INFECTION AND IMMUNITY, June 1996, p. 2010–2018 Vol. 64, No. 6
0019-9567/96/$04.00+0

Biochemical Characterization and Protein Kinase C Dependency of Monokine-Inducing Activities of *Toxoplasma gondii*

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Received 4 January 1996/Returned for modification 22 February 1996/Accepted 8 March 1996

Previous reports have indicated that the early induction of interleukin-12 (IL-12), tumor necrosis factor alpha (TNF-α), IL-1β, and IL-10 is crucial for the establishment and regulation of host cell-mediated immunity to the intracellular protozoan parasite *Toxoplasma gondii*. In this study, we demonstrate that a soluble tachyzoite extract (soluble tachyzoite antigen) can trigger the expression of these four monokines by murine inflammatory macrophages. Further characterization revealed that the parasite molecules in soluble tachyzoite antigen responsible for monokine induction are heat stable at 100°C but differ in sensitivity to protease digestion. Thus, the tachyzoite factors that stimulate TNF-α and IL-1β expression were found to be more resistant to treatment with proteinase K than those responsible for IL-12 and IL-10 induction. Similarly, while the factors responsible for the induction of all four monokines were found to be sensitive to periodate oxidation, the TNF-α-stimulating activity was partially resistant to treatment with the compound at a low concentration (1 mM). A further dichotomy in monokine induction signals was inferred from experiments with isoquinoline sulfonamide protein kinase inhibitors. The latter work suggested that the pathways for TNF-α and IL-1β are protein kinase C dependent, while expression of IL-12 and expression of IL-10 share distinct signal transduction mechanisms involving other kinases. Together, these data argue that monokine induction by *T. gondii* is mediated by glycoproteins that may belong to distinct groups in terms of their biochemical properties and intracellular signaling pathways.

Toxoplasma gondii is an obligate intracellular protozoan that is normally controlled by the host immune system and results in an asymptomatic chronic infection. A distinctive immunological feature of *T. gondii* is the persistent cell-mediated immune response that the parasite stimulates in order to protect the host against rapid tachyzoite replication and subsequent pathology (8, 21). The highly effective resistance that *T. gondii* induces against itself has been shown to be associated with the development of $CD4^+$ and $CD8^+$ T lymphocytes displaying a type 1 cytokine expression profile (8). In particular, the production of gamma interferon (IFN-γ) by these lymphocyte populations has been demonstrated to play a major role in both acquired immunity to acute infection and the control of parasite growth in chronically infected hosts (42, 43). Recent studies have attributed the unusual capacity of *T. gondii* to trigger this strong type 1 cell-mediated immune response to events occurring during the first few days after parasite entry into the host. In particular, the interaction of tachyzoites with macrophages has been shown to lead to the production of a series of cytokines (interleukin-12 [IL-12], tumor necrosis factor alpha [TNF-α], and IL-1β) that drive the synthesis of IFN-γ by both T lymphocytes and natural killer (NK) cells and that appear to be necessary for control of acute infection (11, 13, 17, 37).

Little is known about the mechanisms by which *T. gondii* triggers the induction of the macrophage-produced cytokines (monokines) required for the development of cell-mediated immunity. Previous studies have indicated that the stimulation of these responses occurs independently of the gene controlling macrophage sensitivity to lipopolysaccharide (LPS) and that the genes induced represent a subset of those activated by LPS triggering (11, 26). Nevertheless, exposure to *T. gondii* results in a pattern of tyrosine phosphorylation similar to that induced by LPS, suggesting that the two stimuli may utilize a common signal transduction pathway downstream from receptor engagement (26).

Interestingly, the induction of monokine expression was shown not to require infection with live parasites, since exposure of macrophages to a soluble fraction of sonicated tachyzoites (soluble tachyzoite antigen [STAg]) triggers high levels of the same cytokines (11, 26). The latter observation established the possibility of identifying the parasite molecules responsible for the stimulation of monokine production. In the present study, we report the results of an initial biochemical characterization of the factors in STAg responsible for the expression of IL-12, TNF-α, and IL-1β as well as of IL-10, a cytokine previously implicated in the regulation of IL-12 and TNF-α synthesis in in vitro studies (5, 7, 16) as well as in the

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modulation of *T. gondii*-induced immune responses in vivo (12, 23). A major goal of this work was to determine whether the same or different parasite molecules are responsible for the induction of the different monokine activities and whether the same or different signal transduction mechanisms are involved. To address the latter question, we have compared the effects of isoquinoline sulfonamide protein kinase inhibitors on the expression of the four monokines to assess potential differences in the roles of protein kinases in the pathways leading to gene activation. Our results demonstrate a major role for heat-stable protein and carbohydrate structural components in the induction of IL-12, TNF-α, IL-1β, and IL-10 by *T. gondii* and suggest the existence of a dichotomy in both the parasite molecules and intracellular signaling mechanisms involved in the stimulation of these monokine activities.

MATERIALS AND METHODS

Experimental animals. C3H/HeJ female mice (5 to 6 weeks old) were obtained from The Jackson Laboratory (Bar Harbor, Maine) and were used as a source of inflammatory macrophages. All animals were maintained under specific-pathogen-free conditions and sacrificed within 1 to 2 weeks of receipt.

Parasites and antigen preparation. RH, a virulent strain of *T. gondii*, was used for infection of macrophages in vitro. STAg was prepared from *T. gondii* (RH strain) parasites grown in human foreskin fibroblast cultures. The tachyzoites were pelleted, sonicated, and then centrifuged at 100,000 × *g*, the supernatant was collected, and the protein concentration of each preparation was determined as previously described (10). Supernatants from or extracts of uninfected culture fibroblasts have previously been shown to lack macrophage-stimulatory activity (37).

