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PROJETO DE PESQUISA: “ATIVAÇÃO DE MACRÓFAGOS DURANTE A INFECÇÃO PELO Mycobacterium bovis: O PAPEL DO IFN-GAMA”

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1- INTRODUÇÃO

A tuberculose é uma emergência global, pois afeta cerca de 60 milhões de pessoas no mundo e é responsável por 25% dos óbitos em adultos nos países em desenvolvimento, o que representa mais do que diarreia, malária e AIDS juntas. Essa doença infecciosa, causada pelo *Mycobacterium tuberculosis*, e em menor escala pelo *Mycobacterium bovis*, é responsável por cerca de 3 milhões de óbitos por ano, podendo chegar a 4 milhões no ano 2004. Segundo a Organização Mundial da Saúde, 1.7 bilhões de indivíduos (um terço da população mundial) estão infectados com o *M. tuberculosis*, podendo desenvolver e transmitir a doença (Raviglioni et al, 1995).

A imunidade protetora frente ao *M. tuberculosis* e outras micobactérias, depende da ativação de linfócitos T CD4+ e de citocinas produzidas pelas células Th1. A alta incidência da tuberculose em associação com a síndrome da imunodeficiência adquirida (AIDS), reforça o conceito de que a resistência à infecção por micobactérias depende da capacidade de resposta imune celular do hospedeiro, controlada pela ativação de linfócitos T e macrófagos (Kaufmann, 1998). A ativação de macrófagos foi um dos primeiros mecanismos reconhecidos na resistência contra microorganismos, já tendo sido definida por Mackaness, em 1964 (8), quando estudava a listeriose, e por Lurie, em 1964, em seus estudos sobre a tuberculose. O interferon-gama (IFN-γ) é uma das mais importantes citocinas que atuam na ativação de macrófagos. Teixeira e colaboradores (1995) demonstraram que, após infecção endovenosa de camundongos C57B1/6 com a cepa Phpss da *Mycobacterium bovis*, linfócitos T α/β são as principais células esplênicas responsáveis pela produção de IFN-γ. Foi observado a produção de IFN-γ pelas células T CD4+ e algumas células T CD8+, evidenciando a importância dessa citocina na atividade tuberculostática e tuberculocida, e sustentando a noção de que a ativação macrofágica pelas linfocinas representa um mecanismo importante na proteção contra a tuberculose (Kaufmann 1993). O papel fundamental do IFN-γ no controle de infecções causadas por
micobactérias ficou evidente em experimentos com animais deficientes de IFN-γ ou deficientes do receptor para IFN-γ, por deleção dos genes após recombinação homóloga. Esses animais mutantes se mostram altamente susceptíveis à infecção por M. tuberculosis e M. bovis e desenvolvem uma doença disseminada (Flynn et al., 1992; Cooper et al., 1993; Kamijo et al., 1993; Dalton et al., 1993). Da mesma forma, camundongos mutantes RAG-1 também apresentam maior susceptibilidade à infecção por micobactérias (Ladel et al., 1995), indicando que células T e citocinas estão intimamente envolvidas nos mecanismos de proteção frente a micobactérias.

A clofazmina é uma droga originalmente desenvolvida para o tratamento da tuberculose e que posteriormente foi aprovada também para lepra. (Kansal et al., 1997). A droga é uma tintura iminofenazínica vermelha que inibe o crescimento micobacteriano por se ligar preferencialmente ao DNA das micobactérias causando assim a inibição da sua transcrição. Além desta propriedade antimicobacteriana direta, este agente também proporciona um aumento das atividades microbicida e fagocítica de neutrófilos e monócitos (Van Rensburg et al., 1993). Foi demonstrado que o componente 25kDa do M. tuberculosis inibe a capacidade intracelular dos macrófagos de matar microorganismos. A clofazmina por sua vez é capaz de reverter parcialmente porém significativamente o estado de inibição da ativação de macrófagos. Além disso, culturas de macrófagos que foram pré-estimuladas com clofazmina e outros estimulantes de fagócitos, como por exemplo o interferon-gama, não são afetadas pela fração 25kDa do M. tuberculosis, sugerindo o uso potencial desse agentes como moduladores da função dos fagócitos (Wadée et al., 1995).

O aumento da atividade antimicrobiana da clofazmina pode ser atribuído ao efeito sinérgico da droga e de certas citocinas liberadas por linfócitos T ativados como resultado de uma imunidade específica adquirida (Arbiser et al., 1995). Neste sentido, alguns trabalhos mostram que o IFN-γ sozinho não faz a aceleração do clareamento bacteriano no organismo e sim requer ação de quimioterápicos concomitantesmente para se conseguir redução acelerada no número de bacilos (Sampaio EP, Malta AM, Sarno EN, Kaplan G, 1996). Em um estudo que foi conduzido para se observar a associação de IFN-γ e fator
de necrose tumoral alpha (TNF-α) com a clofazimina, foi visto que esta última sozinha é capaz de reduzir a infecção pelo MAC (Mycobacterium avium complex), porém com as células sendo pré-estimuladas com IFN-γ proporcionava-se uma redução de infecção ainda maior pois o IFN-γ aumenta a captação de clofazimina pelos macrófagos além de potencializar a produção de TNF-α que, por sua vez, diminui o crescimento intracelular do MAC inclusive em macrófagos já infectados (Gomez-Flores et al, 1997).

A comprovação de que é através do TNF-α que há a diminuição de infecção pelo MAC foi conseguida através do uso de pentoxifilina, que inibiu a expressão de TNF-α pelos macrófagos revertendo a inibição da infecção. A pentoxifilina é um derivado metilxantínico e um inibidor da fosfodiesterase que influencia a produção e/ou função de algumas citocinas e na produção de óxido nítrico (Lottis et al, 1997; Lauterbach et al, 1995). Assim, a pentoxifilina tem profundas propriedades imunomodulatórias in vitro, que são associadas com a inibição específica da liberação de citocinas.

Neste projeto estudamos a produção de IFN-γ e a ativação de macrófagos peritoneais de camundongos C57Bl/6, após infecção por Mycobacterium bovis (BCG). A ativação macrofágica foi avaliada através da análise da produção de H₂O₂ e de NO em sobrenadantes de culturas de células peritoneais estimuladas com BCG. Avaliamos a correlação entre o aumento do número de células peritoneais secretoras de IFN-γ com os níveis de produção de H₂O₂ e de NO. Finalmente, avaliamos o papel modulador do tratamento in vivo com clofazimina e in vitro com pentoxifilina sobre a produção de IFN-γ e ativação macrofágica.
2-MATERIAL E MÉTODOS:

2.1-Animais:

Foram utilizados camundongos fêmeas C57Bl/6, com 6 a 8 semanas de idade, obtidos do Biotério do Centro de Biologia da Reprodução (CBR - UFJF). Após aquisição, os animais foram mantidos em biotério próprio, montado recentemente pelo Departamento de Imunologia. A limpeza das gaiolas é realizada duas vezes por semana e os camundongos foram tratados com ração e água à vontade.

2.2-Microorganismo:

Aliquotas liofilizadas contendo 2 mg de Mycobacterium bovis (BCG), estirpe Monreau-Rio de Janeiro, foram obtidas junto à Fundação Ataulpho de Paiva, Rio de Janeiro. O número de bactérias por aliquota foi obtido após plaqueamento em meio M.7H9 e contagem de CFU (unidades formadoras de colônia) (McDonough, 1993). Grupos de camundongos foram injetados intraperitonealmente (i.p.) com diferentes doses de bactérias contidas em 0,5 ml de salina.

2.3-Contagem total e diferencial de células peritoneais:

Aliquotas de 0,9 ml da suspensão de células peritoneais obtidas como acima descrito, são acrescentadas a 0,1 ml de solução de cristal violeta a 0,5%, em ácido acético a 30%. O número total de células no exsudato peritoneal é avaliado em câmara de Neubauer e, as avaliações diferenciais, através da contagem de 200 células entre lâmina e laminula, com auxílio de microscópio de campo claro com objetiva de 100x.

2.4-Produção de H₂O₂:

A produção de moléculas que invibilizam o microorganismo como H₂O₂ e o O₂⁻ dependem do contato do macrófago com o microorganismo e a ativação do burst respiratório (Peetermans et. al.,1993). A importância dos produtos do burst respiratório na atividade microbicida dos fagócitos pode ser demonstrada através da observação in
vitro da ausência de produção destes radicais por leucócitos de pacientes suscetíveis a infecções (Wilson et. al., 1980).

A produção de H$_2$O$_2$ foi analisada através do método descrito por Pick e Mizel (Pick et. al., 1981), adaptada por Russo et. al. (1989).

Aliquotas de 100 ul da suspensão celular são colocadas em placa (Corning) de 96 cavidades (em quadruplicata), acrescido ou não de 0,01 ml de PMA a 0,1 mg/ml (Sigma). Após 1 hora de incubação a 37°C em câmara úmida, a reação é interrompida pela adição de 10 ml de NaOH 1M às cavidades da placa. Em seguida é feita a leitura da absorbância a 620 nm empregando-se o microleitor automático de ELISA (Spectramax, Molecular Devices). A quantidade de H$_2$O$_2$ liberada na reação será calculada de acordo com uma curva padrão estabelecida para cada experimento, utilizando diluições de uma solução estoque (30%) de H$_2$O$_2$ (Sigma) e concentrações variando de 1 a 50 μM. Os resultados serão expressos em nmoles de H$_2$O$_2$/2 x 10$^5$ células peritoneais.

2.5-Produção de NO:

A produção de NO pelos macrófagos é um fator muito importante para a morte de agentes infecciosos intracelulares como o *Mycobacterium bovis* (Kamijo et. al., 1994). Além disso o NO está envolvido em outros processos fisiológicos sendo considerado como importante mediador dos processos homeostáticos.

