Pontifícia Universidade Católica do Rio Grande do Sul
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Dissertação de Mestrado

Efeito do sulfato de deidroepiandrosterona (DHEAS) como adjuvante em vacina de HSP70 de *Mycobacterium tuberculosis* em camundongos idosos e jovens

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Dissertação apresentada ao
Programa de Pós-Graduação
em Biologia Celular e Molecular
como requisito parcial para a
obtenção do grau de Mestre.

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PORTO ALEGRE (RS)

OUTUBRO de 2005

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AGRADECIMENTOS

A Deus.

Ao pessoal do Laboratório de Imunologia Celular e Molecular, especialmente, Rodrigo Pestana Lopes, Cláudia Paiva Nunes, Fábio Maito e Rafael Dincao, pelo apoio no desenvolvimento dos experimentos.

Ao meu amigo Luiz Rodrigues, pelas consultorias à distância, por e-mail e “msn”.

Às minhas amigas, pelos momentos de consolo e apoio, desde agosto de 1998, Raquel Mattos de Oliveira, Jacqueline Piccoli e Cristina Jeckel, mais recentemente a Claudia Paiva Nunes e Carolina Torronteguy, pela cumplicidade singular.

Também às minhas amigas Bibiana Butkus, Carine Paula, Carla Schmitz e Graciela Cordeiro pelos momentos de descontração.

À minha amiga Josiane Bandinelli por compartilhar comigo as oscilações de humor.

Ao professor Moisés Evandro Bauer (Herr Bauer) pelo apoio intelectual, orientação e principalmente paciência.

À professora Cristina Bonorino pelas “chamadas à realidade” e também ao apoio intelectual.

Ao professor Emilio Jeckel pelo incentivo desde meus “primeiros passos” pelo mundo da pesquisa.

À minha mãe por acreditar em mim.

À CAPES pelo suporte financeiro.

LISTA DE ABREVIATURAS

ALUM: hidróxido de alumínio

BALB/c: Linhagem de camundongos albinos

BCG: Bacilo Calmette-Guérin

C57/BL6: Linhagem de camundongos de pelagem preta

CD: Designação de grupo

CMV : Citomegalovirus

DHEAS: Sulfalto deidroepiandrosterona

FITC: Isotiocianato de fluoresceína

HSP: Proteína de choque térmico

HSP 70: Proteína de choque térmico 70

IFA: Adjuvante Incompleto de Freund

IL: Interleucina

IFN: Interferon

MHC: Complexo de Histocompatibilidade Principal

mycHSP70: HSP70 de *Mycobacterium tuberculosis*

ROI: Radicais livres de oxigênio

RNI: Radicais de óxido nítrico

TNF: Fator de necrose tumoral

RESUMO

O sulfato de deidroepiandrosterona (DHEAS) é o principal hormônio esteróide produzido pelas adrenais. Devido a suas propriedades imunomodulatórias, é utilizado como adjuvante em vacinas. Investigamos o efeito adjuvante do DHEAS durante imunização, intraperitonial, com proteína de estresse HSP 70 (*heat shock protein* 70) em camundongos BALB/c idosos (24 meses) e jovens (3 meses). Animais idosos e jovens apresentaram produção de anticorpos semelhantes em resposta à vacinação. Porém, foi observada em camundongos jovens imunizados com mycHSP70 e DHEAS um aumento nos níveis de IgG significante logo no sétimo dia após a imunização e ainda alta proliferação de linfócitos T antígeno-específica, quando comparada aos controles(animais imunizados com IFA). A imunização com mycHSP70 e DHEAS também aumentou a produção de IFN-γ nos dois grupos de idade. O DHEAS não produziu nenhum efeito adjuvante na proliferação de células T antígeno específicas ativadas. Também, foi investigado se a vacinação poderia influenciar a sensibilidade a esteróides *in vitro*. Interessantemente, as células T de camundongos jovens imunizados foram consideravelmente mais resistentes a glicocorticóides e DHEAS em relação às células de animais imunizados somente com o adjuvante controle. Esses efeitos não foram observados nas células dos animais idosos. Os dados sugerem que o DHEAS produz efeitos adjuvantes importantes na resposta imune humoral e celular de camundongos jovens. Esses dados também indicam que a imunização é capaz de alterar a sinalização das células T a esteróides somente nos camundongos jovens.

Palavras-chave: tuberculose, envelhecimento, HSP70, DHEAS, adjuvante, vacina, imunossenescência

1. APRESENTAÇÃO DO TEMA

1.1. Envelhecimento e Infecções Respiratórias

O processo do envelhecimento é caracterizado por alterações ao longo do tempo, que acabam influenciando o funcionamento de diversos sistemas, entre eles, o imune. A imunossenescência é caracterizada por alterações na imunidade inata, processamento de antígenos, resposta humoral e imunidade adaptativa (Pawelec et al., 2002). Por essas razões, o envelhecimento muitas vezes está associado com o declínio da resposta imune a ao aumento da suscetibilidade a doenças infecciosas (Webster, 2000). Entre essas doenças infecciosas, podemos destacar aquelas que possuem como alvo o aparelho respiratório, como gripe, pneumonia e tuberculose que têm chamado atenção nos últimos anos por ter aumentando sua incidência na população idosa (Cohen, 2005; Douglas, 1990; Esposito, 1989; Meyer, 2001).

De acordo com Beers e Berkow (Beers and Berkow, 2000), os idosos são um dos maiores grupos de risco de infecção por influenza, representando pelo menos 50% das hospitalizações mais de 80% do total de mortes por influenza ocorrem em indivíduos com mais de 65 anos de idade. A cada ano, os idosos são priorizados a receber vacinações para influenza, porém, a eficácia dessa vacina nessa população é de somente 30 - 40% em comparação a 70 - 80% nos indivíduos jovens (Stein, 1994; Webster, 2000). Também se tem verificado que a pneumonia é a quarta causa de morte e a primeira entre as doenças infecciosas em idosos (Esposito, 1989; Marston et al., 1997). Além disso, a eficácia das vacinas existentes para pneumonia está entre 44 - 61% em indivíduos com idade acima de 65 anos (Stein, 1994). Outra infecção prevalente em idosos é a tuberculose. A *Mycobacterium tuberculosis* pertencente à família Mycobacteriacea, ordem Actinomycetales, gênero *Mycobacterium*, é considerada uma das mais bem adaptadas e bem sucedidas micobactérias que parasitam os seres humanos. Esse sucesso é devido a facilidade de sobreviver em ambientes hostis, como o interior de um macrófago, mesmo com a possibilidade de uma resposta T específica (Silva et al., 2001).

Estima-se que a tuberculose contribui com quase dois milhões de mortes por ano (Kochi, 1991). Nos países em desenvolvimento, principalmente em áreas onde a ocorrência de indivíduos infectados por HIV é grande, a maioria das mortes por tuberculose ocorre na meia-idade desses indi-

víduos (Kochi, 1991). Por outro lado, em países desenvolvidos da Europa e nos Estados Unidos, os casos de tuberculose ocorrem em indivíduos com 65 anos ou mais (Vesosky and Turner, 2005).

Outro fator que contribui para o desenvolvimento da tuberculose é a internação a longo prazo (Gubser, 1998). As unidades de tratamento a longo prazo tornam-se propícios para o aumento da taxa de transmissão e infecção, uma vez que associa a saúde prejudicada dos indivíduos que estão internados nessas unidades e a proximidade entre as pessoas (Gubser, 1998).

1.2. Resposta Imune a Mycobacterium tuberculosis no Envelhecimento

A tuberculose é adquirida através da inalação da micobacteria *Mycobacterium tuberculosis* . A bactéria é internalizada pelos macrófagos presentes nos alvéolos pulmonares e inicia focos de infecção nos pulmões. Esses focos se expandem através do crescimento da micobactéria e recrutamento de macrófagos e linfócitos, gerando um granuloma que é característico dessa infecção (Flynn and Chan, 2005). Esse granuloma parece limitar o crescimento da micobactéria e previne que a infecção se espalhe. Porém, o granuloma também protege a bactéria do sistema imune e é provavelmente o responsável pela persistência ou pela natureza latente da infecção (Silva et al., 2001) (figura1).

A resposta imune contra *Mycobacterium tuberculosis* se caracteriza por uma resposta celular intensa, que envolve células T CD4 e também T CD8 que secretam IFN-γ para ativar macrófagos que executam inúmeras tarefas como a apresentação e o processamento do antígeno e função antimicobacteriana (revisado em (Flynn and Chan, 2001).

