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Cytokine responses to the native and recombinant forms of the major surface glycoprotein of *Pneumocystis carinii*

S. A. THEUS*†, A. G. SMULIAN*†, D. W. SULLIVAN*† & P. D. WALZER*† *Cincinnati Veterans Affairs Medical Centre, Division of Infectious Diseases, and †Department of Internal Medicine, University of Cincinnati College of Medicine, Cincinnati, OH, USA

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SUMMARY

Pneumocystis carinii is a major opportunistic pathogen and leading cause of morbidity in patients with AIDS. The major surface glycoprotein (MSG) of *P. carinii*, represented by a family of related proteins encoded by unique genes, is highly immunogenic and contains T cell-protective epitopes. We undertook the present study to define the CD4 T helper (Th) response by cytokine secretion to native MSG and a recombinant form of the protein, MSG-B. Spleen cells were collected from Lewis rats and restimulated with both native MSG and MSG-B. Within 24 h, the CD4 cells secreted high levels of interferon-gamma (IFN-γ) in response to both types of antigen, indicative of a Th1 response; however, after 72 h of incubation, only the native MSG stimulated secretion of IL-4 (Th2 response) from the cells. We then investigated whether the presence of IL-4 could alter the predominant Th1 phenotype by the CD4 cells in response to MSG and MSG-B. Cells cultured with native MSG and IL-4 produced low levels of IFN-γ and elevated levels of IL-4. Interestingly, cells incubated with MSG-B and IL-4 reduced production of IFN-γ, but were not stimulated to produce increased levels of IL-4. The presence of anti-IFN-γ antibody in the MSG- or MSG-B-stimulated cultures did not effect the expression of IFN-γ mRNA, suggesting that the generation of Th1 cells in response to MSG or MSG-B was not dependent on IFN-γ. We conclude that native MSG, which contains multiple forms of this antigen, and recombinant MSG elicit different cytokine responses *in vitro*. These data are not only important to studies of MSG, but may also be relevant to the role of MSG in the immunopathogenesis of *P.carinii* infection *in vivo*.

Keywords cytokine pneumonia AIDS

INTRODUCTION

Pneumocystis carinii is a major opportunistic pathogen and a leading cause of morbidity in patients with AIDS. Cell-mediated immunity is believed to be the major mechanism by which the immune-competent host controls infection [1,2]. A variety of cell-mediated functions, including the production of cytokines and different T cell subsets, has been implicated in this protective role [3–7]. Further investigation into protective cellular immune responses has focused attention on the major surface glycoprotein (MSG) of *P. carinii*. Through molecular studies, it has been demonstrated that MSG represents a group of proteins encoded by multiple genes [8–11]. Numerous unique MSG cDNAs have been identified, establishing that multiple mRNAs are transcribed within a population of organisms [8,9,11]. In rat-derived *P. carinii*, MSG expression appears to be regulated. Individual *P. carinii* in a population express numerous MSG genes, but not all genes are expressed [10,11]. MSG is highly immunogenic and elicits both humoral and cellular immune responses [1,2]. Adoptive transfer studies have shown that rat spleen CD4 cells pulsed with MSG proliferate and confer protection against pneumocystosis [7].

The induction of an appropriate CD4 T helper cell response is important in determining a successful immune reaction to pathogens, because these, in general, are best controlled by either a predominately cellular (Th1) or humoral (Th2) immune response [12]. Th cells and the cytokines they synthesize play a crucial role in the responses of B cells, other T cells, and natural killer (NK) cells. Th1 cells are effective mediators of DTH and secrete IL-2 and interferon-gamma (IFN-γ), which are the principal effectors of cell-mediated immunity [13]. In contrast, Th2 cells do not transfer DTH, but cooperate with B cells to generate vigorous IgM, IgG1, IgA and IgE responses [14]. Th2 cells also produce IL-4, IL-5, IL-6 and IL-10, but not IFN-γ or IL-2 [13,15,16]. Cross-regulation of these two T cell subsets, in the form of either activation or suppression, is intricately controlled [17,18]. The presence of IL-4 during priming results in a Th2 phenotype. Conversely, IFN-γ

Correspondence: Peter D. Walzer MD, Research Service, 151 Veterans Affairs Medical Centre, 3200 Vine Street, Cincinnati, OH 45220, USA.

down-regulates Th2 cells, resulting in a predominant Th1 phenotype. Thus it is possible that even after initial induction, the presence of Th2 cytokines can influence the physiologic and autoimmune functions of Th1 effectors.

We undertook the present study to clarify the cytokine profile of rat splenic T cells in response to native MSG and a recombinant form of the protein. Rats are the principal animal model for *P. carinii* infection; although fewer cytokines can be measured here than in mice or humans, these cytokines distinguish Th1 from Th2 phenotypes. Our data suggest that native MSG contains different forms of the protein, which are capable of eliciting both a Th1 and Th2 response from CD4 cells.

