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análise histológica e imuno-histoquímica

"LICHENOID DISEASES OF THE SKIN AND ORAL MUCOSA: histological and immunohistochemical analysis"

CAMPINAS

2014

Universidade Estadual de Campinas
Faculdade de Ciências Médicas

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Orientadora: Profa. Dra. Maria Letícia Cintra

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Tese de Doutorado apresentada à Pós-Graduação em Ciências Médicas da Faculdade de Ciências Médicas da Universidade Estadual de Campinas para obtenção do título de Doutora em Ciências Médicas, área de concentração Anatomia Patológica

Thesis of Doctorate presented to the Graduate Program of Medical Sciences from the School of Medical Sciences, University of Campinas for obtaining the title of Doctor in Medical Sciences, concentration area of Pathologic Anatomy

Este exemplar corresponde à versão final da tese defendida pela aluna **DENISE LAGE**, e orientada pela **PROFA. DRA. MARIA LETÍCIA CINTRA**

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Assinatura da Orientadora

CAMPINAS

2014

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Ciências Médicas
Juliana Ravaschio Franco de Camargo - CRB 8/6631

Lage, Denise, 1979-
L135d Doenças liquenoides da pele e mucosa oral : análise histológica e imuno-histoquímica / Denise Lage. – Campinas, SP : [s.n.], 2014.

Orientador: Maria Letícia Cintra.
Tese (doutorado) – Universidade Estadual de Campinas, Faculdade de Ciências Médicas.

1. Líquen plano. 2. Líquen plano bucal. 3. Erupção por droga. 4. Apoptose. 5. Imunoistoquímica. 6. Perforina. I. Cintra, Maria Letícia,1951-. II. Universidade Estadual de Campinas. Faculdade de Ciências Médicas. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: Lichenoid diseases of the skin and oral mucosa : histological and immunohistochemical analysis
Palavras-chave em inglês:
Lichen planus
Lichen planus, oral
Drug eruptions
Apoptosis
Immunohistochemistry
Perforin
Área de concentração: Anatomia Patológica
Titulação: Doutora em Ciências Médicas
Banca examinadora:
Maria Letícia Cintra [Orientador]
Maria Aparecida Barone Teixeira
Mirian Nacagami Sotto
Paulo Eduardo Neves Pereira Velho
Leandro Luiz Lopes de Freitas
Data de defesa: 20-01-2014
Programa de Pós-Graduação: Ciências Médicas

BANCA EXAMINADORA DA DEFESA DE DOUTORADO

DENISE LAGE

ORIENTADORA PROFA. DRA. MARIA LETICIA CINTRA

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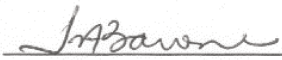
2. **PROFA. DRA. MARIA APARECIDA BARONE TEIXEIRA** 
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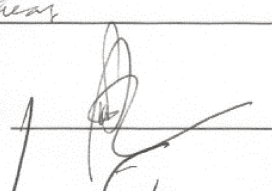
4. **PROF. DR. PAULO EDUARDO NEVES FERREIRA VELHO** 
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Data: 20 de janeiro de 2014

A Deus,
que guia a minha vida em todos os momentos.

Aos meus pais, Helena e Nilton,
pela dedicação,
apoio constante e
amor incondicional em
todos os momentos da minha vida.

Ao meu marido, Sérgio,
o melhor companheiro de todas as
minhas maiores realizações.

AGRADECIMENTOS

À Prof. Dra. Maria Letícia Cintra, minha orientadora, pela simplicidade, pela convivência enriquecedora, pelo incentivo constante e pelos seus preciosos ensinamentos. Reforço meu carinho e extrema admiração.

Obrigada pela confiança.

Ao Prof. Dr. Elemir Macedo de Souza, pela disposição e impecável condução dos pacientes, que permitiram a fundamentação clínica deste trabalho.

Aos meus irmãos Cristina e Juninho e aos meus cunhados, André e Juliane, cuja presença e apoio se configuram em estímulo na realização dos meus objetivos.

A todos os meus sobrinhos que são a alegria da minha vida.

Agradeço aos pacientes que constituíram o elemento indispensável e fundamental deste trabalho.

À estaticista Creusa Maria Roveri Dal Bó, pelos serviços prestados de assessoria estatística.

Ao Prof. Dr. Konradin Metze pela contribuição na análise estatística dos meus resultados.

Ao Dr. Amílcar Castro, pela dedicação e incentivo à pesquisa durante a graduação.

À Profa. Dra. Patrícia Daldon e ao Prof. Dr. Adilson Costa, pelos ensinamentos de Dermatologia e incentivo como pesquisadora, a minha admiração pessoal.

À Dra. Tânia Benetti, pela amizade, por sua generosidade e solicitude em todos os momentos.

À Priscila Bianchi Juliano, pela ajuda na fase inicial do trabalho.

A todos os alunos da pós-graduação pelo apoio, estímulo e incentivo nos momentos mais difíceis.

À bióloga Ana Cláudia Sparapani Piaza, pelo seu sorriso acolhedor, apoio carinhoso e profissionalismo constante que contribuiu de forma fundamental para concretização deste trabalho.

À técnica Luzia Reis pelo cuidado na confecção das lâminas e paciência nos inúmeros recortes adicionais.

Ao técnico de imagem Adilson Abílio Piaza, pelas sugestões e auxílio competente na obtenção e correção de toda a documentação da tese.

À secretária da Subcomissão de Pós-Graduação, Maria do Carmo Machado da Silva, pela infinita ajuda em todas as fases desta jornada, inclusive alertando-me e instruindo-me quanto aos prazos e documentação.

Aos funcionários do Departamento de Anatomia Patológica-FCM/Unicamp, pela ajuda e paciência.

À FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) pela concessão financeira que viabilizou a realização deste trabalho.

A todos que, direta ou indiretamente, contribuíram de alguma forma para a realização deste trabalho.

“A nossa maior glória não reside no fato de nunca cairmos, mas sim em levantarmo-nos sempre depois de cada queda.”

Confúcio

RESUMO

O líquen plano (LP) pode afetar a pele e/ou as mucosas. Histologicamente apresenta infiltrado linfo-histiocitário na junção epitélio-tecido conjuntivo e apoptose de células epiteliais basais. No LP oral (LPO), ocorre erosão frequente pela maior intensidade da necrose. O LP cutâneo (LPC) e o LPO apresentam características histopatológicas similares, mas o curso clínico é diverso. O LPC costuma ter seu curso limitado, enquanto o LPO é frequentemente recidivante.

A erupção liquenoide a droga (ELD) desenvolve-se após semanas da ingestão do medicamento e a resolução do quadro é lenta após a interrupção, dificultando o diagnóstico diferencial com o LP idiopático. Os achados clínicos e histológicos podem ser indistinguíveis daqueles do LP, mas a patogênese da ELD não é conhecida.

Diferenças locais no sistema imune da mucosa oral e pele poderiam explicar a diversidade no comportamento clínico do LP. Quanto à ELD, há poucas publicações sobre as alterações imunes que atuam no seu desenvolvimento.

A citotoxidade celular é mediada, dentre outros mecanismos, por grânulos contendo granzima B e perforina, produzidos por linfocitos T citotóxicos e células *natural killers (*NK).

Com o objetivo de estudar a citotoxicidade celular na patogênese destas doenças, foram analisadas 29 amostras de LPO, 16 de LPC e 6 de ELD. Os cortes foram corados pela H&E e técnica de imuno-histoquímica, para a demonstração de linfocitos $TCD4^+$ e $TCD8^+$, macrófagos HAM 56^+ e MAC 387^+, granzima B, perforina e ICAM-1.

As amostras de LPO apresentaram maior densidade de células granzima B^+ e $perforina^+$, em comparação às do LPC. Nos dois grupos de doenças, quanto maior era o número de células $perforina^+$, maior era o de células granzima B^+. Maior número de células CD4-positivas foi encontrado nas lesões ativas, quando comparado com o das regressivas, no LPO, mas não no LP cutâneo.

À comparação entre o LPC e a ELD, quanto maior o número de células CD8-positivas, maior era o número de células que expressavam a perforina no grupo LPC. Quanto maiores eram os valores da granzima B, maiores os da perforina, no grupo LPC. Quanto maiores eram os valores da granzima B, maiores os de células apoptóticas agregadas, no grupo da ELD. Nas amostras do LPC, quanto maiores os valores das células T, maiores os dos macrófagos HAM56-positivos e vice-versa. Nas amostras da ELD, foi encontrada correlação negativa entre o número de células T e o de histiocitos jovens (MAC 387^{+}). Havia correlação positiva entre o número de células T e o de células CD8, no grupo da ELD. O mesmo não ocorreu, no grupo do LPC.

Concluindo, a expressão aumentada dos grânulos citotóxicos, no LPO, pode estar associada à maior gravidade da doença na mucosa. Os resultados favorecem um papel mais importante da granzima B e linfocitos $TCD8^{+}$, no mecanismo patogenético da ELD, comparativamente com o da perforina, de maior importância no LPC.

É possível que a ação da granzima B esteja ligada ao número abundante de *clusters* encontrado na ELD. Embora o LPC e a ELD apresentem semelhanças clínicas e histológicas, a etiopatogênese parece ser distinta.

Palavras-chave: Líquen plano, líquen plano bucal, erupção por droga, apoptose, granzima B, perforina, imunohistoquímica.

ABSTRACT

Lichen planus (LP) can affect the skin and/or mucous membranes. Histologically it presents lymphohistiocytic infiltrate in the epithelium-connective tissue junction and apoptosis of basal epithelial cells. In oral LP (OLP), erosion occurs frequently by higher intensity of necrosis. Cutaneous LP (CLP) and OLP present similar histopathological features, but the clinical course is diverse. Spontaneous remission is common in CLP, but OLP follows a prolonged course, with periods of remission and relapse.

Lichenoid drug eruption (LDE) develops after weeks of drug intake and the resolution of lesions is slow after drug discontinuation, hampering the differential diagnosis with (idiopathic) LP. Clinical and histological findings of LDE may be indistinguishable from those of LP, but LDE pathogenesis is poorly understood

Local differences in the immune system of the skin and oral mucosa could explain the diversity in the clinical behavior of CLP and OLP. Regarding LDE, there are few publications on the immune changes that act in its development.

Cellular cytotoxicity is mediated, among other mechanisms, by granules containing perforin and granzyme B, produced by cytotoxic T lymphocytes and NK cells.

In order to study cellular cytotoxicity in the pathogenesis of these diseases, we analyzed 29 samples of OLP, 16 of CLP and 6 of LDE. The sections were stained for H&E and immunohistochemically targeted with CD4, CD8, HAM 56, MAC387, granzyme B, perforin and ICAM-1.

OLP specimens exhibited higher density of cytotoxic granules (perforin and granzyme B) when compared with CLP. In both groups of diseases, the greater the number of perforin$^+$ cells, the greater was the number of granzyme B$^+$ cells. Increased number of CD4$^+$ cells was found in active lesions as compared with the regressive ones in OLP but not in the CLP.

The comparison between CLP and LDE revealed that the greater the number of CD8+ cells, the greater the number of cells expressing perforin in CLP group.

The higher were the values of granzyme B, the higher the perforin values in the CLP group; the higher were the values of granzyme B, the higher the number of clusters of apoptotic cells in the LDE group. Within CLP group, the higher were the values of T cells, the greater the number of HAM56+ macrophages and vice versa. In LDE samples, negative correlation was found between the number of T cells and young histiocytes (MAC 387^{+}). There was a positive correlation between the number of T cells and CD8 cells in LDE group, but not in CLP group.

Concluding, increased expression of cytotoxic granules in OLP may be associated with greater mucosa severity. The results favor a greater role of granzyme B and $CD8^{+}$ lymphocytes in the pathogenic mechanism of LDE, when compared with perforin, of greater importance in CLP. It is possible that the action of granzyme B is connected to the abundant number of clusters found in LDE. Although CLP and LDE present clinical and histological similarities, the etiopathogenesis appears to be distinct.

Keywords: Lichen planus, oral lichen planus, drug eruption, apoptosis, granzyme B, perforin, immunohistochemistry.

APC	*Antigen presenting cell*
CL	Células de Langerhans
CTACK	Quimiocina cutânea atraente para célula T
DHC	Doença hepática crônica
ELAM-1	E*ndothelial leukocyte adhesion molecule*
ELD	Erupção liquenóide a droga
Fas	*Fatty acid synthetase*
FasL	*Fatty acid synthetase ligand*
HIV	*Human immunodeficiency vírus*
HLA	*Human leucocyte antigen*
ICAM-1	*Intercellular adhesion molecule-1*
IFN-γ	*Interferon-gama*
IgM	Imunoglobulina M
IgG	Imunoglobulina G
IL	Interleucina
LFA-1	L*eukocyte function-associated antigen-1*
LTC	Linfocito T citotóxico

LP Líquen plano

LPO Líquen plano oral

MAC-1 *Macrophage-1 antigen*

MHC *Major histocompatibility complex*

mRNA *Messenger RNA*

NK *Natural killer*

OR *Odds ratio*

RANTES *Regulated upon activation, normal T-cell expressed and secreted*

Th1 Linfocitos *T helper 1*

Th2 Linfocitos *T helper 2*

TNF-α *Tumor necrosis factor- alpha*

TNF-β *Tumor necrosis factor-beta*

TRAIL *Tumor necrosis factor-related apoptosis-inducing ligand*

VCAM-1 *Vascular cell adhesion molecule-1*

VHC Vírus da hepatite C

VLA-4 V*ery late antingen-4*

SUMÁRIO

1- INTRODUÇÃO

1.1- Aspectos clínicos e histológicos

Dermatites liquenoides são aquelas que se manifestam por pápulas poligonais eritêmato-violáceas, brilhantes, assemelhando-se ao aspecto do líquen das árvores. O exame histológico demonstra infiltrado linfo-histiocitário, localizado na derme papilar, em faixa subepidérmica, que se estende à epiderme e obscurece a interface dermo-epidérmica. O protótipo das dermatites liquenoides é o líquen plano (LP). Dentre os diagnósticos diferenciais, destaca-se a erupção liquenoide a droga (ELD)[1].