Sobrenadantes de culturas, obtidos como descrito anteriormente, foram analisados para quantificar NO$_2^-$ através da reação de Griess (Green et. al., 1982). Em resumo, aliquotas de 50 ml do sobrenadante da suspensão de células peritoneais são incubados com 200 ml de 1% sulfanilamida e 200 ml de 0,1% N-1-naphthylethylendiamina dihidroclorida em 2,5% de H$_3$PO$_4$ (Sigma) em temperatura ambiente por 5 minutos. A absorbência é medida com filtro de 540nm. NO$_2^-$ será quantificado através da comparação com uma curva padrão.

2.6-Produção de Interferon-gama:

O IFN-γ é uma das mais importantes citocinas que atuam na ativação de macrófagos e na inibição da multiplicação de microbactérias fagocitadas (Beckman et. al., 1990; Flesh et. al., 1987).
Para determinar a frequência de células produtoras de IFN-γ, foi utilizado uma adaptação do método de ELISPOT (Teixeira & Kaufmann, 1994). Em resumo, placas de filtração Millipore (STHA09610) de 96 poços, são cobertas com anticorpos monoclonais anti-IFN-γ (R4-6A2, ug./ml, Dianova) diluídos em tampão carbonato 0,05M, pH9.6 (100 ul/poço) e incubadas a 4°C por 16h. Os poços são lavados com PBS e bloqueados com PBS-BSA 1% por 2h a 37°C. As membranas filtradoras da placa são lavadas com PBS e secadas com papel absorvente. A seguir, 100ul de diluições apropriadas de células em RPMI-FCS 10% são adicionadas e incubadas por 16h a 37°C em estufa com 7% de CO₂, em presença ou ausência de antígeno (10⁷ M.bovis) ou 0,1 ug de Con A. As placas são lavadas com PBS contendo Tween 20 0,05% sendo posteriormente, adicionadas aliquotas de 100 ul/poço de anticorpos anti-IFN-γ biotinilados (AN-18.17.24, 0,25 ug/ml, Dianova). Após incubação a 37°C por 2h, as placas são lavadas extensivamente sendo então adicionada uma solução de avidina-fosfatase alcalina (1/20.000 em BSA 0,1% - PBS Tween 20 0,05%). Após incubação a 37°C as placas são novamente lavadas antes da adição do substrato 5-bromo-4-cloro-3-indolil fosfato (Fluka) e do agente catalisador nitroblue tetrazolium (Fluka). Após 15 min, pontos azuis (spots) são revelados na placa e a reação enzimática é terminada com água destilada. A placa é secada e os spots são contados com auxílio de um microscópio de dissecação com aumento de 32x. Cada spot representa uma célula secretora da citocina estudada.
3 - RESULTADOS

1) A dose vacinal de 0,1 mg de BCG i.p. desenvolveu a melhor resposta de produção de H2O2 e NO por células peritoneais de camundongos C57Bl/6, e níveis elevados de ativação de macrófagos peritoneais foram correlacionados com alto número de células secretoras de IFN-γ.

Camundongos C57BL6 foram separados em quatro grupos. Três grupos receberam a inoculação intraperitoneal (i.p.) de *M. bovis* (BCG), nas doses de 0,01mg, 0,1mg ou 1,0 mg, e salina foi inoculada no grupo controle. Aos 7° e 14° dias após infecção após o sacrifício dos animais, o lavado peritoneal foi coletado e submetido às técnicas de produção de H2O2 e NO já descritas. Os resultados mostraram que a dose de 0,1 mg de BCG induz a produção de níveis mais elevados de ativação macrofágica quando comparados com os grupos controle e infectado com 0,01mg, havendo também maior, porém discreta, estimulação em relação à dose de 1 mg de BCG (Figs.1 e 2). Esse alto índice de ativação macrofágica por células peritoneais correlaciona com elevação da produção de IFN-γ, avaliado pelo método do ELISPOT (Fig.3), havendo também melhor eficiência com a dose
de 0,1mg de BCG. A ativação macrofágica e a produção de IFN-γ foi maior no 7º dia após a infecção.

FIG.1- Produção de H₂O₂ por células peritoneais de camundongos C57Bl/6 inoculados i.p. com Mycobacterium bovis (0,01mg, 0,1mg e 1mg BCG Monneau) Após 7 e 14 dias de infecção, as células peritoneais foram coletadas, lavadas em PBS, e plaqueadas (na concentração de 2x 10⁴ células/well). Após uma hora de incubação a 37oC, a produção de H₂O₂, espontânea ou induzida pelo antígeno (BCG, 20 µg/ml) foi bloqueada com NaOH e avaliada através da leitura a 620nm.
FIG.2- Produção de NO por células peritoneais de grupos de camundongos C57Bl/6 inoculados i.p. com diferentes doses de *M. bovis* (BCG Monreau, 0,01mg, 0,1mg e 1mg). As células peritoneais foram coletadas, lavadas e ressuspendidas em meio RPMI e após 48 horas de incubação, a produção de NO foi quantificada através do método de Griess.
FIG. 3 – Produção de IFN-gama em grupos de camundongos C57BL6 inoculados i.p. com diferentes doses de *M. bovis* (BCG Monreau, 0,01mg, 0,1mg e 1mg). As células foram coletadas, lavadas, ressuspensdidas e estimuladas, *in vitro*, com os antígenos (BCG, 20ug/ml) e ConcanavalinaA (10ug/ml). O número de células peritoneais secretoras de IFN-gama foi determinado pelo método de ELISPOT.
2) O número de células secretoras de IFN-γ (IFN-SFC) espontâneo ou induzido por antígeno (BCG), bem como a produção de H2O2 e NO por células peritoneais são elevados nas duas primeiras semanas de infecção com o M. bovis, decrescendo posteriormente.

A cinética da produção de H2O2, NO e IFN-γ, foi avaliada em grupos de camundongos C57BL/6 nos dias 2, 7, 14 e 30 após infecção com o M. bovis (0,1mg de BCG, cepa Montreau). As figuras 4 e 6 mostram que as células peritoneais recuperadas mostraram maior produção de H2O2 e IFN-γ nas duas primeiras semanas, decrescendo posteriormente. Foi observado um pico de produção de H2O2 no dia 7 após infecção, havendo maior estimulação na presença do antígeno (Fig.4). Neste dia, também se observa uma maior produção de NO pelas células peritoneais (Fig.5). A produção de NO é semelhante nos dias 14 e 30 após infecção, sendo significativamente maior que o controle. Células estimulada com o ConA mostraram maior produção de NO e de IFN-γ.

A produção de IFN-γ é maior nas duas primeiras semanas de infecção mas se mantém a níveis significativamente maiores que os apresentados por células de animais controle no 30º dia após infecção, havendo uma melhor estimulação de células secretoras de IFN-γ quando em presença de antígeno ou mitógeno, exceto no 7º dia após infecção, quando a produção expontânea ou induzida pelo antígeno mostraram valores semelhantes (Fig.6).
FIG. 4 Cinética da produção de H₂O₂ em grupos de camundongos que foram inoculados i.p. com 0,1mg de M. bovis (BCG). As células coletadas em diferentes dias após infecção e incubadas na presença ou ausência de antígeno (20µg de BCG).

FIG. 5- A produção de NO em grupos de camundongos C57BL6 que foram inoculados i.p. com 0,1mg de M. bovis (BCG). As células foram recuperadas e ajustadas à concentração de 2x 10⁶/ml e incubadas com BCG (20µg/ml) e ConA (10ug/ml). A quantificação de NO foi obtida pelo método Nessler.
FIG. 6 – A produção de IFN-gama foi avaliada em grupos de camundongos C57BL6 após inoculação i.p. com o *M. bovis* (0,1mg de BCG). As células peritoneais foram recuperadas e ajustadas à concentração de 10⁶/ml e incubadas na presença ou ausência de estímulos: (BCG, 20µg/ml; e, ConA, 10µg/ml).

3) O tratamento in vivo com a clofazimina modificou a cinética da secreção de IFN-γ e a ativação de macrófagos, exibindo um efeito modulador, provavelmente relacionado às suas propriedades anti-inflamatória e bactericida.

Grupos de camundongos C57Bl/6 infectados com o *M. bovis* (BCG, 0,1mg/animal) e tratados com clofazimina foram sacrificados, sendo feita a avaliação da produção de H₂O₂, NO e IFN-γ por células do lavado peritoneal. Observou-se que o grupo infectado e tratado apresentou quantidades inferiores de H₂O₂ (Fig.7) e IFN-γ (Fig.9) quando comparado com os grupos somente infectado e controle. Esse comportamento foi observado mesmo quando se utilizou tempos de infecção distintos, 7° e 14° dias após
infecção. Entretanto, a produção de NO apesar de ser maior no grupo infectado, no 7º dias após infecção, não foi verificada diferença entre os grupos infectados (BCG) ou tratados (BCG + clofazina) no 14º dia após-infecção (Fig.8).

![Bar Chart](image)

**FIG.7-** A produção de H₂O₂ foi quantificada em grupos de camundongos C57BL/6 infectados com *M. bovis* (BCG, 0,1mg) e tratados i.p. a cada dois dias com doses de clofazimina (10µg/dia). As células peritoneais foram recuperadas, lavadas e ressuspensas à concentração de 2x 10⁶/ml e incubadas na presença e ausência de antígeno (BCG, 20µg/ml), nos dias 7 e 14 após infecção.
FIG. 8- A produção de Óxido Nítrico foi avaliada em grupos de camundongos C57BL6 que foram inoculados i.p. com 0,1mg de *M. bovis* e 10μg de clofazimina a cada dois dias. As células foram recuperadas após 7 e 14 dias de infecção, re suspensinadas em meio RPMI, incubadas por 48 horas a 37°C, sendo a produção de NO avaliada por absorbância a 540 nm, segundo o método de Griess.

