Suppression of Gamma Interferon Transcription and Production by Nematode Excretory-Secretory Antigen during Polyclonal Stimulation of Rat Lymph Node T Cells

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Although certain helminth infections preferentially induce type 2 T-cell responses, the immunological mechanisms responsible for type 2 T-cell polarization remain unclear. In the present study, we investigated the effects of excretory-secretory (ES) antigen from the nematode *Nippostrongylus brasiliensis* on cytokine production by mesenteric lymph node (MLN) cells isolated from naive rats. MLN cells produced considerable levels of gamma interferon (IFN-γ) during a 72-h stimulation with concanavalin A (ConA) or with immobilized anti-CD3 plus soluble anti-CD28 antibodies (anti-CD3/CD28). With either stimulation, 10 μg of ES antigen per ml significantly suppressed IFN-γ and interleukin-2 (IL-2) production without cytotoxic activity. The copresence of anti-IL-4, anti-IL-10, or transforming growth factor β (TGF-β) blocking antibodies did not alter the suppressive effect of ES antigen on IFN-γ production. ES antigen did not affect IL-10 production. Kinetic studies of the effect of ES antigen indicated that the antigen suppressed even ongoing IFN-γ production. Reverse transcription-PCR study showed that in the presence of ES antigen, IFN-γ mRNA expression by MLN cells was suppressed 6 and 12 h after ConA or anti-CD3/CD28 stimulation. ES antigen also significantly suppressed IFN-γ production by purified CD4+ or CD8+ T cells during anti-CD3/CD28 stimulation but did not affect IL-4 production by CD4+ T cells. These findings suggested that the nematode antigen suppressed production of IFN-γ and IL-2 but not IL-4 or IL-10 production. ES antigen-mediated suppression of IFN-γ during the initiation of the immune response may provide a microenvironment that helps generation of type 2 T cells.

Naive CD4+ T cells develop into two types of effector cells: Th1 cells produce cytokines, such as interleukin-2 (IL-2), gamma interferon (IFN-γ), tumor necrosis factor beta, and lymphotoxin, while Th2 cells secrete IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 (1, 21). In certain circumstances, CD8+ T cells can also divide into Tc1 and Tc2 cells based on their cytokine profiles (10, 23). It has been reported that the presence of IL-12 or IL-4 during primary activation is critical for naive T cells to differentiate into type 1 or type 2 T cells, respectively (23). IFN-γ secreted mainly from type 1 T cells and NK cells also plays a key role in Th1 and Th2 differentiation. Naive T cells require IFN-γ for maximal IL-12-induced Th1 development during primary activation, and IFN-γ significantly augments IL-12 priming for subsequent IFN-γ production by T cells (30). In addition, it has been reported that IFN-γ directly suppressed IL-4 synthesis by Th2 cells, while IL-12 did not abrogate IL-4 transcription of naive CD4+ T cells or IL-4 production by lymph node T cells (15, 16).

The balance of type 1 and type 2 immune responses contributes to determining the protective immunity and pathogenesis in certain parasite infections (24). In murine leishmaniasis, the development of Th1 responses confers resistance to the parasite, whereas the development of Th2 responses permits lethal pathogen dissemination (9, 20). In human filarial infection, immune responses in asymptomatic and/or microfilaremic patients were strongly associated with Th2-like cytokine production, while symptomatic and/or amicrofilaremic individuals showed Th1-like patterns of cytokine synthesis (8). Infection by intestinal nematodes, such as *Heligmosomoides polygyrus*, *Nippostrongylus brasiliensis*, and *Trichinella spiralis*, induces Th2-skewed immune responses which are closely associated with worm rejection (24, 29). *N. brasiliensis* infection induces dramatic increases of IL-3, IL-4, IL-5, and IL-13 gene expression together with immunoglobulin E (IgE) antibody production and mucosal mastocytosis (11, 12, 25). Finkelman et al. reported that IL-12 administration in *N. brasiliensis*-infected mice suppressed type 2 responses and enhanced adult worm survival and egg production, while anti-IFN-γ antibody inhibited the effects of IL-12, suggesting that not only IL-12 but also IFN-γ levels during the initiation of immune responses are critical for the development of type 1 or type 2 responses (5).