O LP é uma doença inflamatória idiopática da pele e membranas mucosas. Apesar da sua incidência variar de acordo com a localização geográfica, o LP cutâneos (LPC) afeta de 0,22% a 1% da população adulta. Não existe predisposição racial evidente. O surgimento do LP acontece mais comumente durante a quinta e sexta décadas, com dois terços dos pacientes desenvolvendo a doença entre os 30 e 60 anos de idade. Apenas 1 a 4% dos pacientes são crianças e os idosos raramente são afetados; no entanto, estudos recentes sugerem que o LP deve ser mais comum em crianças, especialmente na população árabe, apesar de ser extremamente raro nos primeiros anos de vida. Embora se acredite que não haja predileção por sexo, alguns estudos mostraram que as mulheres são acometidas aproximadamente duas vezes mais que os homens. Relatos de casos familiares são raros, mas o LP pode ocorrer em até 10% de parentes de primeiro grau dos pacientes afetados. Os casos de LP familiar se caracterizam por acometer faixa etária mais jovem, com mais recidivas e acometimento mais frequente da mucosa oral. No entanto, casos descritos de LP concomitante, em gêmeos monozigóticos que viviam juntos, sugerem um fator desencadeante ambiental[2].

O exame dermatológico revela a presença de pápulas poligonais achatadas, de 0,5 a 2mm de diâmetro, eritêmato-violáceas, intensamente pruriginosas, embora alguns pacientes possam ser assintomáticos. As pápulas podem se dispor em arranjo linear, especialmente após escoriações ou outros

traumatismos, o que é conhecido como fenômeno de Koebner. Na superfície da lesão são vistas estrias brancas e delicadas denominadas estrias de Wickham. Frequentemente são encontradas agrupadas nas superfícies flexoras dos punhos e antebraços; face dorsal das mãos, parte anterior das pernas, pescoço, área pré-sacral também são locais comuns. O acometimento ungueal ocorre em 10% dos pacientes com LP e geralmente diversas unhas são afetadas. As anormalidades ungueais características incluem afinamento da parte lateral, estrias longitudinais, fissuras, formação de pterígio e perda permanente da lâmina ungueal se a doença não for tratada. O LP é uma das doenças que se manifestam com distrofia de todas as unhas (síndrome das 20 unhas) e é possível que represente uma variante do LP, neste caso, mais comum em crianças que em adultos. O LP comumente acomete mucosas, como a glande, onde podem ter uma configuração anular e, em especial, a cavidade oral (líquen plano oral - LPO), de forma isolada ou associado ao LPC[2].

O LPO pode se apresentar em pelo menos sete formas: atrófica, bolhosa, erosiva, papulosa, pigmentada, em placa e reticular. Algumas variantes podem ser identificadas em um mesmo paciente. Afeta de 1 a 4% da população adulta e é a doença mais comum nos ambulatórios especializados em estomatologia[2]. O acometimento oral ocorre em, aproximadamente, 60 a 70% dos pacientes com LPC, mas esta pode ser a única manifestação da doença, em 20 a 30% dos casos[3]. Ocorre mais no sexo feminino, na proporção aproximada de 1,4 mulheres para 1 homem, e na faixa etária acima de 40 anos[4]. A forma mais comum e mais característica do LPO é o padrão reticular. É caracterizado por linhas levemente elevadas, esbranquiçadas, com padrão rendilhado, ou em anéis, com pontas radiadas curtas. Essa forma é geralmente assintomática e as lesões costumar ser bilaterais e simétricas. Existe uma maior incidência de lesões semelhantes a placas entre os tabagistas. Geralmente, dor e sensação de queimação acompanham as lesões erosivas, atróficas ou bolhosas.

As regiões lingual, gengival e jugal são as áreas mais frequentemente afetadas da mucosa. O palato, assoalho da boca e coxins retromolares também podem ser acometidos. O acometimento da gengiva é comum e o LPO que afeta exclusivamente a gengiva é observado em 10% dos casos. A lesão gengival pode tomar a forma de estomatite gengival ou gengivite descamativa[2].

Os pacientes com LPO devem ser indagados a respeito de sintomas relacionados ao acometimento esofagiano e examinados à procura de outras lesões mucosas, particularmente lesões genitais, e vice versa, porque aproximadamente 70% dos pacientes com LP da mucosa vulvovaginal têm sinais clínicos de LPO. Existem relatos de que o tipo erosivo ou ulcerado do LPO é menos frequentemente associado ao LPC, em comparação às outras variantes do LPO. As lesões do LPO são mais resistentes à terapia e sofrem remissão espontânea menos frequente do que as lesões cutâneas[2]. Diferenças locais no sistema imune, quando se compara a mucosa oral com a pele, poderiam explicar essa diversidade observada no comportamento clínico do LP, nestas duas localizações.

Existe considerável controvérsia se o LPO tem potencial maligno inerente. Acredita-se que o risco de transformação maligna seja baixo, porém alguns fatores poderiam aumentá-lo, como doença de longa duração, forma atrófica ou erosiva e uso de tabaco. Descreve-se o desenvolvimento de carcinoma espinocelular, *in situ* ou invasor, bem diferenciado, em 0,5 a 5% dos pacientes com LPO, no acompanhamento em longo prazo[3].

Na prática clínica, muitas vezes, o diagnóstico diferencial entre LP e ELD é bastante difícil. Existem algumas diferenças clínicas e histopatológicas que podem auxiliar no diagnóstico (quadro 1). A ELD é caracterizada por lesões individualmente semelhantes às encontradas no LP; no entanto, não respeitam a topografia habitual. São geralmente mais generalizadas, frequentemente desenvolvem aspecto psoriasiforme e tendem a poupar as áreas clássicas do LP. A distribuição em áreas fotoexpostas é frequente, especialmente com o uso de

hidroclortiazida. As membranas mucosas geralmente são poupadas. Ambos os sexos são igualmente afetados e predominam em adultos aproximadamente 10 anos mais velhos do que os com LP idiopático[2].

Habitualmente existe um período de latência, após a ingestão do medicamento, que varia de semanas a meses. Estudo incluindo 17 pacientes com erupções liquenoides induzidas por uma variedade de medicamentos, observou que o período latente médio foi de 12 meses. O período latente varia não somente com a droga precipitante, mas também por outros fatores como frequência de administração, a dosagem e a intensidade da reação individual do paciente à droga precipitante. Por exemplo, o período de latência foi de 2 meses a 3 anos para a penicilamina, de aproximadamente 1 ano para os β-bloqueadores e de 4 a 6 meses para a quinacrina[6]. A resolução das lesões é lenta, variando de semanas a meses, após a interrupção do medicamento, com hiperpigmentação residual pós-inflamatória mais pronunciada, em relação ao LPC idiopático[7].

A ELD associa-se, principalmente, ao uso dos seguintes medicamentos: captopril, enalapril, labetalol, metildopa, propranolol, cloroquina, hidrocloroquina, quinacrina, sais de ouro, clorotiazida, hidroclorotiazida, quinidina, penicilamina (quadro 2)[6].

As lesões do LP caracterizam-se, ao exame histopatológico, por alterações epidérmicas representadas por hiperceratose, hipergranulose em cunha, acantose em “dente de serra” e degeneração hidrópica da camada basal, de intensidade variada. Acompanha infiltrado linfo-histiocitário em faixa subepidérmica, obscurecendo a junção dermo-epidérmica. A formação dos corpos coloides (corpos apoptóticos ou fibrilares ou de Civatte) é decorrente da agressão autoimune aos ceratinocitos da camada basal[1]. Estudos ultra-estruturais no líquen plano[8,9] revelaram que os corpos coloides são exemplos típicos de morte celular por apoptose e a presença de linfocitos junto às células epidérmicas apoptóticas, sugere mecanismo imunocelular na patogênese do LP.

A vacuolização da camada basal pode confluir e resultar em pequenas fendas entre a epiderme e a derme (os chamados espaços de Max-Joseph). Geralmente está presente incontinência pigmentar com múltiplos melanófagos dérmicos[1,2].

As lesões do LPO habitualmente mostram paraceratose ao invés de hiperceratose e o epitélio frequentemente se torna atrófico[2]. Alterações na camada basal são comuns no LPO e incluem interrupções, ramificações e duplicações. Além disso, os elementos que ancoram o ceratinocito basal à derme (hemidesmossomos, filamentos e fibrilas) estão modificados, produzindo fraqueza na junção dermo-epidérmica. As consequências histológicas e clínicas deste fenômeno são, respectivamente, as fendas na interface e as bolhas na mucosa oral (líquen plano bolhoso)[4]. As características histológicas da ELD são similares às do LPC. Classicamente, os critérios histológicos que apontam para o diagnóstico da ELD e/ou desfavorecem o diagnóstico de LPC, incluem a presença de eosinofilos e plasmocitos no infiltrado inflamatório, paraceratose focal, infiltrado perivascular profundo e corpos citóides nas camadas córneas e granulosas. Contudo, o emprego destes critérios, na distinção entre LPC e ELD mostra que, em muitos casos, não há correspondência clínico-patológica[2,5].

1.2- Etiologia e patogênese

A etiologia do LP é desconhecida, mas diversas teorias foram propostas. É provável que tanto fatores endógeno-genéticos quanto exógeno-ambientais, como drogas ou infecções, possam interagir para desencadear a doença.

Existe um crescente número de evidências de que o LP represente o resultado de dano autoimune mediado por células T aos ceratinocitos basais que expressam autoantígenos alterados na sua superfície. Observações clínicas há muito sugerem associação entre a exposição a uma série de antígenos exógenos (vírus, alérgenos e medicações) e o desenvolvimento do LP.

Quanto à teoria infecciosa, vários agentes, incluindo alguns vírus e *Helicobacter pylori,* têm sido associados ao LPO, porém algumas vezes com base em dados duvidosos. Na família dos Herpes vírus, por exemplo, o herpes simples 1, o Epstein-Barr, o citomegalovírus e o herpes vírus 6 têm sido implicados no LPO. Pequenas séries detectaram fragmentos de DNA destes vírus em lesões de LPO, principalmente no tipo erosivo. Contudo, não existe diferença estatisticamente importante na prevalência de anticorpos (IgM e IgG) para alguns destes vírus entre pacientes com LPO e controles. Além disso, muitas vezes não está claro se o vírus atua na patogênese ou se é secundário às lesões de LPO. Poucos casos de lesões liquenoides foram relatados em pacientes infectados pelo HIV e a maioria destas lesões poderia estar relacionada à terapia com zidovudine ou cetoconazol[10].

A frequente associação de LP com doença hepática crônica (DHC) está bem documentada em pacientes do Mediterrâneo, enquanto estudos prospectivos do Reino Unido e da Escandinávia não revelaram qualquer correlação entre o LPO e doenças do fígado. Existem poucos relatos de erupção liquenoide, principalmente cutânea, após a administração de vacinas contra a hepatite B e a maioria dos pacientes com LP e DHC não estão infectados por este vírus. Outras doenças hepáticas como hemocromatose, colangite esclerosante primária, doença de Wilson e deficiência de α1-antitripsina têm sido raramente associadas ao LP, e a relação com cirrose biliar primária parece decorrer principalmente da administração de penicilamina durante o tratamento. A associação entre LP e infecção pelo vírus da hepatite C (VHC) tem sido proposta por diversos estudos. Em uma revisão sistemática recente incluindo estudos controlados, a proporção de pacientes VHC positivos foi maior no grupo de LP quando comparado com o controle em 20 dos 25 estudos, com *odds ratio* (OR) de 4.80 (intervalo de confiança de 3,25-7.09), mostrando uma diferença estatisticamente significante**.** Porém, quando a OR foi calculada para pacientes apenas com LPO, esta diferença não foi substancial, sendo considerável somente em estudos oriundos do Mediterrâneo e do norte europeu e, ainda assim, sem significância estatística[10].

Sensibilizantes de contato e haptenos podem também ter um papel na patogênese do líquen plano. O papel da alergia de contato a diversos metais na exacerbação ou indução no LP foi bem descrito, baseado na exposição a metais de restauração ou de estruturas dentárias, testes de contato positivos e regressão ou clareamento completo após a remoção do metal sensibilizante e reposição com outros materiais[2]. Estudos recentes têm sugerido que as lesões parecem ser decorrentes de hipersensibilidade celular de contato aos materiais odontológicos em indivíduos suscetíveis que foram sensibilizados ao longo do tempo. Estes materiais, em contato com a mucosa oral, poderiam alterar diretamente a antigenicidade dos ceratinocitos basais, pela liberação de mercúrio ou outros produtos. Na hipersensibilidade tipo IV, atuam macrófagos e linfocitos T, que são sensibilizados ao antígeno (hapteno); porém ainda é desconhecido como o mercúrio e outros haptenos metálicos de uso dentários são capazes de ativar a resposta imune[11]. Como os alérgenos envolvidos são dissolvidos e disseminados pela saliva, as reações na mucosa podem se estender além das áreas de contato. Os metais que agravam o LP oral incluem mercúrio da amálgama, cobre e ouro. Apesar de aproximadamente 95% dos pacientes terem melhorado após a remoção do material sensibilizante, 75% de pacientes com teste negativo também relataram clareamento das lesões de LPO após remoção do metal e substituição por outros materiais. Esses resultados indicam que remissões espontâneas podem ocorrer e trazem à tona a discussão sobre a importância da alergia de contato a metais na patogênese do LP. Todavia, alguns autores argumentaram que até mesmo nos pacientes que melhoraram após a remoção, mas tiveram teste de contato negativo, o mercúrio pode atuar como um fator irritante na patogênese do LP (por meio do fenômeno de Koebner). O desenvolvimento de alergia de contato a metais presentes em restaurações dentárias em indivíduos com LP pode ser explicado pela fácil penetração do metal através da mucosa lesada. Logo, a alergia de contato a restaurações dentárias de amálgama deve ser suspeita e testes de contato devem ser realizados nos pacientes com LPO, especialmente se as lesões estiverem em intimo contato com a amálgama[2].