FIG. 9- Produção de IFN-gama em grupos de camundongos C57BL6 inoculados i.p. com *M. bovis* (0,1mg de BCG) e tratados a cada dois dias com clofazimina (10mg/dia), através do método de ELISPOT, com as células re suspensinadas em meio RPMI enriquecido.
4) O tratamento *in vitro* com a pentoxifilina inibiu a produção de IFN-γ e H2O2, mas a produção de IFN-g induzida pela ConA não foi alterada pela PTX.

Avaliamos o efeito do tratamento in vitro com pentoxilina sobre a produção de H2O2, NO e IFN-γ em camundongos C57Bl/6 infectados com o *M. bovis* (BCG, 0,1mg). Culturas de células peritoneais dos grupos estudados foram estimuladas *in vitro* com antígeno (BCG, 20ug/ml) ou PTX (20 ug/ml). Observamos uma diminuição na produção de IFN-γ e de H2O2 nas culturas tratadas com PTX, entretanto, a produção de NO não foi inibida de forma reprodutível, havendo grande variação entre os experimentos realizados, o que não nos deu informação sobre o seu efeito sobre a produção de NO. Fato interessante foi a incapacidade da PTX inibir a produção de IFN-γ por células estimuladas com ConA (dados não mostrados). Os mecanismos que controlam a atividade inibitória da PTX sobre a produção de IFN-γ ainda não estão completamente elucidados.
5 - Efeito do tratamento com anti NK1.1 mAb na produção de IFN-γ induzida pela infecção com *M. bovis*:

Foi analisada a produção de IFN-γ por células peritoneais de camundongos C57Bl/6 tratados com anticorpo monoclonal anti-NK1.1 e, posteriormente, infectados i.p. com 1mg de BCG. Os animais BALB/c foram utilizados como controle por não possuírem a linhagem de células NK1.1. Observou-se que: (i) o tratamento com anti-NK1.1 não modificou a produção de IFN-γ o que sugere que esta linhagem de células não produz IFN-γ na fase inicial nem na fase tardia da infecção pelo *M. bovis*; (ii) os animais BALB/c apresentaram uma maior produção de IFN-γ no 2º dia a.i.

![Diagrama](image)

Fig. 10 Efeito do tratamento *in vivo* com anti NK1.1 mAbs na produção de IFN-γ em camundongos C57Bl/6 durante a infecção com *M. bovis*. Anti NK1.1 mAb foi injetado i.p. nos animais no dia anterior a infecção com BCG. As células foram estimuladas com BCG e o número de células peritoneais produtoras de IFN-γ foi determinado pelo método de ELISPOT.
6) Efeito do tratamento in vitro com ionóforo de cálcio (Ionomycin, Sigma), EDTA e PMA sobre a ativação de macrófagos peritoneais.

Foram feitos experimentos a fim de se avaliar o efeito do PMA, EDTA e Ionóforo de cálcio (Ionomycin, SIGMA) sobre a produção de H2O2, NO e IFN-γ em culturas de células peritoneais provenientes de camundongos C57Bl/6 infectados com o M. bovis (BCG). O PMA foi utilizado em diversos experimentos, sendo considerado um controle positivo da ativação de macrófagos, também estimulando um aumento da produção de IFN-γ por células peritoneais de camundongos infectados com o BCG (dados não mostrados). Os experimentos com uso de Ionomycin não foram bem sucedidos, devido, provavelmente, a erro de diluição do reagente que não mostrou atividade biológica relevante, e houve dificuldades em se obter um novo reagente. Alguns experimentos com EDTA foram realizados, tendo sido observada discreta inibição da produção de NO e maior inibição da produção de H2O2 (dados não mostrados). Finalmente, a técnica de espraiamento de macrófagos em lamínula não foi utilizada, devido a enorme variabilidade dos resultados encontrados em experimentos realizados na fase de padronização das metodologias.
Considerações Finais

Estamos iniciando a redação de um manuscrito que irá enfocar basicamente os itens 1-3 descritos anteriormente. Foram apresentados resumos em congressos/encontros científicos (vide anexos), e os dados aqui apresentados (itens 1-3 de resultados) serão apresentados na próxima reunião da FeSBE (Caxambú, agosto 1999). Estudos de depleção ainda serão refeitos, a fim de se estabelecer quais as populações que participam na produção de IFN-γ observada no início (2 dias) da infecção pelo BCG. Nossos achados preliminares mostraram que a produção de IFN-γ não foi modificada pelo tratamento anti-NK1.1 (Fig.10). Entretanto, infelizmente as análises de citometria de fluxo (realizada no Laboratório de Transplante da Faculdade de Medicina de São Paulo, com a colaboração da Dra. Verônica Coelho e Dr. Jorge Kalil), revelaram presença de células NK1.1+ mesmo nas culturas de células peritonais de camundongos depletados de células NK1+. Desta forma, a inclusão desses achados no manuscrito que estamos preparando depende da realização de novos experimentos, a fim de comprovar a eficiência do método de depleção. Finalmente, a maior importância desse projeto foi ter possibilitado a padronização das metodologias descritas, que conduziram a outras investigações e projetos em desenvolvimento no Laboratório de Imunologia do ICB-UFJF, já havendo uma publicação relacionada ao tema (in press, Braz.J.Med.Biol.Res., em anexo).
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Para: anapaula@icb.ufjf.br <anapaula@icb.ufjf.br>
Data: Segunda-feira, 26 de Julho de 1999 17:22
Assunto: ms3604

MS-3604 The effect of Ascaris suum extract injection on the macrophage activity during the early phase of mycobacterium bovis BCG infection in C57Bl/6 mice

Prezada Dra. Ana Paula Ferreira,

Seu manuscrito acima referido está pronto para composição. No entanto antes de podermos dar andamento ao processo de publicação, precisamos que V.Sa. considere as questões abaixo, levantadas pelo Prof. Dr. Lewis Joel Greene, na preparação do trabalho. Agradeceríamos se a senhora nos enviasse somente os dados solicitados, uma vez que o texto já está preparado para o setor de composição.

Contando com sua colaboração, agradeceríamos se a senhora acusasse o recebimento deste e-mail.
Atenciosamente,

Dalva Pizeta
Editora Executiva

Questões do Prof. Greene:

1. Please provide a running title.

2. Abstract. Since abstracts are published separately by abstracting and indexing services, they should provide informative numerical data.
   a) Please provide some numerical data for NADPH-diaphorase activity, TNFa levels, by day.
   b) Same as a) for NO levels at 2, 7 and 14 days.
   c) Numerical data for decreased BSG (CFU).
   d) Please draw a conclusion which is not simply a restatement of the results.

3. Please provide city and state of Fluka


5. Legends to Table 1 and Figures 3 and 4. Please add statistical test used.

09/06/99
THE EFFECT OF *Ascaris suum* EXTRACT INJECTION ON THE MACROPHAGE ACTIVATION DURING THE EARLY PHASE OF *Mycobacterium bovis* BCG INFECTION IN C57Bl/6 MICE

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Key words: *Mycobacterium bovis, Ascaris suum*, macrophage activation, TNF-α, NADPH diaphorase
ABSTRACT

Injection of *Ascaris suum* extract (Asc) affects both humoral and cellular immune to unrelated antigens when it is co-administered with these antigens. In the present study we evaluated the effect of Asc on macrophage activation at the early phase of *Mycobacterium bovis* BCG (Pasteur strain TMCC 1173) infection in C57Bl/6 mice. C57Bl/6 mice were intraperitonealy (ip) injected with 0,1 mg of BCG (BCG group) or BCG plus 1 mg of Asc (BCG+Asc group). The peritoneal exudates were obtained at 2, 7 and 14 days after infection. Numbers of IFN-γ secreting cells were assessed by the ELISPOT assay. Nitric oxide (NO) production was measured by the Griess method and by the evaluation of NADPH diaphorase activity in the peritoneal exudates. The administration of Asc extract increased NADPH diaphorase activity and TNF-α levels in the early phase of BCG infection. Furthermore, we also observed that Asc co-injection induced a decreased of BCG - CFU in the spleens of BCG infected mice during the first week of infection.
INTRODUCTION

The capacity of helminths to modulate host immune responses to unrelated antigens is well documented (1). High dose immunization with adult *Ascaris suum* worms antigen (Asc) induces a marked production of IL-4 and IL-10 by inguinal and periaortical lymph nodes (LN) cells of DBA/2 mice (2). Asc-injected DBA-2 mice when simultaneously immunized with ovalbumin (OA), exhibit a marked suppression of both OA-specific cutaneous DTH and *in vitro* proliferative capacity of LN cells. In addition, OA-induced IL-2, IFN-γ and IL-4 production were diminished in Asc-treated mice and it was associated with an impairment of OA-specific IgG2a, IgG1 and IgE humoral immune response (2). Similarly, Asc induces a significant decrease of *M. bovis* specific DTH in BCG infected mice 14 days after infection (3). As persons in developing countries who are at risk for mycobacterial infections often have *A. suum* co-infection, these observations may have very important public health implications.

Protection against intracellular pathogens operates in two stages, first an early innate nonspecific response followed by acquired immunity with a strong DTH response (4). During the first week of infection, the early innate immune response is the main mechanism controlling mycobacteria proliferation. After two weeks of infection, cellular immunity develops and immature granulomas are formed. After 4 weeks, the cellular immunity reaches its peak and is represented by epithelioid
granulomas that are associated with the decrease of mycobacterial load (4,5,6). In inbred strains of mice, early innate resistance to M. bovis BCG infection is controlled (5,7,8) by a single dominant gene designated Nramp (natural resistance-associated protein macrophage protein) that regulates the priming activation of macrophages for nitric oxide (NO) production. For example, mice genetically resistant to M. bovis BCG infection (C3H/HeN and A/J) are able to prevent M. bovis proliferation without expressing a strong DTH response. In contrast, genetically susceptible mice (C57Bl/6, B.10.A and BALB/c) only control mycobacterial proliferation by the acquisition of effective cellular immune response (5).