A tuberculose em idosos pode freqüentemente surgir de uma reativação de uma infecção prévia latente (Mackay and Cole, 1984). Geralmente, pessoas saudáveis quando em contato com esse patógeno,não apresentam sintomas imediatos da doença, mas podem armazenar a bactéria latente no pulmão pelo resto da vida. Com o declínio da saúde e as alterações no sistema imune associadas ao envelhecimento (Globerson and Effros, 2000), apresenta-se a oportunidade do organismo latente proliferar levando a doença ativa. Outra causa da tuberculose nos idosos é através de infecção primária com *Mycobacterium tuberculosis* (Dutt, 1992; Stead and Dutt, 1991; Steimke et al., 1993). Essa forma de infecção é particularmente comum em clinicas, onde o risco de exposição a indivíduos que tem a doença ativa é substancialmente aumentado. Estima-se que para cada caso de

tuberculose ativa que ocorre em enfermaria, seis mais pacientes irão se infectar com *Mycobacterium tuberculosis* pela primeira vez (Dutt, 1992). E, ainda, há uma chance entre 8-10%, de que esses indivíduos, progridam rapidamente para a forma ativa da doença (Stead and Dutt, 1991) .

Com o declínio da imunidade a doenças infecciosas e a inabilidade de responder a vacinações, nos idosos, há uma necessidade crescente de identificar estratégias alternativas para o aumento da eficácia das vacinas existentes.

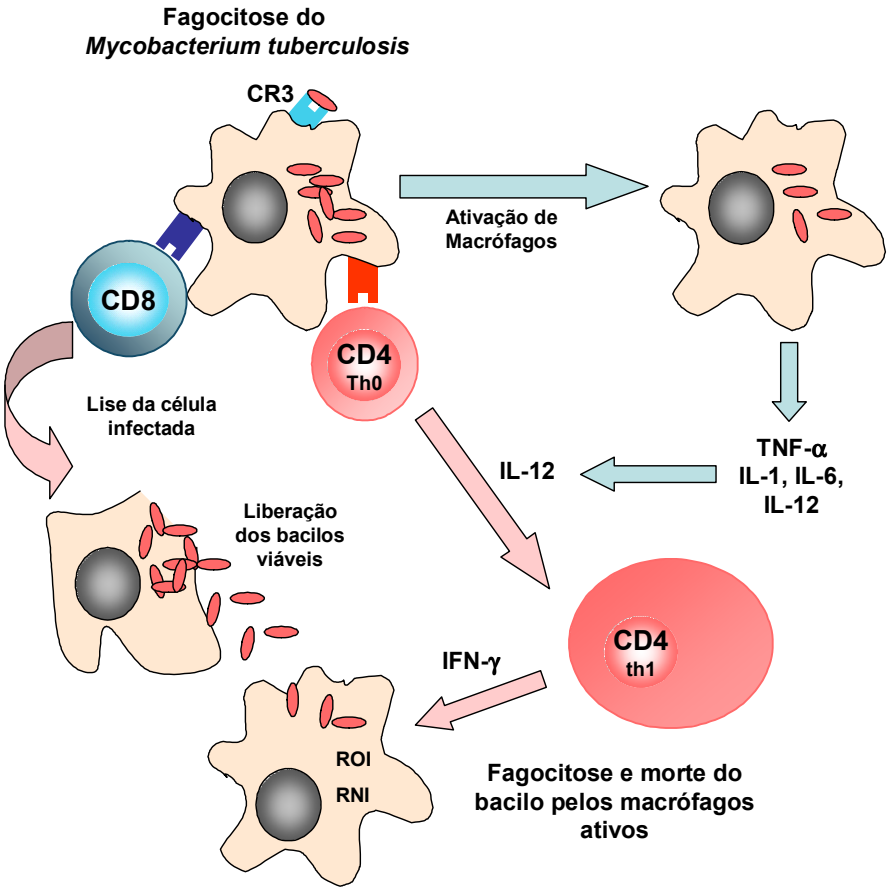

Figura 1. Modelo simplificado dos mecanismos imunológicos contra micobactérias. O bacilo é inicialmente fagocitado pelos macrófagos tissulares, multiplicando-se dentro dessas células. Quando as células T específicas chegam ao tecido infectado, liberam uma grande quantidade de IFN-γ em contato com o antígeno, que é apresentado na superfície celular. O IFN-γ pode ativar as células infectadas para morrer, somente para a bacteriostase. Além disso, essa citocina é capaz, em conjunto com outros fatores, de ativar novos linfócitos recém chegados suficientes para matar a *Mycobacterium tuberculosis* durante a fagocitose. A morte inclui a liberação de radicais livres do oxigênio (ROI) e óxido nítrico (RNI) pelos grânulos das vesículas fagocíticas.

1.3. HSP70 de Mycobacterium tuberculosis

No início do século 20, Calmette e Guérin criaram uma vacina contra tuberculose com a micobactéria atenuada. A BCG (Bacille Calmette-Guérin) é largamente utilizada, sendo administrada em recém-nascidos, reduzindo em torno de 70% a incidência da manifestação da tuberculose na infância. Porém, esta vacina apresenta pouco ou nenhum efeito na tuberculose pulmonar em adultos, que é sua manifestação mais abundante (McCormick et al., 2001),(Young, 2003). Isso subsidia a busca de novas alternativas, quanto à escolha de um antígeno mais eficiente, como também na otimização de adjuvantes.

As HSPs (*Heat Shock Proteins*) são proteínas altamente conservadas na escala evolutiva e são exploradas quanto à sua capacidade imunogênica em várias infecções (Havlir et al., 1991; Pack et al., 2005; Roman et al., 1994; Udono et al., 2004). A família da HSP70 (70kDa) constitui o grupo mais abundante e evolutivamente conservado das proteínas de choque de calor (Parsell and Lindquist, 1993). As HSP70 são normalmente nomeadas pela sua massa molecular e pertencem a famílias multigênicas, em que genes altamente homólogos codificam proteínas com diferentes funções celulares. Através de anticorpos monoclonais foi possível identificar a HSP71 de *Micobacterium tuberculosis* como uma das principais proteínas micobacterianas (Havlir et al., 1991; Mehlert and Young, 1989). A HSP70 de *Mycobacterium tuberculosis*, além de apresentar potencial como adjuvante para a produção de anticorpos (Barrios et al., 1992), também foi caracterizada como um importante antígeno, induzindo um elevado título de anticorpos sete dias após a primeira imunização (Bonorino et al., 1998). Contudo, a resposta a este antígeno é desconhecida em indivíduos idosos.

1.4. Células T, envelhecimento e tuberculose

O repertório de linfócitos T no envelhecimento adquire características peculiares, não somente devido à involução tímica (Douek and Koup, 2000; Hirokawa et al., 1994), como também ao decréscimo na produção de células T virgens e a predominância de células de memória (Callahan et al., 1993; Fulop et al., 2005; Jackola et al., 1994). Além disso, ocorrem alterações na expressão de moléculas co-estimuladoras e nos mecanismos de sinalização celular (Engwerda et al., 1994; Garcia and Miller, 1997; Rea et al., 1996).

A alteração na razão CD4/CD8, é particularmente importante e é caracterizada pelo aumento de células T CD8 de memória e pela diminuição das células virgens. Essa alteração pode estar relacionada às infecções repetitivas, como, por exemplo, o citomegalovirus (CMV) (Looney et al., 1999; Pawelec et al., 2004). Esse fenômeno resulta na exaustão clonal das células T CD8 e no acúmulo de um número reduzido de clones que apresentam características de células senescentes replicativas (Appay et al., 2002,(Effros et al., 1994) (Effros and Pawelec, 1997). Ou seja, a cada divisão, ocorre o encurtamento dos telômeros (Campisi, 2001). Outra característica importante seria a reduzida expressão da molécula CD28, (Batliwalla et al., 1996). Essas células não se dividem mais, mas também não entram em apoptose e, por conseqüência, lotam o compartimento imune (Franceschi et al., 2000a; Franceschi et al., 2000b). A homeostase do sistema imune fica prejudicada e as células tornam-se resistentes a apoptose (Spaulding et al., 1999). Essas alterações (involução tímica, senescência replicativa e resistência a apoptose) levam a inversão da razão CD4/CD8 das células T nos idosos, diminuindo a capacidade de resposta a antígenos novos.

A resposta à tuberculose envolve mecanismos de resposta celular, principalmente os macrófagos e linfócitos T. Ambas as células T CD4 e CD8 estão envolvidas na resposta imune contra a micobactéria. O macrófago infectado apresenta os antígenos micobacterianos, no contexto do MHC de classe II, à célula T CD4, atribuindo a essas últimas células um papel essencial na resposta contra a *Mycobacterium tuberculosis.* As células T CD4 produzem IFN-γ, sendo essa considerada sua função efetora primária que ativa nos macrófagos a produção de NO necessária para controlar ou eliminar os organismos intracelulares (Silva et al., 2001). Embora esse patógeno tenha sido observado no citoplasma dos macrófagos (McDonough et al., 1993), especula-se que ele resida em um vacúolo. Considerando que a apresentação através do MHC de classe I seja mais eficiente para antígenos citoplasmáticos, um possível envolvimento das células T CD8 na resposta imune a *Mycobacterium tuberculosis* recebeu pouca atenção por muitos anos. Há relatos de que os linfócitos T CD8 influenciam no controle da infecção (Muller et al., 1987; Orme and Collins, 1983; Orme and Collins, 1984). Existem evidências que os linfócitos T CD8 migram para os pulmões com uma cinética semelhante aos T CD4 (Feng et al., 1999; Serbina and Flynn, 1999) e são capazes de produzir IFN-γ e lisar macrófagos infectados (Feng et al., 1999; Serbina and Flynn, 1999).