1.3- Autoantígenos

Um modelo murino de LP foi estabelecido ao empregar células T auto-reativas capazes de produzir IFN-γ e TNF-α. A inoculação de células T $CD4^+$ auto-reativas nas patas de ratos singênicos pode induzir alterações histológicas locais semelhantes às do LP ou doenças cutâneas liquenoides. Neste modelo, as células T auto-reativas podem responder a antígenos próprios da classe MHC II expressos de maneira constitucional nos macrófagos e CL, e elas migram para dentro da epiderme, resultando em lesão epidérmica. Estas células T, desta forma, podem induzir lesões semelhantes às do LP, sem nenhuma antigenicidade da epiderme. No processo natural da doença, no entanto, alterações na antigenicidade das células epidérmicas, induzidas por agentes exógenos, como infecções, podem ser um pré-requisito para desencadear a ativação dessas células T. Importante que essas reações autoimunes podem ter a função de eliminação de ceratinocitos alterados por esses agentes exógenos. No entanto, se uma célula T inicialmente responsiva a auto-antígenos modificados por agentes exógenos, subsequentemente reagisse de forma cruzada com alguns epítopos próprios, estas mesmas células T iriam então responder cronicamente aos epítopos próprios previamente ignorados, levando à perpetuação de um ataque autoimune dessas células T, em vez da eliminação dos ceratinocitos anormais[12].

Apesar de numerosos trabalhos relatarem associação do LP com doenças autoimunes, estudos com números maiores de pacientes com LP não mostraram frequência aumentada desta associação. O HLA-DR1 é mais comum em pacientes com LPO e LPC e o HLA-DR9 é mais comum em pacientes com LPO como manifestação única em estudo realizado com pacientes japoneses e chineses. Em uma amostra da população britânica, HLA-B27, B51 e Bw57 foram encontrados associados ao LPO. No entanto, uma verdadeira associação com alelo de HLA específico foi difícil de estabelecer devido à heterogeneidade geográfica significativa. Pacientes com LP geralmente relatam o surgimento e o agravamento dos sintomas em níveis elevados de estresse, mas nenhum dado conclusivo foi publicado[2].

1.4- Células efetoras

A patogênese do LP decorre de um desequilíbrio na regulação imune em que atua, principalmente, a resposta celular. O infiltrado inflamatório consiste primariamente de células T e macrófagos. O ataque autoimune de células T, contra a epiderme, constitui o evento patológico primário na dermatite liquenoide. A gravidade, consequente, da lesão epidérmica, depende do balanço entre o grau de atividade inflamatória, a modulação ou a perpetuação da ativação de células T e a capacidade da epiderme de se proteger contra o ataque.

Existem opiniões conflitantes sobre a população predominante de linfocitos TCD4$^+$ e CD8$^+$[13], com alguns achados de predomínio das células TCD4$^+$ no infiltrado inflamatório, outros de acúmulo de linfocitos TCD8$^+$, junto aos ceratinocitos apoptóticos, especialmente nas lesões tardias[14]. Acredita-se que o infiltrado de linfocitos TCD4$^+$ esteja predominantemente na derme superior, enquanto os linfocitos TCD8$^+$, principalmente na junção epidermo-dérmica[15,16].

Evidências para suportar o papel crucial das células TCD8$^+$ na agressão autoimune aos ceratinocitos basais foram fornecidas pelo isolamento de células TCD8$^+$[13] da pele na área lesada; essas células T exibiam atividade citotóxica específica tanto contra ceratinocitos lesados autólogos como normais[14]. Deve-se dar atenção para o fato de que essa linhagem de células TCD8$^+$ e seus clones podem representar uma seleção e/ou expansão *in vitro* de células T auto-reativas durante seu cultivo com células apresentadoras de antígenos autólogos.

O ataque às células da camada basal, evidenciado pelos restos apoptóticos do DNA, é maior na epiderme intimamente associada às áreas de invasão de células TCD8$^+$ da epiderme. O interferon-gama (IFN-γ) produzido pelas células TCD8$^+$ aumenta a expressão de Fas pelos ceratinocitos, deixando-os suscetíveis à apoptose induzida pelo Fas ligante (FasL) e mediada pelas células T; os ceratinocitos podem ser mortos por meio da ligação cruzada do

receptor de Fas expresso nos próprios ceratinocitos pelos seus ligante (FasL) expresso pelas células $TCD8^+$ e, possivelmente, pelas células *natural killer* (NK). Essa interação desencadeia uma cascata de reações enzimáticas intracelulares resultando na fragmentação do DNA. Além do Fas, a apoptose induzida por receptores de morte celular envolvem os processos de sinalização via TNF-R1, TRAIL-R1 e 2 e DR3 e DR6. Uma vez que as células T *helper* tipo 1 (Th1), como as células $TCD4^+$ no modelo murino, podem produzir grandes quantidades de IFN-γ e TNF-β por sua ativação e dessa forma produzir ou aumentar a expressão de proteínas associadas à apoptose, como o Fas e o TRAIL, eles também podem ter um papel na extensa lesão à epiderme, ao promover a morte, por apoptose, dos ceratinocitos[2].

Além disso, o IFN-γ e TNF-β liberados tanto pelas células $TCD4^+$ quanto pelas células $TCD8^+$, podem induzir a expressão de ICAM-1 pelos ceratinocitos, desta forma tornando essas células mais adesivas aos ceratinocitos e facilitando a exocitose dos grânulos contendo perforina e granzimas. É importante salientar que essas citocinas foram demonstradas em altas concentrações de LP e que essas citocinas pró-inflamatórias também podem ser produzidas por ceratinocitos alterados. Estudos mais recentes mostraram que a exocitose de grânulos, ao contrário do sistema Fas/FasL, é o principal caminho de citotoxicidade mediada pelas células $TCD4^+$ e $TCD8^+$ em humanos[18]. Entretanto, uma combinação dos dois mecanismos é o mais provável, mas o predomínio dependeria do estadio, em particular, do processo da doença[2].

1.5- O acesso das células T efetoras à epiderme

Um evento crítico na iniciação das respostas imunes nas lesões de LP é a migração das células T da circulação para um sítio da pele em particular. Grandes quantidades de citocinas pró-inflamatórias e do tipo 1 como IFN-γ e TNF-α são liberadas pelas células T ativadas e induzem ou aumentam a expressão de E-selectinas e subsequentemente de ICAM-1 e de quimiocinas

associadas à pele, por exemplo quimiocina cutânea para células T (CTACK) no endotélio. Essa expressão sequencial de moléculas é importante por facilitar a transmigração de células T especificas através do endotélio para o espaço intersticial da derme[2].

Um grupo de quimiocinas compostos por IP-10, MCP-1, RANTES e MIG é produzido por ceratinocitos basais nas lesões de LP, em particular nas lesões recentes- e poderiam servir para atrair células T para a junção dermoepidérmica. As quimiocinas então produzidas nos locais de inflamação da pele provavelmente servem para regular a composição dos infiltrados, predominando células Th1 ou Th2. Contudo, células T de memória com uma função de vigilância também podem migrar para as áreas da pele em condições não inflamatórias. Quimiocinas homeostáticas constitutivamente produzidas em condições não inflamatórias podem mediar a migração direcionada à pele dessas células T de vigilância imunológica, levando a remoção de patógenos invasores como vírus. Além disso, células $TCD4^+$ reguladoras que têm a capacidade de suprimir células T ativadas também podem entrar nas áreas inflamadas da pele. Por último, deve-se notar que não existem meios definitivos de distinguir células T de vigilância imunológica das células T regulatórias protetoras das células T patogênicas nas lesões de LP[19].

As lesões do LP apresentam uma distribuição bem definida clinicamente e existe uma demarcação clara entre o tecido acometido e a área sã.

Uma possível explicação para isto é a de que os ceratinocitos expressam o antígeno do LP apenas no sítio da lesão, ou seja, a distribuição clínica do LP seria determinada pela distribuição do antígeno. Após a expressão deste antígeno pelo ceratinocito alterado, as células T CD4+ e CD8+ antígeno-específicas poderiam[1] encontrá-lo na vigilância de rotina do epitélio ao acaso (hipótese do *encontro ao acaso*) ou[2] serem atraídos ao epitélio por quimiocinas produzidas pelo ceratinocito (hipótese da *migração direcionada*). A hipótese do *encontro ao acaso* é apoiada pelo achado de linfocitos T CD8+ na

epiderme humana normal e pela degeneração da camada basal mesmo na ausência de denso infiltrado inflamatório nas lesões de LP. Contrariamente, a hipótese da *migração direcionada* tem suporte na expressão natural de receptores para quimiocinas pelos linfocitos T e pelo fato do infiltrado dérmico de células T preceder o aparecimento intraepitelial de linfócitos e a lesão epidérmica nas lesões de LP[10].

A apoptose tem sido proposta como mecanismo de morte do ceratinocito no LPO[14,20]. No entanto, a forma exata utilizada pelos linfocitos T CD8+ citotóxicos para desencadear este processo ainda é desconhecida. Possíveis mecanismos incluem:

1- TNF-α secretado por linfocito T liga-se ao seu receptor (TNF R1) na superfície do ceratinocito;

2- CD95L (Fas ligante) da superfície do linfocito T liga-se ao CD95 (Fas) na superfície do ceratinocito;

3- Grânulos citotóxicos contendo granzima B e perforina secretados por linfocito T atuam conjuntamente no ceratinocito.

Todos estes mecanismos podem ativar a cascata das caspases, resultando na apoptose do ceratinocito[4,10,11].

A ELD é um diagnóstico diferencial importante e, muitas vezes, difícil de ser obtido, em relação ao LP. Do ponto de vista clínico, é árduo provar que uma erupção foi causada por drogas, a não ser tornando a administrar o agente, o que implica em risco potencial ao paciente. Com exceção da erupção fixa a droga, em todas as lesões passíveis de serem erupções secundárias a drogas, à microscopia, há evidência de sinais que as caracterizam como "prováveis" ou "possíveis". Teoricamente, qualquer doença inflamatória pode ser causada por drogas. Além disso, pode ser encontrado qualquer padrão de doença inflamatória de pele, sendo o padrão mais comum o de dermatite de interface.

Alguns achados histopatológicos permitem, às vezes, diferenciar doença induzida por drogas das não induzidas por drogas. Por exemplo, o infiltrado liquenoide constituído não só por linfocitos, paraceratose focal e muitos ceratinocitos basais necróticos agrupados ("*clusters*") favorecem erupção liquenoide secundária a drogas ao invés do verdadeiro LP. A presença de erosão focal e crostas, no contexto de dermatites liquenoides, é indicativa de erupção liquenoide a drogas. O LP, o qual é notoriamente pruriginoso, induz liquenificação mais que escoriação. A ELD, na qual a epiderme é fina, tem como diagnóstico diferencial o LP atrófico, porém nela o infiltrado é mais denso que no líquen plano, quando a lesão é madura. A presença de eosinofilos não é uma condição necessária para o diagnóstico histopatológico de erupção secundária a drogas. Na realidade, em muitas erupções causadas por drogas, os eosinofilos estão ausentes. A dermatite liquenoide, que não seja o LP idiopático, pode conter extraordinário número de ceratinocitos necróticos agrupados ("*clusters*"), favorecendo o diagnóstico de erupção liquenoide a drogas. Como regra geral, na erupção liquenoide secundária a drogas há infiltrado perivascular tanto em plexos superficiais como profundos. Esta característica isolada, entretanto, não permite a distinção entre o LP verdadeiro e o induzido por drogas. Na ELD, ao invés de hipergranulose, a tendência é encontrar camada granulosa normal e paraceratose, acompanhada por eosinofilos[21].

1.6- O papel dos macrófagos

As células apresentadoras de antígenos (APC) participam do desencadeamento do processo inflamatório em várias dermatoses imunomediadas. Estas células convertem antígenos proteicos em peptídeos e apresentam os complexos peptídeo-MHC em uma forma que pode ser reconhecida pelo receptor antigênico das células T[22]. Esta interação tem um papel crucial na ativação das células T e na resposta imunomediada por estes linfocitos[22,23]. Entre as APC mais conhecidas destacam-se as células dendríticas

e os fagócitos mononucleares (monocitos e macrófagos). As células do sistema fagocitário mononuclear se originam na medula óssea, circulam pelo sangue, amadurecem e são ativadas nos diferentes tecidos. O monocito é o primeiro tipo celular totalmente diferenciado que entra no sangue periférico depois de deixar a medula óssea. Nos tecidos, essas células amadurecem e se tornam macrófagos, recebendo nomes especiais para designar localizações específicas. Na pele, por exemplo, são chamados de histiocitos[22].

O LPC, LPO e a ELD são doenças caracterizadas à histopatologia por apresentar infiltrado inflamatório composto principalmente por linfocitos T, que são reconhecidos como os principais efetores da agressão aos ceratinocitos basais. Além disso, um maior número de macrófagos e células dendríticas é encontrado na interface entre o epitélio e o tecido conjuntivo em ambas as doenças, sugerindo uma participação destas células na apresentação de alo ou autoantígenos e, portanto, na patogênese das mesmas[23,24,25].

1.7- A transmigração celular

O recrutamento dos leucócitos aos sítios da inflamação e o início da resposta imunológica dependem de uma cascata de eventos. Atualmente, sabe-se que a aderência e a transmigração celular são determinadas basicamente pela ligação de moléculas de adesão complementares nas superfícies leucocitária e endotelial, e que os mediadores químicos afetam esses processos modulando a expressão superficial ou avidez dessas moléculas de aderência. Os receptores de adesão pertencem principalmente a quatro famílias moleculares: as selectinas, as imunoglobulinas, as integrinas e as glicoproteínas semelhantes à mucina[26].

