In Vivo Induction of Interleukin-12 mRNA Expression after Oral Immunization with *Salmonella dublin* or the B Subunit of *Escherichia coli* Heat-Labile Enterotoxin

KENNETH L. BOST* AND JOHN D. CLEMENTS

Department of Microbiology and Immunology, Tulane University Medical Center, New Orleans, Louisiana 70112

Received 26 August 1994/Returned for modification 21 October 1994/Accepted 13 December 1994

Mice orally immunized with *Salmonella dublin* EL23, a nonreverting, aromatic-dependent, histidine-requiring mutant transformed with a plasmid which carries a gene that codes for production of the B subunit of the heat-labile toxin (LT-B) of enterotoxigenic *Escherichia coli*, or with purified LT-B alone were compared for their ability to initiate expression of interleukin-12 (IL-12) mRNAs at mucosal sites. At 6 or 20 h following oral immunization, the Peyer's patches and mesenteric lymph nodes were removed, and polyadenylated mRNA was prepared from each tissue. Constitutive expression of an mRNA encoding the p35 subunit of IL-12 was observed in control as well as immunized mice. Conversely, expression of an mRNA encoding the p40 subunit of IL-12 was not detected in control animals but was dramatically upregulated in immunized mice. By using semiquantitative reverse transcription-PCR (RT-PCR) followed by competitive RT-PCR, differences in the magnitude of IL-12 p40 mRNA expression were quantified. Six hours after oral immunization with the *Salmonella* construct, mice had 12.1- and 8.4-fold increases in expression of IL-12 p40 mRNA in the Peyer's patches and mesenteric lymph nodes, respectively, compared with control mice receiving only saline. By 20 h, the pattern of increased mRNA expression was reversed, showing 2.5- and 17.6-fold increases in the Peyer's patches and mesenteric lymph nodes, respectively. Oral immunization with LT-B alone also stimulated IL-12 p40 mRNA expression, but to a lesser extent. The constitutive expression of IL-12 p35 mRNA at these mucosal sites coupled with a rapid and dramatic induction of IL-12 p40 mRNA following immunization with wild-type or attenuated strains of *S. dublin* is consistent with other investigations which support a role for IL-12 in modulating cell-mediated immune responses against intracellular pathogens.

The enteric mucosal immune response can be divided into at least two phases (reviewed in reference 21). First, the inductive phase involves antigen presentation, activation of nonspecific effector cells (e.g., macrophages), and secretion of certain cytokines which dictate the subsequent immune response. Following induction, the effector phase of the mucosal immune response results in proliferation and differentiation of antigen-specific lymphocytes to their effector functions. Progress has been made toward understanding both phases of the mucosal immune response. Most notably, Mosmann and colleagues (7, 24) have described at least two different types of helper T (T_H) lymphocytes, which can be identified based on cytokine secretion. Antigen-specific T_H1 lymphocytes secrete substantial amounts of interleukin-2 (IL-2) and gamma interferon and execute cell-mediated immune responses (e.g., delayed-type hypersensitivity and macrophage activation), whereas antigen-specific T_H2 lymphocytes secrete IL-4, IL-5, IL-6, and IL-10 and assist in antibody production for humoral immunity. Theoretically, then, stimulation of one T helper cell subset and not the other would result in production of a particular set of cytokines, which would define the resulting immune response (1).

Evidence suggests that the mechanisms which initiate the T helper cell response in one direction or another operate soon after exposure to antigen (i.e., the inductive phase) and are undoubtedly influenced by several factors, including the nature of the antigen, route of delivery, and immune status of the

host. Given current knowledge, it would seem logical that an effective mucosal immune response against an intracellular pathogen (e.g., salmonellae) or against a protein antigen (e.g., the B subunit of *Escherichia coli* heat-labile enterotoxin [LT-B]) would result in the initiation of optimal cell-mediated or humoral immune responses, respectively. Understanding how such T_H1 or T_H2 responses are initiated in vivo is developing, and a recently discovered cytokine, IL-12 (17, 26, 30), appears to play an important role in the development of cell-mediated immune responses.

IL-12 is a heterodimer composed of 40- and 35-kDa subunits (13, 35) and has been suggested to play a critical role in the development of cell-mediated immune responses to intracellular pathogens (16, 27, 34). IL-12 not only augments gamma interferon production by T_H1 lymphocytes (15, 16, 18, 28) and natural killer cells (12), but has also been shown to diminish T_H2 lymphocyte development (19, 28). These characteristics suggest that this cytokine plays an important role in directing the initial immune response toward cell-mediated immunity. Consistent with this hypothesis, exogenously administered IL-12 greatly augments immune responses against intracellular pathogens (12, 14, 32), whereas treatment with anti-IL-12 antibodies reduces cell-mediated immunity (32, 33). It is not clear at present to what extent IL-12 contributes to initiation of cell-mediated immune responses in the gut.