Reagents. Protein-free, phenol-water-extracted *Escherichia coli* K235 LPS was prepared as previously described (28). The isoquinoline sulfonamide inhibitors of cyclic nucleotide-dependent protein kinases and protein kinase C (PKC), 1-(5-isoquinolinyl)-2-methylpiperazine (H7), *N*-[2-(methylamino)ethyl]-5-isoquinoline sulfonamide (H8), and *N*-(2-guanidinoethyl)-5-isoquinoline sulfonamide (HA1004) (Seikagaku America Inc., St. Petersburg, Fla.) were dissolved in sterile water and kept in refrigerated 10 mM stock solutions. The specific K_is (micromolar) of the inhibitors (given in parentheses) for the different kinases are as follows: H7 (cyclic AMP [cAMP], 3.0; cGMP, 5.8; PKC, 6.0), H8 (cAMP, 1.2; cGMP, 0.5; PKC, 15), and HA1004 (cAMP, 2.3; cGMP, 1.3; PKC, 40) (15). Proteinase K was purchased from ICN Biomedicals, Inc. (Aurora, Ohio), and cycloheximide and sodium *m*-periodate were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Biochemical characterization of STAg. Approximately 500-μg aliquots of STAg were incubated at 56°C for 30 min or 100°C for 5 min for thermal denaturation analysis. To assess the importance of carbohydrates, STAg (50 to 100 μg) was incubated at 37°C for 2 h in the absence or presence of 1 or 10 mM sodium *m*-periodate (Sigma) and then dialyzed against phosphate-buffered saline (PBS) overnight at 4°C. At these concentrations, sodium *m*-periodate oxidizes polysaccharides without significantly affecting amino acid residues (20). Similarly, to assess the role of protein, STAg (100 to 200 μg) was treated with proteinase K in the presence of 1 mM $CaCl_2$ at an enzyme/STAg weight ratio of 1:50. After incubation at 37°C for 2 to 3 h, the reaction mixtures were treated for 5 min at 100°C to inactivate the protease. All samples were stored at −40°C until use.

Analysis of proteins by SDS-PAGE. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels under reducing conditions as described previously (25). Gels were prepared for silver staining by fixation for 1 h in 6% formaldehyde–26% ethanol, thorough washing in water, and incubation in dithiothreitol (5 μg/ml) for 1 h. They were subsequently incubated for 1 h in 0.1% $AgNO_3$, briefly rinsed in water, and then developed in 3% $NaCO_3$ with 0.02% formaldehyde. The reaction was stopped with 2.3 M citric acid (5 ml/100 ml of developer).

Macrophage preparations. Macrophages were harvested from peritoneal washes 4 days after intraperitoneal injection of 1.5 ml of sterile 3% thioglycolate and plated in RPMI 1640 (Advanced Biotechnologies, Columbia, Md.) in the presence of 2% fetal calf serum, 1% L-glutamine, and 1% penicillin-streptomycin at a concentration of 2×10^6 cells per ml. After 2 to 4 h of incubation (37°C, 5% CO_2) in either 24-well (1 ml per well) or 96-well (100 μl per well) flat-bottom plates (Costar, Cambridge, Mass.), the culture medium was removed and replaced with the appropriate stimulants and/or inhibitors.

Cytokine secretion assays. Measurement of cytokine levels was performed in the macrophage culture supernatants described above by two-site enzyme-linked immunosorbent assays (ELISAs) in Immulon II plates (Dynatech Laboratories, Chantilly, Va.) according to standard procedures. The TNF-α assay was carried out as previously described (36, 41), using hamster monoclonal anti-murine TNF (Genzyme, Boston, Mass.) as the primary antibody, polyclonal rabbit anti-murine TNF (a generous gift from Mary Stevenson, Montreal General Hospital Research Institute, Montreal, Quebec, Canada) as the second antibody, and horseradish peroxidase-conjugated goat anti-rabbit antibody (Bio-Rad, Richmond, Calif.) as the developing reagent. A commercial TNF-α ELISA (DuoSET; Genzyme Diagnostics, Cambridge, Mass.) was substituted in later experiments.

The IL-12 p40 two-site ELISA was performed as previously described (33, 45), using monoclonal antibody C17.15 as the primary antibody, biotinylated monoclonal antibody C15.6 as the secondary antibody, and streptavidin-conjugated peroxidase as the detection reagent. Levels of TNF-α and IL-12 p40 were calculated from standard curves established for each assay by using recombinant cytokines, and the data from triplicate determinations were expressed as picograms per milliliter (mean ± standard deviation). Levels of IL-1β and IL-10 protein secretion were not measured in these studies because of the lack of sufficiently sensitive immunoassays.

After 48 h of incubation, low amounts of IFN-γ were detected by ELISA in the parasite-stimulated macrophage cultures possibly because of minor contamination with other cells. Since similar monokine secretion patterns were observed with macrophages derived from IFN-γ knockout mice (13a), this late, low-level production of IFN-γ was considered not to be a significant factor in the results obtained.

RNA extraction. RNase-free plastic and water were used throughout the procedure. RNA was extracted as previously described (9). Briefly, after the incubation period, adherent cells in 24-well plates were washed twice with warm PBS, homogenized with 0.5 ml of RNA-Stat 60 (Tel-Test, Inc., Friendswood, Tex.), and stored in 1.6-ml microcentrifuge tubes at −70°C. For extraction, tubes were thawed and vortexed, 50 μl of chloroform-isoamyl alcohol (24:1) was added, and the tubes were shaken vigorously for 2 min. They were then incubated at room temperature for 15 min. The suspension was centrifuged at 14,000 × *g* (4°C) for 15 min. The aqueous phase was transferred to a fresh tube, an equal volume of isopropanol was added, and the mixture was incubated at −20°C for 45 min. Samples were centrifuged for 15 min at 14,000 × *g* (4°C). The isopropanol was discarded, and the RNA precipitate was washed with cold 95% ethanol, after which the tubes were once again centrifuged as described above. After the ethanol was carefully discarded, the RNA pellet was dissolved in 25 μl of diethylpyrocarbonate-treated water and RNA was quantitated spectrophotometrically.