Acredita-se que as selectinas atuem na fase inicial da adesão celular entre os leucocitos e o endotélio, resultando numa aderência rápida e relativamente frouxa[26,27].

A família das imunoglobulinas inclui duas moléculas de adesão endoteliais: ICAM-1 (do inglês *intercellular adhesion molecule*) e VCAM-1 (do inglês *vascular adhesion molecule*). Estas interagem com as integrinas encontradas nos leucocitos[26].

As integrinas são glicoproteínas heterodiméricas transmembranosas constituídas por duas cadeias polipeptídicas chamadas cadeias α e β. Os principais receptores de integrinas para a ICAM-1 são as β-integrinas LFA-1 (do inglês *leukocyte function-associated antigen-1*) e MAC-1 (do inglês *macrophage-1 antigen*), e para VCAM-1 é a integrina VLA-4 (do inglês *very late antingen-4*). Estas moléculas atuam na aderência firme entre os leucocitos e as células endoteliais e são necessárias à transmigração celular[26,27].

Existem três mecanismos que modulam a adesão leucocitária durante a inflamação: a redistribuição das moléculas de adesão na superfície celular, a indução de moléculas de adesão no endotélio por citocinas e o aumento da avidez de ligação das integrinas. Neste contexto, por exemplo, a IL-1 e TNF-α aumentam a expressão de ICAM-1 e VCAM-1, que estão presentes em baixos níveis no endotélio normal. Por outro lado, a LFA-1 está presente nos leucocitos (neutrófilos, monocitos e linfocitos), mas não se adere a seu ligante ICAM-1 no endotélio. Para se tornarem firmemente aderidos, os neutrófilos precisam ser ativados, de modo que a LFA-1 seja convertida de um estado de ligação de baixa para um de alta afinidade em relação à ICAM-1, o que ocorre por meio de uma alteração da configuração da molécula de integrina. Os principais agentes responsáveis por essa ativação leucocitária são agentes quimiotáticos (incluindo as quimiocinas) produzidos pelo endotélio ou outras células provenientes do local da lesão. Assim, durante a inflamação, a afinidade aumentada da LFA-1 sobre o leucocito ativado, combinada à maior expressão de ICAM-1 no endotélio, prepara o terreno para uma forte ligação LFA-1/ICAM-1. Esta interação LFA-1/ICAM-1, por sua vez, causa adesão firme ao endotélio e permite à subsequente transmigração celular[26].

1.8- O papel da ICAM-1

O recrutamento dos linfocitos ao sítio ativo das doenças inflamatórias requer uma maior expressão, mediada por citocinas, de moléculas de adesão pelas células endoteliais, bem como de moléculas receptoras pelos linfocitos e macrófagos[25].

As moléculas de ICAM-1 são expressas tanto pelas células endoteliais, como também por ceratinocitos, linfocitos, fibroblastos e por outras células epiteliais. Uma maior expressão destas moléculas também foi encontrada em células de Langerhans (CL), macrófagos e células dendríticas[28,29]. A interação entre ICAM-1 e seu ligante LFA-1 é importante como sinal co-estimulatório durante a ativação dos linfócitos T[29,30] e também para a regulação do contato entre a célula efetora e a célula alvo, incluindo a citólise. Além disso, esta interação é importante na indução da sensibilização de antígenos ao MHC e na rejeição de enxertos[25]. Desta forma, ICAM-1 participa não somente do recrutamento das células inflamatórias, como também do controle de suas funções, da manutenção destas células na pele e da sua transmigração[22].

No LP, a expressão aumentada da ICAM-1 nos ceratinocitos basais está associada ao acúmulo de linfocitos T na camada basal e a ativação de CL, o que sugere a participação desta molécula na etiopatogênese da doença[32,33]. Foi demonstrado também uma maior expressão de ELAM-1 (do inglês, *endothelial leukocyte adhesion molecule*), ICAM-1, VCAM-1 e P-selectina nos compartimentos microvasculares de pacientes com LP quando comparados a fragmentos de tecidos normais, sugerindo a importância dessas moléculas na manutenção ou persistência das lesões[25].

Dados sobre a expressão de moléculas de adesão na ELD são limitados. Apesar disso, também já foi demonstrada maior expressão de ICAM-1 em ceratinocitos da mucosa oral e em leucócitos em amostras de ELD induzidas pelo amálgama[34].

1.9- Perforina e granzima B

1.9.1- A apoptose e o significado funcional da perforina e da granzima B

Linfocitos T citotóxicos (LTC) e células NK reconhecem e eliminam células infectadas por patógenos intracelulares ou células tumorais via exocitose granular e/ou via receptor-ligante[35-37]. Os dois mecanismos requerem contato direto entre a célula efetora e a célula alvo. Na primeira via, grânulos citotóxicos, contendo predominantemente perforina e uma família de proteases estruturalmente relacionadas à serina (granzimas), com vários substratos específicos, são secretados por exocitose na sinapse imunológica formada entre o efetor e o seu alvo. A granzima B, um dos subtipos mais importantes, cliva as proteínas da célula alvo em resíduos específicos de aspartato e leva à morte celular tanto pela ativação da cascata caspase-dependente quanto pela via caspase-independente. Na ausência de perforina, a molécula de granzima pode entrar no citoplasma, mas não consegue provocar a morte celular de forma eficiente. Portanto, estas moléculas trabalham em conjunto para induzir a apoptose da célula alvo. A segunda via cursa com a agregação do receptor da superfície da célula alvo, denominado Fas (CD95), com seu ligante correspondente na superfície da célula citotóxica, chamado ligante de Fas (FasL), resultando na clássica apoptose caspase-dependente. A principal função da via Fas-FasL é eliminar linfocitos T autorreativos[36,37].

A perforina é uma proteína formadora de poro cálcio-dependente de aproximadamente 67 kDa, cuja expressão é regulada durante a diferenciação dos linfocitos por ativação de receptores por meio de sinais (como receptor da célula T e NKG2D) e citocinas (como IL-2, IL-15 e IL-21)[38]. Embora a perforina tenha similaridades a componentes do complemento, particularmente C9, sua sequência primária é única[38,39]. A importância desta proteína tem sido demonstrada por diversos estudos com ratos perforina-deficientes, que são profundamente imunocomprometidos e apresentam grande susceptibilidade a infecções virais e câncer. Pacientes com linfohistiocitose hemofagocítica familiar humana,

causada por mutações bialélicas na perforina, também são intensamente imunodeficientes[39,40,41]. Mais recentemente, tem sido demonstrado *in vivo* o papel da perforina como mediador crucial da vigilância imune de células transformadas espontaneamente e na regulação da homeostase das células B e das células T de memória[36,42,43].

Apesar da importância vital na atividade citotóxica, as funções moleculares e celulares da perforina e a base do sinergismo entre perforina e granzima permanecem pouco compreendidas[36].

1.9.2- Granzima B: como se processa a liberação na sinapse imunológica

Tanto em humanos quanto em roedores, a perforina é indispensável para a liberação de granzimas e seus substratos pró-apoptóticos dentro da célula alvo. Embora exista um consenso geral de que o fluxo de granzimas para dentro do citosol da célula alvo seja dependente da atividade citolítica da perforina, o mecanismo pelo qual a granzima entra na célula permanece controverso[39].

Diversos modelos têm sido propostos desde a década de 80, quando a perforina foi clonada pela primeira vez[36]. À microscopia eletrônica, as células expostas à perforina apresentam poros. Desta forma, no modelo original, a perforina, homóloga ao complemento, liberaria granzima via multimerização na membrana celular, onde formaria poros para a passagem da granzima. Recentemente, este modelo tem sido questionado. Embora, em altas concentrações, a perforina forme grandes poros na membrana que poderiam matar as células por necrose, concentrações sublíticas desta molécula são requeridas para a transferência de granzima, e os poros nestas condições, se é que são formados, podem ser muito pequenos (≤50nm de diâmetro) para garantir a passagem de moléculas globulares tão grandes como as de granzima. Além disso, foi descoberto que granzima B pode se ligar à célula alvo e sofrer endocitose ou macropinocitose independentemente da perforina, que então atuaria como uma endossomolisina[37,39,41].

Em 2000, o receptor manose 6-fosfato foi proposto como candidato a receptor de superfície celular para a granzima B. Embora a existência de um receptor específico para esta molécula fosse interessante, para garantir a eliminação das células-alvos, a granzima B deveria manter a capacidade de se ligar a múltiplas estruturas da superfície celular[44]. Além disso, faltam estudos demonstrando uma associação direta entre a granzima B e um receptor de superfície celular[37]. Assim que, posteriormente, foi proposta a ideia de que um complexo macromolecular contendo perforina, granzima e serglicina (uma proteoglicana primária de grânulos citotóxicos) seria liberado na sinapse entre a célula efetora e a célula-alvo. Esse complexo seria capaz de se ligar e penetrar na célula-alvo por meio de endocitose. A serglicina contribuiria para a apoptose mediada por grânulos por atuar como carreador, facilitando a internalização da granzima e/ou da perforina[44,45]. Dentro das vesículas citosólicas, a perforina alteraria a membrana endocítica, facilitando a liberação da granzima no citosol e o seu transporte em direção ao núcleo. A formação de poros nesta etapa também é controversa na literatura[36,37,39,44].

Mais recentemente um modelo híbrido foi proposto por Pipkin e Lieberman[39], em que a perforina formaria pequenos poros na membrana celular que, então, ativaria o influxo de cálcio para dentro da célula. Como os níveis de cálcio citosólico são normalmente baixos, o aumento representaria um dano à membrana, determinando uma resposta de reparo. Vesículas intracelulares, incluindo endossomos e lisossomos, seriam mobilizadas em segundos, doando suas membranas para tentar restabelecer a área lesada. O próximo passo incluiria rápida co-endocitose de granzima e perforina em endossomos gigantes, seguido da liberação de granzima mediada pela perforina no citosol. Já foi demonstrado que quando a perforina encontra-se em concentrações consideradas sublíticas, como acontece no ataque por linfocitos T citotóxicos, uma rápida resposta de reparo é ativada com pronta restauração da integridade da membrana plasmática, seguida pela coliberação de granzimas que, por sua vez, induzem um lento processo de apoptose. Quando a dose de perforina é lítica, a resposta de reparo é incapaz de competir com o dano à membrana,

assim o influxo de cálcio persiste e a célula morre rapidamente por necrose. Quando a resposta de reparo da membrana é inibida, células tratadas com granzima B e perforina, mesmo em concentrações sublíticas, têm mais chance de morrer por necrose do que por apoptose. Mesmo no modelo híbrido, algumas questões permanecem desconhecidas, como o que ativaria a rápida endocitose das moléculas citotóxicas, se poros de perforina desestabilizariam as membranas dos endossomos causando o seu rompimento ou se os poros formados nessas membranas seriam largos o suficiente para permitir a passagem das moléculas de granzima para o citosol[39].

1.9.3- O papel da granzima B e da perforina no LP, LPO e ELD

Considerando as evidências atuais, a citotoxicidade mediada por linfocitos T tem sido proposta como mecanismo de apoptose nas lesões orais do LP e da ELD. Estudos ultraestruturais têm demonstrado que os corpos coloides encontrados nestas estomatites de interface são formados no processo de morte dos ceratinocitos basais e suprabasais, e apresentam características morfológicas típicas da apoptose[46,47].

Um importante marcador da atividade citolítica destas células é a expressão da granzima B e da perforina *in situ*. Estudos imuno-histoquímicos demonstraram infiltrados celulares, principalmente de linfocitos CD8+, contendo granzima B e perforina nas lesões de LP[47,48,49]. Santoro et al.[50] encontraram um número significantemente maior destas moléculas citotóxicas nas lesões de LPO quando comparadas à mucosa normal e mesmo às lesões cutâneas de LP. Por meio de microscopia eletrônica, moléculas de granzima B foram observadas sendo secretadas de um linfócito para um ceratinocito em processo de apoptose[47]. Técnicas capazes de mensurar a expressão *in situ* de mRNA de granzima B demonstraram ser esta 100 a 200x maior nas lesões de LP comparativamente à pele normal[51].

Já foi demonstrado também que a expressão de linfocitos T contendo perforina é muito maior na fase de exacerbação do líquen plano quando comparada à fase de remissão da doença ou ainda à pele normal de indivíduos controles[48,49]. Esses achados sugerem um importante papel da perforina e granzima B no processo de apoptose dos ceratinocitos no LP.

Não foram encontrados trabalhos sobre o papel destas moléculas citotóxicas em amostras de ELD na literatura.

2- OBJETIVOS

Os objetivos deste trabalho foram:

Capítulo 1:

Estudar, comparativamente, em 16 amostras de tecido de LPC e 6 de ELD, o papel dos grânulos citotóxicos de granzima B e perforina e analisar alguns achados histológicos e imuno-fenotípicos do infiltrado inflamatório no LPC e ELD.

Capítulo 2:

Estudar, comparativamente, em 29 amostras de tecido de LPO e 16 de LPC, o papel dos grânulos citotóxicos de granzima B e perforina.

3- CAPÍTULOS

International Journal of Dermatology

Report

Lichen planus and lichenoid drug-induced eruption: a histological and immunohistochemical study

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Abstract

Introduction Lichenoid drug eruption (LDE) shares similar features with lichen planus (LP), that could reflect the same pathogenesis. In LP, an autoimmune attack is accepted and cytotoxic T-lymphocytes (CD8+) predominate, especially in late lesions. Apoptosis of keratinocytes may be mediated by CD8+ T and NK cells in two distinct ways: by the release of cytotoxic molecules such as perforin and granzyme B or by the Fas/FasL system. The immunological mechanisms involved in LDE are not yet fully established.

Objectives Investigate immunohistological features in LP and LDE to add clues to better understand their pathogenesis.