Thus, the immune mechanisms associated with the regulation of resistance and susceptibility in the innate early phase of BCG infection clearly involves cytokines and NO production (4,9). As mentioned above, injection of A. suum extract decreases M. bovis-specific immune responses during the stages of infection controlled by cellular immunity (3). To evaluated whether it also affects early innate responses to these organisms, we studied whether co-injection of A. suum extract alters NO production in a model of intraperitoneal (i.p.) BCG infection in C57Bl/6 mice. In these study, we measurement of NADPH diaphorase activity as a histochemical marker for nitric oxide synthase (iNOS; NOS2), the enzyme which catalyzes oxidation of L-arginine to L-citruline and the production of NO (10,11). As, several studies have demonstrated that IFN-γ and TNF-α (6,12,13) are important cytokines correlated with the development of NO-mediated non-specific early host defense, we also chose to measure these cytokines. Surprisingly, our
findings suggest that Asc administration in *M. bovis* BCG infected mice induces the increase of both NADPH diaphorase activity and the TNF-α production by peritoneal cells as well as correlates with a reduction of BCG - CFU numbers in spleens.
MATERIAL AND METHODS

Animals

C57Bl/6 female mice were obtained from the Reproduction Biology Center – UFJF and were maintained in the animal facility of the Biological Sciences Institute – UFJF, were housed in plastic cages with unlimited access to food and water. Mice were used in experiments at 6-8 weeks of age. Three main groups of mice were studied: Group BCG - three mice i.p. injected with BCG; Group BCG+Asc - three mice i.p. injected with BCG + Asc; Group Asc - three mice i.p. injected only with Asc.

*M. bovis* BCG infection and CFU enumeration

*M. bovis* BCG, strain Pasteur TMCC 1173, was thawed, washed in PBS, then inoculated at a dose of 0.1 mg in 0.5 ml PBS by i.p. injection. At 2, 7 and 14 days postinfection, BCG-CFU enumeration in spleens was performed. In brief, spleens were collected and homogenized individually in 1 ml PBS using a teflon coated tissue homogenizer (Glas-Col Apparatus Co., Terre Haute, IN). Serial ten fold dilutions of organ homogenates were made in PBS and plated onto Middlebrook 7H10 Bacto Agar (Difco Laboratories, Detroit, MI) plates, in triplicate. Plates were sealed in plastic bags, incubated at 37°C for 14 days, and the number of bacteria per organ were determined.
**Ascaris suum** extract preparation and injection. The animals were i.p. injected with 1mg of adult worm extract from A. suum, prepared as described by Macedo & Mota (14). In summary, live worms obtained from pigs intestines were washed with PBS and mixed with equal volume of borate-buffered saline (BBS) pH 8.0. After being homogenized in an Ultra-Turrax apparatus and centrifuged at 10,000 rev/min for 1 h, the precipitate was resuspended in 400 ml of BBS and stirred overnight at 4°C. This suspension was centrifuged again and the supernatant was dialysed against distilled water overnight in the cold. A clear supernatant obtained after centrifugation at 10,000rev/min for 2 h. The supernatant was aliquoted and lyophilized.

**Diaphorase Citochemistry** - Peritoneal cavities were washed with 5 ml of PBS to remove resident macrophages. Cell suspensions were washed once time and resuspended to obtained 10^6 cells/ml. Slides were prepared using cytopin (FANEM) with 2x10^5 cells per well, left dry at room temperature, fixed in cold acetone for 10 minutes, wrapped in plastic film and kept at -20°C prior to use. Slides were stained with 50 mM Tris, pH 8, with 0,3mM nitroblue tetrazolium (Sigma), 1mM NADPH, 0,2% Triton X-100 for 30 min at 37°C, washed, mounted in glycerol, and dark-blue cells were enumerated by using an optical microscope at 40x magnitude. Macrophages-like cell line RAW 264-7 (ATCC) were stimulated with 1 µg/ml of LPS (Sigma) and used as positive control for NADPH diaphorase activity.
**Measurement of nitrite release** - NO release was quantified by the accumulation of nitrite in the supernatants of peritoneal cell culture after 48 h in culture with 10μg/ml of BCG antigen, using the standard Griess method (15). In brief, Griess reagent was freshly made prior to use and added 1:1 with supernatant and left 5 min at room temperature. Standards were prepared using sodium nitrite (2-100 μM) and were included on each assay plate. Absorbances at 570 nm were read on a spectrophotometer (Titertek Uniskan II, Flow, Inc., Lugano, Switzerland)

**Determination of TNFα levels** - Peritoneal cells were collected and cultured for 48 h in RPMI- 1640 medium with 10 μg/ml of BCG antigen. Supernatants were collected and TNFα was quantitated by double sandwich ELISA, using 2μg/ml of purified anti-mouse TNF-α Ab (Pharmlingen, San Diego, CA) and 1μg/ml to biotin rat anti-mouse TNF-α Ab (Pharmlingen, San Diego, CA). Binding of biotinylated antibodies was detected using streptavidin–biotinylated horseradish peroxidase complex (Southern Biotechnology Associates, Inc.), OPD and H2O2 and stopped with 50 μl of 0,2 M citric acid. The plates were read at 450nm on ELISA reader (Titertek Uniskan II, Flow, Inc., Lugano, Switzerland). Samples were quantitated by comparison with standard curves of purified recombinant TNF-α (Pharmlingen, San Diego, CA)

**Determination of IFN-γ** - For frequency of IFN-γ producing peritoneal cells, the ELISPOT assay was used as previously described (16). Briefly, milliliter filtration
plates (Millipore STHA09610, Bedford, MA) were coated overnight at 4°C with anti-IFN-γ (R4-6A2, 1µg/ml). Wells were blocked with 1% BSA-PBS for 2 hrs at 37°C. Appropriate dilutions of peritoneal cells in 5% FCS-RPMI were added in a volume of 100 µl and incubated with 10 µg/ml of BCG antigen for 16 h at 37°C in 7% CO₂. Plates were thoroughly washed with PBS-Tween 20 (0.05%), before adding aliquots of 100µl/well of biotinylated anti-IFN-γ mAb (AN-18.17.24, 0.5µg/ml; Kindly provided by Dra Mahasti Sahidi de Macedo, USP, São Paulo). After incubation at 37°C for 2 hrs, plates were washed with PBS-Tween 20 several times and then avidin-alkaline phosphates (Dianova) were added. After 1-h incubation at 37°C, plates were washed 8 times with PBS-Tween 20 and twice with alkaline phosphates buffer, pH 9.5. Then, the substrate, 5-bromo-4-chloro-3-indoyl phosphate (Fluka, Basel, Switzerland), was added. After 15 min, blue dots had developed and the reaction was terminated with distilled water. Individual spots were counted under a dissecting microscope at 32X magnification.

Statistical analysis – The Student’s t-test was used to determine significant differences between the different groups of mice with the level of significance set at p<0.05.
RESULTS

Ascaris suum extract increased NADPH diaphorase activity

A high proportion of peritoneal macrophages from BCG-infected animals injected or not with Asc extract were positive by diaphorase cytochemistry. However, the BCG-infected mice injected with Asc extract increased 2-fold the capacity to induce diaphorase activity (P<0.05). Control and Asc injected mice showed a reduced percentage of positive cells (Figure 1).

Nitrite production in culture of peritoneal cells

Supernatants of peritoneal cells from animals injected with BCG, BCG+Asc or Asc were collected after 48 h of culture with BCG antigen, and the amount of NO was evaluated (Figure 2). Low levels of NO production were detected at 2 and 7 days after BCG infection, increasing at 14 days p.i. No significant difference (p<0.05) was observed between levels of NO production by peritoneal cells of BCG injected mice and BCG+Asc injected mice. In addition, it was also detected a high level of NO production in mice injected with Asc on 14th day.
Effect of Asc on TNF-α production in culture of peritoneal cells

The kinetics of TNF-α production was measured in supernatant culture during the early phase of BCG infection (Figure 3). TNF-α was detectable in the supernatants at 2, 7 and 14 days p.i. At 2 days after BCG infection TNF-α was observed in mice injected with BCG and mice injected with BCG+Asc. However, at day 7 we demonstrated a significant increase (p<0.05) in TNF-α levels produced from mice injected with BCG+Asc compared with mice injected only with BCG. At the end of the early phase of BCG infection (day 14) there was detectable lower levels of TNF-α in mice injected with BCG+Asc. In mice injected only with Asc higher TNF-α levels were observed at 14 days after Asc injection.