The use of attenuated *Salmonella* strains as vaccine delivery vehicles for heterologous antigens has been extensively studied in a number of animal species and in humans (see reference 3 for a review). Oral immunization with these multivalent vaccines is an effective and safe way to elicit the production of serum and mucosal antibodies as well as cell-mediated immunity against both the *Salmonella* carrier and the foreign antigen

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Tulane University Medical Center, 1430 Tulane Avenue, New Orleans, LA 70112. Phone: (504) 582-7956. Fax: (504) 588-5144.

(2, 4, 5). There are, however, a number of fundamental questions remaining to be answered regarding the underlying mechanisms associated with oral administration of these organisms. In particular, it is not clear which cytokines must be expressed at mucosal sites in vivo for initiation of the most effective immune responses. In the present study, we demonstrate the ability of one attenuated *Salmonella* carrier expressing a recombinant foreign antigen (LT-B) or LT-B itself to stimulate expression of IL-12 mRNA in the Peyer's patches and mesenteric lymph nodes of mice within hours of oral administration. By using quantitative competitive reverse transcription-PCR (QC-RT-PCR), we demonstrated dramatic up-regulation of IL-12 p40 mRNA levels following oral immunization with the *Salmonella* vector and, to a lesser extent, with the soluble recombinant antigen alone.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Salmonella dublin* EL23 is a derivative of *S. dublin* SL1438, a nonreverting, aromatic-dependent, histidine-requiring mutant (kindly provided by B. A. D. Stocker, Stanford University School of Medicine) transformed with a 3.5-kb plasmid (pJC217) which carries a gene that codes for production of the B subunit from the heat-labile toxin (LT-B) of enterotoxigenic *E. coli* (9). *S. dublin* SL1438 was derived from the wild-type *S. dublin* SL1363.

Oral immunization and removal of Peyer's patches and mesenteric lymph nodes. Female BALB/c mice (Charles River) weighing 20 to 23 g were housed in filter-topped cages containing sterile bedding and fed sterile chow and sterile water for at least 2 weeks prior to treatment. Groups of mice were inoculated intragastrically with a feeding tube containing 250 μ l of sterile saline, 200 μ g of LT-B (9) in 250 μ l of sterile saline, or 10^8 log-phase *S. dublin* EL23 (10) in 250 μ l of sterile saline. At 6 or 20 h following oral immunization, the mice were killed. Six to eight Peyer's patches from the entire length of the small intestine and the mesenteric lymph nodes were removed from each animal for isolation of polyadenylated [poly(A)⁺] RNA.

Isolation of poly(A)⁺ RNA. Total RNA was first isolated by homogenizing tissue (TissueMiser; Tekmar, Cincinnati, Ohio) in Tri-Reagent (Molecular Research Center, Cincinnati, Ohio). This isolation procedure is based on the single-step acid guanidinium thiocyanate-phenol-chloroform extraction method described by Chomczynski and Sacchi (8). After centrifugation at $13,000 \times g$ for 15 min, the RNA-containing aqueous phase was precipitated in isopropanol. RNA precipitates were washed once in 75% ethanol and then resuspended in 85 μ l of RNase-free water. Poly(A)⁺ RNA was isolated by first heating the RNA in solution to 65°C for 5 min and then sequentially adding 20 μ l of binding buffer (60 mM Tris-HCl, 3 M NaCl, 6 mM EDTA [pH 7.5]) and 10 μ l of polystyrene latex-oligo(dT) beads (Oligotex-dT; Qiagen, Chatsworth, Calif.). The suspension was incubated at room temperature for 30 min, after which the beads were washed in 10 mM Tris-150 mM NaCl-1 mM EDTA (pH 7.5). Poly(A)⁺ RNA was eluted from the polystyrene latex-oligo(dT) with RNase-free water at 65°C. This isolation procedure produced poly(A)⁺ RNA that had A_{260}/A_{280} ratios of between 1.9 and 2.0, with no detectable 28S and 18S rRNA bands on ethidium bromide-stained formaldehyde-agarose gels. Poly(A)⁺ RNA was quantified for each sample by using multiple dilutions applied to DNA Dipsticks (Invitrogen).

Determining the sensitivity and range of linearity for detecting IL-12 p40 mRNA by using semiquantitative RT-PCR. To determine the sensitivity and range of linearity for amplifying the mRNA encoding p40 of IL-12 by RT-PCR, it was necessary to have a known quantity of this message. To this end, positive-strand RNA for IL-12 p40 was synthesized in vitro from a plasmid constructed as described below.