A avaliação da expressão de antígenos de superfície é um meio para avaliar indiretamente a função efetora dos linfócitos (Hara et al., 1986). Sabe-se que o CD69 é um marcador de ativação recente de linfócitos antígeno-específicos *in vitro* (Testi et al., 1994). Uma vez expresso na superfície, ele atua como molécula co-estimuladora na ativação e proliferação de células T (Ziegler et al., 1994). Além disso, o CD69 pode servir como auxiliar no diagnóstico da tuberculose (Avgustin et al., 2005; Skoberne et al., 2000), que é feito através de um teste cutâneo de hipersensibilidade tardia na pele (*tuberculin skin test*- TST).

1.5. IFN-γ e IL-2 no envelhecimento e na resposta a Mycobacterium tuberculosis

Quando as células T são desafiadas com um determinado antígeno infeccioso, ocorre a produção de citocinas do perfil linfócito T auxiliar tipo 1 (Th1): IL-2 e IFN-γ (caso o desafio seja um alergeno, no entanto, o padrão de citocinas é do tipo linfócito T auxiliar tipo 2 (Th2), com produção de IL-10, IL-4 e IL-5. Da mesma forma, foi verificado um desvio no padrão de citocinas do perfil Th1 para Th2 no envelhecimento (Ginaldi et al., 2001; Globerson and Effros, 2000; Pawelec, 1999). Esse desvio se caracteriza pela redução da resposta pró-inflamatória em favor de uma antiinflamatória, que tem como conseqüência uma menor resistência a infecções bacterianas.

O IFN-γ é a principal citocina no controle da infecção por *Mycobacterium tuberculosis,* produzida tanto pelas células T CD4 como CD8 (Lalvani et al., 1998; Orme et al., 1992; Orme et al., 1993; Serbina and Flynn, 1999), mas também por células NK e macrófagos infectados pela micobactéria (Fenton et al., 1997). Seu papel principal é ativar e recrutar macrófagos que apresentam propriedades bacteriostásticas (Howard and Zwilling, 1998). Porém a micobactéria desenvolveu mecanismos que impedem a sinalização de IFN-γ aos macrófagos, limitando a ativação destes (Ting et al., 1999). Isso garante a sobrevivência do patógeno sob forma latente no interior dos macrófagos não ativados, mas em situações em que o sistema imune possa se encontra debilitado, pr exemplo, em indivíduos idosos.

A produção de IL-2 poderia ser importante para a expansão clonal tanto de células T CD4 como T CD8 Células, possibilitando, assim o aumento da produção de mais células expressando IFN-γ. Contudo os linfócitos T CD4 de camundongos idosos parecem não responder a essa citocina, devido a uma baixa expressão de receptores de membrana (Negoro et al., 1986).

1.6. Alterações neuroendócrinas no envelhecimento

Os hormônios são responsáveis pela regulação do metabolismo, reprodução, síntese de proteínas, funções imunes, crescimento e comportamento. Ou seja, estão intimamente ligados em todas as etapas do desenvolvimento do organismo. O fato mais marcante do desenvolvimento humano é o declínio da capacidade reprodutiva que é regulada pelo sistema neuroendócrino (Hayflick, 1994).

Outras alterações caracterizam o envelhecimento do sistema endócrino, como a diminuição da produção de diversos hormônios, entre eles o hormônio do crescimento, glicocorticóides (GC), testosterona, progesterona, aldosterona e DHEA. A forma sulfatada deste hormônio (DHEAS), é inativa e se converte a DHEA perifericamente passando a apresentar atividade biológica. O DHEAS é um dos principais hormônios esteróides produzidos pelo córtex da adrenal, com declínio marcante durante o envelhecimento (Migeon et al., 1957; Orentreich et al., 1992; Sulcova et al., 1997) . Além dos efeitos androgênicos, o DHEA apresenta efeitos imunomodulatórios. O uso do DHEA promoveu melhora em pacientes com lupus eritematoso (Derksen, 1998; van Vollenhoven et al., 1998). O declínio da produção do DHEA parece influenciar a etiologia de doenças que estão associadas ao envelhecimento (James et al., 1997; Williams, 2000), como por exemplo na produção de IL-6 em camundongos (Daynes et al., 1993). O aumento dessa citocina pode estar relacionado com alterações clínicas como osteoporose, formação de auto-anticorpos, alterações malignas das células B e alterações patológicas ligadas a doença de Alzheimer (Ershler, 1993).

A administração de DHEA a roedores foi capaz de reverter algumas alterações celulares da imunossenescência, tais como aumentar os níveis de secreção de IL-2 (Daynes et al., 1990) e a citotoxicidade das células NK (Corsini et al., 2002; James et al., 1997; McLachlan et al., 1996; Risdon et al., 1991; Williams, 2000). O mesmo fenômeno foi observado em estudos *in vitro* (Solerte et al., 1999; Suzuki et al., 1991). Além disso, o DHEAS mostrou-se um importante adjuvante em vacinas para influenza em fêmeas de camundongos da linhagem C57/BL/6, entre 16-24 meses de idade (Danenberg et al., 1995a; Danenberg et al., 1995b), como também para a hepatite B, em camundongos fêmeas e machos C3H/HeN MTV$^-$, C57BL/6, e (C3H/HeN X C57BL/6)F1 ([C3HXBL/6]F1), muito idosos (21-26 meses) (Araneo et al., 1993).

Considerando as infecções respiratórias, a administração (subcutânea) do DHEAS foi capaz também de aumentar os títulos de IgG em camundongos BALB/c imunizados com Pnu-Immune ,um antígeno, presente nas vacinas contra pneumococcus (Garg and Bondada, 1993).

Levando em consideração a baixa eficácia da BCG na tuberculose pulmonar em idosos (Young, 2003), procuramos explorar neste estudo o impacto da administração de DHEAS como adjuvante em vacina de HSP70 de *Mycobacterium tuberculosis* em camundongos velhos (24 meses) em relação à resposta imune celular e humoral.

2. OBJETIVOS

Avaliar o efeito da administração de DHEAS como adjuvante em vacina de HSP70 em camundongos velhos e jovens em relação à:

1. Cinética e intensidade de resposta humoral após vacinação.
2. Proporção de subtipos de células T antígeno-específicas.
3. Sensibilidade de esplenócitos a glicocorticóides e DHEAS
4. Produção das citocinas IL-2 e IFN-γ

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4. ARTIGO CIENTÍFICO

Submetido a *Mechanisms of Ageing and Development*

THE ADJUVANT EFFECT OF DEHYDROEPIANDROSTERONE SULPHATE DURING IMMUNIZATION TO *MYCOBACTERIUM TUBERCULOSIS* HEAT SHOCK PROTEIN 70 IN OLD BALB/C MICE

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5. Abstract

Dehydroepiandrosterone sulphate (DHEAS) is the major steroid hormone produced by the adrenals with reported immunomodulatory properties. We investigated the adjuvant effects of DHEAS during intraperitoneal immunization to *Mycobacterium tuberculosis* heat shock protein 70 (mycHSP70) in old (24 mo) as well as young (3 mo) BALB/c mice. Aged and young animals had similar antibody response to vaccination. However, young mice co-immunized with mycHSP70-DHEAS presented an early increase in IgG levels and had higher antigen-specific splenocyte proliferation compared to non-immunized animals. Co-immunization with mycHSP70-DHEAS also increased IFN-gamma production in both age groups. DHEAS treatment did not produce any effect on the percentage of antigen-specific activated T-cells. We then investigated whether vaccination would impair T-cell sensitivity to steroids in vitro. Interestingly, T cells of young immunized mice were consistently more resistant to glucocorticoids and DHEAS than cells of animals immunized to control adjuvant only. These effects were not observed in cells of aged mice. Taken together, our data suggest that DHEAS produced stronger adjuvant effects upon humoral and cellular immune responses of young mice. Our data also indicate that immunization is capable of changing T-cell signalling to steroids in young mice only.

Key words: tuberculosis, ageing, HSP70, DHEAS, vaccine, immunosenescence

6. Introduction

The ability of elderly individuals to mount an effective immune responses for vaccination for new antigens is diminished when compared to younger subjects. This is related to the decline of immune function (immunosenescence) in many aspects, including altered production of T cell progenitors, decrease levels of newly-generated mature T cells (thymic involution), ageing of resting immune cells, activation pathways disrupted of immune cells and replicative senescence of clonally expanding cells (Pawelec et al., 2002). One strategy to improving the response of elderly people to immunization is through the use of more effective vaccine adjuvants. Hormones may be such candidates.