Reverse transcription-PCR (RT-PCR). The primer (sense and antisense) and probe sequences for TNF-α, IL-12 p40, IL-1β, IL-10, and hypoxanthine phosphoribosyltransferase (HPRT) have been reported elsewhere (9). Reverse transcription of RNA was performed as previously described (47). Briefly, 1 μg of total RNA extracted from macrophage cell cultures was reverse transcribed by using Superscript reverse transcriptase (Life Technologies, Gaithersburg, Md.) in a 25-μl reaction mixture in diethylpyrocarbonate-treated H_2O containing 250 μM deoxynucleoside triphosphate (dNTP), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, and 0.5 U of random hexamers. The reaction mixture was incubated at 37°C for 60 min, heated at 90°C for 5 min to denature the reverse transcriptase, and cooled at 4°C for 5 min. The samples were then diluted to 200 μl, and 10 μl of the diluted product was used for specific amplification of cytokine mRNA, using *Taq* DNA polymerase. The following components were present in the PCR mixture: 250 μM dNTP, 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 80 ng of sense and antisense primer mixture in diethylpyrocarbonate-treated water containing 1.5 mM Mg, 10 μl of cDNA, and 1 U of *Taq* polymerase. After initial incubation at 95°C for 3 min, temperature cycling was initiated with each cycle as follows: 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min. Negative controls (H_2O) were included in each assay to confirm that none of the reagents were contaminated with cDNA or previous PCR products. For each gene product, the optimum number of cycles was determined experimentally and was defined as the number of cycles that would achieve a detectable concentration that was well below the saturating conditions. The number of cycles selected for each cytokine amplification are as follows: IL-12 p40, 28; TNF-α, 24; IL-1β, 22; and IL-10, 30. To verify that equal amounts of RNA were added in all PCR mixtures within an experiment and to verify a uniform amplification process, mRNA of *HPRT* (a consitutively expressed housekeeping gene) was also reverse transcribed and amplified (24 cycles) for each sample (44).

Detection and quantitation of PCR products. Detection of PCR products was performed as previously described (9) by running samples in 1% agarose gels, transferring them to a Hybond membrane by standard blotting procedures (32), hybridizing them as described for the ECL (enhanced chemiluminescence) gene detection kit (Amersham International, Amersham, England), and performing autoradiography with Hyperfilm-ECL (Amersham). The quantitation of mRNA was performed with a densitometer and normalized to the *HPRT* level, and when appropriate, the results were expressed relative to the RNA level in positive control macrophage cultures.

RESULTS

Comparison of monokine gene expression induced by live tachyzoites and STAg. Our previous studies demonstrated that *T. gondii* activates macrophages to produce the proinflammatory cytokines TNF-α and IL-12 (11). Since IL-10 and IL-1β

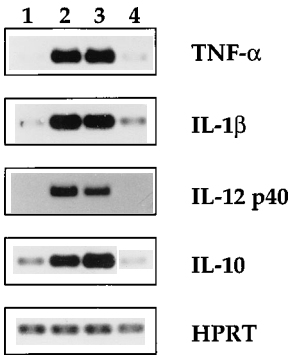

FIG. 1. Both STAg and live tachyzoites induce C3H/HeJ inflammatory macrophages to express genes encoding TNF-α, IL-1β, IL-12 p40, and IL-10. Murine peritoneal macrophages harvested 4 days after intraperitoneal thioglycolate injection were cultured for 6 h in the presence of medium alone (lane 1), STAg (25 μg/ml) (lane 2), live *T. gondii* at a tachyzoite/macrophage ratio of 1:1 (lane 3), or *E. coli* K235 LPS (100 ng/ml) (lane 4) as a control for the endotoxin hyporesponsiveness of the C3H/HeJ macrophages. Total RNA was harvested, subjected to reverse transcription, and amplified by PCR for Southern blot analysis of gene expression. As a control for equal amplification and gel loading, the constitutively expressed *HPRT* message was assayed simultaneously.

have also been hypothesized to play regulatory functions in resistance to *T. gondii*, we examined the ability of the parasite as well as a soluble tachyzoite extract (STAg) to stimulate the induction of mRNAs encoding these monokines. Inflammatory macrophages from LPS-hyporesponsive C3H/HeJ mice were used to minimize effects due to possible endotoxin contamination. As shown in Fig. 1, both STAg (lane 2) and live tachyzoites (lane 3) induced the expression of mRNAs for IL-10 and IL-1β, as well as IL-12 p40 (the regulated subunit of the IL-12 p70 molecule) (6, 46) and TNF-α, as detected by RT-PCR at 6 h after stimulation, whereas LPS (lane 4) had no effect on monokine gene expression. Because STAg was similar to live parasites in its ability to trigger monokine expression, it was used in all subsequent experiments characterizing the stimulation of these activities by *T. gondii.*

To ensure that we were not using toxic or inhibitory concentrations of parasite extract, dose-response curves were established for both cytokine secretion and gene expression. When added to macrophages at increasing doses, STAg induced similar quantitative cytokine secretion patterns for IL-12 p40 and TNF-α, with higher doses (>10 to 25 μg/ml) being inhibitory (Fig. 2A). Similarly, as shown in Fig. 2B, a dose titration of STAg activity measured by RT-PCR for the various cytokines revealed a range of 1 to 10 μg/ml to be optimal for inducing a nonsaturating response. We therefore chose 5 μg/ml as the STAg concentration to be used in all subsequent experiments. Interestingly, the RT-PCR dose-response profiles for TNF-α and IL-1β closely resembled each other. Similarly, IL-12 p40 and IL-10 displayed comparable titration patterns although distinct from those observed with the other two cytokines.