Presence of IFN-γ producing cells during intraperitoneal BCG infection

For frequency determinations of IFN-γ producing peritoneal cells, the ELISPOT assay was used (Table I). 10^6 cells were cultured with 10 μg/ml of BCG antigen for 24h. Numbers of spontaneous IFN-γ spot-forming cells (SFC) were evaluated at 7 days after BCG infection with or without Asc injection. No significant differences (p<0.05) were demonstrated between mice injected with BCG or BCG+Asc. On contrary, mice injected with Asc presented significant (p<0.05) lower numbers of SFC when compared with mice injected with BCG or BCG+Asc.
Asc interferes with the proliferation of BCG in spleen

The number of colony forming units (CFU) was investigated in the early phase of BCG infection (Figure 4). A significant reduction in the number of BCG-CFU was observed during the first week of BCG infection in mice injected with BCG+Asc when compared with mice injected only BCG (2 and 7 days p.i.). However, by the end of the early phase of BCG infection there is not a significant difference number of BCG-CFU between BCG group and BCG+Asc group.
DISCUSSION

The present experiment investigated the effect of Asc extract during the early phase of intraperitoneal (i.p) BCG infection in C57Bl/6. The immune response in BCG infected mice was evaluated based on five parameters (NO production, NADPH diaphorase activity, numbers of IFN-γ secreting cells, TNF-α production by peritoneal cells and number of BCG-CFU in the spleen). The data demonstrated that BCG infection (i.p.) induced NADPH diaphorase activity, NO production, TNF-α and IFN-γ production during the development of the early phase of infection (2 weeks p.i.) in C57Bl/6 mice. The administration of Asc extract (group II) increased NADPH diaphorase activity and TNF-α levels in the early phase of BCG infection. Furthermore, we also observed that Asc injection induced a decrease of BCG-CFU in the spleens of BCG infected mice during the first week of infection.

BCG administration in BCG-susceptible mice (C57Bl/6, BALB/c and B10.A) generated an efficient protective early response to the challenge with homologous BCG and heterologous pathogens (Lysterisa monocytogenes) (5). It was also demonstrated that BCG administration restricted Mycobacterium avium proliferation and, at the same time, promoted expression of TNF-α mRNA and IFN-γ mRNA in spleen cells suggesting that these cytokines act in an additive or synergistic fashion in the induction of bacteriostasis (17). In vitro TNF-α was produced by macrophages upon stimulation with rIFN-γ and mycobacterial infection and both cytokines are crucial for activation of macrophage functions (18). Our
data demonstrated that Asc extract in BCG-susceptible mice (C57Bl/6) is associated with the development of a protective early response to BCG represented by a reduction of BCG-CFU in the spleen. In addition, the present results suggested that the enhancement of TNF-α production and NADPH diaphorase activity is probably associated with the induction of a protective immune response.

The murine resistance to intracellular pathogen upon genetic regulation of Nramp gene is correlated with the priming/activation of macrophages linked with TNF-α-dependent production of antimicrobial NO (19). Using NADPH diaphorase histochemistry and PCR was observed that macrophages in the lungs of people with active *Mycobacterium tuberculosis* often express catalytically competent nitric oxide synthase (20). Yoshida et al (1995) showed that C57Bl/B10 mice presented high levels of iNOS protein and mRNA at 3 and 6 days after intravenously BCG infection. Our results in C57Bl/6 mice also demonstrated the participation of iNOS in the early phase of BCG infection. Additionally, the results also support the hypothesis that the production of TNF-α is closely linked with the levels of NADPH diaphorase activity.
Concerning NO production, in the present study was not observed significant differences between groups BCG and BCG+Asc. Some studies have been described in humans (21) and mice macrophages the presence of iNOS protein but associated with little or no NO synthesized. In our experiment it is possible that nitrite produced in supernatants of peritoneal cells was utilized to macrophages in their activation during the culture time.

Many studies have been demonstrated that Asc impaired important T cell functions for cell-mediated and humoral responses to other antigens through the induction of a predominantly Th2 type response with increase to IL-4 and IL-10 (2, 22, 23). Interestingly, it was recently found that the mechanism by which IL-10 inhibits macrophage killing of Shistosomes involves suppression of TNF-α production, and can be reversed by addition of exogenous TNF-α (24). The ability of mice to develop DTH after subcutaneous BCG immunization was inhibited by the administration of Asc extract (3). In the present study, we investigated the effect of Asc injection at the early phase of BCG infection during the development of innate unspecific host immune response. We detected that the administration of Asc induces the enhancement of resistance against BCG infection in a susceptibility strain of mice. Therefore, our data suggested that the effect of Asc might be influenced by the stage of the host response developed during BCG infection. Finally we postulate that Asc present different effects on innate immune response and acquired immune response in BCG infected mice and that these responses are correlated between them.
ACKNOWLEDGMENT

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<thead>
<tr>
<th>Groups</th>
<th>Number of IFN-γ SFC/10^6 Peritoneal cells</th>
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<tbody>
<tr>
<td></td>
<td>(Mean ± SD)</td>
</tr>
<tr>
<td>Control</td>
<td>15 ± 1,2</td>
</tr>
<tr>
<td>BCG</td>
<td>706 ± 25,6</td>
</tr>
<tr>
<td>BCG+Asc</td>
<td>749 ± 6,2</td>
</tr>
<tr>
<td>Asc</td>
<td>400 ± 10,0*</td>
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LEGENDS

Table 1 – Frequencies of IFN-γ SFC in C57Bl/6 at 7 days p.i. Results are reported as mean ± SD from duplicates per data point of one representative experiment.

* Significant differences were observed when compared with groups BCG and BCG+Asc. (Student's t-test)

Figure 1- Effect of Ascaris suum extract in NADPH diaphorase activity in cells obtained from C57Bl/6 mice injected with BCG, BCG+Asc or Asc. Results are reported as percentual of dark cells indicated NADPH diaphorase activity from duplicates per data point of one representative experiment.

Figure 2- Effect of Ascaris suum extract in NO production in C57Bl/6 mice. NO was measured with Griess reactive. 2×10⁵ peritoneal cells were incubated for 48 h with 10 µg/ml of BCG antigen, at 37°C, in 5% CO₂ and absorbance was determinate at 540nm. Results are reported as mean ± SD from duplicates per data point of one representative experiment.
Figure 3- Effect of Ascaris suum extract on TNF-α production in culture of peritoneal cells of C57Bl/6 mice during BCG infection. Supernatants were harvested after 48 h with 10 μg/ml of BCG antigen stimulation and measured to two-sandwich ELISA. Kinetic TNF-α data are expressed as mean TNF-α (pg/ml) ± SD from quadruplicates per data point of one representative experiment.

* Significant differences were observed when compared with groups BCG and BCG+Asc. (Student’s t-test)

Figure 4- Effect of Ascaris suum extract in the number of BCG-CFU in the spleen of BCG infected mice. At indicated periods, spleens were recovered and bacterial counts (BCG-CFU) performed as described. Data represent mean ± SD of BCG-CFU enumeration (two animals per data point) from triplicates of one representative experiment. * Significant difference was observed when compared with BCG group. (Student’s t-test)
De: Cadernos de Saúde Pública <cadernos@ensp.fiocruz.br>
Para: Henrique C. Teixeira <henri@icb.ufjf.br>
Data: Segunda-feira, 21 de Junho de 1999 08:46
Assunto: Re: informações sobre trabalho submetido

Prezado Dr. Henrique C. Teixeira:

Seu artigo acaba de seguir pelo correio, para o endereço que nos forneceu para correspondência. Nossos consultores recomendaram a sua publicação, desde que atendidas algumas solicitações de mudanças. Desta forma, passamos a aguardar uma resposta de sua parte.

Atenciosamente,

Mauricio Leite
Editor assistente

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At 15:16 18/06/99, you wrote:
> Senhor Editor,
> > Recebi em 14/04/99 um postal informando que o trabalho intitulado:
> > "The impact of multidrug therapy on the epidemiology of leprosy in 
> > Juiz de Fora" foi recebido e encaminhado para parecer do Conselho 
> > Editorial. Tendo já passado mais de 2 meses, solicito informações 
> > sobre o andamento do processo (0612), e coloco-me a disposição para 
> > quaisquer informações necessárias.
> > > Atenciosamente,
> > > Dr. Henrique Couto Teixeira
> > > Lab. de Imunologia - ICB - UFJF
> > >
> > CADERNOS DE SAÚDE PÚBLICA/REPORTS IN PUBLIC HEALTH
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> > ---
THE IMPACT OF MULTIDRUG THERAPY ON THE EPIDEMIOLOGIC PATTERN OF
LEPROSY IN JUIZ DE FORA, BRAZIL.

Running Title: Impact of MDT on Leprosy

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ABSTRACT

We investigated the impact of multidrug therapy (MDT) on the epidemiological pattern of leprosy in Juiz de Fora, Brazil from 1978 to 1995. Evaluation of 1283 medical charts was undertaken, according to the therapeutic schedule used in two different periods. Since introduction of MDT in 1987, the prevalence of leprosy decreased from 22 patients/10,000 inhabitants to 5.2 patients/10,000 inhabitants in 1995. Moreover, incidence rate of leprosy was lower in period II (1987-1995) than in period I (1978-1986). The decreasing prevalence and incidence appears to be related to drug efficacy rather than decreased infection identification as both self-initiated and health care professional referral sought treatment increased markedly from period I to period II. For both periods, multibacillary leprosy was the most frequent clinical form of the disease (±76%) and the main known risk factor for infection was contact in the home. Although leprosy is predominantly manifested in adults, an increase in the percentage of very old and very young patients was observed in period II. In conclusion, the results indicate that the MDT program has been effective in combating leprosy and has increased awareness of the disease. Elements that might impair success of the MDT leprosy control program are also discussed.

Keywords: Leprosy. Multidrug therapy. Epidemiology. Prevalence. Incidence
RESUMO

Investigamos o impacto da poliquimioterapia (PQT) no perfil epidemiológico da hanseníase em Juiz de Fora, Brasil, de 1978 a 1995. Fizemos uma avaliação de 1283 prontuários, de acordo com o esquema terapêutico adotado em 2 diferentes períodos. Desde a introdução da PQT em 1987, a prevalência da hanseníase caiu de 22 pacientes/10.000 habitantes para 5,2 pacientes/10.000 habitantes em 1995. Além disso, a incidência da doença foi menor no período II (1987-1995) em comparação ao período I (1978-1986). A diminuição da prevalência e da incidência está mais relacionada à eficácia das drogas do que a uma queda na identificação da infecção, já que tanto a procura espontânea quanto os encaminhamentos aumentaram significativamente do período I para o período II. Em ambos os períodos, a forma clínica mais frequente foi a multibacilar (±76%), e o contato intradomiciliar foi o maior fator de risco conhecido associado à infecção. Um aumento na porcentagem de pacientes muito velhos ou muito novos foi observado no período II. Concluindo, os resultados indicam que o esquema PQT tem sido eficaz no combate à hanseníase e tem aumentado a conscientização e o conhecimento da doença. Fatores que podem comprometer o sucesso da PQT também são discutidos.