Ageing is associated with significant neuroendocrine changes that may be related to immunosenescence. From both human and animal studies, it has been demonstrated a decline in growth hormone, sex hormones and dehydroepiandrosterone (DHEA) with ageing (Migeon et al., 1957; Orentreich et al., 1992; Sulcova et al., 1997). DHEA is the major secretory product of human adrenal. The hormone is uniquely sulphated (DHEAS) before entering the plasma, and this prohormone is converted to DHEA and its metabolites in various peripheral tissues (Labrie et al., 1998). Concentrations of DHEA and DHEAS decline after the third decade of life, reaching 10-20% of the peak level in the elderly (Wickens, 1998). We have previously shown that strictly healthy ageing is associated with increased cortisol levels and lower DHEA (Collaziol et al., 2004; Luz et al., 2003). Ageing of the endocrine system (endocrinosenescence) may be close related to immunosenescence. For instance, the increased cortisol/DHEA ratio has been negatively associated to blunted T-cell proliferation and cellular sensitivity to glucocorticoids (Bauer, 2005). Hormonal replacement therapies may thus have an important restoring immunological effects during ageing.

In particular, DHEA has been proposed as exerting restoring effects on immunosenescence, including adjuvant effect on the immunization of aged mice with recombinant hepatitis B surface antigen (Araneo et al., 1993) or influenza (Danenberg et al., 1995a; Danenberg et al., 1995b). These restoring effects were related to the immunomodulatory properties of DHEA, including increased antibody titres, higher mitogen-stimulated IL-2 production (Daynes et al., 1990; Suzuki et al., 1991) diminished TNF-α or IL-6 production (Di Santo et al., 1996; Straub et al., 1998), inhibition of natural killer cell differentiation (Risdon et al., 1991) or lymphocyte proliferation (Padgett and Loria, 1994).

However, there is no data in the literature assessing the DHEA/S adjuvant effects on the immunization to tuberculosis thus far. This may have critical importance for the frail elderly, in which the incidence of this opportunistic infection has recently increased (Meyer, 2001).

The *Mycobacterium tuberculosis* heat shock protein 70 (mycHSP70) has been suggested as important antigen in the tuberculosis vaccine (Havlir et al., 1991). The HSP70 family is characterized for its well conserved protein sequence during the evolution (Parsell and Lindquist, 1993). In addition, HSP70 has been described as important antigen in other infections, such as *Trypanosoma cruzy, Leishmania major* and *Plasmodium falciparum* (Schoel and Kaufmann, 1996). Moreover, mycHSP70 induced a strong humoral response mediated by TCD4+ cells, after the first immunization (Bonorino et al., 1998). However, the impact of ageing on the mycHSP70 immunization has not been explored so far.

The objective of this study was to describe the adjuvant effects of DHEAS on both humoral and cellular immune functions of aged mice immunized with mycHSP70. We also investigated the age-related neuroimmunomodulatory effects of vaccination. In view of evidence that during ageing lymphocytes become resistant to the immunosuppressive effects of steroids (Bauer, 2005), we particularly examined whether vaccination for mycHSP70 would render *ex vivo* changes in T-cell sensitivity to steroids.

7. Materials and Methods

7.1. Mice

Three-month old female BALB/c mice, 20-25 g, were purchased from FEPPS (Porto Alegre, Brazil) and group housed (n=5 per cage) under standard housing conditions with food and water available *ad libitum* and a set light dark cycle. The temperature of the colony room was maintained between 22-23°C. Care and use of the mice were in accordance with protocols approved by the Home Office of Pontifical Catholic University of Rio Grande do Sul (PUCRS, Porto Alegre, Brazil). Aged mice were housed in the animal facility for 24 months before use. The ages of the mice were 3 (young) and 24 months (aged) at the onset of the experimental vaccination protocols. Old mice that had tumors or

splenomegaly or were weak or sick were excluded from all analyses. The study protocol was approved by both scientific and ethics committees (PUCRS).

7.2. Vaccine design

Recombinant protein of *Mycobacterium tuberculosis* HSP70 was purified from cultures of *Escherichia coli* (BL21), transformed with plasmid pY3111, as describe (Menoret, 2004). The Lipopolysaccharide (LPS) was eliminated by a column with polymyxin B (Sigma). DHEAS were diluted in propylene glycol at a concentration of 1mg/mL, as previously described (Araneo et al., 1993), and emulsified in mineral oil (IFA), mycHSP70 and PBS in a final volume of 200µl per mice.

7.3. Immunization regimens

Mice of 3 or 24 months of age (n=10 per group) were given three intraperitoneal (i.p.) immunizations of either incomplete Freund´s Adjuvant vaccine (IFA, control group), vaccine (5 µg of mycHSP70 + IFA) or vaccine plus 10 µg DHEAS in a final volume of 200 µl. DHEAS was used since it is not as rapidly degraded as DHEA (Daynes et al., 1993). The sulphated form of the hormone is then taken up by the macrophages and converted into hormone and is released into the local environment and circulation.

Each animal were injected with the respective treatment at day 1, 7 and 14 and sacrificed at the 17th day. Blood samples were taken by tale venopuncture prior and following each immunization. The sera were isolated and stored at –20°C prior to analyses.

7.4. Antibody response

Mouse sera were tested for mycHSP70-especific IgG antibody responses by enzyme-linked immunosorbent assays (ELISAs) on 96-well microtiter plates (Nunc,USA). Plates were coated overnight with 5 µg/mL of purified mycHSP70 in washing buffer (0.5% phosphate buffer saline-Tween 20, pH 7.5). Plates were blocked with 3% bovine serum albumin 0.05% Tween 20 for 1h and washed three times. Samples were diluted 1:100 in blocking buffer, incubated for 1h at 37°C and washed three times. Total IgG was detected by using streptavidin–horseradish peroxidase-conjugated antibody (Southern Biotechnology Associates, Inc., Birmingham, USA). Plates were incubated by 1h at 37°C and washed three times. The horseradish-peroxidase activity was detected with 3,3',5,5; - TetraMetil-

Benzidine (TMB, Zymed, San Francisco, California, USA) substrate solution (100 µl/well). The reaction was stopped by the addition of 50 µl/well of 1N HCl for 10 minutes in the dark. The optical density was read at 450/655nm in an ELISA plate reader (BioRad, USA).

7.5. Tissue collection and cell cultures

Spleens were aseptically removed and splenocytes isolated by gently grinding small pieces of spleen between the frosted ends of two sterile slides. Red cells removed by incubation for 2 min with lysing buffer (NH_4Cl) and washed twice in RPMI-1640. Cells were counted by means of microscopy (100 x) and viability always exceeded 95%, as judged from their ability to exclude trypan blue (Sigma).

To investigate antigen-specific T-cell proliferation, splenocytes ($2X10^5$ cell/well) were stimulated *in vitro* with mycHSP70 (0.05 – 0.3 µg) and cultured (duplicates) in complete RPMI-1640 medium (i.e. supplemented with gentamicin 0.5%, glutamine 1%, fungizone 0.1%, HEPES 1% and fetal calf serum 10%) for 48h at 37°C with 5% CO_2. To assess *in vitro* T-cell sensitivity to steroids, dexamethasone (selective type II adrenal receptor agonist), corticosterone (natural steroid which binds to both types of adrenal receptors) or DHEAS (all 10^{-9} to 10^{-4} M, Sigma) were added in duplicates to cultures stimulated with phytohemagglutinin (PHA) 1% for 48h at 37°C, 5% CO_2. Data are presented as percentage of basal proliferation (PHA 1% without steroids/mycHSP70).

7.6. Cell proliferation/viability assay

The proliferative responses were determined by a modified colorimetric assay (Mosmann, 1983). In the last 4h of culture, 100 µl of the supernatant was gently discarded and 30 µl of freshly prepared MTT (3-(4,5-diamethyl 2-thiazolyl) 2,5 diphenyl-2H-tetrazolium, Sigma) solution (5 mg/ml in RPMI-1640) was added to each well. The dehydrogenase enzymes in metabolically active cells convert this substrate to formazan, producing a dark blue precipitate. The cell cultures were incubated for 4 h at 37°C in 5% CO_2 atmosphere. After completely removal of the supernatant, 100 µl of dimethyl sulfoxide (Sigma) was added to each well. The optical density (OD) was determined using Biorad ELISA plate reader at a wavelength of 570 and 630 nm.

7.7. Cytokines

To measure the cellular production of IL-2 and IFN-γ, splenocytes (1x10 cells/mL) were stimulated with 2.0 µg of mycHSP70 and cultured in 24-well plates for 24h in complete RPMI-1640 at 37°C, 5% CO_2. After the incubation, the supernatant were collected and stored at –80°C until assayed for cytokine content. Assays were performed in according to the manufacturer's specificities (DuoSet ELISA R&D Systems,Inc.).

7.8. Immunophenotyping

T-cell early activation markers were assessed by immunofluorescent antibody staining of splenocytes and subsequent analyzed using a flow cytometer (FACScalibur, BD Pharmingen, USA). For the two-colour staining, fluorescein (FITC) or phycoerythrin (PE) labelled monoclonal antibodies were added to 12 x 75-mm test tubes and analysed as previously described (Collaziol et al., 2004). Cells were labelled with the following panel of monoclonal antibodies: isotype controls (IgG_1-FITC and IgG_1-PE), anti-CD4-FITC, anti-CD8-FITC and anti-CD69-PE (all from BD Pharmingen).