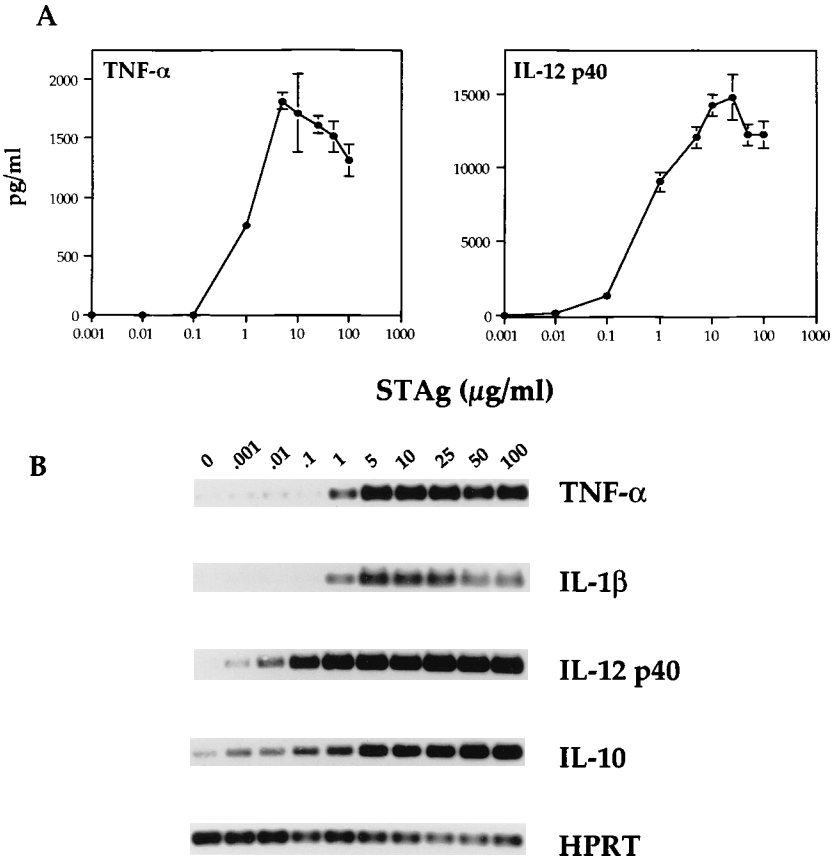

FIG. 2. Dose dependency of STAg-induced monokine secretion and mRNA expression. Macrophages were cultured for 6 h for TNF-α ELISA or for 18 to 20 h for IL-12 p40 ELISA in the presence of STAg at the indicated concentrations. Supernatants were assayed for cytokine production by ELISA (A), or cells were assayed for gene expression by RT-PCR and Southern blot analysis on mRNA harvested at 6 h (B). Values in panel A represent the means ± standard deviations of triplicate samples. Similar results were obtained in a second experiment performed.

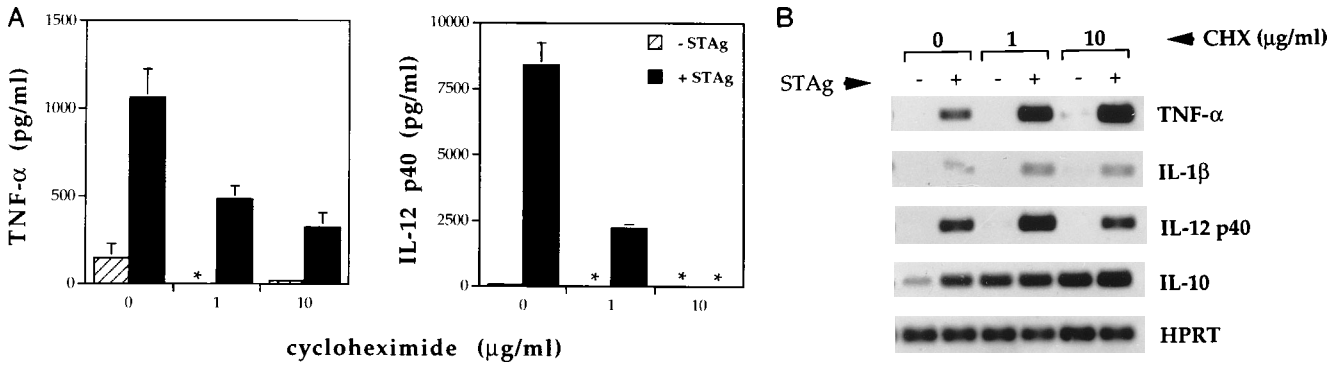

FIG. 3. Induction of monokines by STAg does not require de novo protein synthesis. Macrophages were cultured for 6 h for TNF-α ELISA or for 18 to 20 h for IL-12 p40 ELISA in the presence of cycloheximide (CHX) at the indicated concentrations with or without STAg (5 μg/ml). Supernatants were assayed for cytokine production by ELISA (A), or cells were assayed for gene expression by RT-PCR and Southern blot analysis on mRNA harvested at 6 h (B). Values in panel A represent the means ± standard deviations of triplicate samples. Asterisks indicate values below the limits of detection (<4 pg/ml for TNF-α and <10 pg/ml for IL-12 p40).

Gene expression induced by STAg does not require de novo protein synthesis. To determine if the induction of each monokine is due to direct triggering by STAg rather than the consequence of the synthesis of another cellular factor, macrophage stimulation was performed in the presence of cycloheximide. The effectiveness of drug treatment was evidenced by its complete inhibition of IL-12 p40 protein synthesis at 10 μg/ml and 70% inhibition of TNF-α secretion at the same dose (Fig. 3A). RT-PCR analysis, however, demonstrated no inhibition of monokine gene expression by cycloheximide (Fig. 3B), arguing that de novo protein synthesis is not a requirement for induction of these cytokines. Interestingly, treatment of macrophage cultures with cycloheximide alone induced the expression of IL-10 message, suggesting repression of this monokine by a constitutively synthesized factor in our macrophage populations. Similarly, in the presence of cycloheximide, STAg appeared to superinduce IL-1β and TNF-α mRNAs but not IL-12 p40 mRNA, arguing for protein synthesis-dependent negative regulation of message levels for the former cytokine genes.

Differential effects of protein kinase inhibitors on expression of monokines induced by STAg. Previous studies have implicated PKC-dependent pathways in the induction of TNF-α expression by monocytes/macrophages (24). To examine the roles of such pathways in the induction of monokine expression by STAg, we used the isoquinoline sulfonamide inhibitors H7, H8, and HA1004, which differ in their affinities for cAMP-dependent protein kinases (PKA), cGMP-dependent protein kinases (PKG), and phospholipid/Ca^{2+}-dependent protein kinase (PKC) (15).