Material and methods Twenty-two patients fulfilled all clinical, laboratory, histopathological, and follow-up features of lichen planus (n = 16) and lichenoid drug eruption (n = 6). Classic histological features favoring LP or LDE were evaluated by two observers. HAM56, MAC387, UCHL-1, OPD4, CD8, Granzyme B, Perforin, and ICAM-1 antibodies were used to decorate the immune infiltrate. Results were analyzed through Pearson correlation, Student's t-test, and linear discriminant analysis.

Results A higher number of necrotic keratinocytes as well as plasma cells and eosinophils within inflammatory cells were associated with LDE diagnosis. Only in LDE, a correlation was found between the number of T and CD8+ cells and between the number of granzyme B+ cells and apoptotic keratinocytes.

Conclusion Our findings suggest a more important role of CD8+ granzyme B-containing cells in LDE group, being its synthesis associated with more intense apoptosis. So, LP and LDE may have a somewhat distinct pathogenesis.

Introduction

Lichenoid drug-induced eruption (LDE) resembles lichen planus (LP) on a clinical and histological basis. The most characteristic histological substrate of both is subepidermal band-like cytotoxic lymphocyte infiltration and apoptosis of the basal keratinocytes. Several histological criteria have been used to differentiate LDE from LP, but there is not a good clinicopathological correlation. Regarding etiology, in LP, an autoimmune attack is generally accepted, and it was demonstrated that the inflammatory infiltrate is composed mainly of T-lymphocytes, with varying populations of CD4+ and CD8+ cells.[1–4] Some studies have shown a predominance of cytotoxic T-lymphocytes (CD8+), especially in late lesions.[2,5–8] Apoptosis of keratinocytes is accepted to be mediated by CD8+ T- and natural killer (NK) cells in two distinct ways: by the release of cytotoxic molecules, such as perforin[4,9] and granzyme B;[10,11] or by the Fas/FasL system.[12] The immunological mechanisms involved in LDE are not yet fully established. This study examined some histological and immunohistochemical features of the cell infiltrate in LP and LDE lesions. Our results may provide clues to better understand pathogenesis in order to identify new therapeutic strategies for LDE and LP.

Materials and methods

The files of the Pathology Department, School of Medical Sciences – UNICAMP, were searched for all skin biopsies with a diagnosis of lichenoid dermatitis during the period 1996–2008. The nosologic diagnoses, defined by clinical, laboratory,

histopathological, and follow-up data, were obtained from these medical records. Patients whose diagnosis could not be accurately achieved were excluded from the study. Twenty-two cases fulfilled all characteristics of LP (n – 16) and LDE (n – 6). Only patients whose eruptions began when the implicated medication was started, and improved when it was stopped, were included in the LDE group. The medication was discontinued or replaced and, one week later, at the next office visit, the lesions were required to be clear and could not subsequently flare up or relapse. If a cause and effect relationship could not be safely defined or if the patient's lesions improved, even while continuing to use the same medication, the patient was excluded from either group. No case was rechallenged with the implicated medication. Paraffin-embedded tissue was cut for H&E and immunohistochemical stain. With the aim of identifying significant histological findings for the diagnosis of LP or LDE, in each H&E specimen, the following histopathological features were blindly evaluated by two observers: stratum corneum: 1 – orthokeratosis only/ 2 – focal parakeratosis; granular layer: 0 – agranulosis or hypogranulosis/1 – "v" hypergranulosis or normal; quality of inflammatory infiltrate: 0 – lymphohistiocytic only/1 – eosinophils and plasma cells among inflammatory cells; inflammatory infiltrate: 0 – mild/1 – moderate/remarkable; inflammatory infiltrate level: 0 – band-like dermoepidermal junction/1 – both band-like dermoepidermal junction and lower reticular dermis; acanthosis: 0 – present (saw tooth)/1 – mild/absent; incontinentia pigmenti: 0 – conspicuous/1 – not conspicuous; epidermal/dermal Civatte bodies (apoptotic cells): 0 – in large numbers and grouped (clusters) together (Fig. 1)/1 – not too many; signs of regression: 0 – absent/1 – present (scanty hyperkeratosis, parakeratosis, and acanthosis, flattened epidermis, dermal fibrosis, and sparse inflammatory infiltrate). Aiming to improve the statistical power of the results, the data were stratified into two categories (present or absent), and the discordant results were reviewed for consensus achievement. A quantitative analysis for the number of single or clusters of apoptotic bodies was done, by the same observer, using an Olympus CH30 optical microscope in high (400×) magnification. Each high-power field all along the sample was analyzed, and the values were recorded in mm^2.

Immunohistochemical staining

Briefly, 4 μm-thick sections were dewaxed and rehydrated in graded ethanol. The primary antibodies used were HAM56 (MO 632; Dako, Carpenteria, CA, USA) at a dilution of 1:50, MAC387 (MO 747; Dako) at a dilution of 1:100, UCHL-1 (CD45RO, MO747; Dako) at a dilution of 1:100 (Fig. 2), CD8 (M7103; Dako) at a dilution of 1:50, OPD4 (59375; Santa Cruz, Santa Cruz, CA, USA) at a dilution of 1:50 (Fig. 3), perforin (5B10, Novocastra, Burlingame, CA, USA) at a dilution of 1:50 (Fig. 4), and granzyme B (M7235, Dako) at a dilution of 1:100 (Fig. 5). For all antibodies, a steamer was used for epitope retrieval with either citrate buffer (HAM56, MAC387, UCHL1, perforin) or Tris-EDTA buffer (CD8, OPD4, and granzyme B). The Advance polymer (K 4068; Dako) was used as a reaction amplifier. Visualization of the antibody complex was achieved using 3.3-diaminobenzidine tetrahydrochloride (K 3468; Dako) according to the manufacturer's instructions. Sections were counterstained with Mayer hematoxylin. Appropriate negative

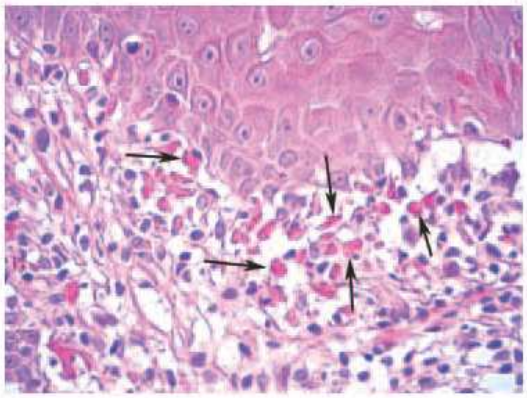

Figure 1 LDE: many Civatte bodies at the papillary dermis, some of them indicated by arrows (original magnification ×640)

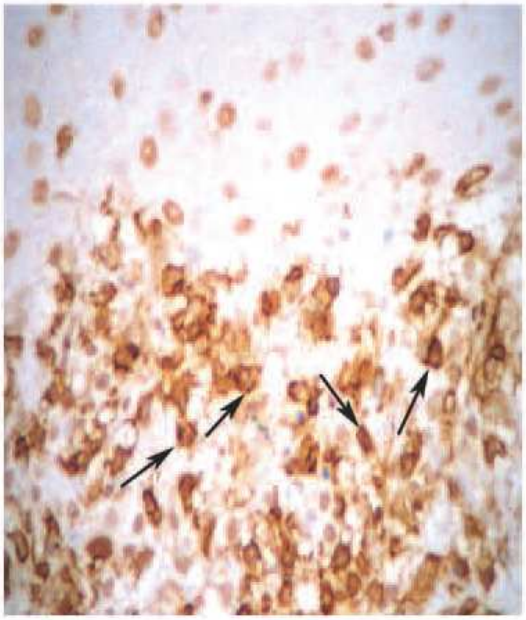

Figure 2 LDE: UCHL-1-positive T-lymphocytes at the dermo-epidermal interface, some of them indicated by arrows (original magnification ×640)

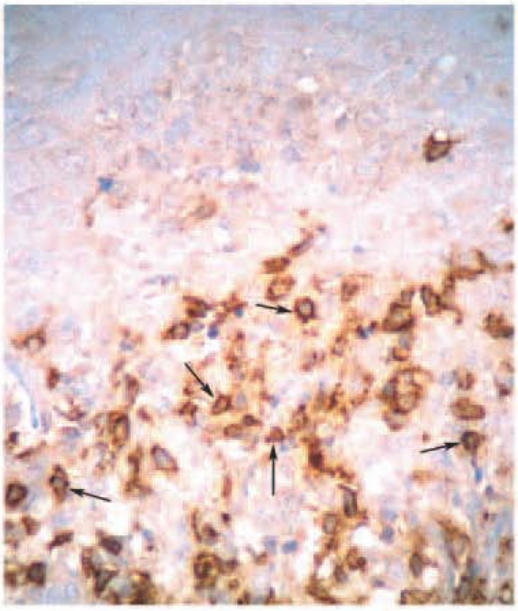

Figure 3 LDE: CD8-positive T-lymphocytes at the dermo-epidermal junction and papillary dermis, some of them indicated by arrows (original magnification ×640)

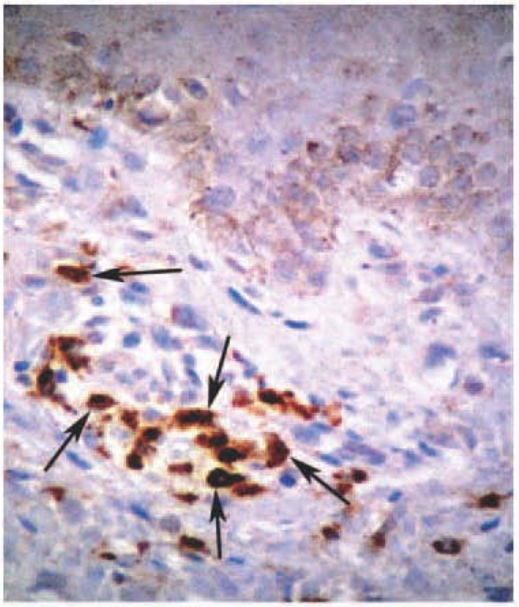

Figure 5 LDE: granzyme B-positive inflammatory cells at the papillary dermis, some of them indicated by arrows (original magnification ×640)

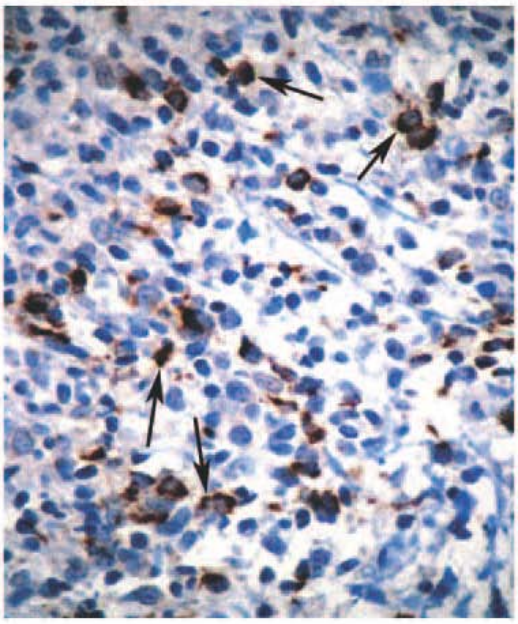

Figure 4 LP: perforin-positive inflammatory cells at the papillary dermis, some of them indicated by arrows (original magnification ×640)

and positive controls were included in each assay. Perforin and granzyme B quantification was performed as follows: for each antibody, the immunostained cells were blindly counted for apoptotic cells by one observer, in the whole extension of the biopsy, in high (400×) magnification. HAM56, MAC387, UCHL1, CD8, and OPD4 quantification were performed as follows: 10 blindly and randomly selected images of the stained sections were obtained, and a quantitative analysis of the expression of each marker was performed using computer-assisted image analysis (IMAGELAB analysis software, Softium, Sao Paulo, Brazil, 2000). All positive and negative nuclei of inflammatory cells were counted in each image. The expression of these markers was calculated as the percentage of positivity in relation to the total amount of nuclei counted per section. To standardize the counting, the epidermis basal layer was positioned in the middle of the field.

Statistical methods

To test the correlation between variables, according to the particular disease studied (LP or LDE), the Pearson correlation was used. To compare the two disease groups, according to the variables analyzed, we used the Student's *t*-test. To evaluate the discriminatory power of the analyzed variables relatively to the two groups of diseases, we used linear discriminant analysis.

Ethics

This study was approved by the Research Ethics Committee of the State University of Campinas (protocol # 660/2006).

Results

There were no significant differences between the groups in relation to sex and age of the patients (LP: 18–73 years, average 48 years; LDE: 14–64 years, average 44 years).

An increased number of necrotic keratinocytes (grouped into clusters) and plasma cells and eosinophils in the infiltrate were shown to have statistical significance for the diagnosis of LDE (Table 1). In order to evaluate the discriminatory power of the variables between the groups, we used discriminant analysis. The variable "cluster" (studied by logarithmic transformation) classified correctly 90.9% of LP cases and 81.8% of LDE ($P = 0.005$). Namely, there was, a higher number of apoptotic cells clusters in the LDE group.

The results of the other morphological criteria (routine stained specimens) did not show statistical significance for the diagnosis of LDE.

We observed a significant positive correlation between the number of T-cells and CD8 cells only in the LDE group (Fig. 6). Nevertheless, the number of CD4 cells did not show significant correlation with any other variables in either of the two groups. The number of CD8 cells showed a significant positive correlation with the number of inflammatory cells that expressed perforin, though only in the LP group.

The higher the values of granzyme B, the higher were those of perforin in the LP group and higher the number of clusters in the LDE group (Fig. 7; Table 2).