7.9. Statistical analysis

All variables were tested for normality of distribution by means of the Kolmogorov-Smirnov test. Comparisons between groups and treatments were done by two-way repeated measures ANOVA that included two variables between-subjects (AGE GROUP X TREATMENTS) and one variable within-subjects (mycHSP70, immunization days or steroid levels). FACS data was evaluated by one-way ANOVA. Multiple comparisons among levels were checked with Tukey post hoc test. The significance level was set at α=0.05 (two-tailed) and a computer statistics package (SPSS 11.5, USA) was used for statistical analyses in this study. Data are expressed as mean ± SEM in all figures.

8. Results

8.1. The effects of DHEAS on IgG levels

To assess the adjuvant effect of DHEAS, serum IgG levels were monitored in BALB/c mice inoculated three times with mycHSP70 (Fig.1). IgG levels were increased significant following each immunization day, $F(3,123) = 211.40$, $p < 0.0001$. Confirming the effectiveness of our vaccine, the

IgG levels differed significantly between immunized and control animals, $F(1,41) = 90.04$, $p < 0.0001$. Aged and young animals had similar antibody response to vaccination. A trend was found between TIME X AGE GROUP, indicating that IgG levels were significantly higher at the 7th day in the young groups compared to aged mice, $F(3,123) = 2.38$, $p = 0.07$. Although DHEAS did produce any adjuvant effect in the immunization to mycHSP70, this early increase in IgG production was only noticed in the young animals treated with DHEAS.

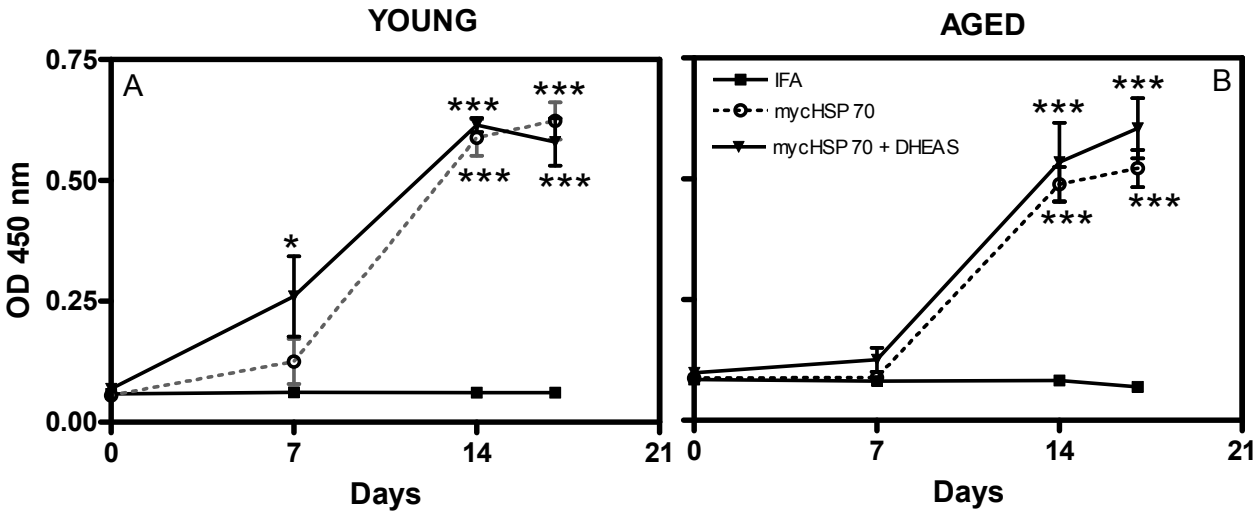

Fig. 1. Dynamics of IgG levels. Aged and young mice were immunized i.p. with IFA (control), IFA + mycHSP70 and IFA + mycHSP70+DHEAS. Each animal were injected with the respective treatment at day 1, 7 and 14. Sera were obtained from tail artery blood and IgG levels evaluated by ELISA. Statistically significant differences are indicated: ***$p < 0.0001$ and *$p < 0.05$ versus non-immunized (IFA) group.

8.2. Antigen-specific cellular proliferation

Antigen-specific splenocyte proliferation was evaluated here as general index of cell-mediated immunity to vaccination. As shown in Fig. 2, the levels of mycHSP70-specific cell proliferation responses varied across the mycHSP70 concentrations, $F(3,96) = 48.76$, $p < 0.0001$. Splenocytes of aged animals had a higher proliferation rate compared to young mice, $F(1,32) = 11.51$, $p = 0.002$. In addition, there was a significant interaction between mycHSP70 concentrations X TREATMENTS: as shown in Fig. 2A, the DHEAS treatment produced a significant adjuvant effect on splenocyte proliferation of young mice, $F(6,96) = 2.87$, $p = 0.01$. The DHEAS treatment was unable to produce any significant effect on proliferation of cells from aged animals.

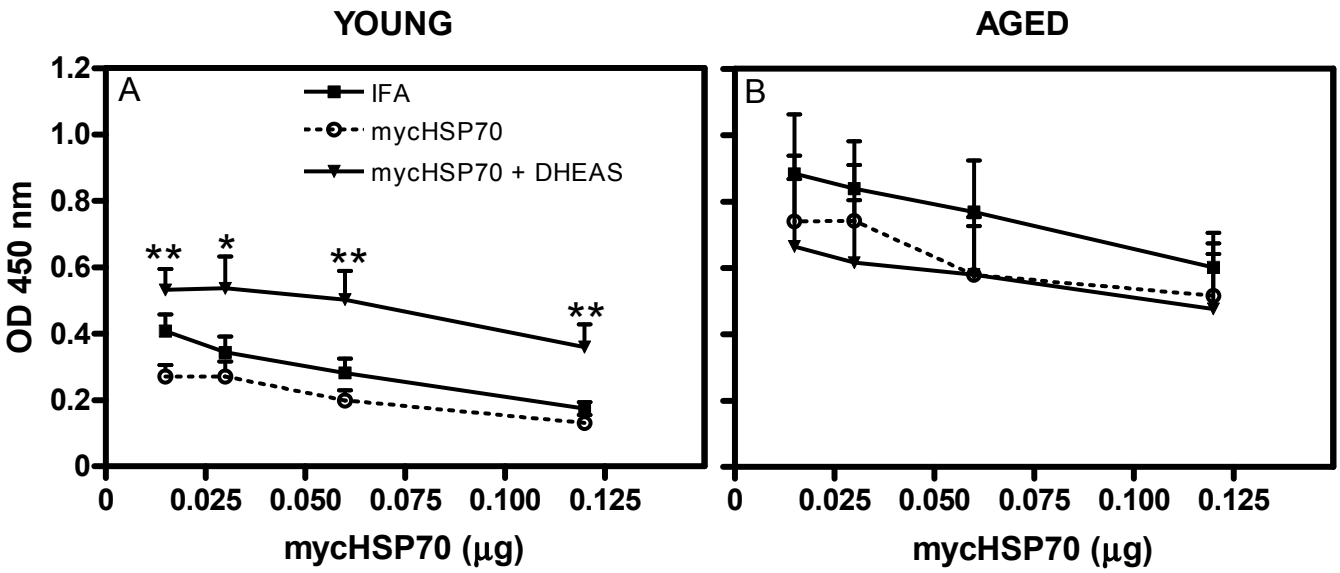

Fig. 2. Antigen-specific splenocyte proliferation. Cells obtained from spleen were stimulated with purified mycHSP70 for 48h and the proliferation/viability was determined in the last 4h by colorimetric assays. Statistically significant differences are indicated: **p < 0.01 and *p < 0.05 versus non-immunized (IFA) group.

8.3. Cytokine production

Splenocytes were stimulated with mycHSP70 for 24h and IL-2 and IFN-γ measured in the supernatants (Fig. 3). These cytokines play a key role in several processes of cell-mediated immunity, including T-cell proliferation. We found that IL-2 levels differed significantly between unstimulated and stimulated cells, $F(1,26) = 76.00$, $p < 0.0001$. The splenocytes of old mice produced higher stimulated IL-2 levels than young animals, $F(1, 26) = 18.72$, $p < 0.0001$. However, IL-2 levels did not differ between the experimental groups.

We also found that IFN-γ differed significantly between unstimulated and stimulated cultures, $F(1,39) = 33.07$, $p < 0.0001$. The splenocytes of old mice produced similar stimulated IFN-γ levels than young animals Furthermore, mice immunized with mycHSP70 + DHEAS age-independently increased IFN-γ levels compared to other groups, $F(2,39) = 3.33$, $p < 0.05$.