Previously, we showed that stimulation of mesenteric lymph node (MLN) cells with allergen-rich excretory-secretory (ES) antigen of *N. brasiliensis* enhanced ongoing IgE production and suppressed IFN-γ secretion (28). However, it has not been elucidated whether ES antigen can modify the lymphoid tissue cytokine milieu during the initiation period of immune responses. In the present study, we investigated the effect of ES antigen of *N. brasiliensis* on cytokine production by MLN cells obtained from naive rats. The results indicated that the nematode antigen suppressed production of IFN-γ and IL-2 by MLN T cells but did not affect IL-4 or IL-10 production.

**MATERIALS AND METHODS**

Animals and antigen. Specific-pathogen-free Fischer-344/NsIC male rats were purchased from Japan SLIC Inc. (Hamamatsu, Japan). *N. brasiliensis* was main-
tained for years in our laboratory by serial passage in rats. To obtain ES antigen, living adult worms were collected from the small intestines of rats 8 days after inoculation and were incubated at 37°C for 24 h in Dulbecco's phosphate-buffered saline (pH 7.4; Nissui Pharmaceutical, Tokyo, Japan) as described previously (31). The supernatants were centrifuged several times using a centrifugal concentrator (Amicon, Inc., Beverly, Mass.) to remove low-molecular-mass substances (<3 kDa) and to concentrate higher-molecular-mass molecules. After passage through a 0.22-μm-pore-size filter, the protein concentration was measured by the Bradford method and the supernatants were stored at −80°C until use. All experimental procedures were carried out according to the guidelines of the Committee for Animal Research, Kyoto Prefectural University of Medicine.

Cell preparation. MLNs were removed from naive 8-week-old animals and forced through a stainless steel wire mesh in Hanks balanced salt solution (Nissui). After discontinuous Ficoll gradient centrifugation, mononuclear cells from two or three rats were pooled and suspended in Hanks balanced salt solution containing 5% fetal calf serum. CD4+ and CD8+ T cells were purified from MLN cells by negative selection as described previously (12). In Brief, MLN cells were incubated for 30 min on ice with a mixture of the following mouse monoclonal antibodies: anti-rat CD45RA, specific for B cells (OX-33; Serotec, Oxford, United Kingdom), and anti-rat CD8 (OX-8; Serotec) for CD4+ T-cell selection or anti-rat CD45RA and anti-rat CD4 (W3/25; Serotec) for CD8+ T-cell selection. After washing, cells were incubated with a suspension of magnetic beads coated with goat anti-mouse IgG antibody (PerSeptive Diagnostics, Cambridge, Mass.) for 60 min at 4°C. The cells which were bound to beads and the free magnetic beads were then removed using a magnetic field for two 10-min sessions. The remaining cells were passed through glass bead columns coated with polyclonal anti-rat IgG (heavy plus light chains) and anti-mouse IgG (heavy plus light chains) antibodies (Biotest Laboratory, Edmonton, Canada). The purity of CD4+ and CD8+ T-cell populations thus obtained was >98%, as determined by flow cytometric analysis with FACS Calibur (Becton Dickinson, San Jose, Calif.).

Cell culture. RPMI 1640 containing 10% fetal calf serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 5 × 10−5 M 2-mercaptoethanol, 2 μg of t-glutamine per ml, and 15 mM HEPES was used as the culture medium. Cells (105/ml) suspended in the medium were stimulated in triplicate with concanavalin A (ConA, 2 μg/ml; Boehringer Mannheim Biochimica, Mannheim, Germany) or plate-bound anti-rat CD3 and soluble anti-rat CD28 (anti-CD3/CD28) mouse monoclonal antibody (G4.18 [10 μg/ml] and J319 [1 μg/ml], respectively; Pharmingen, San Diego, Calif.) in the presence or absence of ES antigen at the indicated doses. After 72 h, supernatants were harvested and the protein concentration was measured by the Bradford method and the supernatants were stored at −80°C until use.