To amplify the gene encoding IL-12 p40, 200 ng of poly(A)⁺ RNA was isolated as described above from murine peritoneal macrophages activated in vitro with lipopolysaccharide (LPS). Reverse transcription was performed in the presence of random hexamers with 200 U of RNase H-minus murine leukemia virus reverse transcriptase (Promega, Madison, Wis.) and the buffer supplied by the manufacturer. The reaction was carried out at room temperature for 30 min, followed by 90 min at 37°C. The reverse transcriptase was inactivated at 95°C for 5 min, and the cDNA was precipitated in ethanol. After the precipitate was washed in 75% ethanol, the reverse-transcribed RNA was amplified by PCR with primers specific for murine IL-12 p40.

PCR amplification of IL-12 p40 included combining macrophage cDNA with 2.5 U of *Taq* polymerase, 0.02 mM each of the four deoxynucleoside triphosphates (dNTPs), 1 μ g of each primer, and PCR buffer containing 2 mM MgCl₂ (Promega). Reactions were assembled with positive-displacement pipettors and brought to 70°C prior to the addition of *Taq* polymerase. Samples were placed in a thermal cycler (Robocycler 40; Stratagene, La Jolla, Calif.), with 95°C denaturation, 58°C annealing, and 72°C extension temperatures, with the first 3 of 30 total cycles having extended denaturation and annealing times.

PCR performed on macrophage cDNA used positive- and negative-strand primers capable of amplifying a 334-bp fragment of IL-12 p40 (TAGTCTA

GAGCCACTCACATCTGCTCCAC and GATTCTAGACAGTTCAATGGGCA GGGTCTCCTC, respectively). In addition to the IL-12 p40-specific sequences (26), shown in italics, these PCR primers contained *Xba*I restriction endonuclease sequences (underlined). After PCR amplification, the IL-12 p40 gene fragment was isolated from ethidium bromide-stained agarose gels (Prep-a-Gene; Bio-Rad, Richmond, Calif.) and digested with *Xba*I (Promega) for 4 h at 37°C. After phenol-chloroform extraction, this fragment was ligated into the *Xba*I site of pGEM3Z and used to transform *E. coli* JM83. To ensure that the correct gene sequence had been cloned and to determine orientation, the insert was sequenced with dideoxynucleotides, Sequenase version 2.0, and the instructions supplied by the manufacturer (U.S. Biochemicals [USB], Cleveland, Ohio).

Positive-strand RNA was transcribed from plasmid DNA linearized with *Hind*III by using T7 RNA polymerase and an in vitro transcription reaction (Ambion, Austin, Tex.). After transcription, plasmid DNA was digested with DNase, and the RNA was isolated by electrophoresis on polyacrylamide gels. RNA eluted from the gel was quantified by the A_{260} and by using DNA Dipsticks. Limiting dilutions of IL-12 p40 transcripts (0 to 100 pg) were added to individual tubes, each containing 200 ng of poly(A)⁺ RNA isolated from a cell line that expressed no detectable IL-12 p40 message, as assayed by RT-PCR. The RNA in each tube was reverse transcribed and amplified by PCR as described above with the following modifications.

First, the positive and negative PCR primers derived from the published sequence of IL-12 p40 (26) were CCATCTACATCTGCTGCTCCACAAG and ACTTCTCATAGTCCCTTTGGTCCAG, respectively. These primers amplified a 266-bp fragment of IL-12 p40 internal to the fragment cloned into pGEM3Z. These primers were designed by using Oligo 4.0 primer analysis software (National Biosciences, Inc., Plymouth, Minn.) and based on their location in different exons of the genomic sequence of IL-12 p40, as well as the lack of significant homology with all other sequences present in GenBank version 70 (MacVector Sequence analysis software; IBI, New Haven, Conn.). Second, PCR amplifications were performed for 30 cycles in the presence of the P-33 labeled positive-strand IL-12 p40 primer. This primer was end labeled with T4 polynucleotide kinase (Promega) and gamma-P-33 ATP (New England Nuclear, Boston, Mass.). After PCR, 20% of the total reaction mix was electrophoresed on ethidium bromide-stained agarose gels and visualized under UV light so that a comparison of amplified gene fragments with DNA standards (*Hae*III-digested ϕ X174 DNA; Promega) electrophoresed on the same gel could be made. Amplified fragments were excised, and radioactivity was counted in scintillation cocktail (BioSafe II; RPI Inc., Mount Prospect, Ill.). Results were plotted as total counts per minute (cpm) in each PCR mix versus the amount of IL-12 p40 RNA transcripts added.