Macrophage cultures were stimulated with STAg in the presence of the inhibitors at various concentrations and subsequently assayed for gene expression and monokine production as described in Materials and Methods. These drugs, either in the absence or in the presence of STAg, had no toxic effects on the cultured macrophages at the concentrations used, as measured by exclusion of trypan blue (data not shown). As shown in Fig. 4, H7, which among the inhibitors has the highest affinity for PKC, strongly inhibited in a dose-dependent manner TNF-α production measured in culture supernatants by ELISA, while HA1004, a weak PKC inhibitor, was without effect. H8 was intermediate in its suppression of TNF-α secretion. The 50% inhibitory concentrations (IC_{50}s) for H7 (6 μM) and H8 (12 μM) are consistent with previously published data demonstrating that H8 has a 2.5-fold weaker affinity for PKC than H7 (15), as well as previously described effects of these inhibitors on PKC-dependent pathways in murine macrophages (30). In contrast, H7 and H8 were weaker inhibitors of IL-12 p40 production and were quantitatively similar in their

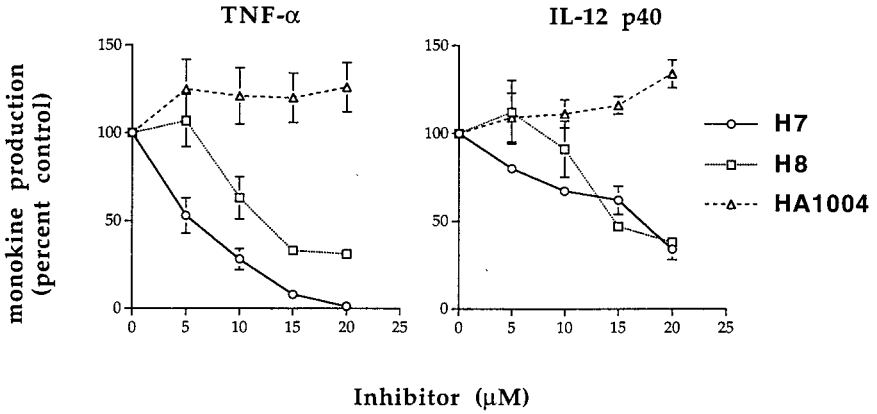

FIG. 4. Effects of protein kinase inhibitors on monokine secretion by STAg. Macrophages were cultured for 6 h in the presence of STAg and the inhibitors at the indicated concentrations for TNFα or for 18 to 20 h for IL-12 p40 protein assays. Supernatants were assayed for cytokine production by ELISA. Values are the means and standard errors for three pooled experiments.

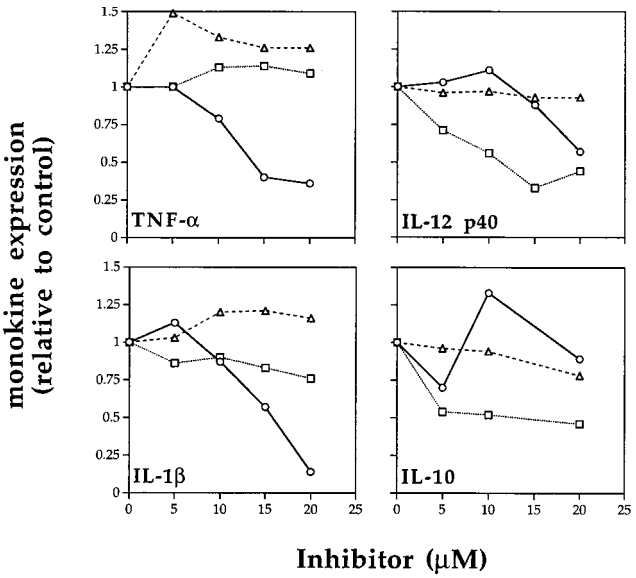

FIG. 5. Effects of protein kinase inhibitors on monokine gene expression induced by STAg. Macrophages were cultured for 6 h in the presence of STAg and the inhibitors at the indicated concentrations. Cellular RNA was harvested, RT-PCR was performed, and the data were quantitated as described in Materials and Methods. Similar results were obtained in a second experiment performed. ○, H7; □, H8; △, HA1004.

effects (H7 IC_{50} = 17 μM; H8 IC_{50} = 15 μM). As observed with TNF-α, HA1004 failed to inhibit production of IL-12 p40.

RT-PCR analysis of macrophage cultures was performed to characterize the effects of the inhibitors on expression of the genes for all four monokines (Fig. 5). TNF-α gene expression was significantly inhibited by H7 in a dose-dependent manner, while H8, in contrast to the results of the cytokine protein assays, failed to reduce TNF-α message levels (Fig. 5, top left). The pattern of inhibition by H7 and H8 for IL-1β closely resembled that observed for TNF-α. In contrast, a different inhibition profile was observed when the effects of these drugs were measured on IL-12 p40 and IL-10 gene expression, H8 having the most potent effects on cytokine mRNA levels. HA1004 failed to significantly inhibit expression of any of the four monokine genes studied.

Thermostability of monokine-inducing factors in STAg. To assess the heat lability of the monokine-inducing molecule(s) in *T. gondii*, aliquots of STAg were heat treated and subsequently assayed for the ability to induce TNF-α, IL-12, IL-1β, and IL-10 gene expression. Heating at 56°C for 30 min or boiling for 5 min failed to cause significant reductions in the stimulatory activity of STAg assayed by RT-PCR for each of the cytokines studied (Table 1). In the case of IL-12 and TNF-α, the thermostability of the cytokine-inducing factors was confirmed by ELISA measurements performed on culture supernatants (data not shown).

TABLE 1. Thermostability of monokine-inducing factors in STAg

Monokine assayed	Relative mRNA expression[a]	
	56°C, 30 min	100°C, 5 min
TNF-α	1.17 ± 0.09	1.02 ± 0.12
IL-12	1.06 ± 0.11	1.01 ± 0.26
IL-1β	1.06 ± 0.13	0.91 ± 0.16
IL-10	1.27 ± 0.36	0.80 ± 0.06

[a] Macrophages were stimulated with STAg (5 μg/ml) untreated or heated under the conditions indicated, and gene expression was measured by RT-PCR as indicated previously. Values represent intensities of RT-PCR products relative to that of products from control macrophages stimulated with untreated STAg, which was assigned a value of 1. Values are means ± standard deviations of determinations from two experiments performed. Neither of the treatments had a statistically significant effect on monokine mRNA levels induced by STAg.