MATERIALS AND METHODS

Animals

Adult male Lewis rats (viral antibody-positive) were bred and maintained under standard husbandry conditions in open-topped cages in a conventional animal room at the Veterans Affairs Medical Centre at Cincinnati, Ohio, as described [2]. These animals had ample environmental exposure to *P. carinii*, which was confirmed by the presence of antibodies to MSG and other antigens by immunoblot analysis.

Antigen purification

The MSG was isolated as previously described [2]. Briefly, *P. carinii* organisms were isolated from the lungs of corticosteroid-treated Lewis rats following 8–10 weeks of immunosuppression. Lungs were removed, minced, and ground through a 60-mesh wire screen in PBS. Erythrocytes were lysed by exposure to 0·85% ammonium chloride, centrifuged, and the pellet washed twice in PBS. Samples of homogenates were streaked on Mueller-Hinton and Sabouraud dextrose agar plates for detection of any bacterial or fungal contamination. Specimens with no detectable contamination were pooled and digested with Zymolyase T100 (ICN Biomedicals, Inc., Costa Mesa, CA) for 30 min at room temperature prior to differential centrifugation. MSG was purified from the 100 000 ***g*** supernatant by high performance liquid chromatography (HPLC) on the basis of size by using a Macrosphere GPC 150 column (Alltech Associates Inc., Deerfield, IL) under isocratic conditions in 0·1 M KH_2PO_4 pH 7·0, 0·2 M NaCl, as previously described [2]. MSG migrated as a band with a molecular weight of 120 kD on SDS–PAGE under reducing conditions. The purity of the preparation was shown by coomassie blue staining, reactivity with MSG-specific MoAbs, and by the absence of endotoxin, as verified by the *Limulus* amebocyte assay (< 0·125 U/ml; Whittaker M.A. Bioproducts, Walkersville, MD).

Recombinant MSG expression and purification

A λ clone, rp 3–1, containing three MSG gene sequences, was generously provided by Dr James Stringer (University of Cincinnati) [10]. A single MSG gene, MSG-B, was subcloned and expressed using the pMAL expression system (New England Biolabs, Beverly, MA). A 3-kb *Ava* I-*Hind* III fragment of a single MSG gene isolated from rat-derived *P. carinii* was cloned into pMAL C2 between the *Sal* I and *Hind* III sites downstream of the maltose-binding protein (MBP) gene, malE. The construct, pMAL/MSG-B, encoded a 1065 amino acid protein which lacks only the 40 NH terminus amino acids. Recombinant MSG was produced as a fusion with MBP and purified by affinity chromatography over an amylose resin column. Following column purification, fractions were analysed for the presence of recombinant protein by SDS–PAGE and immunoblot analysis.

Cell purification and culture

Spleen cell suspensions were prepared from individual animals essentially as described [2]. Briefly, spleens were excised from male Lewis rats, placed in PBS without divalent cations, and then passaged through a stainless steel screen to obtain a single-cell suspension. Erythrocytes in the cell preparation were lysed in 0·85% ammonium chloride. The cells were washed, enumerated with a haemocytometer, and viability determined by trypan blue exclusion. Cells were cultured at 10^6/ml in RPMI 1640 containing 2 mM L-glutamine, 100 U penicillin/ml, 100 μg streptomycin/ml, 5×10^{-5} M β-mercaptoethanol, and 10% heat-inactivated fetal calf serum (FCS) (RPMI complete). Only cells with ≥ 90% viability were used. For purified T cell populations, macrophages were removed by adherence for 2 h in RPMI complete. B cells were then effectively removed from the non-adherent cell population by separation using a Cellect Rat T Cell immunocolumn (BioTex Labs, Edmonton, Canada) according to the manufacturer's instructions. The non-adherent cell population was pelleted, washed twice in pyrogen-free PBS, and cell concentration adjusted to 5×10^7 cells/ml before loading onto the column. Purified T cells were eluted in the flow-through from the column. Samples were then analysed for purity by flow microfluorometry as previously described [7]. The purity of the T cell population was > 97% with < 1% B cells with a viability of ≥ 95% by trypan blue exclusion.

Preparation of antigen-presenting cells

T cell-depleted splenocytes were prepared from the spleens of *P. carinii*-naive Lewis rats by removing the anti-CD4 and anti-CD8 staining cells by using negative selection with Cellect immunocolumn (BioTex Labs). The resulting population was >98% CD4- and CD8-negative by flow microfluorometry. Antigen-presenting cells (APC), 10^6/ml, were incubated with 25 μg mitomycin (Sigma, St Louis, MO) before culture.