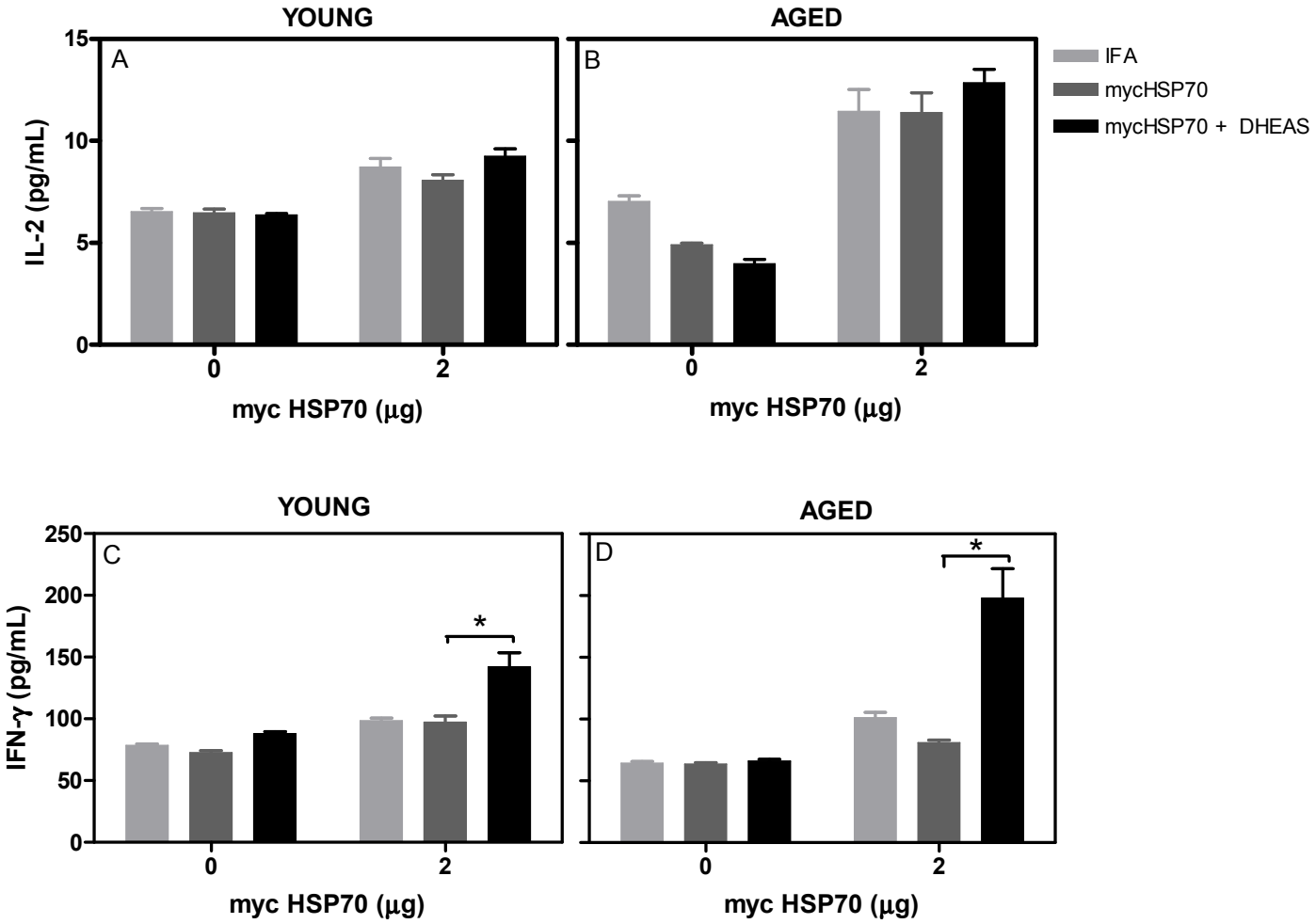

Fig. 3. Production of cytokines. Splenocytes were stimulated with purified mycHSP70 for 24h at 37°C, 5% CO_2 and the supernatant were collected and stored at –80°C. Cytokines were assayed by ELISAs. Statistically significant differences are indicated: *p < 0.05 versus non-immunized (IFA) group.

8.4. Analysis of activated T cells

In this study we investigated whether DHEAS co-immunization produced any effect on antigen-specific T-cell subsets recently activated by *in vitro* exposure to mycHSP70. As shown in Fig.4A and B, there was a trend for a lower percentage of CD4+CD69+ cells in the aged groups compared to young mice, F (1,39) = 3.74, p = 0.06. In contrast, there was a trend for a higher percentage of CD8+CD69+ cells in the aged groups compared to young mice, F (1,39) = 3.13, p = 0.08. The immunization treatments did not produce any effect on the percentage of activated cells. The expression of CD69 on T-cell subsets was also assessed by analysis of mean fluorescence intensity (MFI, Fig. 4C

and D). However, the CD69 expression did not differ among different age groups or immunization regiments.

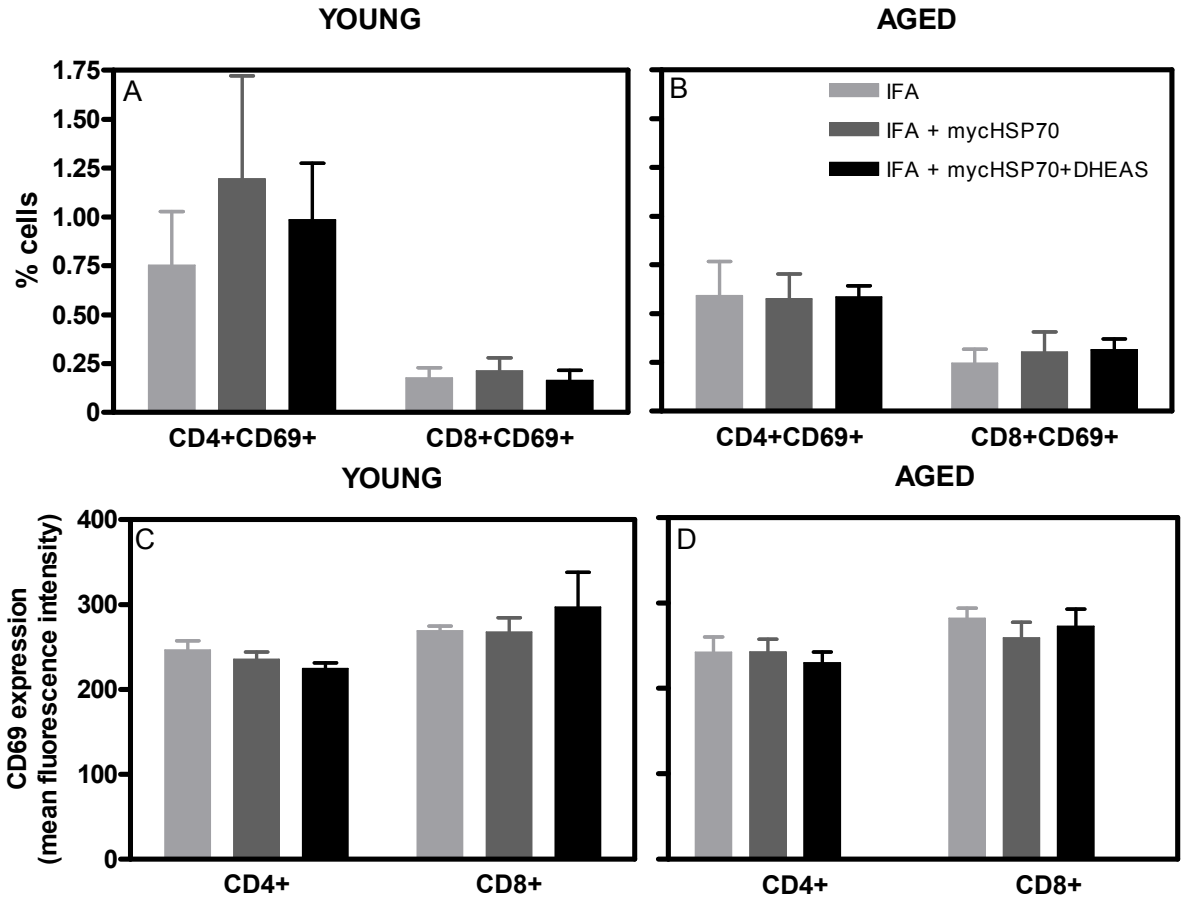

Fig. 4. Analysis of activated T cells. Splenocytes were stimulated with purified mycHSP70 and cultured for 24h. Cells were labelled with monoclonal antibodies for anti-CD4-FITC, anti-CD8-FITC and anti-CD69-PE.

8.5. T-cell sensitivity to steroids

In view of evidence that during ageing lymphocytes become resistant to the immunosuppressive effects of steroids (Bauer, 2005), we examined whether vaccination to mycHSP70 would render changes in T-cell sensitivity to these hormones. This was explored by stimulating splenocytes with a common polyclonal stimulus for T cells (i.e. PHA) and co-incubating with dexamethasone, corticosterone and DHEAS. As shown in Fig. 5, all studied steroids produced a significant dose-dependent suppression of T-cell proliferation (all $p < 0.0001$). We first assessed T-cell sensitivity to steroids in animals immunized with control adjuvant IFA only. We found that T-cells of aged animals had similar cellular sensitivities to dexamethasone or DHEAS and decreased sensitivity to corticosterone treatment compared to young mice, although this only approached statistical significance, $p = 0.08$.