Table 1 Histological features in LP and LDE: comparative analysis

Histological features	LP ($n = 16$)	LDE ($n = 6$)	P
Number of single apoptotic cells	4.31 + 7.56	6.30 + 3.67	0.086[a]
Number of clusters of apoptotic cells	1.51 + 3.01	4.95 + 3.62	**0.005**[a]
Civatte bodies (apoptotic cells) – few	15 (93.8%)	1 (16.7%)	**0.001**[b]
Orthokeratosis only	6 (37.5%)	0 (0.0%)	0.133[b]
"V" hypergranulosis or normal	15 (93.8%)	5 (83.3%)	0.481[b]
Eosinophils and/or plasma cells	2 (12,5%)	4(66,7%)	**0.025**[b]
Moderate/remarkable inflammatory infiltrate	13 (81.3%)	4 (66.7%)	0.585[b]
Band-like and deep inflammatory infiltrate	4 (25.0%)	3 (50.0%)	0.334[b]
Mild/absent acanthosis	1 (6.3%)	0 (0.0%)	1.000[b]
Incontinentia pigmenti – not conspicuous	6 (37.5%)	2 (33.3%)	1.000[b]
Signs of regression	6 (37.5%)	4 (66.7%)	0.348[b]
Photodamage absent	6 (37.5%)	1 (16.7%)	0.616[b]

[a]Student's t-test, descriptive level of probability.
[b]Fisher's exact test, descriptive level of probability.
LDE, lichenoid drug-induced eruption; LP, lichen planus.
Bold values represent statistically significant P-values.

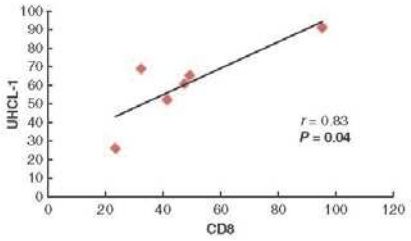

Figure 6 Lichenoid drug eruption: positive Pearson correlation between UCHL-1- positive T cells and CD8 (significant P-values in bold)

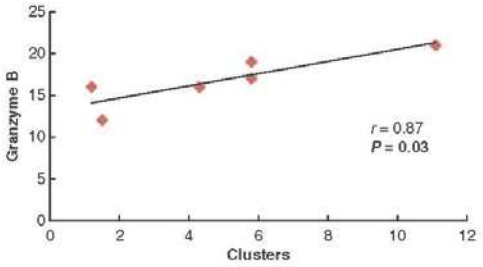

Figure 7 Lichenoid drug eruption: positive Pearson correlation between granzyme B and apoptotic clusters (significant P-values in bold)

Table 2. Pearson correlation between granzyme B and the variables "clusters" and "perforin" in LP and LDE

Group	Clusters	Perforin
LP ($n = 16$)		
R	0.22	0.68
P	0.41	**0.002**
LDE ($n = 6$)		
R	0.87	−0.12
P	**0.03**	0.82

Clusters: number of apoptotic clusters/mm^2.
Granzyme B or perforin: percentage of positivity in relation to the total number of cells counted per section.
LDE, lichenoid drug-induced eruption; LP, lichen planus.
Bold values represent statistically significant P-values.

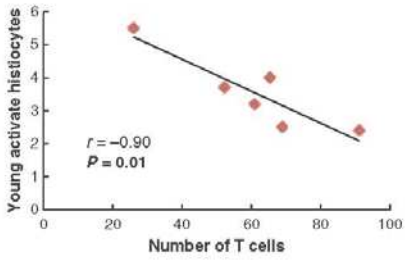

Figure 8 Lichenoid drug eruption: negative Pearson correlation between T cells and MAC387 positive young activated histiocytes (significant P-values in bold)

No correlation was found between the values of perforin and the other variables in either group. Numerous cells expressed the ICAM in both groups, but no significant differences were found between groups or correlation with other variables.

HAM56-positive macrophages were present in large numbers in the infiltrate in both groups. In the LP group, a number of these cells showed positive and significant correlation with a number of T-cells. In the LDE group, this correlation was not repeated. We found a significant negative correlation between the number of T-cells and young activated histiocytes (MAC387-positive) in the LDE group (Fig. 8).

Discussion

LP and LDE are similar skin diseases. To distinguish between them, most authors recommend a correlation between the anamnestic, clinical, and histological findings.[13,14] The distinction is very important, because the mere interruption (or replacement) of the drug can lead to the resolution of the lesions.[15] However, as (idiopathic) LP may undergo spontaneous remission, it could be necessary to reintroduce the drug for the diagnosis of LDE to be substantiated, which may not be acceptable. In addition, the drug can trigger the eruption, which eventually will not regress, even after its withdrawal. Also, determining that a particular medication caused an eruption is often difficult when the patient is taking multiple drugs and for which most cannot be easily interrupted or replaced.[16] Moreover, cross-reactions may occur, for example, between the different antihypertensive drugs available for substitution.[15] Finally, the lesion may disappear even when the drug is still being administered.[17] Considering the strictness of the inclusion criteria for LDE or LP diagnosis, in our sample the analysis was limited to 22 patients for a period of 12 years, 16 with LP and only six with LDE, which limited the statistical power of results. Of these six patients, five had used an anti-inflammatory or anti-hypertensive drug, and only one used solely isotretinoin.

Regarding histological findings on routine stained sections, it is known that LDE features are frequently indistinguishable from typical LP,[14] especially if the lesions are photodistributed.[18] Van den Haute *et al.* studied various histopathological features in skin biopsies of idiopathic LP and LDE. No criterion was found to be pathognomonic for distinguishing LP from LDE. However, focal parakeratosis, focal interruption of the granular layer, and cytoid bodies in the cornified and granular layers were present in more than 50% of LDE and never in idiopathic LP.[19] In fact, the presence of parakeratosis argues against a diagnosis of LP.[14] We found focal parakeratosis in 100% of LDE and in 66.7% of LP, but the differences were not significant, perhaps due to the limitation of our sample. All but one LDE specimen displayed signs of photodamage. Actually, only the higher number of grouped necrotic keratinocytes (clusters) and presence of plasma cells and eosinophils in the infiltrate showed statistical significance for the diagnosis of LDE (Table 1). Through discriminant analysis, the variable cluster of apoptotic keratinocytes classified correctly 91% of cases of LP and 82% of LDE. So, massive apoptosis may be an indication of drug etiology in lichenoid lesions. Our results are in line with the literature. According to Ackerman *et al.*,[20] LDE tends to present numerous individual necrotic keratinocytes in the epidermis, and eosinophils may accompany lymphocytes in the dermal infiltrate. Apoptosis, the process of programmed cell death not followed by autolysis, can be triggered by external stimuli through the activation of specific cell death receptors present on the cell surface (extrinsic pathway) or by intracellular mechanisms (intrinsic or mitochondrial pathway).[21] Among the mechanisms that have been postulated to trigger apoptosis in interface drug dermatitis are the attacks of cytotoxic T-lymphocytes and/or NK cells via toxic granules.[22] Toxic granules, especially those containing perforin, a 70-kd protein capable of breaking the cell membrane, and granzyme B, are preformed specialized lysosomes.[22–29] Granzyme B has been identified within lymphocytes in various types of interface dermatitis.[30] Perforin is involved in activation or inhibition of activated T-cells, in the control of immunoglobulin production and Th1/Th2 balance, in the control of antiviral defense mechanisms, and in the destruction of cancer and virus-infected cells. Therefore, the dysfunction in the formation and transport of their secretory vesicles can cause both immunodeficiency and autoimmunity.[31] The expression of perforin was shown to be significantly increased in LP lesions, pointing to the potential role of perforins in apoptosis of basal keratinocytes.[3,8,32] Choi *et al.*,[33] studying

lesions of fixed drug eruption, another example of interface drug dermatitis, found that the combined expression of Fas and FasL was associated with apoptosis, and many inflammatory cells were CD8+, but perforin was rarely found. In our study sample, all specimens of LDE and LP inflammatory cells expressed perforin and granzyme, in variable numbers. We found, only in the LDE group, a significant positive correlation between the number of inflammatory cells that expressed granzyme B and the number of clusters of apoptotic keratinocytes. On the other hand, only in the LP group, we found that the greater the number of granzyme B-positive cells, the greater the number of those expressing perforin. Granzyme B may be considered a specific marker of direct cytotoxic damage. Our findings suggest that, in LDE, the apoptosis of keratinocytes is predominantly linked to cytotoxicity mediated by granzyme B.

Regarding the subpopulation of inflammatory cells, various studies have shown that, in lichenoid dermatitis, both subtypes of T-lymphocytes (CD4+ and CD8+) are present and act synergistically[3,7,9] but probably by different mechanisms. CD8+ T-lymphocytes are considered to be responsible for injury to basal keratinocytes and, therefore, important in the pathogenesis of LP.[5] We found, in LDE but not in LP, a significant positive correlation between the number of T- (UCHL-1-positive) and CD8-positive cells. On the other hand, in the LP group, the greater the number of CD8-positive lymphocytes, the greater the number of cells that expressed perforin and vice versa. In LP, granzyme B+ CD8 T-cells were considered capable of inducing apoptosis of keratinocytes.[34] However, recent studies have shown that in LP, granzyme B plays a role in cell death by CD4+ Th2-type lymphocytes and probably plays a role in the *in vivo* regulation of Th2-cell responses.[35] Our results failed to find any significant correlation between the number of CD4-positive cells and any other studied variables, in either the LP or LDE group.

A high density of macrophages (CD68+) in LP lesions[36] was demonstrated. We studied the role of macrophages in LP and LDE through the use of two antibodies: HAM56 and MAC387. The HAM56 antibody reacts strongly with macrophages and does not stain any T- or B-lymphocytes. As might be expected in our work, the HAM56-positive macrophages were present in large numbers in the infiltrate in both groups. In the LP group, a number of these cells showed positive and significant correlation with the number of T-cells (UCHL-1-positive). In the LDE group, this correlation was not repeated, but it is possible that this finding is due to the small number of patients in the sample. The monoclonal antibody MAC387 identifies small activated histiocytes, newcomers from the blood to the inflammatory site. Macrophages stain with MAC387 only in the early stages of differentiation and lose the immunostaining in later stages.[37] Among the functions of macrophages are digestion and presentation of antigens to T-lymphocytes. In the LDE group, we found a significant negative correlation between the number of T-cells (UCHL-1-positive) and young histiocytes (MAC387-positive). Thus, as these young macrophages phagocyte antigens and undergo maturation, they activate T-cells leading to clonal proliferation of these cells, triggering the lichenoid drug reaction.

In short, apoptotic keratinocytes were observed in greater number and in clusters in lesions of LDE, as compared with LP. Most T-cells were CD8-positive in the LDE group. Furthermore, significant correlation was found between the number of inflammatory cells expressing granzyme B and the number of apoptotic cell clusters. This correlation seems to indicate that in LDE, granzyme B has a more important role than perforin. Its release could be linked to the most abundant number of apoptotic keratinocytes in the lesions found in this group. Our results suggest that the pathogenesis of LP and LDE is somewhat distinct. Systematic studies using a greater number of cases of LDE are needed to further our understanding on this topic.

Acknowledgments

The authors acknowledge Ms. Ana Cláudia S. Piaza for technical assistance, Mr. Adilson A. Piaza for assistance in photographic documentation, and Ms. Diane Ellis, BS, for her aid with English grammar usage. Dr. Konradin Metze receives a grant as researcher of CNPq. This study was supported by a grant from FAPESP (Sao Paulo Research Foundation), Sao Paulo, Brazil. The authors have no relevant financial interest in this article.

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3.2- Capítulo II

J Cutan Pathol 2011: 38: 973–978
doi: 10.1111/j.1600-0560.2011.01781.x
John Wiley & Sons. Printed in Singapore

Journal of
Cutaneous Pathology

Perforin and granzyme B expression in oral and cutaneous lichen planus – a comparative study

Background: Although cutaneous and oral lichen planus (LP) share similar histopathological features, oral LP often follows a recalcitrant course while LP skin lesions tend to be self-limiting. Apoptosis, mediated by cytotoxic T-cells in LP, may be triggered by the release of molecules such as perforin and granzyme B. As variation in clinical behavior can reflect differences in LP immune expression, we studied the role of those cytotoxic molecules in oral and cutaneous LP.
Methods: We analyzed 16 cases of cutaneous LP and 29 of oral LP. The sections were studied on hematoxylin and eosin, CD4, CD8, perforin and granzyme B staining.
Results: The mean number of immunostained cells expressing each cytotoxic molecule was significantly higher in oral LP than in cutaneous LP. A higher number of single necrotic keratinocytes (apoptotic bodies) was found in oral LP lesions when compared to cutaneous LP. Only in oral LP lesions, a higher number of CD4-positive cells was found in active lesions when compared to regressive lesions.
Conclusions: Our results confirm increased expression of granzyme B and perforin in oral LP lesions as compared to cutaneous LP. The increased expression suggests a relationship with the clinical behavior of the disease.

Keywords: apoptosis, CD4, CD8, cytotoxic granules

Lage D, Pimentel VN, Soares TCB, Souza EM, Metze K, Cintra ML. Perforin and granzyme B expression in oral and cutaneous lichen planus – a comparative study.
J Cutan Pathol 2011; 38: 973–978. © 2011 John Wiley & Sons A/S.

