 O índice de proliferação celular foi maior na hiperplasia pseudoepiteliomatosa em relação ao epitélio normal; não foram encontrados pleomorfismo celular e atividade proliferativa na camada espinhosa características apenas da displasia epitelial.

7.3 Houve diferença na distribuição das células inflamatórias nos granulomas organizados, áreas não granulomatosas e microabscessos

7.3.a. as células CD68 foram mais freqüentes no centro dos granulomas organizados, ao seu redor foram encontrados linfócitos (CD45+), principalmente o fenótipo CD4. Também na periferia dos granulomas foram descritos poucos linfócitos T CD8+ e B (CD20+), células dendríticas (S100+), neutrófilos (CD15+) e plasmócitos;

7.3.b. nas áreas não granulomatosas, também predominaram as células CD68 assim como os linfócitos, porém não houve diferença estatística entre as células CD4/CD8. As células dendríticas foram observadas no epitélio e no tecido conjuntivo subepitelial. Neutrófilos foram abundantes nas áreas ulceradas; alguns eosinófilos e plasmócitos também foram encontrados entre as células inflamatórias descritas;

7.3.c. nos microabscessos intraepiteliais, predominaram os neutrófilos, seguidos pelas CD68+ e CD45+. As demais células inflamatórias foram encontradas ocasionalmente nessa área.

7.4. Um dos principais diagnósticos diferenciais da Pmicose é o carcinoma espino celular, devido às semelhanças de suas características clínicas e histopatológicas, principalmente em lesões isoladas.

1. Afonso J.E., Nery L.E., Romaldini R., Bogossian M., Ribeiro-Ratto O. Função pulmonar na paracoccidioidomicose (Blastomicose Sul Americana). *Rev Inst Med Trop S Paulo* 1979;21(6):269-80.
2. Almeida O. P.; Jorge Jr. J.; Scully C.; Bozzo L. Oral manifestations of paracoccidioidomycosis (South American blastomycosis). *Oral Surg Oral Med Oral Pathol* 1991;72(4):430-5.
3. Almeida O. P.; Jorge Jr J.; Scully C. Paracoccidioidomycosis of the mouth: An emerging deep mycosis. *Crit Rev Oral Biol Med* 2003;14(5):377-83.
4. Benard G.; Romano C.C.; Cacere C.R.; Juvenale M.; Mendes-Giannini M.S.J.; Duarte A.J.S. Imbalance of IL-2, IFN-γ and IL-10 secretion in the immunosupression associated with human paracoccidioidomycosis. *Cytokine* 2001;13(4):248-52.
5. Bicalho R.N.; Santo M.F.E.; de Aguiar M.C.; Santos V.R. Oral - paracoccidioidomycosis : a retrospective study of 62 Brazilian patients. Oral disease 2001;7(1):56-60.
6. Brummer E.; Castaneda E.; Restrepo A. Paracoccidioidomycosis: an Update. *Clin Microbiol Reviews* 1993;6(2):89-117.
7. Coelho K.I.R.; Takeo K.; Yamaguchi M.; Sano A.; Kurita S.; Yoshida S. *et al.* Experimental paracoccidioidomycosis in hamster: transmission electron microscopy of inoculation site lesion. *Rev Inst Med Trop São Paulo* 1994;36:217-23.
8. Del Negro G., Wajchenberg B.L., Pereira V.G., Shnaider J., Ulhoa Cintra A.B., Marques De Assis L., Prado Sampaio S.A. Addison`s disease associated with South American Blastomycosis. *An Int Med* 1961;54(2):189-97.
9. Franco M.; Montenegro M.R.; Mendes R.P.; Marques S.A; Dillon N.L.; Mota N.G. Paracoccidioidomycosis: a recently proposed classification of its clinical forms. *Rev Soc Bras Med Trop* 1987;20(2):129-32.

*De acordo com a norma da FOP/UNICAMP, baseada no modelo Vancouver. Abreviatura dos periódicos em conformidade com o MedLine.

10. Franco M.; Peraçoli M.T.S.; Soares A.M.V.C.; Montenegro M.R.; Mendes R.P.; Meira D.A. Host-parasite relationship in paracoccidioidomycosis. Review. *Curr Trop Med Mycol* 1993;5:115-49.

11. Gómez B.L.; Nosanchuk J.D.; Díez S.; Youngchim S.; Aisen P.; Cano L.E *et al.* Detection of melanin-like pigments in the dimorphic fungal pathogen *Paracoccidioides brasiliensis in vitro* and during infection. *Infect Immun* 2001;69(9):5760-7.