We then investigated whether vaccination would impair T-cell sensitivity to steroids. Interestingly, T cells of young mice immunized to mycHSP70 or mycHSP70 + DHEAS were found significantly more resistant to steroids than cells of animals immunized to control adjuvant IFA (Fig. 5; all $p < 0.01$). This phenomenon was not observed in the aged animals. These data suggest that immunization is capable of changing T-cell signalling to steroids in young mice.

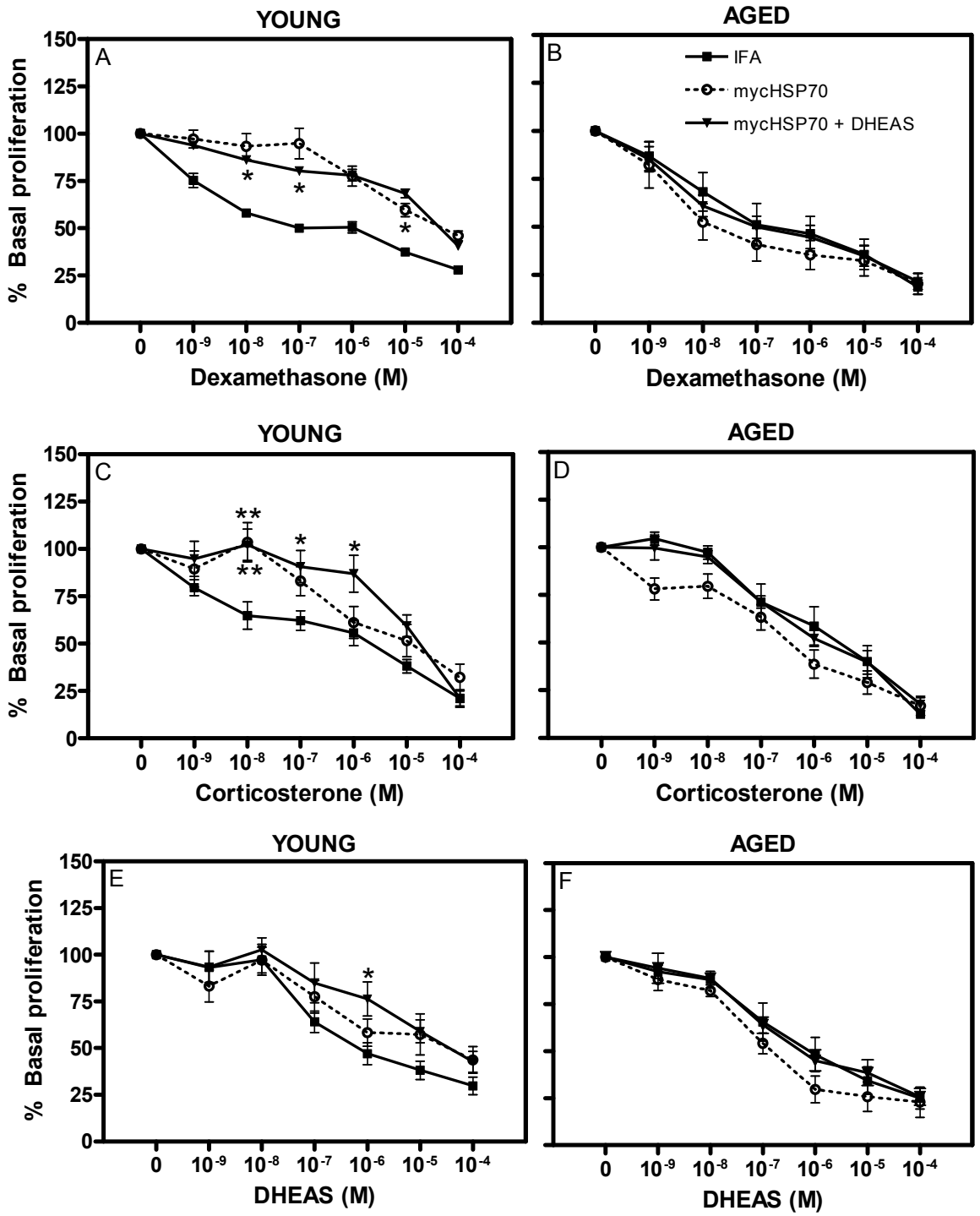

Fig. 5. T-cell sensitivity to steroids. Splenocytes were stimulated with phytohemagglutinin 1% and various steroids. Cells were cultured for 48h at 37°C, 5% CO_2 and proliferation/viability was determined in the last 4h by colorimetric assays. Sensitivity to steroids is shown as percentage of suppression of basal proliferation (PHA 1%). Statistically significant differences are indicated: **p < 0.01 and *p < 0.05 versus non-immunized (IFA) group.

9. Discussion

The DHEA and DHEAS immunomodulatory properties have been implicated in the reversal of age-related decline of immune responses. This includes the important adjuvant effects on the immunization of aged mice against hepatitis B (Araneo et al., 1993) or influenza (Danenberg et al., 1995a; Danenberg et al., 1995b). In this study, we described the adjuvant effects of DHEAS on both humoral and cellular immunological functions of aged mice immunized with a recombinant antigen of *Mycobacterium tuberculosis*. We also investigated the age-related neuroimmunomodulatory effects of vaccination, assessing *ex vivo* changes in T-cell sensitivity to glucocorticoids and DHEAS.

In this study, we observed that aged and young animals presented a similar antibody response to vaccination. This could be of biological significance for this vaccine since ageing is commonly associated to impaired humoral response to new antigens (Pawelec et al., 2002). The DHEAS did not produce significant adjuvant effects on the immunization to mycHSP70. These results contrast with previous work showing increased antibody titres following DHEAS treatment (Danenberg et al., 1995a; Danenberg et al., 1995b). However, our data should be interpreted with caution because of differences in strains, antigens and route of immunization used in previous studies. We observed that young mice co-immunized with mycHSP70-DHEAS presented an early increase in IgG levels. The presence of an early IgG response could be antigen-related since this is a characteristic of HSP70 as previously described (Bonorino et al., 1998). The antigen-responding cells are mostly T $\gamma\delta$ and B cells. Old C57/BL6 mice (16 months) immunized to influenza showed higher antibody titres when receive DHEA subcutaneous 4h before immunization (i.p.), with similar levels to the young control group (6-8 weeks) without DHEA (Danenberg et al., 1995a). Although the DHEA treatment produced an attenuated adjuvant effect in older animals (24 months), they still had higher antibody titres than non-treated old mice. Aged BALB/c mice that were given DHEAS subcutaneous and immunized to a pneumococcal vaccine in the same route 24h later showed a greater antibody response than animals that received DHEAS at 3h following vaccination in a different route (Garg and Bondada, 1993). Taken together, these observations suggest that strain and the route of immunization/DHEAS treatment play important roles on the humoral response to vaccines.

In addition to antibody levels, both T cells and activated macrophages are required as cellular mechanisms to control and eliminate the micobacteria. Here, the splenocyte proliferation was

evaluated as index of cell-mediated immunity to vaccination. It was observed that aged groups, independently of treatment, presented a higher proliferation than young mice. This could be related to higher IL-2 levels observed in supernatants of stimulated splenocytes of aged mice. These data are in contrast to a previous study that observed lower IL-2 levels in splenocytes of aged (24-26 months) C57BL/6 mice stimulated with anti-CD3 and anti-CD28 antibodies compared to young animals (Wakikawa et al., 1999). However, these animals were not immunized and constitute a different mouse strain. Considering the latter, BALB/c mice tend to have a constant IL-2 production throughout the life span (Gorczynski et al., 1993) whereas C57/BL6 mice present a reduced IL-2 production with ageing (Kubo and Cinader, 1990; Wakikawa et al., 1999). These differential strain features could partially explain in IL-2 secretion differences observed between studies. Araneo et al. (1993) have demonstrated that oral DHEAS treatment enhanced the IL-2 production in splenocytes of C3HXBL/6 mice immunized to hepatitis B. In our study, the DHEAS co-immunization significantly increased the antigen-specific splenocyte proliferation in the young mice only. These data suggest that young animals are more sensitive to the DHEAS immunomodulation than aged mice.

The infection of *Mycobacterium tuberculosis* requires antigen-specific CD4+ T cell response and production of Th1 cytokines such as IL-12, TNF and IFN-γ. It has been observed that CD4 as well as CD8 cells have an important role in controlling micobacterial infection, recruiting and activating macrophages by IFN-γ secretion (Flynn and Chan, 2001). In this study, both aged and young animals immunized with mycHSP70 and DHEAS presented significantly higher levels of IFN-γ. This corroborates previous data showing increased IFN-γ levels in retrovirus infected aged C57/BL6 mice that received DHEAS orally (Araghi-Niknam et al., 1997). It has been shown that immunization to proteins in IFA or alum as adjuvants induced polarized Th2 immune responses in neonatal and young adult mice, characterized by the production of antigen-specific IL-4, IL-5, IgG1, and IgE, but not IFN-γ or IgG2a (Chu et al., 1997; Yip et al., 1999). Thus, it is likely that DHEAS in our system could have yielded a shift towards a Th1 profile, with increasing IFN-γ levels.