Protease sensitivity of monokine-inducing factors. We next examined the sensitivity of the monokine-inducing factors to protease digestion. STAg was incubated with proteinase K for 2 to 3 h and heat treated for 5 min at 100°C to inactivate residual protease prior to addition to macrophage cultures. Analysis by SDS-PAGE confirmed the nearly complete digestion of the protein as well as the successful heat inactivation of the enzyme under the conditions used (Fig. 6).

A dose-response analysis of cytokine secretion indicated that the protease-digested STAg lost nearly all of its IL-12 p40-inducing activity (Fig. 7A). In contrast, protease digestion caused only a partial loss in TNF-α stimulation by the same samples. This highly reproducible difference between the induction of TNF-α and IL-12 secretion by proteolytically cleaved STAg was reflected in the RT-PCR analysis of mRNA levels for the same cytokines (Fig. 7B), although in the latter assay, perhaps because of its greater sensitivity, no effect of protease treatment on TNF-α induction was evident. The same analysis indicated that the IL-1β-inducing activity of STAg, in common with the TNF-α-inducing activity, is relatively protease resistant, whereas the IL-10-inducing factor, like the IL-12-inducing factor, is more sensitive to protease digestion. The enzyme inactivation controls confirmed that the observed results are not due to the artifactual effects of residual protease activity.

Sensitivity of monokine-inducing factors to periodate oxidation. To investigate the contribution of carbohydrate to monokine induction, STAg was treated with different concentrations of sodium periodate at 37°C for 2 h and subsequently dialyzed to remove the oxidant and its released products. STAg treated with 10 mM periodate showed a nearly complete loss of its ability to stimulate TNF-α and IL-12 p40 protein production in

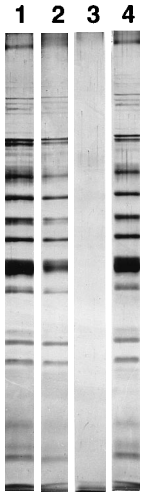

FIG. 6. Protease digestion of STAg. Aliquots of STAg were incubated at 37°C for 2 h in the absence (lane 2) or presence (lane 3) of proteinase K (enzyme/STAg weight ratio of 1:50) and then boiled for 5 min or incubated at 37°C for 2 h with proteinase K previously boiled for the same period (lane 4) as a control for heat inactivation of the enzyme. These samples along with untreated STAg (lane 1) were subjected to SDS-PAGE, and the gel was silver stained as described in Materials and Methods. Similar results were obtained in three additional experiments.

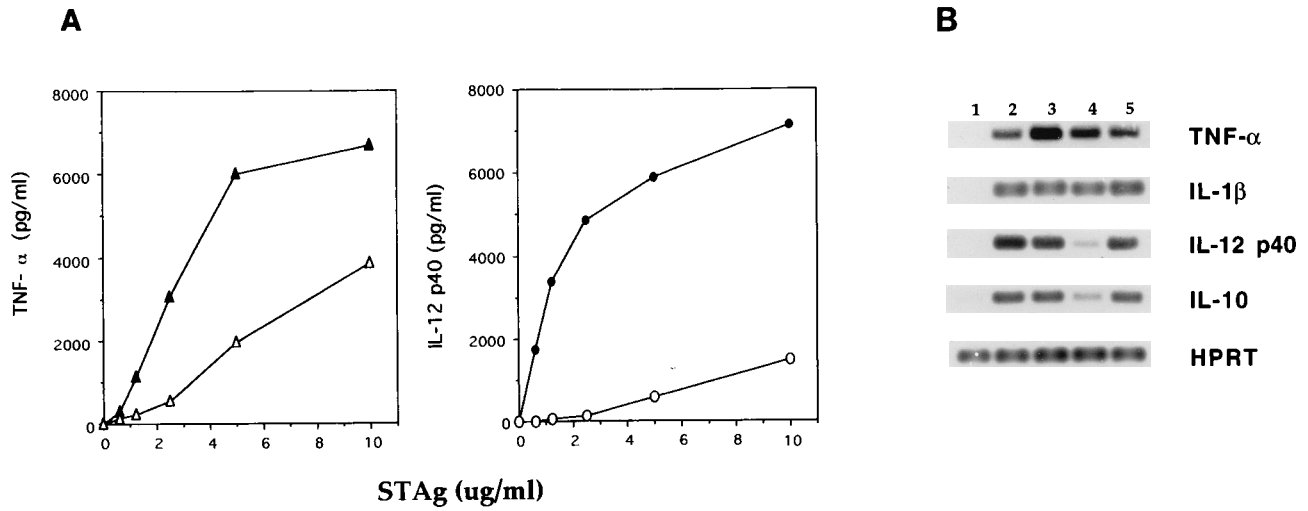

FIG. 7. Effects of protease digestion on monokine induction by STAg. Macrophages were cultured for 6 h for either RNA extraction or TNF-α ELISA or for 18 to 20 h for IL-12 p40 ELISA in the presence of protease-treated or control STAg preparations at doses (based on predigestion concentration) ranging from 0.062 to 10 μg/ml for protein secretion assays and at 5 μg/ml for RT-PCR assays. (A) Results of ELISA measurements. The closed symbols represent the responses induced by control samples incubated without enzyme, and the open symbols represent the responses stimulated by protease-digested STAg. The experiment presented is representative of five performed with similar results. (B) RT-PCR analysis performed on macrophages incubated with the following addition: medium alone (lane 1), control STAg (lane 2), STAg incubated at 37°C for 2 h in the absence (lane 3) or presence (lane 4) of proteinase K followed by boiling for 5 min, or STAg incubated at 37°C for 2 h with proteinase K previously boiled to confirm inactivation of the enzyme (lane 5). The experiment shown is representative of three performed.

vitro (Fig. 8A). Similarly, STAg treated with 1 mM periodate also failed to stimulate significant IL-12 secretion. In contrast, TNF-α production by the same preparations was only partially (approximately 60%) ablated. This finding, which was observed in three different experiments, indicated a possible difference in the periodate sensitivities of the parasite factors responsible for TNF-α and IL-12 induction.

The sensitivity to periodate oxidation at 10 mM of the *T. gondii* TNF-α- and IL-12-inducing molecules was confirmed by measurement of gene expression by RT-PCR (Fig. 8B) and was shown to extend to the factors responsible for IL-1β and IL-10 transcription. However, perhaps because of the increased sensitivity of the RT-PCR assay, no significant reductions were observed in the induction of the different monokine gene activities as a consequence of treatment of STAg with 1 mM periodate.