Bioassay of cytokines

Purified T cells (10^6/ml) with and without APC (10^6/ml), were incubated for 24–72 h in the presence and absence of native (10 μg/ml), recombinant MSG (10 μg/ml) or MBP (10 μg/ml). The concentrations used were found to be optimal following a dose–response curve (0·1 μg/ml to 500 μg/ml) of lymphocyte proliferative response and IL-2 secretion. In some cultures, anti-cytokine MoAbs or cytokines were added during the primary culture period. Supernatants were harvested, filtered through a 0·45 μm pore size filter, and stored frozen at −70°C until assayed for cytokine levels. The presence of IFN-γ was determined by a rat IFN-γ ELISA kit (GIBCO, Gaithersburg, MD) as previously described [19]. IL-4 activity in the supernatants was measured by the ability to sustain growth of HT2 cells (American Type Culture Collection, Rockville, MD). Serial two-fold dilutions of the samples were placed in triplicate wells of a 96-well microtitre plate along with 5×10^3 HT2 cells. Cultures were incubated at 37°C in 5% CO_2 for 18 h, pulsed with 1 μCi of ^{3}H-thymidine per well (2 Ci/mmol; New England Nuclear, Boston, MA), and harvested 6 h later. Units of IL-4 per ml were calculated from the 50% end point of a reference curve constructed from a commercial IL-4 preparation free of lectin (Genzyme, Cambridge, MA). The samples were tested in the presence of rat anti-IL-4 MoAb (PharMingen, San Diego, CA) or rat anti-IL-2 MoAb (PharMingen) so that residual activity could

be attributed to the presence of IL-2 or IL-4, respectively. Controls included recombinant rat IL-2 and IL-4 assayed in the presence of anti-IL-2, anti-IL-4, or both MoAbs.

Preparation of cDNA

Total RNA was isolated from enriched T cell suspensions incubated in RPMI complete with or without MSG (10 μg/ml) for various time points by using RNAzol (Tel-Test Inc., Friendswood, TX). Briefly, cells were lysed in 2 ml of RNAzol per 10^7 cells by passage of the lysate through a pipette. Chloroform (200 μl) was added to each 2-ml homogenate. Samples were placed on ice for 15 min before centrifugation at 12 000 ***g***, 4°C for 15 min. The aqueous phase was collected and precipitated. The quantity and integrity of the RNA was routinely tested by absorbance at 260 nm and by agarose gel electrophoresis. RNA (4·0 μg) from each sample was reversed transcribed in 50 mM Tris–Cl pH 8·0, 50 mM KCl, 5 mM $MgCl_2$ (RT buffer) containing 5 μg of oligo dT (12–18 mer), 20 U AMV reverse transcriptase, 0·5 mM each dNTP, 0·1 mg/ml bovine serum albumin (BSA), 10 mM DTT, and 40 U Rnase Inhibitor. Tubes were incubated for 20 min at room temperature followed by 30 min at 42°C before storage at −70°C.

Polymerase chain reaction amplification

Polymerase chain reaction (PCR) primers for rat IL-4 were designed from the published rat IL-4 cDNA sequence [20, 21]. The β-actin and IFN-γ primers were commercially available (Clontech, Palo Alto, CA). The sense oligonucleotide primer used for IL-4 was 5′ TAG TAG TCT AGA GAG GCC ACC ATG GGT CTC AGC CCC CAC 3′ and the anti-sense primer was 5′ T AGT AGG GAT CCG TTA GGA CAT GGA AGT GC 3′, which amplify a 478 base pair IL-4 fragment; the sense primer used for IFN-γ was 5′ ATC TGG AGG AAC TGG CAA AAG GAC G 3′ and the anti-sense primer was 5′ CCT TAG GCT AGA TTC TGG TGA CAG C 3′, which amplify a 288 base pair IFN-γ fragment; and the sense primer used for actin was 5′ AGA AGA GCT ATG AGC TGC CTG ACG 3′ and the anti-sense primer was 5′ CTT CTG CAT CCT GTC AGC CTA CG 3′, which produce a 236 base pair product derived from the rat β-actin sequence. The β-actin primers were used as a control for both reverse transcription and the PCR reaction itself, and for comparing the amount of products from samples obtained with the same primer. PCR reactions consisted of 5 μl cDNA in RT buffer containing 40 pM each primer, 200 μM dNTP, 2·5 mM $MgCl_2$, and 1·2 U AmpliTaq polymerase (Perkin-Elmer, Hayword, CA). Reaction mixtures were overlayed with mineral oil and transferred to a thermocycler (Perkin-Elmer) with the following parameters: 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C for 35 cycles. The PCR fragments were analysed by 1·5% agarose gel electrophoresis and visualized by ethidium bromide staining. If levels were too low for detection by ethidium bromide visualization, slot blot analysis was performed. Fragments were transferred onto nylon membranes (Hybond N), the membranes baked at 80°C for 1 h, and hybridized to a γ-^{32}P-5′ end-labelled primer (IL-4, IFN-γ, or β-actin). The amounts of amplified products were determined from the densities of the corresponding bands in the autoradiograms using a densitometer (Hoefer Scientific, San Francisco, CA). The results were arbitrarily normalized to the signals of the β-actin cDNA, which were amplified in the same tubes for cytokine cDNA amplification. PCR-assisted mRNA amplification was repeated at least twice for at least two separately prepared cDNA samples for each experiment. Data shown are representative of at least three different experiments.