We have also observed that aged animals had a reduced ratio of CD4/CD8 cells recently activated by in vitro challenge with antigen. This phenomenon may represent an immunosenescence characteristic across species and could be due to a reduced production of newly thymic emigrants (thymic involution) as well as increased peripheral numbers of memory CD8+ cells, as consequence

from repeated antigen exposure, including cytomegalovirus (CMV) (Looney et al., 1999; Pawelec et al., 2004). It has been shown that CD8+ cells loses the capacity to divide and are unable to suffer apoptosis and fill up the immunological compartment (Franceschi et al., 2000a; Franceschi et al., 2000b). The homeostasis is further impaired considering that CD4+ cells are more susceptible to apoptosis (Pawelec et al., 1996). In this study, neither mycHSP70 vaccination nor DHEAS co-treatment produced changes on the percentage of activated T cells as well as CD69 expression. We speculate that there were a few antigen-specific splenocytes to respond to the *in vitro* antigenic challenge.

Interestingly, we demonstrated that non-immunized aged animals (control IFA group) had lymphocytes more resistant to corticosterone treatment *in vitro* than young mice. These data gives further support of a previous study showing that strictly human ageing is associated with increased lymphocyte resistance to the immunosuppressive action of steroids (Bauer, 2005; Luz et al., 2003). This phenomenon could be directly associated to age-related neuroendocrine changes, namely the increased production of peripheral glucocorticoids (corticosterone in rodents), rendering cells more resistant to the increased concentration of the ligand. However, stress hormones were not measured here. Here, we also investigated whether vaccination would impair T-cell sensitivity to steroids. It was found that T cells of young immunized mice were remarkably more resistant to glucocorticoids and DHEAS. These data suggest that immunization is capable of changing T-cell signalling to steroids in young mice. It has been shown that rodents immunized to sheep red blood cells presented a significant increase of serum corticosterone levels, simultaneously to the time of the humoral immune response, measured by the number of antibody producing plaque forming cells (Besedovsky et al., 1977). These data should be discussed in light of the “corticosterone milieu” of the cells. Considering that young mice have lower corticosterone levels than aged mice, their lymphocytes are expected to be more sensitive to steroid modulation compared to cells of aged mice. Therefore, T cells of young mice are rapidly protected from increasing glucocorticoids produced by stress or immunological challenge (vaccination or infection) through their capacity to become less responsive to steroid signalling. This phenomenon should be further explored due the importance of hypothalamic-pituitary-adrenal axis in controlling immune responses.

Several efforts have been made to improve the efficacy of existing vaccines to tuberculosis. In various attempts, however, the strategies were insufficient to trigger protection against the disease. These strategies included the insertion of mammalian genes encoding cytokines or mycobacterial genes encoding bacilli proteins that did not contribute to improve the efficacy of protection of the original vaccine (Bao et al., 2003; Dhar et al., 2004). In addition to the efforts in designing new vaccines, persistent infections such as tuberculosis still represent a greater risk to human health. It has been shown that immunization against a recombinant protein with dominant immunogenic epitopes may yield better results and avoids the risk of using vaccine with whole organisms (O'Hagan et al., 2001). However, some recombinant proteins may have a lower immunogenicity and the only adjuvant licensed for human use (aluminum hydroxide, alum) is not always effective. Much progress has been made on the development of recombinant of recombinant subunit tuberculosis vaccines. In particular bacterial plasmid DNAs containing *Mycobacterium tuberculosis* genes have been extensively tested (Baldwin et al., 1998; Lowrie and Silva, 2000; Lowrie et al., 1999). Future studies are necessary to explore different immunization routes and new adjuvants to improve the cellular response against this infection. This will certainly contribute for healthier elderly individuals, free of opportunistic infections.

10. Acknowledgements

This study was supported by grants from CAPES (F.R.) and CNPq (551180/01-3, M.E.B.).

11. CONSIDERAÇÕES FINAIS

Neste estudo, os camundongos idosos apresentaram uma resposta humoral à vacinação semelhante àquela observada nos animais jovens. Este dado ilustra a integridade imunológica dos camundongos BALB/c envelhecidos em responder para HSP70 de *Mycobacterium tuberculosis*. O próximo passo seria verificar a eficiência desta imunização na sobrevivência dos animais infectados com a micobactéria. Além disso, poderíamos investigar se esta boa resposta humoral é mantida em outras linhagens bem como frente a outros antígenos de *Mycobacterium tuberculosis*.

Em relação à produção de citocinas, verificamos que os animais idosos apresentaram níveis mais aumentados de IL-2 e níveis inalterados de IFN-γ em relação aos jovens. Esses dados são contrários da literatura e especula-se que os camundongos da linhagem BALB/c apresentam níveis invariáveis de IL-2 ao longo do desenvolvimento (Gorczynski et al., 1993). Isso não acontece com outras linhagens que apresentam um perfil de citocinas pró-inflamatórias, como é o caso do C57BL/6, que adquirem um perfil mais Th2 ao longo do envelhecimento (Wakikawa et al., 1999). Para verificar se essas características estariam influenciando em nosso modelo de vacina, seria interessante comparar animais imunizados de linhagem diferentes, além disso, imunizar animais BALB/c em idades intermediárias.

Além das alterações ligadas a imunossenescência, verificamos neste estudo alguns efeitos adjuvantes do DHEAS em camundongos jovens imunizados com a proteína recombinante (HSP70) de *Mycobacterium tuberculosis.* Em particular, verificamos um aumento da proliferação de esplenócitos (possivelmente células B e T $\gamma\,\delta$) (Bonorino et al., 1998) e níveis aumentados da citocina essencial para a resposta contra o *Mycobacterium tuberculosis*, o IFN-γ. Esta última alteração foi verificada independentemente da idade dos animais imunizados com mycHSP70 + DHEAS. Embora o antígeno HSP micobacteriana produza resposta humoral, foi demonstrado que esse fato não implica em proteção. (Johnson et al., 1997), porém em nosso estudo a combinação com o hormônio foi capaz de aumentar a produção do IFN-γ, esse achado é muito importante, devido a dificuldade dos indivíduos idosos em responder a antígenos novos (Pawelec et al., 1996). O próximo passo seria desafiar os animais para verificar a eficácia da vacina.

Este estudo também indicou mudanças de sensibilidade a glicocorticóides associadas ao processo de envelhecimento e a imunização. Os animais idosos não imunizados apresentaram uma maior resistência a corticosterona (glicocorticóide endógeno) do que os animais jovens não imunizados. Esses dados apóiam um estudo anterior no qual linfócitos de humanos idosos também apresentaram resistência à ação de glicocorticóides (Bauer, 2005; Luz et al., 2003). Isso poderia ser uma característica da endócrinossenescência, na qual os níveis de cortisol aumentam com o envelhecimento, conferindo às células maior resistência a concentração do ligante. E ainda, os animais jovens imunizados com mycHSP70 e este combinado com o DHEAS, tornaram-se mais resistentes aos efeitos imunossupressores dos esteróides. Esse dado pode confirmar mais uma vez a estreita ligação entre as alterações endócrinas e imunológicas. Provavelmente a vacina alterou a sinalização das células T nos camundongos jovens, comprovando o que foi demonstrado anteriormente (Besedovsky et al., 1977). As células dos animais jovens poderiam ser mais susceptíveis a ação dos glicocorticóides primeiramente porque os animais jovens não se encontram tão “adaptadas” a presença de grandes quantidades de corticosterona, como ocorre nos idosos. Mais estudos são necessários para explorar essas complexas relações e suas implicações para a resposta imune do hospedeiro frente a desafios naturais.

Além disso, mais estudos são necessários para confirmar as alterações observadas nestes experimentos. Poderíamos explorar, por exemplo, outros regimes de imunização, o que não foi possível no momento devido à quantidade de animais envelhecidos disponíveis. Importante ressaltar que todos os animais foram envelhecidos nas nossas instalações. Em outro estudo, poderíamos estudar o uso de outro adjuvante, ao invés do IFA, como o hidróxido de alumínio. Além disso, poderíamos investigar outras rotas de imunização bem como tratamento com DHEAS via oral ou subcutâneo antes das imunizações. Torna-se igualmente fundamental o estudo de adjuvantes não hormonais (e.g. CpG DNA e citocinas) na vacina contra tuberculose.

Outra estratégia que vem sendo explorada em nosso laboratório é o uso do gene da HSP70 como adjuvante em outras vacinas, incluindo a hepatite B. Tem-se investigado as maneiras de otimizar a expressão do gene da HSP70 em células eucarióticas. Assim, poderíamos verificar as regiões mais imunogênicas dessa proteína, com a intenção de aumentar a resposta celular, visto que a HSP70, sem duvida é um antígeno capaz de produzir ótima resposta humoral.

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