Palavras-chave: Hanseníase, Poliquimioterapia, Epidemiologia, Prevalência, Incidência
1) INTRODUCTION

Leprosy is a chronic infectious disease that represents a major public health problem affecting about 1.8 million people worldwide (WHO 1994). An estimated 2 to 3 million individuals suffer from physical disabilities as a result of having contracted leprosy (Van Beers et al. 1994). Although it is one of the oldest disease afflicting humans, only the advent of sulfones in the 1940's provided the possibility for its treatment and cure. However, the outcomes experienced with the use of sulfones were not satisfactory. Dapsone failed to prevent the growth of resistant bacillus (Dharomendra 1986) and new potent drugs like clofazimine (Browne & Hogerzeil 1962) and rifampicin (Levy et al. 1976; Opromolla 1963) could not control the dissemination of the disease as monotherapeutic agents. In 1981, World Health Organization (WHO) began to recommend a multidrug therapy (MDT) of dapsone, clofazimine and rifampicin. MDT was an effective strategy and allowed for reduced treatment times compared to monotherapy: from five to two years for the lepromatous (L) or dimorphus (D) multibacillary forms (MB) and from 2 years to 6 months for the tuberculoid (T) or indeterminate (I) paucibacillary forms (PB). MDT was introduced in Brasil in 1986, and it was authorized for leprosy treatment in 1992.

Based on the efficacy of MDT for leprosy, WHO has target elimination of the disease as a world wide public health problem by the year 2000. To do so, it has enrolled the authorities of countries with high rates of disease. Brazil currently has the second highest prevalence and incidence of leprosy in the world. The Brazilian government, through its Health State Offices, has been working to reduce the prevalence of the disease to less than 1 case per 10,000 inhabitants, which is considered a low rate by WHO.
Juiz de Fora, a city with a population of 414,520 inhabitants (population estimate for 1995), has many patients with leprosy. However, the epidemiologic pattern of the disease in the city has not been described. This article analyses the main characteristics of leprosy in Juiz de Fora and investigates the impact of MDT on the epidemiologic pattern of the disease.
2) PATIENTS AND METHODS

Juiz de Fora is the home of a regional health directorate of the state of Minas Gerais and serves a large number of nearby cities by providing drugs and human resources for leprosy treatment and control. The city has two health centers that care for patients with leprosy: Dr. Antonio Carlos Pereira Filho - Dermatology Sector and Universitary Hospital. We studied the epidemiologic patterns of patients with leprosy over two defined periods, differing in their use of MDT and in some characteristics of the control program, as described in table 1.

In the first phase of the study, medical charts of 1283 patients were analysed to determine annual incidences and prevalences of disease. These patients were treated at leprosy health centers in Juiz de Fora, representing 375 cases of leprosy registered before 1978 plus 534 new cases from period I (1978-1986) and 374 new cases from period II (1987-1995). Less than 10% of the patients of period I were treated with MDT and about 90% of the patients had sulphone monotherapeutic treatment from the National Sanitary Dermatology Division (DNDS). In contrast, approximately 90% of the patients of period II were treated with MDT and less than 10% with the DNDS schedule. Leprosy classification was based on clinical presentation, bacilloscopy, Mitsuda test and classic histopathology (Madrid system, 1953) in both periods. Until 1993, patients were considered cured after completion of the treatment if clinical signs of the disease and bacilloscopy were both negative; otherwise they remained under observation for an additional period of 5 years (MB) or 2 years (PB). Fixed duration treatment was introduced in 1993. Statistical criteria for discharge was adopted in 1987. Patients absent from health units for a period of a year or more whose treatment was not completed were considered as non-compliant and were discharged by statistical criteria after completing 2 years (PB) or 5 years (MB) of abandonment.
Efficacy of the therapy used was evaluated by the number of discharges resulting from cure of the disease, and by the comparison of prevalence and incidence rates between the two periods studied. In the second phase of the analysis, the case identification strategy, the most frequent clinical presentation, the age and sex distribution and the infection source were determined by analysis of randomly selected sampled medical charts. 314 files were selected in each one of the two periods studied, totalling 628 new case protocols.

The data were analysed using the computer program EPI Info 6.03. First we did a descriptive statistical analysis for the two groups enrolled in this study. The results of phase one (table 2) were compared by analysis of variance of the means using the non-parametric Kruskal-Wallis method. For the results of phase two (figures 1-3), the Chi-square statistics were used for analysis of the data. The significance level was 5%.
3) RESULTS

3.1) THE IMPACT OF MDT ON THE NUMBER OF PATIENTS WITH LEPROSY IN JUIZ DE FORA.

MDT was introduced in Juiz de Fora in 1987 and caused a reduction of treatment time; besides being accompanied by improvements in the leprosy surveillance system, including more frequent contact examination and professional training. In addition, a statistical criteria was introduced for patient removal from the registry due to non-compliance with the treatment, and BCG was adopted in 1989 in an attempt to stimulate anti-mycobacterial protective responses in leprosy contacts (table 1).

From 1978 to 1986 (period I), the number of new cases registered in Juiz de Fora exceeded the number of discharges of patients on active file from the city health units, so that the total number of registered cases increased every year peaking at 787 in 1986 (table 2). The disease prevalence increased from 14.4 patients/10,000 inhabitants in 1978 to 22 patients/10,000 inhabitants in 1986. During this time, 534 new individuals were added to the register and only 99 were removed. The reasons for removal from registry were 49.5% for leprosy cure, 42.4% for locality transfer and 8% by patient death. In contrast, from 1987 to 1995 (period II) the number of discharges (removal from the registry) overcame the number of additions. The total number of cases in 1987 was 810 but decreased to 264 by 1995. Accordingly, during period II, 959 discharges occurred. This was almost 10 times the number of period I. 63.8% of the patients were discharged due to cure, 23.3% by statistical criteria, 9.6% because of locality transfer and 4.3% by patient death. This results suggest that the use of MDT improved the epidemiologic control of the disease as it reverted the flow of new registrations and discharges, it demonstrated an statistical significant improvement.
in the number of discharges as a result of cure (p=0.0003) and it reduced the overall disease prevalence from 19.5 cases/10,000 inhabitants in 1987 to 5.2 cases/10,000 inhabitants in 1995. Table 2 also shows that the incidence rate of leprosy in Juiz de Fora varied but showed lower incidence during period II compared to period I (p=0.0051), suggesting that the incidence of leprosy reduced after introduction of MDT, perhaps as a result of the decreased number of individuals infectious to others.

3.2) COMPARISON OF NEW CASE IDENTIFICATION IN PERIOD I VS. PERIOD II.

Figure 1 shows the frequency of how new cases of leprosy were identified in Juiz de Fora in the two treatment periods. Patient initiated pursuit of treatment at health units was the most frequent method of new cases identification in Period II (48.4%). This was clearly higher than the patient initiated treatment sought during period I (11.1%). Similarly, the percentage of patients identified by health care professionals increased from 2.2% during Period I to 26.8% during Period II. Case identification through tests of previously identified patient contacts also became more prevalent in Juiz de Fora in Period II (10.2%) compared with Period I (3.5%). In contrast, there was a reduction in the percentage of case identified by mass survey (3.2% to 1.3%) as well as a reduction in the percentage of patients whose case identification mechanism was not determined during Period II (13.4%) compared with Period I (79.9). The overall distribution of case identification has changed in a statistically significant manner ($\chi^2 = 300.27$, 4 df, p<0.0001).
3.3) THE MULTIBACILLAR FORMS OF LEPROSY PREDOMINATE OVER THE PAUCIBACILLAR FORMS.

It is well known that leprosy manifests as a spectrum of different clinical forms. In our study, it was observed that the multibacilar forms (lepromatous and dimorphous) predominate over paucibacilar forms (tuberculoid and indeterminded) in both periods studied (figure 2). A reduction of lepromatous leprosy frequency (60.8% to 33.8%) from Period I to Period II was accompanied by a raise of dimorphous leprosy cases (1.9% to 38.5%). In contrast, the percentage of patients who had tuberculoid leprosy did not show an important variation, remaining at ≈19.5%. Patients with indeterminded leprosy represent less than 15% of the total number of patients, showing a drop from 14% to 11.8% between the periods studied. The overall distribution showed statically significant change from period I to period II ($\chi^2 = 133.37, 3$ df, $p<0.0001$).

3.4) THE INCIDENCE OF INCREASED LEPROSY AT EXTREME AGES.

Figure 3 shows that although leprosy was diagnosed mainly in adults (15 to 59 years of age), the percentage of patients 60 and over with clinical presentation of the disease increased from 6.1% to 10.2%, as did the percentage of clinical presentations in patients below 15 years of age also raised (1.0% to 4.6%). The overall change was significant ($\chi^2 = 12.52, 5$ df, $p=0.0283$).
4) DISCUSSION

The epidemiologic features of leprosy were analysed in Juiz de Fora, Brazil, focusing on the impact of the introduction of the MDT protocol on the epidemiologic control of the disease. Our results show that the use of MDT in Juiz de Fora since 1986 caused a sharp decrease in disease prevalence as well as a significant increase in the number of discharges resulting from leprosy cure. These data suggest the efficacy of MDT schedule for treatment of leprosy and confirm the prevalence studies described in other regions of Brazil (Nogueira et al. 1995; Ministério da Saúde, 1998). In accordance, recent data indicate that prevalence rates of leprosy in Juiz de Fora were reduced to 1.8 patients/10,000 inhabitants in 1996 and 1.6 patients/10,000 inhabitants in 1997 and 1998 (A.F.M. Pimentel, personal communication). Moreover, our results indicate that the incidence rate of leprosy in Juiz de Fora is decreasing, because the annual detection rates of period II were lower in comparison with those of period I. These findings are in contrast to the increased caseload of leprosy in Brasil during the 1986 to 1997 period (Ministério da Saúde, 1998), which may be explained by large differences between the detection of leprosy in different regions of Brasil.