Detection of cytokines. Cytokine production in culture supernatants was measured using enzyme-linked immunosorbent assay (ELISA) kits for IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10, and IL-12 (Biosource International, Camarillo, Calif.), or IL-10 (Biosource International), according to the manufacturers’ instructions. The levels of IL-4 production were detected by ELISA using mouse anti-rat IL-4 monoclonal antibodies (OX-81 and biotinylated B1-3; Pharmingen). The detection limits were 250 pg/ml for IFN-γ, 7.8 pg/ml for IL-4, and 5.0 pg/ml for IL-2 and IL-10.

Evaluation of proliferation and viable cell numbers. Proliferation of ConA-stimulated MLN cells was determined by the relative incorporation rate of [3H]thymidine by beta scintillation counting. Triplicate cultures were pulsed with [methyl-3H]thymidine (1 μCi/10 μl/well; Amersham, Little Chalfont, Buckinghamshire, England) for 6 h, and cells were harvested onto fiber filter paper for scintillation counting. Viability of cells was determined by trypan blue exclusion or by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay. For MTS assay, 40 μl of MTS solution (Promega, Madison, Wis.) was added to each well containing 200 μl of cell suspension, and the optical density values at 490 nm were measured after the additional 2-h incubation.

RT-PCR. The levels of IL-4 and IFN-γ mRNA expression were analyzed by reverse transcription (RT)-PCR as described previously (11, 12). Briefly, RNA extracted from cultured MLN cells was reverse transcribed in reverse transcriptase buffer containing oligo(dT) primer (Pharmacia, Uppsala, Sweden). RNase inhibitor (Toyobo, Osaka, Japan), deoxynucleoside triphosphate, dithiothreitol, and Superscript II reverse transcriptase (Gibco BRL). The cDNA samples were added to the amplification solution containing deoxynucleoside triphosphate, Perfect Match DNA polymerase enhancer (Stratagene, La Jolla, Calif.), sense and antisense primers, [α-32P]dCTP, and Vent DNA polymerase (New England Biolabs, Beverly, Mass.). The amplified samples were electrophoresed on 5% polyacrylamide gels in 10% methanol-10 mM gelatin acid solution, and dried. Autoradiograms were analyzed using a Bioimage analyzer (Fuji Photo Film Co., Tokyo, Japan).

RESULTS

Effect of ES antigen on cytokine production by MLN cells. The effect of N. brasiliensis ES antigen on the production of IFN-γ was studied using freshly isolated MLN cells from naive animals. A low level of IFN-γ was detectable in 72-h cultures of MLN cells (5 × 105/ml) without polyclonal stimulation, and this spontaneous IFN-γ production was suppressed in the presence of ≥10 μg of ES antigen per ml in the culture medium (Fig. 1). Next, we examined the effect of ES antigen on MLN cells stimulated with ConA or with anti-CD3/CD28. In the absence of ES antigen, IFN-γ production was increased as early as 24 h after the start of polyclonal stimulation and plateaued after 48 h (data not shown). Therefore, to examine the effect of ES antigen, MLN cells were cultured with polyclonal stimulation in the presence or absence of the antigen, and levels of IFN-γ in the supernatants were determined after 72 h. As shown in Fig. 2, the presence of ≥10 μg of ES antigen per ml suppressed the induction of IFN-γ production by MLN cells stimulated with either ConA or anti-CD3/CD28. It is possible that ES antigen stimulated release of IL-4 from certain populations of MLN cells and IL-4 caused the suppression of IFN-γ production in MLN cells. However, IL-4 was undetectable in the supernatants of cultures in the absence or presence of ES antigen (data not shown). In addition, anti-IL-4 antibody which was added to the cultures did not affect the IFN-γ levels in MLN cell cultures in the presence or absence of ES antigen (Fig. 2). The presence of anti-IL-10 or anti-TGF-β blocking antibody did not affect the suppressive effects of ES antigen on IFN-γ production (Fig. 3).

The presence of ≤10 μg of ES antigen per ml did not affect either the viability or the proliferation of MLN cells, although more than 30 μg of ES antigen per ml induced suppression of polyclonal activation-driven cell proliferation (Fig. 4).