Statistical analysis

The two-tailed Student's *t*-test or analysis of variance, followed by a multicomparisons test, was used to determine if differences existed between data sets, as appropriate, by INSTAT (GraphPad Software for Science, San Diego, CA). Significance was accepted when $P < 0{\cdot}05$.

RESULTS

Patterns of cytokine production in P. carinii*-exposed Lewis rats to native MSG and one form of the recombinant MSG*

To determine if Th1- and Th2-like cytokine secretion patterns could be detected in response to the native *P. carinii* antigen MSG and one form of the recombinant MSG (MSG-B), CD4 cells were purified from the spleens of *P. carinii*-exposed Lewis rats. The cells were then cultured with MSG or MSG-B and APC. Mitomycin-treated splenic APC from naive animals were used at a concentration previously determined to give optimal cytokine production. Supernatants were collected at 24, 48 and 72 h for cytokine measurements. IFN-γ was measured to indicate Th1-like development and IL-4 was measured as an indicator of Th2-like development.

As shown in Fig. 1a, the production of IFN-γ was significantly increased following culture with native MSG when compared with cells incubated in medium alone ($P < 0{\cdot}01$); this suggested that the cytokine secretion observed was due to the antigen preparation and that the response was Th1-like. In addition, high levels of IFN-γ were also detected in cultures incubated with the recombinant MSG within 24 h. These levels remained elevated over the 72-h incubation period. To investigate whether the native MSG antigen and MSG-B could also induce a Th2 response in these cells, supernatants were measured for IL-4 content. Figure 1b demonstrates that these cells, when stimulated with both phorbol myristate acetate (PMA; 10 ng/ml) and ionomycin (400 ng/ml), secreted levels of IL-4 which were significant within 24 h compared with cells incubated in media ($P < 0{\cdot}01$). In contrast, 72 h of incubation were required with the native MSG preparation for detectable and significant levels of IL-4 ($P < 0{\cdot}01$) to be present in the supernatants. No detectable levels of IL-4 were found at any time point in the supernatants in response to MSG-B. No detectable levels of either cytokine were observed following incubation with MBP. Increasing the concentration of MSG-B by 50-fold had no effect on the secretion of IL-4 from the CD4 cells (data not shown).

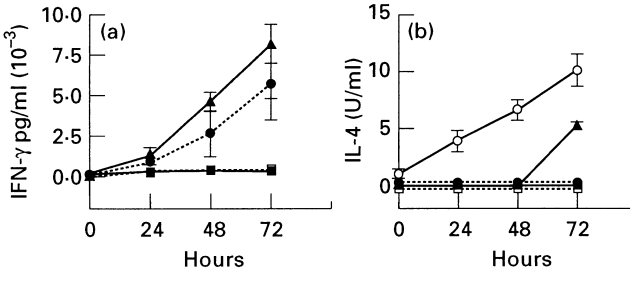

Fig. 1. *In vitro* production of IFN-γ (a) and IL-4 (b) following induction with native major surface glycoprotein (MSG) or MSG-B. CD4 cells were isolated from *Pneumocystis carinii*-exposed Lewis rats and then cultured with antigen-presenting cells in the presence of medium (■), native MSG (▲), MSG-B (●), maltose-binding protein (□), or phorbol myristate acetate (PMA) and ionomycin (○) (b) for 24 h, 48 h or 72 h. Supernatants were then evaluated for cytokine production. Values represent the means ± s.e.m. of three separate experiments.

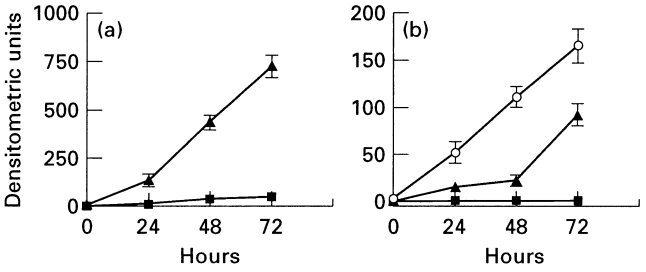

Fig. 2. Expression of IFN-γ (a) and IL-4 (b) mRNA in CD4 cells following induction with native major surface glycoprotein (MSG) or MSG-B (b). CD4 cells were isolated from *Pneumocystis carinii*-exposed Lewis rats and then cultured with antigen-presenting cells in the presence of medium alone (■), native MSG (▲), MSG-B (●), or phorbol myristate acetate (PMA) and ionomycin (○) for 24 h, 48 h or 72 h before assessment of mRNA levels by reverse transcriptase-polymerase chain reaction (RT-PCR). Values represent means ± s.e.m. of three independent experiments.