RT-PCR for IL-12 p35 and p40 mRNA expression. Poly(A)⁺ RNA (200 ng) isolated from the Peyer's patches or mesenteric lymph nodes of treated mice was reverse transcribed as described above. Then 20% of the cDNA reaction mix was subjected to PCR as described above for 30 cycles; 20% of each amplification reaction mix was then electrophoresed on ethidium bromide-stained agarose gels and visualized under UV illumination. Negative controls for RT-PCR included amplification of experimental RNA from which the reverse transcriptase was omitted from the RT reaction mix. The positive- and negative-strand PCR primers used to amplify IL-12 p35 mRNA were AAGACATCACACGGGAC CAAACCA and CGCAGAGTCTCGCCATTATGATTC, respectively (26).

QC-RT-PCR for IL-12 p40 mRNA. To more accurately quantify the amount of IL-12 p40 mRNA expressed in a particular sample, QC-RT-PCR was developed. Central to the successful development of QC-RT-PCR was the production of competitor DNA which was similar to but detectably different in size from IL-12 p40 cDNA but which could be amplified by the same pair of primers. To produce such a competitor, RT-PCR was used to amplify RNA isolated from LPS-activated murine macrophages with the PCR primers containing *Xba*I restriction endonuclease sites described above. The amplified 334-bp IL-12 p40 fragment was isolated by Prep-a-Gene from ethidium bromide-stained agarose gels. To produce a fragment of smaller size, a 78-bp internal segment was removed from the 334-bp fragment by cleavage with *Nla*III (USB). End segments were then isolated by Prep-A-Gene and ligated, resulting in the construction of an IL-12 p40 competitor which had 78 bp deleted. This competitor was cloned into the *Xba*I site of pGEM3Z. The plasmid construct (designated pKB12p40- Δ 78) was sequenced with dideoxynucleotides, Sequenase version 2.0, and the instructions supplied by the manufacturer (USB) to identify the orientation and confirm that the appropriate segment had been deleted.

For QC-RT-PCR, various dilutions of cDNA from a particular tissue were added to individual tubes, each containing the same amount of IL-12 p40 competitor DNA which had been isolated from cleavage of pKB12p40- Δ 78 with *Eco*RI and *Hind*III (Promega). PCR was carried out as described above with the primer pair that amplified a 266-bp IL-12 p40 fragment in the presence of the P-33-labeled positive-strand primer. The number of PCR cycles was limited to 28 to ensure linear amplification of the experimental and competitor gene fragments. After PCR, 20% of each reaction mix was electrophoresed on ethidium bromide-stained agarose gels, amplified fragments were excised, and radioactivity was counted with a scintillation cocktail. The results were plotted as total cpm for the corresponding fragment in each PCR versus the dilution of cDNA added.

Direct sequencing and restriction mapping of PCR-amplified products. To confirm the identity of PCR-amplified IL-12 p40, a cycle sequencing protocol was used (fmol DNA Sequencing System; Promega). Briefly, amplified fragments were isolated from ethidium bromide-stained agarose gels by using Prep-A-

Gene. At least 10 ng of DNA, buffer, 5 U of sequencing-grade *Taq* polymerase, a mixture of the appropriate dNTP and ddNTP, and a P-33 end-labeled primer specific for IL-12 p40 were added to each tube. Thermal cycling at 94, 58, and then 72°C was performed for 35 cycles, and the reaction mix was loaded onto a 6% acrylamide–6 M urea sequencing gel. After drying, gels were exposed to film for 2 days so that the sequence could be confirmed as that of murine IL-12 p40 (MacVector sequence analysis software; IBI Inc.). In addition, multiple gel isolates of the 266-bp IL-12 p40 fragment were subjected to cleavage with the restriction endonuclease *Hpa*II (Promega) to ensure that the correct sequence was being amplified. To confirm the identity of PCR-amplified IL-12 p35, multiple gel isolates of the 319-bp amplified fragment were subjected to cleavage with the restriction endonuclease *Sph*I or *Sly*I (Promega).

RESULTS

Semiquantitative RT-PCR for IL-12 p40 mRNA. To begin to investigate the inductive phase of the mucosal immune response with respect to the use of attenuated *Salmonella* strains as vaccine carriers, the in situ expression of IL-12 p40 mRNA was examined following oral immunization with *S. dublin* EL23, a nonreverting, aromatic-dependent, histidine-requiring mutant transformed with a 3.5-kb plasmid (pJC217) which carries a gene that codes for production of the B subunit from the heat-labile toxin (LT-B) of enterotoxigenic *E. coli*, or with the purified soluble protein antigen (LT-B) alone. RT-PCR is a valuable tool, since RNA from small amounts of tissue is sufficient for analyses of immunological events occurring in vivo. To compare the kinetics, magnitude, and in vivo location of IL-12 mRNA expression upon exposure to orally introduced antigens, it was necessary to ensure the semiquantitative nature of RT-PCR for this message. A variety of factors influence the sensitivity and linearity of RT-PCR; therefore, we investigated these limits for amplifying IL-12 p40 mRNA by using known quantities of in vitro RNA transcripts in RT-PCR.