To determine whether ES antigen affects the production of other cytokines, levels of IL-10 and IL-2 in the supernatants of MLN cell cultures were examined. IL-10 production was first detected at 24 h and then increased until at least 72 h, resulting in much higher levels of IL-10 production with anti-CD3/CD28 stimulation than with ConA. Addition of ES antigen to culture medium, however, did not significantly alter the kinetics or levels of IL-10 production by MLN cells (Fig. 5). Much higher levels of IL-2 production by MLN cells were induced with anti-CD3/CD28 than with ConA stimulation. The addition of

FIG. 1. Effect of ES antigen on spontaneous IFN-γ production of MLN cells isolated from naive rats. Pooled MLN cells (5 × 105/ml) were cultured with ES antigen at the indicated doses. After 72 h, supernatants were harvested and IFN-γ levels were determined by ELISA. The data are the means ± standard deviations of triplicate cultures. The results are representative of three independent experiments.
10 μg of ES antigen per ml resulted in decreased IL-2 production, although the rates of suppression of IL-2 production (less than 35%) were lower than those of IFN-γ production (Fig. 6).

Kinetic analyses of effects of ES antigen on IFN-γ production by MLN cells. To further analyze the suppressive effects of ES antigen on IFN-γ production, ES antigen (10 μg/ml) was added to culture wells at various time points after the start of ConA stimulation, and the supernatants were harvested at 72 h. Addition of ES antigen within 12 h after the start of ConA stimulation suppressed IFN-γ production as efficiently as the addition of ES antigen from the start of culture did (Fig. 7). However, addition of ES antigen later than 24 h, when IFN-γ production and/or secretion had already started, resulted in partial suppression, suggesting that ES antigen could at least partially suppress ConA-driven ongoing IFN-γ production. Next, we examined whether preincubation of MLN cells with ES antigen modified the potential of MLN cells to produce IFN-γ in response to ConA stimulation. MLN cells were incubated for 1, 3, or 6 h with ES antigen (10 μg/ml) alone, and after being washed, the cells were stimulated with ConA for 72 h. MLN cells lost the ability to produce IFN-γ after the 6-h preincubation with ES antigen, while a shorter period of preincubation did not affect ConA-driven IFN-γ production (Fig. 8).

Effect of ES antigen on cytokine gene expression. Expression of the IFN-γ and IL-4 genes was examined by RT-PCR in MLN cells stimulated with ConA or with anti-CD3/CD28 in the presence or absence of ES antigen (10 μg/ml). IFN-γ mRNA expression was markedly suppressed by ES antigen 6 or 12 h after polyclonal stimulation (Fig. 9). In contrast, IL-4 gene expression was low and did not change in the presence or absence of ES antigen (data not shown).

Effect of ES antigen on cytokine production by CD4+ or CD8+ T cells. To clarify whether ES antigen acts directly on T cells, CD4+ and CD8+ T cells purified from MLN cells were incubated with or without ES antigen under stimulation with anti-CD3/CD28. In the presence of ES antigen (10 μg/ml), the levels of IFN-γ production by both CD4+ and CD8+ T cells were suppressed (Fig. 10A). However, IL-4 production, a low level of which was detectable in CD4+ T-cell cultures but not in CD8+ T-cell cultures, was not affected by ES antigen (Fig. 10B). In both T-cell subsets, there was no decrease in cell viability in the presence of ES antigen (Fig. 10C). Fluorescence-activated cell sorter analysis using annexin V also revealed that ES antigen did not induce apoptosis of T cells (data not shown).