12. Gómez B.L.; Nosanchuk J.D. Melanin and fungi. *Cur Opin Infect Dis* 2003;16(2):91-6.

13. Greer D. L.; Restrepo A.M. La epidemiologia de la paracoccidioidomicosis. *Boletin de la Oficina Sanitaria Panamericana* 1977;Mayo(4):28-45.

14. Januário A.; Pietro R.C.; Rodrigues E.L.; Silva C.L. Paracoccidioidomicose. *Biotecnologia Ciência e Desenvolvimento* 1999;8:10-1.

15. Joseph E. A.; Mare A.; Irving Jr W.R. Oral South American Blastomycosis in the United States of America. Report of a case. *Oral Surg Oral Med Oral Pathol*, 1966;21(6):732-7.

16. Lacaz C.S. South American Blastomycosis. *An Fac Med SP* 1956;7:120. *Apud* Ref. 42.

17. Lazow S. K.; Seldin D.R.; Solomon M.P. South American Blastomycosis of the maxilla: Report of a case. *J Oral Maxillofac Surg* 1990;48(1):68-71.

18. Limongelli W. A.; Rothstein S.S.; Smith L.G.; Clarck M.S. Disseminated South American blastomycosis (paracoccidioidomycosis): report of case. *J Oral Surgery* 1978;36(8):625-30.

19. Londero A.T., Ramos C.D., Lopes J.O.S. Progressive pulmonary paracoccidioidomycosis: a study of 34 cases observed in Rio Grande do Sul (Brazil). *Mycopathologia* 1978;63(1):53-6.

20. Loose D.S.; Stover E.P.; Restrepo A.; Stevens D.A.; Feldman A. Estradiol binds to a receptor-like cytosol binding protein and initiates a biological response in *Paracoccidiodes brasiliensis*. *Proc Natl Acad Sci USA* 1983;80:7659-63.

21. Lutz A. Uma mycose pseudococcidica localisada na bocca e observada no Brazil. Contribuição ao conhecimento das hyphoblastomycoses americanas. *Brazil-Medico* 1908;22:121-124.

22. McEwen J.G.; Bedoya V.; Pantino M.M.; Salazar M.E.; Restrepo A. Experimental murine paracoccidioidomycosis induced by the inhalation of conidia. *J Med Vet Mycol* 1987;25(3):165-75.

23. Mendes-Gianinni M.J.S; Bueno J.P; Shikani-Yasuda M.A.; Stolf A.M.S.; Masuda A.; Amato Neto V. *et al.* Antibody response to the 43 kDa glycoprotein of *Paracoccidiodes brasiliensis* as a marker for the evaluation of patient under treatment. *Am J Trop Med Hyg* 1990;43:200-6.

24. Meneses-Garcia A.; Mosqueda-Taylor A.; Luz R.M.; Rivera L.M.R.G. Paracoccidioidomicosys: Report of 2 cases mimicking squamous cell-carcinoma. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2002;94(5):609-13.

25. Migliari D.A.; Sugaya N.N.; Mimura M.A.; Cucé L.C. Periodontal aspects of the juvenile form of paracoccidioidomycosis. *Rev. Inst Med Trop S Paulo* 1998;40(1):15-8.

26. Moscardi-Bacchi M.; Soares A.; Mendes R.; Marques S; Franco M. *In situ* localization of T lymphocyte subsets in human paracoccidioidomycosis. *J Med Vet Mycol* 1989;27(3):149-58.

27. Mota L.C.; Pupo J.A. Granulomatose paracoccidióica ("Blastomycose brasileira"). I- Estudo anátomo-clínico das lesões cutâneas. II- Estudo clínico das blastomicoses tegumentares. *An Fac Med SP* 1936;12:407-26,. *Apud* Ref. 42.

28. Mota N.G.S.; Peçaroli M.T.S.; Mendes R.P.; Gattass C.R.; Marques S.A.; Soares A.M.V.C. *et al.* Mononuclear cell subsets in patients with different clinical forms of paracoccidioidomycosis. *J Med Vet Mycol* 1988;26(2):105-11.

29. Musatti C.M.; Peçaroli M.T.S.; Soares A.M.V.C.; Rezkallah-Iwasso M.T. Cell-mediated immunity in patients with paracoccidioidomycosis. *In:* Franco M.; Lacaz C.M.; Restrepo-Moreno A.; Del Negro G. *Paracoccidioidomycosis* CRC Press, Boca Raton Cap. 11;p.175-184;1994.