To closely duplicate the reaction conditions to be used later for investigating RNA extracted from Peyer's patches and mesenteric lymph nodes but at the same time eliminate the uncertainty of IL-12 p40 mRNA content in these tissues, positive-strand IL-12 p40 RNA was transcribed in vitro. It was then possible to add precise amounts (0 to 100 pg) of IL-12 p40 mRNA to 200 ng of poly(A)⁺ RNA from a murine lymphoid cell line which expressed no detectable IL-12 message. After reverse transcription of tubes containing limiting dilutions of synthesized IL-12 mRNA and the same amount of poly(A)⁺ RNA, 20% of each reaction mix was amplified by PCR. Amplification reactions were performed with radiolabeled primers specific for a 266-bp fragment of murine IL-12 p40. Electrophoresis of 20% of each PCR mix on ethidium bromide-stained agarose gels shows a sequential decrease in the presence of amplified material (Fig. 1A). Furthermore, excision of each fragment to quantify the incorporation of radiolabeled primer allowed a comparison with the amount of input IL-12 p40 mRNA transcripts (Fig. 1B). As shown in Fig. 1B, less than 100 fg of input IL-12 p40 mRNA could be detected by RT-PCR even in the presence of 200 ng of "irrelevant" poly(A)⁺ RNA. These results not only demonstrate the sensitivity of RT-PCR of IL-12 p40 mRNA but also establish a limit of detectability for this message.

Figure 1B also shows that there is a range of approximately linear amplification when between 100 fg and 10 pg of input IL-12 p40 mRNA was used. At concentrations above or below these, nonlinear amplification of the message was evident. From these investigations, it was possible to define limitations for RT-PCR and demonstrate the semiquantitative nature of this technique.

Expression of IL-12 p40 mRNA in Peyer's patches and mesenteric lymph nodes. Oral immunization with *S. dublin* EL23 or soluble LT-B results in both humoral and cell-mediated

FIG. 1. Sensitivity and linearity of semiquantitative RT-PCR. Various amounts (0 to 100 pg) of IL-12 p40 mRNA transcribed in vitro were added to individual tubes, each containing 200 ng of poly(A)⁺ RNA from a cell line which contained no detectable IL-12 p40 message. The contents of each tube were reverse transcribed, and 20% of each reaction mix was amplified by PCR. (A) Twenty percent of each PCR amplification mix was electrophoresed on ethidium bromide-stained agarose gels. Each lane contained the following amounts of input IL-12 p40 mRNA transcripts: lane 1, 100 pg, no reverse transcriptase; lane 2, 100 pg; lane 3, 10 pg; lane 4, 1 pg; lane 5, 0.1 pg; lane 6, 0.01 pg; lane 7, 0.001 pg; lane 8, none. (B) Amplified fragments were isolated from the agarose gel, and incorporation of radiolabeled primer was determined. Results are presented as counts per minute incorporated versus amount of input IL-12 p40 mRNA. This experiment was performed three times with similar results.

immune responses. While the effector phases of these developing immunological responses have been studied in some detail, especially with respect to the humoral response, their induction is not well defined. IL-12 has been suggested to be important in initiating T_H1-type cell-mediated immune responses. To begin to define its importance with respect to use of attenuated *Salmonella* strains as vaccine carriers, mice immunized with *S. dublin* EL23 or soluble LT-B were evaluated for expression of IL-12 p40 mRNA in the Peyer's patches and mesenteric lymph nodes at 6 or 20 h following oral administration.

Groups of mice were orally immunized with sterile saline, 200 µg of LT-B, or 10⁸ *S. dublin* EL23. Six hours after immunization, RNA was extracted from the Peyer's patches and mesenteric lymph nodes; 200 ng of poly(A)⁺ RNA from each tissue was reverse transcribed, and 20% of the cDNA was amplified by PCR with primers specific for IL-12 p40. Figure 2A shows the results of one such experiment, with two mice in each group. IL-12 p40 mRNA was below the level of detectability in both the Peyer's patches and mesenteric lymph nodes of control mice receiving only saline. The sensitivity of this RT-PCR (Fig. 1) allows us to conclude that constitutive production of this mRNA in these mucosal lymphoid organs is minimal. Upon exposure to LT-B, IL-12 p40 mRNA increased to detectable levels in the Peyer's patches of both mice, but was still not detectable in the mesenteric lymph nodes (Fig. 2A). Mice inoculated with *S. dublin* EL23 showed dramatic increases in IL-12 p40 message in both the Peyer's patches and mesenteric lymph nodes (Fig. 2A). These differences could not be ascribed to differences in the amount of RNA used or to the efficiencies of reverse transcription. Not only was the amount of poly(A)⁺ RNA quantified prior to reverse transcription, but