DISCUSSION

The stimulation of host monokine synthesis by parasites appears to play an important role in the regulation of both the resistance mechanisms and the pathologic effects induced by these organisms (29, 38). Nevertheless, little is known about the parasite molecules and macrophage-signaling mechanisms involved in this important group of host responses. Previous studies in this area have largely focused on the molecules in *Plasmodium falciparum* responsible for the induction of TNF-α, a cytokine implicated in malaria pyrogenesis (2) as well as resistance to infection (4). Protease-resistant, phosphatidylinositol-containing structures have been implicated as the *Plasmodium* factors that stimulate macrophages to produce the latter monokine (2). In the case of IL-12 induction by *Leishmania braziliensis*, however, a recombinant protein has been

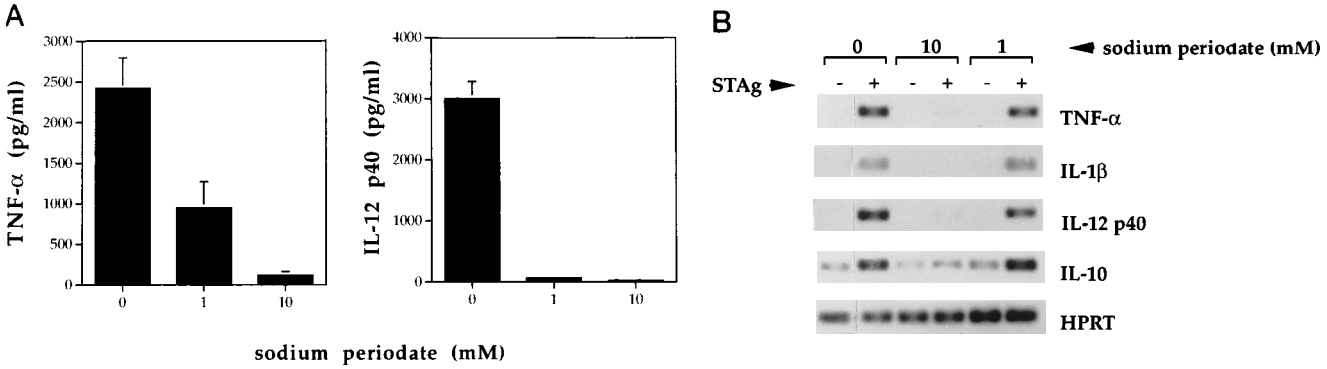

FIG. 8. Effects of oxidation with sodium periodate on monokine induction by STAg. STAg treated with sodium periodate (10 or 1 mM) and subsequently dialyzed was used to stimulate macrophages, and monokine protein (A) and mRNA responses (B) were measured as for previous figures. The background (medium alone) stimulation in protein production assays was below the limits of detection of the ELISAs (not shown). These results, including the differential effects on monokine secretion after oxidation with 1 mM sodium periodate, were reproduced in two additional experiments.

shown recently to be the relevant parasite molecule responsible for monokine induction (40).

In *T. gondii* infection, the induction of monokines has been demonstrated to be essential for the control of initial parasite growth, as evidenced by experiments in which IL-12 or TNF-α had been depleted in vivo by administration of monoclonal antibodies (9, 13, 18, 22). This early resistance mechanism appears to rely on the induction of IFN-γ by NK cells and early $CD4^+$ T cells as a consequence of triggering by IL-12, TNF-α, and IL-1β (11, 13, 17, 38). In turn, parasite-induced monokine synthesis appears to be down-regulated by IL-10, a response which is likely to play an important role in preventing pathologic effects due to overproduction of these potentially toxic host molecules (12).

Our initial analysis of *T. gondii* molecules responsible for monokine induction was indirect, focusing on NK cell activation for IFN-γ synthesis, an end function of the monokine response cascade. The latter experiments suggested that the relevant parasite factors are heat labile, exist in both soluble and membrane-associated forms in tachyzoites, and do not represent contaminating bacterial endotoxin (37). In later work, the direct stimulation of macrophage monokine (IL-12 p40 and TNF-α) synthesis was studied and shown again to be triggered by both live parasites and a soluble extract (STAg) independently of macrophage LPS sensitivity (11, 26).

In the present report, we have extended these findings by initiating a more direct biochemical characterization of the *T. gondii* factors responsible for monokine stimulation. We began this analysis by confirming that STAg was comparable to live tachyzoites in its ability to induce monokine synthesis and formally demonstrating that IL-1β and IL-10 are expressed in response to both parasite stimuli. An analysis of mRNA expression in the presence of cycloheximide (Fig. 3) suggested that de novo protein synthesis is not required for the induction of IL-12 p40, TNF-α, IL-1β, and IL-10, arguing that the activation of each monokine gene is a direct consequence of parasite stimulation rather than the synthesis of another cytokine or associated translation product. Surprisingly, cycloheximide alone was found to induce IL-10 mRNA expression, suggesting that in murine inflammatory macrophages, this cytokine is held in a repressed state by the action of a protein inhibitor of gene expression. The apparent superinduction by STAg of TNF-α and IL-1β mRNAs in the presence of the drug may indicate the existence of similar regulatory influences on the expression of the latter cytokines as well.

The observed simultaneous expression of IL-10 with IL-12 and TNF-α mRNAs in *T. gondii*-stimulated macrophages at 6 h is somewhat paradoxical since the former monokine is supposed to down-regulate the expression of the latter mediators. Nevertheless, coexpression of these transcripts is not unique to the *T. gondii* system (e.g., references 3 and 31) and may not accurately reflect the kinetics of cytokine secretion. Indeed, using our existing ELISA, we were unable to detect IL-10 protein production during the 24-h incubation period of our cultures, suggesting that although its message is evident at 6 h, the monokine itself accumulates gradually in vitro. Careful studies comparing the kinetics of secretion of IL-10 with those of the other monokines will be necessary to formally interpret their functional interrelationship.