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Accepted for publication July 18, 2011

Lichen planus (LP) is a chronic inflammatory disease involving cutaneous and mucosal surfaces. The oral mucosa is commonly affected and may be the sole site of involvement.[1] Oral LP develops in 1–2% of the general adult population and is the most common noninfectious oral mucosal disease in patients referred to Oral Medicine and Oral Pathology clinics.[2] Cutaneous lesions develop in 2–3% of patients with oral LP.[3] The etiology of LP is unknown, but factors such as genetics, infections, allergies, drugs, stress and autoimmunity have been proposed as being contributory.[2,4] A cell-mediated cytotoxic response is the primary suspected mechanism of LP. It is characterized histopathologically by a subepithelial band-like lymphocytic infiltrate, with necrosis (apoptosis) of basal keratinocytes.[2,4] It has been shown that the inflammatory infiltrate is composed mainly of CD4+ and CD8+ T-lymphocytes.[5,6,7] Although cutaneous and oral LP share similar histopathological features, they are distinguished by heterogeneity in their clinical course. In general, oral LP follows a chronic and recalcitrant course and may persist for a very long period of time,[1] whereas spontaneous remission occurs after 1 year in the majority of cutaneous LP patients.[8]

Local differences in the immune system, when comparing oral mucosa and skin, could help to explain the observed variation in clinical behavior. Apoptosis is accepted to be mediated by CD8+ T-cells and natural killer cells. Among the known mechanisms is the release of cytotoxic molecules such as perforin[6,9] and granzyme B.[10,11] Perforin (described originally as a pore-forming protein) and the granzymes (a family of serine proteases) are the principal contents of cytotoxic granules.[12] Perforin is required to deliver granzymes into the cytosol of the target cell. Granzyme B leads to cell death through either caspase cascade-dependent or caspase cascade-independent pathways.[11–15] There are some data in the literature suggesting the importance of the cytotoxicity mediated by perforin and granzyme in LP.[6,16–18] This study was undertaken to evaluate the role of granzyme B and perforin comparatively in cutaneous and oral lesions of LP.

Material and methods

Routinely formalin-fixed and paraffin-embedded specimens of cutaneous and oral LP were selected from the files of the Department of Pathology, State University of Campinas. The patients had been admitted to the University Hospital between 1996 and 2008. Clinical information was obtained from their medical charts. Cutaneous and oral LP diagnoses were based on the clinical history, histopathological findings and follow-up data. Patients whose clinical data were incomplete or lacked follow-up information were excluded. Pathological material insufficient for further cuts was also excluded. In the end, specimens of 16 cutaneous LPs and 29 oral LPs entered the study.

Morphological analysis

Additional sections, stained with hematoxylin and eosin, were obtained for all cases. A qualitative analysis was blindly undergone by an observer for the lesion development stage (active or regressive) and intensity of the inflammatory infiltrate (light/moderate/severe). A lesion was considered as 'active' when presenting intense inflammatory infiltrate and apoptosis, with pronounced vacuolar (hydropic) degeneration of the basal layer. It was considered as 'late' or 'regressive' when the following observations were present: sparse inflammatory infiltrate, melanophages within collagenized subepithelial connective tissue, flattened epithelium and areas in which the basal cell layer had regenerated and the dermal infiltrate was no longer kept in close proximity to the epithelium.[4,19] A quantitative analysis for the number of single or clusters of necrotic keratinocytes (apoptotic bodies) was performed by the same observer, using an Olympus CH30 optical microscope (Olympus, Center Valley, PA, USA) in high (×400) magnification. Each high-power field was analyzed all along the sample, and the values were recorded in square millimeters. To standardize the counting, the basal layer was positioned in the middle of the field.

Immunohistochemical staining

Four micrometric thick sections were obtained, dewaxed and rehydrated. The primary monoclonal antibodies used were perforin (5B10; Novocastra Lab. Ltd., New Castle-upon-Tyne, UK) at a dilution of 1 : 20, granzyme B (GrB-7; Dako Cytomation, Carpenteria, CA, USA) at a dilution of 1 : 100, CD8 (M7103; DAKO) at a dilution of 1 : 50 and OPD4 (59375; Santa Cruz Biotech., Inc, Santa Cruz, CA, USA) at a dilution of 1 : 50. A steamer was used for epitope retrieval with either citrate buffer (perforin) or Tris-EDTA buffer (granzyme B, CD8 and OPD4). The sections for granzyme B were incubated at 37°C with a serum-free protein block (DAKO). The Advance™ HPR Enzyme (DAKO) was used as a reaction amplifier. Staining was performed according to the supplier's recommendations. Visualization of the antibody complex was achieved using 3,3-diaminobenzidine tetrahydrochloride (DAKO) according to the manufacturer's instructions. Sections were counterstained with Mayer hematoxylin. Samples from known perforin, granzyme B, CD8 and OPD4-positive lymph node sections were used as a positive control. The omission of the primary antibody provided a negative control.

Immunohistochemical quantification

Perforin and granzyme B quantification was performed as follows: for each antibody, one observer blindly counted the immunostained cells throughout the entire biopsy using high (×400) magnification and the values were recorded in square millimeters. CD8 and OPD4 quantification was performed as follows: 10 blindly and randomly selected images of the stained sections were obtained and a quantitative analysis of the expression of each marker was performed using computer-assisted image analysis (Imagelab analysis software, 2000, ImageLab, Softium, Fortaleza, CE, Brazil). The nuclei of all immunostained and negative inflammatory cells were counted in each image. The expression of these markers was calculated as the percentage of positivity in relation to the total amount of nuclei counted per section.

Statistical analysis

Differences between groups were analyzed using the Chi-square method for the categorical variables and Student's t-test for the other variables. Correlation among the variables was assessed through Pearson's correlation coefficient analysis and multiple linear regression, and $p < 0.05$ was considered a significant level.

Results

No statistical differences were found between groups in relation to (i) patient's sex and age (cutaneous LP: 8 females and 8 males, 18–73 years, average of 48 years and oral LP: 13 females and 16 males, 25–69 years, average of 47 years), (ii) qualitative variables regarding the histopathological lesion development stage, to wit, active or regressive (in cutaneous LP, 62% of the samples were in the active stage and in oral LP, 45%) and the intensity of the epithelium/connective tissue inflammatory infiltrate (Table 1). Concerning quantitative analysis, the mean number of immunostained cells expressing each cytotoxic molecule was significantly higher in oral LP ($p < 0.001$ for perforin and granzyme B) compared to cutaneous LP (Table 2 and Fig. 1A,B). In both groups, the analysis through Pearson's correlation coefficient showed that the higher the number of perforin-positive cells, the higher the number of granzyme B-positive cells ($p < 0.05$). Furthermore, a significantly higher number of single apoptotic bodies was found in oral LP lesions, either within or beneath the epithelium (<0.001), as compared to cutaneous LP (Table 2). Through multiple linear regression analysis, it was found that apoptotic bodies clusters had a positive correlation with regressive lesions ($p = 0.01$) only in the oral LP group (Fig. 1C). Although no differences were found between groups with respect to CD4 : CD8 cells ratios, a higher number of CD4-positive cells was found in active vs. regressive lesions ($p = 0.03$) in the oral LP group, but not in cutaneous LP (Fig. 1D).

Table 1. Values of absolute and relative frequencies of patients' sex, stage of lesion development and intensity of inflammatory infiltrate in cutaneous and oral LP

		cutaneous LP		oral LP		
Variable	Category	N	%	N	%	p
Sex	M	8	50.0	16	55.2	0.765 (C)
	F	8	50.0	13	44.8	
Stage	0	10	62.5	13	44.8	0.353 (C)
	1	6	37.5	16	55.2	
Intensity of inflammatory infiltrate	0	3	18.8	1	3.4	0.121 (F)
	1	13	81.3	28	96.6	

Descriptive level of probability of Chi-square test (C) and Fisher exact test (F).
Intensity of inflammatory infiltrate: 0, light and 1, moderate to severe; LP, lichen planus; N, number of patients; sex: M, male and F, female; stage of lesion development: 0, active and 1, late.

Discussion

Diseases affecting the skin and mucous membranes manifest distinct clinical appearances in each of these two different organs, and this is probably due to variations in their structure and function. Oral mucosa and skin differ in keratinization patterns, resistance to external pressure and moist vs. dry environment.[20] Cultured normal oral keratinocytes locomote significantly faster than skin keratinocytes and exhibit faster growth.[21] During wound repair,

Table 2. Patients' ages, number of clusters of apoptotic cells, CD4/CD8 ratio, number of perforin and granzyme B-positive cells and single apoptotic bodies – comparative analysis between CLP and OLP

Variable	Group	N	Mean	SD	Median	Minimum	Maximum	p
Age	CLP	16	48.94	15.36	50.50	18.00	73.00	0.653 (S)
	OLP	29	46.97	13.18	48.00	25.00	69.00	
Clusters	CLP	16	1.51	3.01	0.20	0.00	11.70	0.626*
	OLP	29	0.83	1.02	0.47	0.00	3.92	
CD4/CD8	CLP	16	1.39	1.64	0.94	0.19	6.78	0.155*
	OLP	29	1.48	1.05	1.15	0.47	4.16	
Granz	CLP	16	18.94	3.84	19.50	12.00	26.00	<0.001*
	OLP	29	121.83	107.48	89.86	15.19	390.78	
Perf	CLP	16	17.50	3.48	17.00	11.00	24.00	<0.001*
	OLP	29	83.45	74.71	59.08	11.39	398.65	
Apop	CLP	16	4.31	7.56	2.10	0.30	31.80	<0.001*
	OLP	29	24.24	22.75	17.09	0.00	81.52	

Descriptive level of probability of Student t-test (S) and Mann–Whitney nonparametric test (*).
Apop, single apoptotic bodies; CLP, cutaneous lichen planus; Granz, granzyme B; N, number of patients; OLP, oral lichen planus; Perf, perforin; SD, standard deviation.

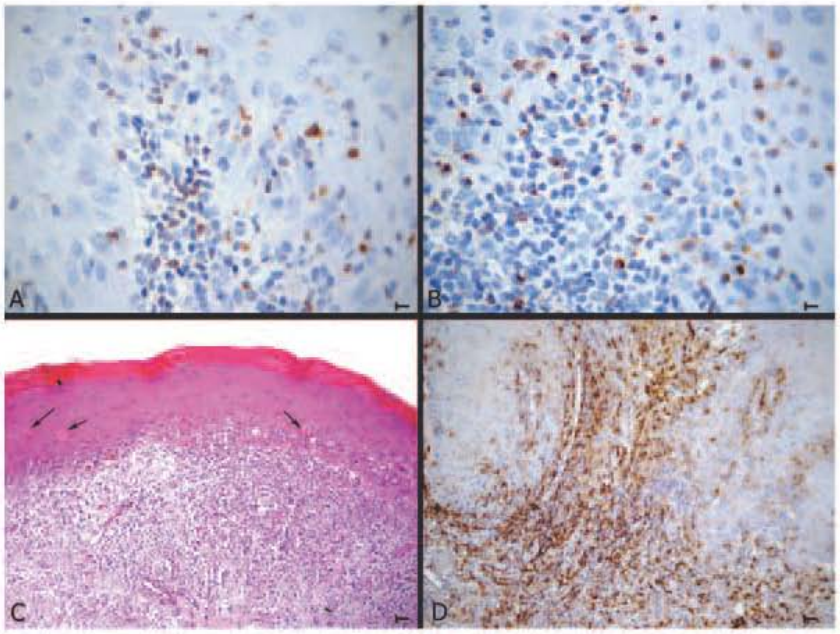

Fig. 1. Perforin (A) and granzyme B-stained cells (B) in oral lichen planus (LP), (C) clusters of necrotic keratinocytes (apoptotic bodies; see arrows) in a regressive lesion of oral LP, (D) CD4-positive cells in active oral LP. Bar = 0.012 mm (A and B) and 0.029 mm (C and D).

re-epithelialization and neoformation of supporting tissue are completed earlier in oral mucosa and mononuclear phagocytic activity reaches higher levels than in skin.[22]

Most inflammatory diseases of the skin and oral mucosa are because of immunological events mediated by T-cells. Comparative studies of the mechanisms by which T-cells react in these tissues must be carried out in this context. As oral mucosa, compared with the skin, is exposed to large amounts of antigen, whether from food, bacteria, virus or fungi, it is possible that this antigenic load interferes with its immunocompetent cells.[23] This might explain the clinical observation that while cutaneous LP is self-limited, having pruritus as its primary discomfort, oral LP is chronic in nature. Oral LP rarely undergoes spontaneous remission and is the cause of severe morbidity. Oral LP lesions often last for years, with alternating periods of exacerbation and quiescence, and both erythematous and ulcerated areas are often sensitive or painful.[24,25]

Hasséus et al.[23] found that oral Langerhans cells have a higher capacity to provide costimulatory help and to stimulate alloreactive T-cells, as compared to skin Langerhans cells. Santoro et al.[17] hypothesized that a mechanism that could be responsible for long-lasting inflammatory processes in oral LP could be the activation of nuclear factor kappa B (NF-kappaB). It is expressed on basal and suprabasal keratinocytes in all cases of LP. Oral LP shows higher numbers of NF-kappaB-positive keratinocytes in comparison to cutaneous LP. NF-kappaB expression correlates with the recruitment of cytotoxic T-cells that express perforin.[17] T-cell cytotoxicity mediated by perforin and granzyme B has been shown in the control of tumor growth, in viral and intracellular bacterial infections, in autoimmunity, in the rejection of foreign tissue and, more recently, in the pathogenesis of chronic inflammatory diseases.[12–14,26,27]

To further explore if these molecules could account for differences in clinical behavior, we evaluated the expression of perforin and granzyme B in a series of oral and cutaneous LP. The two groups were similar with respect to patient sex and age, the histopathological stage of lesion development (active or regressive) and the severity/intensity of inflammation. We found a significantly higher number of cells expressing granzyme B and perforin in oral LP in comparison to cutaneous LP. It is known that the lymphocytic infiltrate in oral LP is composed almost exclusively of T-cells, and that most T-cells within the epithelium and adjacent to damaged basal keratinocytes are activated CD8+ T-lymphocytes.[28] These findings suggest that CD8+ T-cells are involved in disease pathogenesis, and that activated CD8+ T-cells may trigger keratinocyte necrosis/apoptosis in oral LP.