FIG. 2. Induction of IL-12 p40 mRNA expression in Peyer's patches and mesenteric lymph nodes 6 h following oral immunization with LT-B or *S. dublin* EL23. Two mice per group were given sterile saline, 200 μ g of LT-B, or 10^8 *S. dublin* EL23. Six hours following administration, RNA was extracted from both the Peyer's patches and mesenteric lymph nodes of each animal; 200 ng of poly(A)⁺ RNA was reverse transcribed and then amplified by PCR. (A) Twenty percent of the total cDNA reaction mix from each tissue was subjected to PCR amplification for the expression of IL-12 p40 mRNA. Following amplification, 20% of each PCR reaction mix was electrophoresed on ethidium bromide-stained agarose gels. The presence of a 266-bp fragment of IL-12 p40 mRNA in the Peyer's patches or mesenteric lymph nodes is compared for control mice administered saline (lanes 2 and 3), mice immunized with LT-B (lanes 4 and 5), and mice immunized with *S. dublin* EL23 (lanes 6 and 7). The migration of DNA standards electrophoresed on the same gel is shown to the right (in base pairs). In addition, a negative control is shown in lane 1 of each gel, representing the lack of PCR-amplified product when reverse transcriptase was omitted from the RT reaction mix. (B) Ten percent of the total cDNA reaction mix from each tissue was subjected to PCR amplification for expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH). Following amplification, 20% of each PCR mix was electrophoresed on ethidium bromide-stained agarose gels. Each lane corresponds to cDNA taken from the same reaction as that described for panel A. The presence of a 452-bp fragment of G3PDH in each sample demonstrates that similar efficiencies of reverse transcription occurred in each cDNA reaction. In addition, a negative control is shown in lane 1 of each gel, representing the lack of PCR-amplified product when reverse transcriptase was omitted from the RT reaction mix.

RT-PCR for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase showed no such increases (Fig. 2B).

To further investigate the kinetics of IL-12 p40 mRNA expression, additional groups of mice were orally inoculated with saline, *S. dublin* EL23, or soluble LT-B, and their Peyer's patches and mesenteric lymph nodes were removed 20 h later. RT-PCR was performed to detect the presence of IL-12 p40 mRNA in these tissues, as shown in Fig. 3. Constitutive levels

FIG. 3. Induction of IL-12 p40 mRNA expression in Peyer's patches and mesenteric lymph nodes 20 h following oral immunization with LT-B or *S. dublin* EL23. Two mice per group were given sterile saline, 200 μ g of LT-B, or 10^8 *S. dublin* EL23. Twenty hours following administration, RNA was extracted from both the Peyer's patches and mesenteric lymph nodes of each animal; 200 ng of poly(A)⁺ RNA was reverse transcribed, and then 20% of the total cDNA reaction mix from each tissue was subjected to PCR for the expression of IL-12 p40 mRNA. Following amplification, 20% of each PCR mix was electrophoresed on ethidium bromide-stained agarose gels. The presence of a 266-bp fragment of IL-12 p40 mRNA in the Peyer's patches or mesenteric lymph nodes was compared for control mice administered saline (lanes 2 and 3), mice immunized with LT-B (lanes 4 and 5), and mice immunized with *S. dublin* EL23 (lanes 6 and 7). The migration of DNA standards electrophoresed on the same gel is shown to the right (in base pairs). In addition, a negative control is shown in lane 1, representing the lack of PCR-amplified product when reverse transcriptase was omitted from the RT reaction mix.

of IL-12 p40 message were not detected in control mice, demonstrating again that minimal expression occurs without stimulation. Mice inoculated orally with LT-B showed barely detectable amounts of IL-12 p40 mRNA expression in the Peyer's patches and more significant expression in the mesenteric lymph nodes. The expression of IL-12 p40 mRNA in the Peyer's patches and mesenteric lymph nodes of mice immunized with *S. dublin* EL23 was the most dramatic among the three groups.

While the data in Fig. 2 and 3 are representative, RT-PCR for IL-12 p40 mRNA has been performed on poly(A)⁺ RNA from the Peyer's patches and mesenteric lymph nodes of a total of (i) 10 control mice given only saline, (ii) 5 mice treated with LT-B for 6 h, (iii) 5 mice treated with *S. dublin* EL23 for 6 h, (iv) 6 mice treated with LT-B for 20 h, and (v) 6 mice treated with *S. dublin* EL23 for 20 h. The following conclusions can be made.