DISCUSSION

In the present study, we investigated the effects of *N. brasiliensis* ES antigen on cytokine production by freshly isolated MLN cells from naive rats. The results clearly showed that the addition of ES antigen to culture medium induced suppression of spontaneous IFN-γ production as well as ConA- or anti-CD3/CD28-driven IFN-γ production by rat MLN cells. ES antigen also suppressed IL-2 production by MLN cells, al-
though the effect of the antigen was more intense for IFN-γ production than for IL-2 production. The suppression of IFN-γ and IL-2 production does not appear to be due to the cytotoxicity of ES antigen to lymphoid cells, since lymphoid cell proliferation was not inhibited and a substantial amount of IL-10 was produced at the concentrations of ES antigen employed in the present study (≥10 μg/ml). Since IL-4, IL-10, and TGF-β are known to inhibit IFN-γ production (1, 22), it is possible that production or secretion of these cytokines was first induced in certain populations of MLN cells in response to the stimulation of ES antigen and that these cytokines suppressed the production of IFN-γ. ES antigen, however, did not induce detectable levels of IL-4 production by naive MLN cells, and the copresence of anti-IL-4, anti-IL-10, or anti-TGF-β antibody did not affect the suppression of IFN-γ production by ES antigen. In addition, the kinetics and levels of IL-10 secretion by MLN cells did not differ between cultures supplemented with ES antigen or left unsupplemented. These results suggest that the suppression of IFN-γ or IL-2 production by ES antigen is the consequence not of increases of IL-4, IL-10, or TGF-β but rather of selective inhibition of IFN-γ and IL-2 production.

Kinetic studies of the effect of ES antigen showed that a 6-h preincubation of MLN cells with ES antigen or the addition of ES antigen within 12 h after the start of ConA stimulation fully abrogated IFN-γ production by MLN cells, while the addition of ES antigen after 24 h, by which time secretion of IFN-γ had significantly increased, induced partial suppression of IFN-γ production. These results suggest that ES antigen inhibits not only the induction of IFN-γ production but also the ongoing IFN-γ production initiated by polyclonal stimulation. RT-PCR study further showed that IFN-γ mRNA expression 6 or 12 h after stimulation with ConA or anti-CD3/CD28 was significantly suppressed in the presence of ES antigen, indicating that the nematode antigen could quickly suppress the transcription of IFN-γ.

It is known that IFN-γ is secreted mainly by T cells and NK cells (18). In naive rats, however, very low numbers of NK cells are distributed in peripheral lymph nodes, including MLNs (6). The present results showed that production of IFN-γ in the presence of ES antigen by purified CD4+ and CD8+ T cells stimulated with anti-CD3/CD28 was suppressed significantly, but IL-4 production by CD4+ T cells was not affected. However, the levels of suppression of IFN-γ production in purified T cells were not as high as in total MLN cells, suggesting that
there may be a mechanism other than the direct action of ES antigen on T cells. It is possible that ES antigen acts on macrophages to decrease production of IL-12, which enhances IFN-γ production, or to produce certain mediators, such as prostaglandin E₂, which inhibits IL-12 production (2, 3). Effects of ES antigen on antigen-presenting cells should be clarified in future studies.

Certain helminth infections induce strong type 2 immune responses, such as elevation of total serum IgE, intestinal mastocytosis, and peripheral eosinophilia in humans, mice, and rats (4, 11, 12, 24, 25, 27, 28). ES antigen derived from *N. brasiliensis* enhanced IL-4 production by MLN cells from infected rats (R. Uchikawa et al., unpublished data), and intraperitoneal injection of the antigen induced the elevation of total IgE in naive rats (27). ES antigen has also been reported to contain many target molecules of specific IgE (7, 31). These findings have implied that certain helminth antigens preferentially induce type 2 T-cell development. The differentiation of type 1 and type 2 T cells is also controlled by genetic and environmental factors at the level of antigen presentation, such as the route of entry, the physical properties, and the dose of antigen.
were cultured with or without ES antigen under stimulation with anti- 
with high-level infection, animals with low-level infection by nematodes do not sufficiently reject the worms (19, 26). Thus, it is interesting to speculate that in low-level infections, the ES antigen concentration in the lymphoid tissues would not attain a level as high as that which suppresses IFN-γ production, and this would lead to a rather weak type 2 response possibly resulting in sustained infections. Taken together, the results show that *N. brasiliensis* ES antigen may play a crucial role in the development of type 2 effector T cells by modulating the cytokine milieu during the initiation period of the immune response.

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REFERENCES


Editor: J. M. Mansfield