Our data, however, are consistent with the results described by others using retrospective analysis of data on the occurrence of leprosy in other countries (Myint, T. & Htoon. M.T., 1996; Meima. A. et al. 1997). We are aware that the reduction in treatment time and implementation of statistical criteria for being discharged from the active file (both adopted after 1986) may have some influence on the interpretation of the success of MDT. Increased BCG coverage also could have affected our data by lowering the incidence of the lepromatous form of leprosy, since it has been demonstrated that BCG confers protection against leprosy and is able to decrease the proportion of MB forms (Rodrigues et al, 1992). Yet, we believe that the implementation of the MDT program in Juiz de Fora has brought about major changes
in patient treatment and cure and was accompanied by an improvement in the surveillance and in health service availability and quality.

The increase in frequency of patients seeking treatment for care of leprosy at health units in Juiz de Fora suggests that following the initiation of the MDT program there was an improvement in the population's knowledge of the early signs and symptoms of the disease and the availability of medical care. On the other hand, improved recognition of the disease by health professionals and increased surveillance of patient contacts resulted in a higher percentage of case discovery by professional reference. The reduction on the percentage of patients identified by mass survey confirms other studies which showed that this high cost new case find strategy has been discontinued (Theuvenet et al. 1994).

As expected, the percentage of patients who acquired the disease by home contact (≈24%) was higher than those who acquired the disease by other known source (≈7%), in both periods. The majority of patients (≈68% in both periods) could not identify the source of infection. The occurrence of new cases of leprosy for which the source of infection is not known might be explained by the close association of the patients with assymptomatic leprosy carriers living in endemic areas of disease. This possibility is enhanced by the long viability of M. leprae outside the human body (Van Beers et al. 1995). These data implicate a role of genetic features and prolonged contact with assymptomatic carriers in the spread of disease (Kyriakis et al. 1994; Mehra et al. 1995). However, there is still very little evidence of the main sources of infection in these situations.

The multibacillary forms of leprosy predominated during both periods, however, the frequency of dimorphous leprosy and lepromatous leprosy differed. Such variation may be explained by changes in the evaluation criteria of clinical presentations and by improved diagnostic accuracy. Furthermore, most dimorphous cases are, in fact, skin-smear-negative patients (89.3% of period II), in comparison
with the smear-positive patients who were predominant among the lepromatous patients of period II (70.8%), showing a highly significant difference ($\chi^2$=20.92, 1 df, p<0.0001). Hence, we cannot exclude the possibility that the described shift in dimorphous frequency and the high detection rate for MB leprosy may, in some degree, reflect a conservative behavior in which clinicians prefer to choose longer treatment for leprosy patients (Martelli et al., 1994). The incidence of leprosy in females (44.5%) was lower than in males (55.5%), but it was not statistically significant ($\chi^2$=1.17, df=1, p=0.2783); interestingly, a higher percentage of MB leprosy was observed in males (73.7% versus 59.6%), in both periods ($\chi^2$=13.99, 1 df, p<0.0002). These findings reinforce the concept that immunologic responses against *M. leprae* is stronger in females than in males (Olrich et al.1993).

Although leprosy predominates between 15 to 60 years of age, the current data suggest a larger age distribution of leprosy in this population. An increased frequency of leprosy in patients 60 years of age or older was observed, similar to the results found by others (Smith, T.C. and Richardus, J.H. (1993). The percentage of children under 15 with the disease changed from low to moderate according to WHO criteria. The same results were also described elsewhere (Ministério da Saúde, 1998) and considered an indicator of a higher rate of disease occurrence, but it may be associated with detection improvements in some way.

During period II, two-hundred and twenty four patients were discharged by statistical criteria and therefore were lost to follow-up along with 284 assymptomatic leprosy carriers who have a significant risk of disease development in the future. Nevertheless, the use of MDT as a new treatment protocole for patients with leprosy reduced the non-compliance rate in Juiz de Fora from 90% in 1986 to 56% in 1995 and 19% in 1998 (A.F.M. Pimentel, personal communication). These non-compliance rates are still considered high and might jeopardize the leprosy control program as the lack
of completed patient treatment and contact follow-up could lead to development of resistant bacilli.

The MDT program shows good prospects for the cure of leprosy and its elimination as a public health problem (WHO, 1994). However, it cannot be forgotten that the control and the eradication of the disease rely on different collective efforts that can interfere with the spread and evolution of the disease. Such efforts are not restricted to the use of drugs against *M. leprae* but can also employ integrated nutritional programs, BCG coverage, educational curricula and sanitary conditions. These factors may also influence the efficacy patient's immunologic response, the clinical presentation and the therapeutic results.
Acknowledgments: Dr. Evan Secor for critical review of the manuscript. Francisco Teixeira for his expert help on the Epi Info6 program. This work was supported by grants from Conselho Nacional de Pesquisa (CNPq) and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG).
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LEGENDS

Table 1 – Characteristics of Period I and Period II.

Table 2 – Number of admissions and discharges from file of leprosy in Juiz de Fora in two different periods.

Fig. 1 – Case Identification strategy of leprosy in Juiz de Fora in the two different periods.

Fig. 2 – Clinical distribution of leprosy in the two different periods.

Fig. 3 – Age distribution of leprosy in the two different periods.
<table>
<thead>
<tr>
<th></th>
<th>Period I</th>
<th>Period II</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Time period</strong></td>
<td>01/01/78 to 12/31/86</td>
<td>01/01/87 to 12/31/95</td>
</tr>
<tr>
<td><strong>Main drugs and</strong></td>
<td>DNDS</td>
<td>MDT</td>
</tr>
<tr>
<td><strong>Length of treatment</strong></td>
<td>MB: Rifampin + Dapsone (first 3 months) and Dapsone alone (through the rest of 5 years)</td>
<td>MB: Rifampin + Dapsone + Clofazimine (normally 24 months, up to 36 months)</td>
</tr>
<tr>
<td></td>
<td>PB: Dapsone (2 years)</td>
<td>PB: Rifampin + Dapsone (normally 6 months, up to 9 months)</td>
</tr>
<tr>
<td><strong>Discharge by Statistical criteria</strong></td>
<td>Not performed</td>
<td>Performed</td>
</tr>
<tr>
<td><strong>Exam of contacts</strong></td>
<td>Less frequent</td>
<td>More frequent</td>
</tr>
<tr>
<td><strong>BCG vaccination</strong></td>
<td>Not performed</td>
<td>Performed</td>
</tr>
<tr>
<td><strong>Professional training</strong></td>
<td>Less frequent</td>
<td>More frequent</td>
</tr>
</tbody>
</table>

*BCG was introduced in 1989 for all MB contacts and in 1993 for all leprosy contacts. Before 1989 it was only used for scar negative contacts (usually children ranging from 0 to 4 years old).*  
*Rif = Rifampin; Dds = Dapsone; Clof = Clofazimine*
Table 2 - Number of Admissions and discharges from file of leprosy in the city of Juiz de Fora

<table>
<thead>
<tr>
<th>Year</th>
<th>Number of cases</th>
<th>Admission&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Discharges&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Population&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Incidence /100,000 hab</th>
<th>Prevalence /10,000 hab</th>
</tr>
</thead>
<tbody>
<tr>
<td>1978</td>
<td>375</td>
<td>49</td>
<td>0</td>
<td>293.000</td>
<td>17</td>
<td>14</td>
</tr>
<tr>
<td>1979</td>
<td>424</td>
<td>91</td>
<td>0</td>
<td>300.000</td>
<td>30</td>
<td>17</td>
</tr>
<tr>
<td>1980</td>
<td>515</td>
<td>66</td>
<td>1</td>
<td>307.525</td>
<td>21</td>
<td>19</td>
</tr>
<tr>
<td>1981</td>
<td>578</td>
<td>36</td>
<td>4</td>
<td>314.658</td>
<td>11</td>
<td>19</td>
</tr>
<tr>
<td>1982</td>
<td>606</td>
<td>45</td>
<td>20</td>
<td>321.789</td>
<td>14</td>
<td>19</td>
</tr>
<tr>
<td>1983</td>
<td>627</td>
<td>60</td>
<td>13</td>
<td>328.920</td>
<td>18</td>
<td>20</td>
</tr>
<tr>
<td>1984</td>
<td>672</td>
<td>95</td>
<td>4</td>
<td>336.051</td>
<td>28</td>
<td>22</td>
</tr>
<tr>
<td>1985</td>
<td>753</td>
<td>50</td>
<td>6</td>
<td>343.182</td>
<td>14</td>
<td>23</td>
</tr>
<tr>
<td>1986</td>
<td>787</td>
<td>42</td>
<td>1</td>
<td>350.313</td>
<td>12</td>
<td>22</td>
</tr>
<tr>
<td>TOTAL</td>
<td>534</td>
<td>49</td>
<td>8</td>
<td>99</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> number of leprosy cases in the active file in the end of the previous year
<sup>b</sup> number of new cases registered
<sup>c</sup> number of cases excluded from the active file because of cure, death, locality transfer or statistical criteria
<sup>d</sup> population estimate for the year
<table>
<thead>
<tr>
<th></th>
<th>Period I</th>
<th>Period II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Health Advise</td>
<td>2.2 (07)</td>
<td>26.8 (84)</td>
</tr>
<tr>
<td>Self-Decision</td>
<td>11.1 (35)</td>
<td>48.4 (152)</td>
</tr>
<tr>
<td>Test of Contact</td>
<td>3.5 (251)</td>
<td>10.2 (42)</td>
</tr>
<tr>
<td>Mass Survey</td>
<td>3.2 (11)</td>
<td>1.3 (32)</td>
</tr>
<tr>
<td>Not Determinated</td>
<td>79.9 (10)</td>
<td>13.4 (04)</td>
</tr>
</tbody>
</table>
### Figure 2