First, constitutive levels of IL-12 p40 mRNA were below the level of detection in both the Peyer's patches and mesenteric lymph nodes of BALB/c receiving no oral immunogen. The sensitivity of this RT-PCR for IL-12 p40 mRNA would therefore suggest that the presence of this mRNA in these tissues must be induced. There is an important technical point which should be made here. Upon receipt of mice from the manufacturer, they were housed for at least 2 weeks in filter-topped cages and given sterile food and water. We have found in preliminary experiments that some, but not all, animals used soon after receipt or given nonsterile laboratory chow and water had detectable levels of IL-12 p40 mRNA in the Peyer's patches and/or mesenteric lymph nodes. The exact stimulus inducing IL-12 p40 mRNA in some animals is not clear. However, limiting the exposure of otherwise normal mice to environmental antigens or stress eliminates background mRNA expression.

Second, while IL-12 p40 mRNA must be induced, its induction in the Peyer's patches and mesenteric lymph nodes is rapid, occurring as soon as 6 h after oral administration of either LT-B or *S. dublin* EL23. Furthermore, at 6 h there is more IL-12 p40 mRNA expression in the Peyer's patches than in the mesenteric lymph nodes. The magnitude of this expression is qualitatively reversed at 20 h, with more message being present in the mesenteric lymph nodes. This observation is consistent with sequential movement of antigen and/or activated leukocytes from the Peyer's patches to the mesenteric nodes after mucosal exposure.

Constitutive expression of IL-12 p35 mRNA in Peyer's patches and mesenteric lymph nodes of control and immunized mice. Bioactive IL-12 is secreted as a heterodimer, consisting of p35 and p40 subunits. Therefore, a prerequisite for secretion of this cytokine at mucosal sites is the expression of mRNA for both subunits. Using RT-PCR, we studied whether the mRNA for the p35 subunit was expressed in the Peyer's patches and mesenteric lymph nodes and whether its expression was inducible or constitutive. While it was clear that IL-12 p40 mRNA expression required induction by oral immunization, this was not the case for expression of IL-12 p35 mRNA in these mucosal tissues. When 200 ng of poly(A)⁺ RNA was subjected to RT-PCR for the presence of IL-12 p35 mRNA expression, constitutive expression was observed in both the Peyer's patches and mesenteric lymph nodes of control animals (Fig. 4, lanes 2 and 3). Furthermore, IL-12 p35 mRNA was also detected in the Peyer's patches 6 h postimmunization and in the mesenteric lymph nodes 20 h postimmunization with LT-B (Fig. 4, lanes 4 and 5) or *S. dublin* EL23 (Fig. 4, lanes 6 and 7), respectively. In Fig. 4 and in additional studies (not shown) with limiting amounts of input RNA, there may be increases in

FIG. 4. Expression of IL-12 p35 mRNA in Peyer's patches and mesenteric lymph nodes 20 h following oral immunization with LT-B or *S. dublin* EL23. Two mice per group were given sterile saline, 200 μ g of LT-B, or 10^8 *S. dublin* EL23. RNA was extracted from the Peyer's patches 6 h postadministration or from the mesenteric lymph nodes 20 h postadministration. Poly(A)⁺ RNA (200 ng) was reverse transcribed, and then 20% of the total cDNA reaction mix from each tissue was subjected to PCR for the expression of IL-12 p35 mRNA. Following amplification, 20% of each PCR mix was electrophoresed on ethidium bromide-stained agarose gels. The presence of a 319-bp fragment of IL-12 p35 mRNA in the Peyer's patches or mesenteric lymph nodes was compared for control mice administered saline (lanes 2 and 3), mice immunized with LT-B (lanes 4 and 5), and mice immunized with *S. dublin* EL23 (lanes 6 and 7). The migration of DNA standards electrophoresed on the same gel is shown to the right (in base pairs). In addition, a negative control is shown in lane 1, representing the lack of PCR-amplified product when reverse transcriptase was omitted from the RT reaction mix.

IL-12 p35 mRNA expression in some of the immunized animals compared with controls. We have not yet performed QC-RT-PCR to quantify any such differences. However, it is clear from Fig. 2, 3, and 4 that upon oral immunization, mRNAs for both the p35 and p40 subunits of IL-12 are expressed in the Peyer's patches and mesenteric lymph nodes of these mice.

Quantification of IL-12 p40 mRNA expression in Peyer's patches and mesenteric lymph nodes by QC-RT-PCR. To more precisely quantify increases in IL-12 p40 mRNA expression in the Peyer's patches and mesenteric lymph nodes with respect to use of attenuated *Salmonella* strains as vaccine carriers, QC-RT-PCR was developed. A competitor, 78 bp smaller than the 266-bp IL-12 p40 fragment amplified by RT-PCR, was constructed (see Materials and Methods). When limiting dilutions of cDNA were amplified by PCR in the presence of a constant amount of competitor, as shown in Fig. 5A, it was possible to precisely determine the relative amounts of IL-12 p40 cDNA present in a particular sample. As the amount of IL-12 p40 cDNA present in the experimental sample was decreased, a point of equivalence was reached, representing equal amounts of competitor and cDNA (Fig. 5A). From such competition, it was possible to quantify relative differences in IL-12 p40 mRNA expression in different samples.