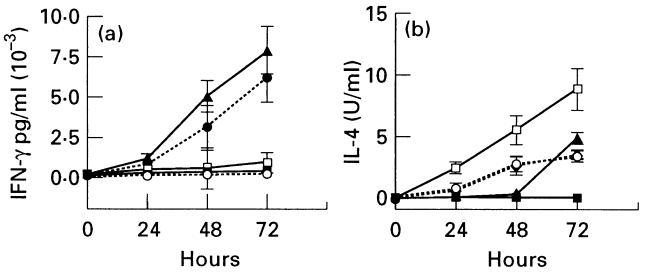

Fig. 3. Conversion of a major surface glycoprotein (MSG)-stimulated Th1 cytokine production pattern to a Th2 pattern requires the presence of IL-4. CD4 cells were isolated from *Pneumocystis carinii*-exposed Lewis rats and incubated with antigen-presenting cells in the presence of medium (■), MSG (▲), MSG-B (●), MSG-B and IL-4 (○), or MSG and IL-4 (□) for 24, 48, or 72 h. Supernatants were collected and assessed for IFN-γ (a) or IL-4 (b) expression. Values represent the means ± s.e.m. of three separate experiments. ◆, CD4 cells cultured with IL-4.

To clarify these findings at the level of mRNA, Th1 and Th2 cytokine expression were assessed using the RT-PCR assay described. Spleen cells were isolated from *P. carinii*-exposed Lewis rats and incubated for 24 h, 48 h and 72 h with and without MSG. As seen in Fig. 2a, low, yet detectable, levels of IFN-γ mRNA were present at all time points under all conditions. However, there was significantly more IFN-γ mRNA present, starting at the 24 h time point, in cells incubated with MSG compared with those incubated in medium alone ($P < 0{\cdot}01$). These findings correlate with the appearance of IFN-γ in the supernatants of these culture conditions.

Figure 2b demonstrates the levels for IL-4 mRNA. These experiments were performed to investigate the possibility that the bioassay was not sensitive enough to detect low levels of IL-4 produced in response to MSG-B. As shown, no IL-4 mRNA was detected at any time point in the cells incubated in medium alone. Significant levels of IL-4 mRNA were present in the cells incubated with PMA and ionomycin within 24 h ($P < 0{\cdot}001$). The level of mRNA increased proportionately over the incubation time with the appearance of the protein in the supernatant. Within 24 h of incubation of the cells with MSG, IL-4 mRNA was detected. However, the levels of IL-4 mRNA isolated from cells incubated with MSG were only significantly greater than the levels demonstrated from cells incubated in medium alone following 72 h of incubation ($P < 0{\cdot}01$). The level of IL-4 mRNA in these samples correlated with the presence of the protein in the supernatants. There were no detectable levels of IL-4 mRNA present in the cultures incubated with MSG-B. Within the 72-h incubation period, no detectable levels of mRNA for either cytokine were present following incubation with MBP (data not shown). These data demonstrate that the cells are capable of making IL-4 mRNA and the protein, but not in response to MSG-B.

Cytokine secretion patterns of MSG-specific cells can be changed in vitro

It has been shown that the products of Th1 and Th2 cells may negatively regulate the functions of each other [17]. In humans, there is evidence that IFN-γ and IL-4 are important regulators of the development of the Th1 or Th2 phenotype [22]. To investigate whether the presence of a specific cytokine, IL-4, could alter the predominant phenotype displayed by the CD4 cells in response to MSG, these cells were incubated in the presence of APC, MSG or MSG-B and IL-4 (20 ng/ml). The production of IFN-γ by MSG-stimulated CD4 cells was significantly reduced within 24 h by culturing the cells in the presence of IL-4 (Fig. 3a). Incubation of the cells with MSG-B and IL-4 resulted in the disappearance of IFN-γ in the supernatants.

To measure IL-4 production in the supernatants from these cultures, a bioassay was performed using the IL-4-dependent cell line, HT-2, as described in Materials and Methods. Titered amounts of anti-IL-4 antibody were added to the supernatants until the activity of the exogenously added IL-4 was neutralized in the bioassay. The values shown represent IL-4 secreted by the CD4 cells in excess to the exogenously added IL-4. Figure 3b shows that CD4 cells incubated in the presence of IL-4 alone secreted significant levels of the protein within 24 h of incubation, compared with those incubated in medium alone ($P < 0{\cdot}01$). When cells were cultured in the presence of MSG and IL-4, significantly