<table>
<thead>
<tr>
<th></th>
<th>Period I</th>
<th>Period II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lepromatous</td>
<td>60.8 (191)</td>
<td>33.8 (106)</td>
</tr>
<tr>
<td>Dimorphous</td>
<td>1.9 (6)</td>
<td>38.5 (121)</td>
</tr>
<tr>
<td>Tuberculoid</td>
<td>23.2 (73)</td>
<td>15.9 (50)</td>
</tr>
<tr>
<td>Indeterminate</td>
<td>14 (44)</td>
<td>11.8 (37)</td>
</tr>
</tbody>
</table>
**Fig. 3**

<table>
<thead>
<tr>
<th>Age of detection</th>
<th>Period I</th>
<th>Period II</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 to 14</td>
<td>3.2 (10)</td>
<td>7 (22)</td>
</tr>
<tr>
<td>15 to 29</td>
<td>27.1 (85)</td>
<td>25.2 (79)</td>
</tr>
<tr>
<td>30 to 44</td>
<td>36.3 (114)</td>
<td>30.9 (97)</td>
</tr>
<tr>
<td>45 to 59</td>
<td>26.1 (82)</td>
<td>24.8 (78)</td>
</tr>
<tr>
<td>60 to 74</td>
<td>6.1 (19)</td>
<td>10.2 (32)</td>
</tr>
<tr>
<td>75 to 89</td>
<td>1.3 (04)</td>
<td>1.9 (06)</td>
</tr>
</tbody>
</table>
Efeito do EXTRAÇÃO DE AÇO NAS SUMIN "MACROFAGOS"

Artigos do INMUNOLOGIA EXPERIMENTAL MUNHA

Procedimentos Experimentais Munha

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Procedimentos Experimentais Munha

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La correlación entre la ingesta de grasas saturadas y la enfermedad cardiovascular es un tema de investigación en curso. Los estudios han mostrado que una alta ingesta de grasas saturadas puede aumentar el riesgo de enfermedades cardiovasculares, incluyendo enfermedades del corazón y enfermedades cerebrovasculares. Además, se ha asociado con la presencia de factores de riesgo como el sobrepeso y la obesidad.

En el caso de la enfermedad del corazón, las grasas saturadas pueden aumentar el colesterol malo (LDL) y disminuir el colesterol bueno (HDL), lo que puede llevar a la formación de placas en las arterias, obstruyendo el flujo de sangre. Por otro lado, el consumo de grasas saludables como las grasas mono-insaturadas y poli-insaturadas puede reducir el riesgo de enfermedades cardiovasculares.

En resumen, es importante controlar la ingesta de grasas saturadas para reducir el riesgo de enfermedades cardiovasculares. Las recomiendaciones incluyen limitar la ingesta de alimentos procesados, golosinas, y productos lácteos y carne procesados que generalmente contienen altos niveles de grasas saturadas.
INTERDISCIPLINA EN ATLAS.

COMUNICACIONES.

DEPORTES Y RECREO.

PROYECTO DE EDUCACIÓN FÍSICA.

INVESTIGACIÓN DE PROPORCIONALIDADES.

VERIFICACIÓN DE NIGOSDINA EN MOVIMIENTO.

USO DA NIGOSDINA NA PREVENÇÃO DE HIPERTROFIA.

MOBILIZAÇÃO DO SISTEMA MONITORINGFÓRMOFICO NA

DEFICIENTE DE ÁGUA EXPERIMENTAL NA TROMBOSICURA.
DETECTION OF INTRACYTOPLASMIC CYTOKINES IN PERIPHERAL BLOOD CELLS OF AMERICAN TEGUMENTARY LEISHMANIASIS (ATL) PATIENTS BY FLOW CYTOMETRY.

It is well established that T cell cytokine phenotypes play an important role in regulating the immune response in leishmaniasis. The production of cytokines by activated peripheral blood cells has been studied either at mRNA or protein level. Nevertheless, these methods do not permit the analysis of the T cell cytokine profile at a single cell level, unless performing the cell cloning which can influence the phenotype of T cell cytokine production.

A flow cytometric method was described by Andersson et al. (J.Immunol.Methods, 1988) for detection of cytokines at a single cell level. However, the use of flow cytometry to quantitate intracytoplasmic cytokines in T cells from ATL patients has not been reported yet. In this work we show our preliminary results of a flow cytometric method to detect intracellular IL-2, IL-4 and IFN-γ produced by T cells of ATL patients upon in vitro stimulation with Leishmania braziliensis antigen (LbAg). The method consists of cell fixation with paraformaldehyde, permeabilization with saponin, staining with monoclonal antibodies anti-IL-2, anti-IL-4 and anti-IFNγ conjugated with FITC and analysis on a flow cytometer.

The kinetic analysis of intracellular IL-2, IL-4 and IFN-γ suggests that the best time point to detect these cytokines is 16 hours after cell stimulation with LbAg in culture. In order to assure that these cytokines producing cells were specific to L braziliensis, we have performed cultures of unstimulated cells, and these results were subtracted from those of the stimulated cultures. We have also tested the use of Brefeldin A, which interrupts intracellular transport processes leading to an accumulation of the cytokine in the cell, to increase the specific staining. The method described above is a rapid, easy and semiquantitative assay and still permits the simultaneous determination of cytokine production and the cell population. Therefore, this method can be used to evaluate the functional role of the T cell subsets in leishmaniasis as well as in other diseases.

INTRAPERITONEAL INOCULATION OF MYCOBACTERIUM BOVIS BCG INDUCES HIGH FREQUENCIES OF NK1-INDEPENDENT EARLY IFN-γ-SECRETING CELLS.
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Interferon-γ (IFN-γ) production is considered important for protective immunity against intracellular bacterial infections and both natural killer (NK) cells and T cells are major IFN-γ producers. It was proposed that different intracellular bacteria, such as Listeria monocytogenes (NK cell activator) and M. bovis (NK cell non-activator) differ in their capacity to stimulate IFN-γ-producing NK1.1+ cells (Teixeira, H.C.; Munk, M.E.; Kaulmann, S.H.E., Immunol. Letters, 46, 1995). In the present study, we used the ELISPOT assay to determine frequencies of IFN-γ producers in the peritoneal cavity of susceptible mice during M. bovis BCG infection. The results show that numbers of spontaneous and antigen (BCG) induced IFN-γ-producing peritoneal cells remain notably high in C57Bl/6 mice (f=1/1000) during the first 2 weeks after intraperitoneal M. bovis BCG infection decreasing thereafter. Moreover, in vivo depletion of NK1.1+ cells with PK136 MAb did not modify IFN-γ production by peritoneal cells during infection. In addition, early IFN-γ production was also observed in BALB/c mice (NK1.1-) during i.p. M. bovis BCG infection. Our findings indicate that i.p. inoculation of M. bovis (BCG) induces high frequencies of NK1-independent early IFN-γ-secreting cells. This early IFN-γ production seems to be important for local macrophage activation and protection. The cellular source involved in this early IFN-γ production still remains to be determined.

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INTRODUÇÃO: A clofazimina é uma droga utilizada no tratamento da hanseníase e da tuberculose, sendo sua atividade quimioterapêutica associada a ações imunomoduladora e antimicrobiana. Sendo a produção de Interferon-gama (IFN-γ) e a ativação de macrófagos comumente associados à proteção contra a infecção por micobactérias, estudamos a influência do tratamento in vivo com clofazimina sobre esses parâmetros imunológicos, em animais infectados com o Mycobacterium bovis (BCG). MATERIAL E MÉTODOS: Diferentes doses de BCG foram usadas (0,01mg – 0,1mg – 1mg, cepa Monreau, Fundação Ataulpho de Paiva, RJ) para estimular a produção de IFN-γ por células peritoneais de camundongos susceptíveis C57Bl/6, tratados ou não tratados com clofazimina (100ug/cam. cada dois dias). A ativação de macrófagos foi avaliada pelo aumento da produção de peróxido de hidrogênio (H₂O₂) e óxido nitrico (NO). RESULTADOS: Nossos resultados mostram que a dose de 0,1 mg de BCG induziu ótima produção de H₂O₂ e NO, e os elevados índices de ativação macrofágica foram correlacionados com o aumento do número de células secretoras de IFN-γ, avaliadas pelo método de ELISPOT. Foi também demonstrado que a produção de IFN-γ, experimental ou induzida pela adição do Ag (BCG) nas culturas, foi maior durante as duas primeiras semanas de infecção, decrescendo posteriormente. O tratamento in vivo com a clofazimina modificou a cinética da produção de citocinas e a ativação de macrófagos, induzindo uma diminuição da produção de citocinas e ativação macrofágica durante as 2 primeiras semanas de infecção. Em conjunto, os resultados mostram que existe uma correlação entre a ativação de macrófagos e a produção de IFN-γ induzida pelo BCG, e sugerem que a associação entre a produção de IFN-γ induzida pelo M. bovis e o tratamento com a clofazimina podem ter um efeito sinérgico no clearance bacteriano.

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