30. Oliveira S.J.; Mamoni R.L.; Musatti C.C.; Papaiordanou P.M.O.; Blotta M.H.S.L. Cytokines and lymphocyte proliferation in juvenile and adults forms of paracoccidioidomycosis: comparison with infected and non-infected controls. *Microbes Infection* 2002;4(2):139-44.

31. Restrepo A.M. the ecology of *Paracoccidioides brasiliensis*: a puzzle still unsolved. *Sabouraudia J Med Vet Mycol* 1985;23(5):323-34.

32. Restrepo A.; McEwen J.G.; Castañeda E. The habitat of Paracoccidioides brasiliensis: how far from solving the riddle? Review article. *Med Myc*ol 2001;39(3):233-41.

33. Romano C.C.; Mendes-Giannini M.J.S.; Duarte A.J.S.; Benard G. IL-12 and neutralization of endogenous Il-10 revert the in vitro antigen-specific cellular immunosupression of paracoccidioidomycosis patients. *Cytokine* 2002;18(3):149-57.

34. Salman L.; Sheppard S.M. South American Blastomycosis. *Oral Surg Oral Med Oral Pathol* 1962;15:671-6.

35. San-Blas G.; San-Blas F. *Paracoccidioides brasiliensis*: Cell wall structure and virulence. A review. *Mycopathologia* 1977;62(2):77-86.

36. San-Blas G. *Paracoccidioides brasiliensis:* cell wall glucans, and pathogenicity dimorphism. *Curr. Trop. Med Mycol* 1985;1:235-57.

37. Scully C.; Paes de Almeida O. Orofacial manifestations of the systemic mycoses. *J Oral Pathol Med* 1992;21(7):289-94.

38. Shikanai-Yasuda M.A.; Benard G.; Higaki Y.; Del Negro G.M.B.; Hoo S.; Vaccari E.H *et al.* Randomized trial with itraconazole, ketaconazole and sulfadiazine in paracoccidioidomycosis. *Med Mycol* 2002;40(4):411-7.

39. Sposto M.R.; Scully C.; Almeida O.P.; Jorge J.; Graner E.; Bozzo L. Oral paracoccidioidomycosis. A study of 36 South American patients. *Oral Surg Oral Med Oral Pathol* 1993;75(4):461-65.

40. Sposto M.R.; Mendes-Giannini M.J.; Moraes R.A.; Branco F.C.; Scully C. Paracoccidioidomycosis manifesting as oral lesions: clinical, cytological and serological investigation. *J Oral Pathol Med* 1994;23(2):85-7.

41. Stambuk B.U.; Puccia R.; de Almeida M.C.L.; Travassos L.R.; Schenkman S. Secretion of the 43 kDa glycoprotein antigen by *Paracoccidioides brasiliensis. J Med Vet Mycol* 1988;26(6):367-73.

42. Villalba H. *Características microscópicas da Paracoccidioidomicose bucal.* Piaracicaba, 1998 [Dissertação (Mestrado) em Biologia e Patologia Buco-Dental – Faculdade de Odontologia de Piracicaba, Universidade Estadual de Campinas].

UNICAMP

COMITÊ DE ÉTICA EM PESQUISA

UNIVERSIDADE ESTADUAL DE CAMPINAS

FACULDADE DE ODONTOLOGIA DE PIRACICABA

CERTIFICADO

Certificamos que o Projeto de pesquisa intitulado "Paracoccidioidomicose bucal. Estudo imunohistoquímico e ultra-estrutural", sob o protocolo nº **122/2001**, da Pesquisadora **Estela Kaminagakura**, sob a responsabilidade do Prof. Dr. **Oslei Paes de Almeida**, está de acordo com a Resolução 196/96 do Conselho Nacional de Saúde/MS, de 10/10/96, tendo sido aprovado pelo Comitê de Ética em Pesquisa – FOP.

Piracicaba, 19 de dezembro de 2001

We certify that the research project with title "Oral paracoccidioidomycosis. Immunohistochemical and ultrastructural study", protocol nº **122/2001,** by Researcher **Estela Kaminagakura**, responsibility by Prof. Dr. **Oslei Paes de Almeida**, is in agreement with the Resolution 196/96 from National Committee of Health/Health Department (BR) and was approved by the Ethical Committee in Resarch at the Piracicaba Dentistry School/UNICAMP (State University of Campinas).

Piracicaba, SP, Brazil, December 19 2001

Prof. Dr. Pedro Luiz Rosalen
Secretário
CEP/FOP/UNICAMP

Prof. Dr. Antonio Bento Alves de Moraes
Coordenador
CEP/FOP/UNICAMP