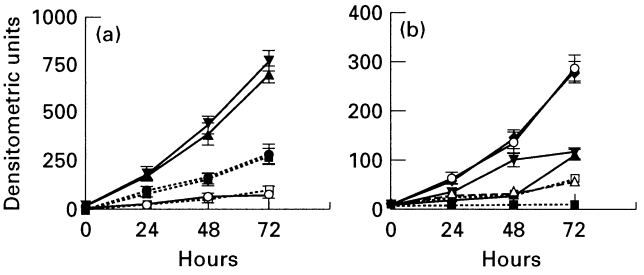

Fig. 4. (a) Expression of IFN-γ mRNA was not effected by the presence of anti-IFN-γ in the major surface glycoprotein (MSG)-stimulated cultures. CD4 cells were isolated from *Pneumocystis carinii*-exposed Lewis rats and cultured with antigen-presenting cells (APC) in the presence of MSG (▲), MSG-B (●), MSG and anti-IFN-γ (▼), MSG-B and anti-IFN-γ (■), MSG, anti-IFN-γ and IL-4 (○), MSG-B, anti-IFN-γ, and IL-4 (□), MSG and IL-4 (◆), or MSG-B and IL-4 (△) for 24 h, 48 h and 72 h before assessment of mRNA levels by reverse transcriptase-polymerase chain reaction (RT-PCR). Values represent the means ± s.e.m. of three separate experiments. (b) Expression of IL-4 mRNA in MSG- or MSG-B-stimulated cultures. CD4 cells were isolated from *P. carinii*-exposed Lewis rats and cultured with APC in the presence of MSG (▲), MSG-B (●), MSG and anti-IFN-γ (▼), MSG-B and anti-IFN-γ (■), MSG, anti-IFN-γ and IL-4 (○), MSG-B, anti-IFN-γ, and IL-4 (□), MSG and IL-4 (◆), or MSG-B and IL-4 (△) for 24 h, 48 h and 72 h before assessment of mRNA levels by RT-PCR. Values represent the means ± s.e.m. of three separate experiments.

greater levels of IL-4 were detected in the supernatants above the exogenously added concentration within 24 h of stimulation. These levels continued to elevate with increasing incubation time. When these cells were incubated with MSG-B and IL-4, no additional IL-4 secretion was noted, suggesting that the observed increase in IL-4 secretion was due solely to the presence of exogenous IL-4 in the supernatant and not to the presence of MSG-B.

The IL-4 mRNA coincides with the protein data. Although there were no differences observed in the level of β-actin mRNA at any time point, there was a steady increase in the level of IL-4 mRNA over the 72-h incubation period in the presence of MSG and IL-4. Similar to the protein results, an increase in IL-4 mRNA was observed only after 72 h following incubation with MSG (data not shown). No additional increase in IL-4 mRNA was observed following incubation with MSG-B and IL-4 (data not shown). When comparing the addition of IL-4 alone or IL-4 and MSG-B to the CD4 cells with MSG and IL-4 to the CD4 cells, there was a 2·5–5-fold increase in the observed level of secreted IL-4. Although incubation of the CD4 cells with MSG did stimulate low levels of Th2-secreting CD4 cells, these cells became the dominant phenotype when incubated with exogenous IL-4. These data suggest that IL-4 was able to maintain the Th2 phenotype over the incubation period.

Neutralization of IFN-γ does not affect IL-4

Several lines of evidence suggest that IFN-γ is necessary for the induction and maintenance of the Th1 response, and that this response would inhibit Th2 cells [15,17]. To examine whether neutralization of IFN-γ could result in a Th2 response, endogenous IFN-γ was blocked in the MSG-stimulated or MSG-B-stimulated CD4 cultures by rat anti-IFN-γ MoAb. As shown in Fig. 4a, the presence of anti-IFN-γ in the MSG-stimulated cultures did not effect the expression of IFN-γ mRNA. This same result was seen when the CD4 cells were incubated in the presence of anti-IFN-γ and MSG-B. Upon incubation of the MSG- or MSG-B-stimulated CD4 cells with IL-4 and anti-IFN-γ, there was a significant decrease in IFN-γ mRNA ($P < 0{\cdot}01$).

Figure 4b demonstrates that there was a slight, yet not significant, increase in IL-4 mRNA at 48 h in the cultures incubated with MSG and anti-IFN-γ compared with those incubated with MSG alone. However, by 72 h of incubation, there was no difference in the levels of IL-4 mRNA between these two groups. Incubation of the cells in the presence of MSG, anti-IFN-γ, and IL-4 resulted in a significant increase in IL-4 mRNA ($P < 0{\cdot}01$), similar to the protein results obtained in the bioassay and the RNA results following incubation with MSG and IL-4 alone. IL-4 mRNA was not detected at any time point in the cultures incubated with either MSG-B or MSG-B and anti-IFN-γ. As observed previously, CD4 cells in the presence of IL-4 had a significant increase in levels of IL-4 mRNA within 24 h of incubation regardless of the presence of anti-IFN-γ. Levels of β-actin expression showed no significant differences between cultures. These results suggest that the generation of Th1 cells in response to MSG is not dependent on IFN-γ; yet, the results also suggest that the conversion from an MSG-stimulated Th1 response to a Th2 response is IL-4-dependent.