Possible mechanisms used by CD8+ cytotoxic T-cells to cause keratinocyte apoptosis in oral LP

include (a) T-cell-secreted TNF-α binding TNF-α receptor 1 (TNF R1) on the keratinocyte surface, (b) T-cell surface CD95L (Fas ligand) binding CD95 (Fas) on the keratinocyte surface or (c) T-cell-secreted granzyme B entering the keratinocyte via perforin-induced membrane pores. All of these mechanisms may activate the keratinocyte caspase cascade, thereby resulting in keratinocyte apoptosis.[29] We also found that the higher the number of perforin-positive cells, the higher the number of granzyme B-positive cells either in oral or cutaneous LP. In fact, in the absence of perforin, the granzyme molecule can enter the cytoplasm but it cannot trigger cell damage. The two molecules work cooperatively to induce target–cell apoptosis.[14,15] Furthermore, although no differences were found between groups with respect to CD4 : CD8 cell ratios, a higher number of CD4-positive cells was found in active lesions as compared to regressive lesions only in the oral LP group. Also, significantly higher numbers of single necrotic keratinocytes (apoptotic bodies) were found in oral LP lesions, either within or beneath the epithelium, as compared with cutaneous LP. Only in the oral LP group, apoptotic body clusters had a positive correlation with regressive lesions. These data point to the active status of oral LP lesions.

In oral LP, while the majority of intraepithelial lymphocytes are CD8+ cytotoxic T-cells, most lymphocytes in the lamina propria are CD4+ helper T-cells.[28,30] An early event in oral LP lesion formation may be the presentation of MHC class II antigens to CD4+ helper T-cells, which is followed by keratinocyte necrosis/apoptosis mediated by CD8+ cytotoxic T-cells. Some studies have shown a predominance of cytotoxic T-lymphocytes (CD8+) in late lesions.[5,31–34] The balance between immunological help and suppression might determine the clinical behavior of the disease.[35] Langerhans cells or keratinocytes in oral LP may present antigens associated with MHC class II to CD4+ helper T-cells, which then are stimulated to secrete the Th1 cytokines IL-2 and IFN-γ. Local production of IFN-γ may maintain keratinocyte MHC class II expression, thereby contributing to disease chronicity.[36] Wenzel et al.[37] found strong expressions of type I IFN in chronic cytotoxic inflammation of LPs, triggering the influx of cytotoxic effector lymphocytes.

Our work confirms the role of granzyme B and perforin in LP pathogenesis. We have observed increased expression in oral LP, and we postulate a correlation between cytotoxic enzymes and the clinical behavior of the disease. Insights gained from these results could potentially be applicable to the differential diagnosis of oral LP and inflammatory early phase of leukoplakia. Costa et al.[38] reported their findings in premalignant oral lesions. They found that the density of cells expressing granzyme B was significantly lower in a dysplastic leukoplakia group in comparison to an LP group as well as significantly higher than in a nondysplastic leukoplakia group. From a histopathological perspective, the observance of several single necrotic keratinocytes in active lesions favors the diagnosis of LP.

Acknowledgements

The authors acknowledge Ms Ana Cláudia S. Piaza for technical assistance, Mr Adilson A. Piaza for assistance in photographic documentation and Ms Diane Ellis, BS, for her aid with English grammar usage. Dr Konradin Metze receives a grant as researcher of CNPq. This work is supported by FAPESP.

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4- CONCLUSÕES GERAIS

Com relação à análise comparativa entre o líquen plano cutâneo e a erupção liquenoide a droga:

1- O número de ceratinocitos apoptóticos agregados foi maior na ELD em relação ao LPC e no grupo da ELD foi encontrado maior número de biopsias com eosinofilos e plasmócitos no infiltrado. Estes achados podem ser úteis no diagnóstico diferencial entre as doenças.

2- O número e a distribuição dos monocitos, histiocitos jovens, células T e seus subtipos não apresentou diferenças entre os grupos. Ambos foram encontrados, em grande número, nas biopsias dos 2 grupos de doenças. A expressão da ICAM-1 foi intensa em ambos os grupos; não foram encontradas diferenças significantes, entre os grupos, no número de células que expressavam este marcador. Não foram encontradas diferenças significantes no número ou na distribuição das células inflamatórias que expressavam as proteases citotóxicas granzima B e perforina.

3- No grupo do LPC, quanto maior era o número das células T, maior o dos macrófagos HAM56-positivos. No grupo da ELD, foi encontrada correlação negativa entre o número de células T e o de histiocitos jovens (MAC 387-positivos). Foi observada correlação positiva entre o número de células T e o de células CD8-positivas, no grupo da ELD. O mesmo não ocorreu, no grupo do LPC.

4- O número de células CD8-positivas apresentava correlação positiva e significante com o número de células inflamatórias que expressavam a perforina, apenas no grupo LPC. O número de células inflamatórias que expressava a granzima B apresentava correlação positiva e significante com a variável perforina, apenas no grupo LPC e com o número de ceratinocitos apoptóticos agregados, apenas no grupo ELD. Assim quanto maiores eram os valores da granzima B, maiores os da perforina, no grupo LPC e maiores os números de células apoptóticas agregadas, no grupo ELD.

Estes resultados favorecem um papel mais importante da granzima B e linfocitos TCD8$^+$ no mecanismo patogenético da ELD, comparativamente com o da perforina, de maior importância no LPC. É possível que a ação da granzima B esteja ligada ao número abundante de células apoptóticas agregadas encontradas na ELD. Embora o LPC e a ELD apresentem semelhanças clínicas e histológicas, a etiopatogênese parece ser distinta.

Com relação à análise comparativa entre o líquen plano cutâneo e o líquen plano oral:

1- Foi encontrada maior densidade de grânulos citotóxicos de granzima B e perforina, nas lesões do LPO, em comparação com as do LPC. A expressão aumentada destas moléculas pode estar associada à diferença no comportamento clínico da doença.

2- No LPO, o número de corpos apoptóticos agrupados mostrou correlação positiva com as lesões em fase regressiva, resquício de acentuada atividade citotóxica em fases mais ativas das lesões.

3- Não houve diferença entre as amostras de LPO e LPC com relação à razão CD4: CD8. Contudo, maior número de células CD4-positivas foi encontrado nas lesões ativas, quando comparado com o das lesões regressivas no LPO, mas não no LPC, apontando para diferenças na expressão imune da doença nas duas localizações.

Estes resultados favorecem uma correlação entre as enzimas citotóxicas e o comportamento clínico distinto entre estas doenças com manifestações liquenoides.

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6- ANEXOS

6.1- Parecer do Comitê de Ética em Pesquisa da Unicamp

CEP, 29/01/07.
(Grupo III)

PARECER PROJETO: Nº 660/2006 (Este nº deve ser citado nas correspondências referente a este projeto)
CAAE: 0530.0.146.000-06

I-IDENTIFICAÇÃO:

PROJETO: **DERMATITES LIQUENÓIDES: ASPECTOS HISTOLÓGICOS E IMUNO-HISTOQUÍMICOS**
PESQUISADOR RESPONSÁVEL: Denise Lage
INSTITUIÇÃO: HC/UNICAMP
APRESENTAÇÃO AO CEP: 01/11/06
APRESENTAR RELATÓRIO EM: 04/12/07 (O formulário encontra-se no *site* acima)

II - OBJETIVOS

Analisar os achados morfológicos e imuno-histoquímicos da pele dos pacientes com líquem plano e demais dermatites liquenóides tratados no Serviço de Dermatologia da Faculdade de Ciências Médicas da UNICAMP no período de 1996 a 2006, correlacionando entre si e com os achados clínicos.

III - SUMÁRIO

Estudo retrospectivo com dados extraídos da observação clínica arquivadas no SAME (Serviço de Arquivo Médico) de pacientes que tenham sido acometidos pelo líquem plano ou outras dermatites liquenóides dos quais tenha sido coletada amostra de pele. Além dos dados de observação clínica serão extraídos dados da leitura histológica. Novos cortes serão tratados para icam-1, granzima e perforina (estudo imuno-histoquímicos) e analisados em imagens digitalizadas. Os resultados serão tabulados e sua significância avaliada por testes estatísticos.

IV - COMENTÁRIOS DOS RELATORES

A pesquisadora solicita dispensa do Termo de Consentimento Livre e Esclarecido por tratar-se de trabalho retrospectivo, sendo empregados dados e material anátomo-patológico de arquivo a partir de biópsias da rotina dos ambulatórios da disciplina de dermatologia. Consideramos pertinente a solicitação de dispensa.

V - PARECER DO CEP

O Comitê de Ética em Pesquisa da Faculdade de Ciências Médicas da UNICAMP, após acatar os pareceres dos membros-relatores previamente designados para o presente caso e atendendo todos os dispositivos das Resoluções 196/96 e complementares, resolve aprovar sem

Comitê de Ética em Pesquisa - UNICAMP
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restrições o Protocolo de Pesquisa, bem como ter aprovado todos os anexos incluídos na Pesquisa supracitada.

O conteúdo e as conclusões aqui apresentados são de responsabilidade exclusiva do CEP/FCM/UNICAMP e não representam a opinião da Universidade Estadual de Campinas nem a comprometem.

VI - INFORMAÇÕES COMPLEMENTARES

O sujeito da pesquisa tem a liberdade de recusar-se a participar ou de retirar seu consentimento em qualquer fase da pesquisa, sem penalização alguma e sem prejuízo ao seu cuidado (Res. CNS 196/96 – Item IV.1.f) e deve receber uma cópia do Termo de Consentimento Livre e Esclarecido, na íntegra, por ele assinado (Item IV.2.d).

Pesquisador deve desenvolver a pesquisa conforme delineada no protocolo aprovado e descontinuar o estudo somente após análise das razões da descontinuidade pelo CEP que o aprovou (Res. CNS Item III.1.z), exceto quando perceber risco ou dano não previsto ao sujeito participante ou quando constatar a superioridade do regime oferecido a um dos grupos de pesquisa (Item V.3.).

O CEP deve ser informado de todos os efeitos adversos ou fatos relevantes que alterem o curso normal do estudo (Res. CNS Item V.4.). É papel do pesquisador assegurar medidas imediatas adequadas frente a evento adverso grave ocorrido (mesmo que tenha sido em outro centro) e enviar notificação ao CEP e à Agência Nacional de Vigilância Sanitária – ANVISA – junto com seu posicionamento.

Eventuais modificações ou emendas ao protocolo devem ser apresentadas ao CEP de forma clara e sucinta, identificando a parte do protocolo a ser modificada e suas justificativas. Em caso de projeto do Grupo I ou II apresentados anteriormente à ANVISA, o pesquisador ou patrocinador deve enviá-las também à mesma junto com o parecer aprovatório do CEP, para serem juntadas ao protocolo inicial (Res. 251/97, Item III.2.e)

Relatórios parciais e final devem ser apresentados ao CEP, de acordo com os prazos estabelecidos na Resolução CNS-MS 196/96.

VII - DATA DA REUNIÃO

Homologado na II Reunião Extraordinária do CEP/FCM, em 04 de dezembro de 2006.

Profª. Drª. Carmen Silvia Bertuzzo
PRESIDENTE DO COMITÊ DE ÉTICA EM PESQUISA
FCM / UNICAMP

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Quadro 1- Características para distinção entre erupção liquenoide a droga e líquen plano

	ELD	**LP**
Idade média	66 anos	50 anos
Localização	Distribuição mais generalizada; geralmente poupa as áreas clássicas do LP	Punhos, parte flexora dos antebraços, região pré-sacral, membros inferiores, genitália
Morofologia	Mais eczematosa, psoriasiforme ou semelhante à pitiríase rósea	Pápulas brilhantes, achatadas, poligonais ou violáceas
Estrias de Wickham	Incomuns	Presentes
Hiperpigmentação	Muito comum, algumas vezes persistentes	Comum
Distribuição em áreas fotoexpostas	Frequente*	Incomum
Mucosas	Geralmente poupadas	Geralmente acometidas
Histolopatologia	Graus variáveis de infiltrado eosinofílico e/ou plasmocitário. Infiltrado profundo pode estar presente. Paraceratose	Eosinófilos e plasmocitos são incomuns. Infiltrado linfocitico denso em faixa. Sem paraceratose

*Especialmente com medicamentos como a hidroclorotiazida

Quadro 2- Medicamentos associados à erupção liquenoide a droga

ANTIMICROBIANOS

- Etambutol
- Griseofulvina
- Isoniazida
- Cetoconazol
- Primetamina
- Estreptomicina
- Sulfametoxazol
- Tetraciclinas

ANTIHIPERTENSIVOS

- **Captopril**
- **Enalapril**
- **Labetalol**
- **Metildopa**
- **Propranolol**
- Diazóxido
- Doxazosina
- Prazosina

ANTIMALÁRICOS

- **Cloroquina**
- Hidroxocloroquina
- Quinacrina

ANTIDEPRESSIVOS, DROGAS ANSIOLÍTICAS, ANTIPSICÓTICOS E ANTICONVULSIVANTES

- Amitriptilina
- Carbamazepina
- Clorpromazina
- Imipramina
- Levomepromazina
- Lorazepan
- Metopromazina
- Fenitoína

DIURÉTICOS

- **Clorotiazida**
- **Hidroclorotiazida**
- Furosemida
- Espironolactona

AGENTES HIPOGLICEMIANTES

- Clorpropamida
- Gliburida (glibenclamida)
- Tolazamida
- Tolbutamida

METAIS

- **Sais de ouro**
- Arsênico
- Bismuto
- Mercúrio
- Paládio

AINES

- Ácido acetilsalicílico
- Benaxoprofeno
- Diflunisal
- Fenclofenaco
- Flurbiprofeno
- Ibuprofeno
- Indometacina
- Naxop

MISCELÂNEA

- Alopurinol
- Amifenazol
- Cinarizina
- Clanamida
- Dapsona
- Gemfibrozil
- Hidroxiureia
- Imatinibe
- Infliximabe
- Interferon-α
- Iodetos
- Isotretinoína
- Levamisole
- Lítio
- Mercapto-propionoglicina
- Mesalina
- Meticrano
- Nifedipina
- Omeprazol
- Orlistat
- Penicilamina
- Procainamida
- Piritoxina
- Sivastatina
- Quinina
- Quinidina
- Sildenafil
- Sulfasalazina
- Triexifenidil

Os medicamentos mais comumente associados estão em negrito