Further investigation of the monokine-inducing factors in STAg revealed that the relevant molecules are heat stable, resisting denaturation at 100°C for 5 min. The latter finding is in direct contrast to the previously noted heat lability (56°C for 30 min) of the STAg activity responsible for NK cell stimulation (37), a response which itself is dependent on IL-12, TNF-α, and IL-1β synthesis. Since this heat sensitivity was also exhibited by the parasite extracts used in the present study (data not shown), it would appear that an additional heat-labile factor present in STAg, not necessary for monokine induction, may be required for NK cell activation.

Previous studies by Sharma and colleagues (14, 35) had indicated that the factors in *T. gondii* sonic extracts responsible for the induction of NK cell cytolytic activity are sensitive to protease digestion and periodate oxidation. The results of a similar analysis performed on the monokine-inducing factors in STAg indicated that the bioactive components of the molecules responsible for induction of all four monokines are sensitive to oxidation by 10 mM periodate and therefore are likely to contain carbohydrate. Although the factors responsible for stimulating IL-12, TNF-α, IL-1β, and IL-10 share heat stability and sensitivity to periodate at high concentrations, a more detailed analysis revealed reproducible differences in the biochemical properties of the relevant parasite molecules. Thus, the factors in STAg responsible for TNF-α secretion were partially resistant to oxidation by periodate at 1 mM, whereas the IL-12-inducing activity was found to be completely sensitive to the same treatment. Similarly, the parasite factors responsible for TNF-α and IL-1β production were consistently found to be more resistant to protease digestion than those stimulating IL-12 and IL-10 responses.

Taken together, these initial biochemical characterization experiments suggest that the bioactive structures in *T. gondii* responsible for monokine induction are likely to be protein-associated glycoconjugates. In the case of the IL-12- and IL-10-inducing factor(s), the activity of the relevant carbohydrate moieties is highly dependent on the associated protein. The TNF-α- and IL-1β-inducing activities, in contrast, appear to be less contingent on the protein component of the structure. Recent evidence from our laboratory (unpublished data) indicates that lipids also play an important role in determining mononokine induction, and our current view is that the relevant structures are protein-anchored glycolipids similar to the phosphatidylinositol-containing molecules in the malaria parasite responsible for the stimulation of TNF-α (2).

A second approach used in this study to discriminate between the different monokine-inducing activities of *T. gondii* involved an examination of protein kinase function in the intracellular triggering of these responses. The signal transduction pathways for cytokine induction in macrophages are known to involve specific kinase activities which may be distinct for the different cytokine genes studied. PKC is a Ca^{2+}-sensitive, phospholipid-dependent serine/threonine kinase which has been shown to be required for the induction of TNF-α expression in macrophages by LPS (24, 34) or taxol (19). Similarly, inhibitor studies have implicated PKC in LPS-induced IL-1β expression in monocytes, although it is not clear whether the kinase acts to regulate transcription of the cytokine gene or secretion of its protein product (1). In contrast, stimulation with phorbol ester, a known inducer of PKC activity, fails to stimulate IL-12 production in human macrophages, suggesting that the latter pathway is PKC independent (6).

The findings presented here argue that STAg-induced TNF-α synthesis by murine inflammatory macrophages is PKC dependent, given its strong suppression (at both the mRNA expression and protein secretion levels) by the high-affinity inhibitor H7 and appropriately weaker inhibition by the low-affinity drug H8. Although not analyzed at the level of secretion, a quantitatively similar pattern of inhibition by H7 versus H8 was seen for IL-1β mRNA expression, suggesting that the induction of both monokines involves a PKC-dependent pathway. In contrast, H8 was more effective at inhibiting IL-12 p40 gene expression than H7 and was difficult to distinguish from

H7 in its weak suppression of p40 protein secretion. Similarly, IL-10 mRNA expression was best inhibited by H8 rather than H7. Thus, PKC does not appear to play a dominant role in STAg-induced IL-12 and IL-10 responses, although the inhibitor data do not rule out a partial contribution of the kinase in the activation of these genes. On the other hand, their inhibition by H8 suggests the involvement of cyclic nucleotide-dependent kinases which have a high affinity for this drug. Clearly, further studies are necessary to characterize the specific kinase requirements of the STAg-induced IL-12 and IL-10 responses as well as confirm by means of comparisons with additional inhibitors our hypothesis that the induction of TNF-α and IL-1β occurs through a signal transduction pathway different from that involved in IL-12 and IL-10 triggering.

Although awaiting formal corroboration, the concept of dual pathways for monokine induction by *T. gondii* may make good biologic sense. Thus, TNF-α and IL-1β are frequently coordinately expressed early by activated macrophages (27, 39), and when stimulated by *T. gondii*, both potentiate the ability of IL-12 to induce IFN-γ synthesis by NK cells (11, 17). Such behavior is consistent with both monokines being triggered by a common parasite component through the same macrophage receptor and signal transduction pathway. In contrast, IL-12 and IL-10 are typically secreted later after macrophage stimulation and together regulate many of the same immunologic processes (5, 6). Thus, a common parasite-triggering molecule and macrophage-signaling pathway for this pair of monokines would be consistent with their linked, although opposing, biologic activities.

An important reason for investigating the monokine-inducing molecules in *T. gondii* relates to their potential use as immunomodulators. In particular, one would predict the factor in tachyzoites which stimulates IL-12 production to be particularly potent and therefore likely to be particularly useful as an adjuvant for inducing cell-mediated immunity. In this context, the evidence presented here arguing that IL-12 induction and TNF-α induction involve distinct parasite signals is encouraging since it suggests that it may be possible to isolate a *T. gondii* ligand or synthesize a derivative that triggers IL-12 synthesis without the potentially toxic effects of simultaneous TNF-α production. Further characterization and purification of these biologically relevant and potentially medically useful parasite-produced immunomodulators are now in progress in our laboratories.

ACKNOWLEDGMENTS

We are grateful to Lesley Pesnicak for technical assistance and thank Tom Wynn, Eric Denkers, and Tanya Scharton for their invaluable advice and discussions.

This work was supported in part by NIH grant AI-18797 to S.N.V. E.G. was sponsored by the Howard Hughes Medical Institute-National Institutes of Health Research Scholars Program.

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Editor: J. M. Mansfield