DISCUSSION

Patterns of susceptibility and resistance to protozoan, helminth, and fungal infections in experimental models have been associated with distinct, mutually exclusive profiles of cytokine production [13,15–18,22]. An example is mice genetically susceptible to *Leishmania major*, which have relatively high levels of IL-4 and low levels of IFN-γ, whereas the genetically resistant strains have the opposite cytokine pattern [18]. The dual nature of these responses implies that protective immunity depends on the activation of the appropriate T helper subset that can be down-regulated by inhibitory cytokines produced by the other subset. The ability to control the emerging Th phenotype following exposure to a specific antigen offers the potential to induce a suitable response to a pathogen with minimum pathology.

The present experiments address the question of whether cytokine production by Th1 and Th2 populations can be changed once a population has been established to a specific antigen, MSG. In these experiments, CD4 cells from animals with previous exposure to *P. carinii* were used. Upon restimulation of these cells with MSG, a predominately Th1-like cytokine pattern was established; however, IL-4, a Th2 cytokine, was also detected. The data suggest that within this population of responding CD4 cells there is not a complete separation into a Th1 or Th2 phenotype.

In contrast, a recombinant form of MSG, MSG-B, appeared to induce only a Th1 response upon restimulation of the CD4 cell population. Since the IFN-γ and IL-4 cytokine measurements were performed early in culture and the same population of APC was used, the cause for the observed difference between native MSG- and MSG-B-stimulated cytokine secretion by the CD4 cells was not due to the cytokines present in culture, nor to the type of APC. It is possible that IL-4 was not detected due to the sensitivity of the bioassay. Yet, examination of the IL-4 mRNA within the T cell population in response to MSG-B demonstrated that this was not the case.

Another observation of particular interest was that the predominate established Th1 response by the CD4 cells to native MSG could be switched to a Th2 response by culture with IL-4 within 24 h. Upon restimulation, the addition of IL-4 to *in vitro* culture of CD4 cells in the presence of antigen directs the cells into an apparent Th2 population characterized by the secretion of high levels of IL-4 [18,21]. The effects of IL-4, which are most potent within the first 24 h of culture, include the suppression of the development of IFN-γ-secreting effector cells [13]. It has been recently demonstrated that highly polarized Th1 cells from *Leishmania*-infected mice can be converted to the Th2 phenotype by exposure to IL-4 *in vitro* [18]. The experiments demonstrated here expand these observations to include the *P. carinii* antigen, MSG.

Although high levels of IFN-γ were produced early in culture in response to MSG and MSG-B, blocking of endogenous IFN-γ activity with anti-IFN-γ antibody had no effect on the ability of CD4 cells to make IFN-γ, and did not result in a Th1 to Th2 shift. In addition, the presence of anti-IFN-γ in the culture did not enhance the ability of IL-4 to induce the shift to the Th2 phenotype. The generation of Th1-like cells in this culture system does not appear to be dependent on the presence of IFN-γ.

There are several possible explanations for the different cytokine responses to the native and recombinant MSG preparations. Since MSG represent a family of proteins, it is likely that the native preparation contains multiple forms of the antigen; some forms might elicit a Th1 response, while other forms might elicit a Th2 response. Native MSG is heavily glycosylated, whereas prokaryotically expressed recombinant MSG is not. There is also evidence that native MSG undergoes additional post-translational modifications. Any and/or all of these variables could result in different

conformations of the proteins between the native and recombinant MSG. These differences could influence how the antigen is processed or presented to T cells. One way to study this question would be to compare the cytokine responses of the same recombinant MSG preparations expressed in non-glycosylated (e.g. bacterial) and glycosylated (e.g. yeast, baculovirus) systems.

It is well accepted that CD4 cells are critical in resistance to infection with *P. carinii* [6,7]. Some studies have shown that IFN-γ plays a role in clearance of *P. carinii* from the lungs [3], but others have shown that IFN-γ is not required for the resolution of *P. carinii* infection [23]. At present, there is no direct evidence for the involvement of Th2 cells and Th2-type cytokines in *P. carinii*. However, Beck *et al.* [24], using intact and CD4-depleted mice, demonstrated that while Th1 cytokine responses were necessary for defence against *P. carinii*, they were not sufficient. In addition, early Th2 responses were associated with protection in intact mice. It appears clear that in animal models, both Th1 and Th2 cells have a role in clearance. It is possible that *P. carinii* could undergo antigenic polymorphism in MSG that results in a Th2 cytokine response; such a process could be an important mechanism whereby the organism evades the host. There are numerous therapeutic implications with this hypothesis. Anti-*Pneumocystis* strategies could include immunotherapy with cytokine antagonists, vaccination with cytokines as the adjuvant, or MSG-specific immunotherapy to induce tolerance to disease-promoting epitopes.

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REFERENCES

1 Fisher DJ, Gigliotti F, Zauderer M, Harmsen AG. Specific T cell response to *Pneumocystis carinii* surface glycoprotein (gp120) after immunization and natural infection. Infect Immun 1991; **59**:3372–6.
2 Theus SA, Linke MJ, Andrews RP, Walzer PD. Proliferative and cytokine responses to a major surface glycoprotein of *Pneumocystis carinii*. Infect Immun 1993; **61**:4703–9.
3 Beck JM, Liggitt HD, Brunette EN *et al.* Reduction in intensity of *Pneumocystis carinii* pneumonia in mice by aerosol administration of interferon-gamma. Infect Immun 0000; **59**:3859–62.
4 Kolls JK, Beck JM, Nelson S *et al.* Alveolar macrophage release of tumor necrosis factor during murine *Pneumocystis carinii* pneumonia. Am J Respir Cell Mol Biol 1993; **8**:370–6.
5 Mandujano JF, D'Souza NP, Nelson S *et al.* Granulocyte macrophage colony stimulating factor and *Pneumocystis carinii* in mice. Am J Respir Crit Care Med 1995; **151**:1233–8.
6 Roths JB, Sidman CL. Both immunity and hyperresponsiveness to *Pneumocystis carinii* result from transfer of CD4 but not CD8 T cells into severe combined immunodeficient mice. J Clin Invest 1992; **90**:673–8.
7 Theus SA, Andrews RP, Steele P, Walzer PD. Adoptive transfer of lymphocytes sensitized to the major surface glycoprotein of *Pneumocystis carinii* confers protection in the rat. J Clin Invest 1995; **95**:2587–93.
8 Kovacs JA, Powell F, Edman JC *et al.* Multiple genes encode the major surface glycoprotein of *Pneumocystis carinii*. J Biol Chem 1993; **268**:6034–40.
9 Linke MJ, Smulian AG, Stringer JR, Walzer PD. Characterization of multiple unique cDNAs encoding the major surface glycoprotein of rat-derived *Pneumocystis carinii*. Parasitol Res 1994; **80**:478–86.
10 Sunkin SM, Stringer SL, Stringer JR. A tandem repeat of rat-derived *Pneumocystis carinii* genes encoding the major surface glycoprotein. J Eukaryot Microbiol 1994; **41**:292–300.
11 Wada M, Kitada K, Saito M *et al.* cDNA sequence diversity and genomic clusters of major surface glycoprotein genes of *Pneumocystis carinii*. J Infect Dis 1993; **168**:979–85.
12 Tonnetti L, Spaccapelo R, Cenci E *et al.* Interleukin 4 and -10 exacerbate candidiasis in mice. Eur J Immunol 1995; **25**:1559–65.
13 Perez VL, Lederer JA, Lichtman AH, Abbas AK. Stability of Th1 and Th2 populations. Int Immunol 1995; **7**:869–75.
14 Finkelman FD, Holmes J, Katona IM *et al.* Lymphokine control of *in vivo* immunoglobulin isotype selection. Ann Rev Immunol 1990; **8**:303–33.
15 Noble A, Staynov DZ, Kemeny DM. Generation of rat Th2-like cells *in vitro* is interleukin 4 dependent and inhibited by interferon-gamma. Immunol 1993; **79**:562–7.
16 Swain SL, Weinberg AD, English M, Huston G. IL-4 directs the development of Th2-like helper effectors. J Immunol 1990; **145**: 3796–806.
17 Powrie F, Coffman RL. Cytokine regulation of T cell function: potential for therapeutic intervention. Immunol Today 1993; **14**:270–4.
18 Mocci S, Coffman RL. Induction of a Th2 population from a polarized *Leishmania*-specific Th1 population by *in vitro* culture with IL-4. J Immunol 1995; **154**:3779–87.
19 Theus SA, Sullivan DW, Walzer PD, Smulian AG. Cellular responses to a 55-kilodalton recombinant *Pneumocystis carinii* antigen. Infect Immun 1994; **62**:3479–84.
20 McKnight AJ, Classon BJ. Biochemical and immunological properties of rat recombinant interleukin-2 and interleukin-4. Immunol 1992; **75**:286–92.
21 Matsuda S, Uchikawa R, Yamada M, Arizono N. Cytokine mRNA expression profiles in rats infected with the intestinal nematode *Nippostrongylus brasiliensis*. Infect Immun 1995; **63**:4653–00.
22 Carvalho EM, Bacellar O, Brownell C *et al.* Restoration of interferon-gamma production and lymphocyte proliferation in visceral leishmaniasis. J Immunol 1994; **152**:5949–56.
23 Garvy BA, Ezekowitz RA, Harsen AG. Role of gamma interferon in the host immune and inflammatory responses to *Pneumocystis carinii* infection. Infect Immun 1997; **65**:373–9.
24 Beck JM, Preston AM, Warmington KS, Chensue SW. Th1 and Th2 cytokine responses during experimental *Pneumocystis carinii* pneumonia in mice, Abstr. 127:112. In: Abstracts of the Joint Meeting of The American Society of Parasitologists and The Society of Protozologists, 1996, Tucson, AZ.