As an example, QC-RT-PCR was used to quantify differences between IL-12 p40 mRNA expression in the Peyer's patches 6 h after administration of saline to a control mouse (cDNA the same as in Fig. 2A, lane 2) versus administration of *S. dublin* EL23 (cDNA the same as in Fig. 2A, lane 7). Limiting dilutions of cDNA were amplified in the presence of a constant amount of IL-12 p40 competitor DNA. As shown in Fig. 5B, a 266-bp IL-12 p40 fragment readily competed in a concentration-dependent manner for amplification with the competitor in the mouse receiving *S. dublin* EL23. In contrast, amplification of an experimental IL-12 p40 fragment in the saline-treated mouse was not detectable, with only the 188-bp competitor being observed on ethidium bromide-stained agarose gels (Fig. 5B).

Since QC-RT-PCR was performed in the presence of P-33-labeled primers, it was possible to quantify the amount of amplified IL-12 p40 cDNA by scintillation counting. The area of gel corresponding to the 266-bp fragment was excised, the

FIG. 5. QC-RT-PCR for the expression of IL-12 p40 mRNA. (A) To demonstrate our ability to perform QC-RT-PCR, 200 ng of poly(A)⁺ mRNA was isolated from LPS-activated murine macrophages and reverse transcribed with random hexamers. Various percentages of the total cDNA reaction mix (lane 1, 60%; lane 2, 20%; lane 3, 6%; lane 4, 2%; lane 5, 0.6%; lane 6, 0.2%; lane 7, none) were each PCR amplified in the presence of 1 pg of IL-12 p40 competitor DNA. After amplification, 20% of each PCR mix was electrophoresed on ethidium bromide-stained agarose gels. The presence of an experimental 266-bp fragment of IL-12 p40 decreased with sequential dilution of the amount of cDNA added in the presence of a constant amount of a 188-bp IL-12 p40 competitor. (B) As an example of QC-RT-PCR applied to experimental samples, 200 ng of poly(A)⁺ RNA isolated from the mesenteric lymph nodes of one animal receiving saline and one animal receiving *S. dublin* EL23 was reverse transcribed. Various percentages of the total cDNA reaction mix from each (lane 1, 30%; lane 2, 6%; lane 3, 1.2%; lane 4, 0.24%) were PCR amplified in the presence of 1 pg of IL-12 p40 competitor DNA and P-33 radiolabeled primers. After amplification, 20% of each PCR mix was electrophoresed on ethidium bromide-stained agarose gels. The locations of the 266-bp experimental IL-12 p40 fragment and the 188-bp competitor are shown. (C) The 266-bp IL-12 p40 fragment was excised from each lane of the agarose gels shown in panel B. The incorporation of radiolabeled primers in each fragment was determined by scintillation counting. Radiolabel incorporated is plotted versus the dilution of cDNA added.

radioactivity was counted, and the results were plotted versus the dilution of cDNA added (Fig. 5C). This graph represents the magnitude of difference in the presence of IL-12 p40 mRNA between samples. As expected, incorporation of radiolabel is a more sensitive method of detection than ethidium bromide staining, which explains the presence of radiolabel in the control sample while such amplification could not be visualized under UV illumination.

QC-RT-PCR was then performed on the remaining portions of each cDNA sample used for semiquantitative PCR in Fig. 2 and 3. Following QC-RT-PCR, data were analyzed in a manner similar to that shown in Fig. 5C, and these results are summarized in Table 1. Perhaps the most intriguing finding was the magnitude and rapidity of induction of IL-12 p40 mRNA expression. At 6 h post-oral immunization, *S. dublin* EL23 initiated 12.1-fold and 8.4-fold increases of this message in the Peyer's patches and mesenteric lymph nodes, respectively. Soluble LT-B alone induced significantly less IL-12 p40 mRNA expression in the Peyer's patches (2.5-fold) and mesenteric lymph nodes (1.1-fold) than did the *Salmonella* carrier expressing this antigen. Therefore, while either antigen was capable of initiating rapid increases in IL-12 p40 mRNA expression, *S. dublin* EL23 was dramatically effective at such induction.

TABLE 1. Increases in IL-12 p40 mRNA expression in mice treated orally with LT-B or *S. dublin* EL23, as determined by QC-RT-PCR^a

Time post-administration (h)	Source of poly(A) ⁺ RNA	Treatment group	Fold increase in IL-12 p40 mRNA ^b
6	Peyer's patches	Saline (control)	1.0 (± 0.0)
		LT-B	2.5 (± 0.4)
		<i>S. dublin</i> EL23	12.1 (± 2.4)
	Mesenteric lymph nodes	Saline (control)	1.0 (± 0.1)
		LT-B	1.1 (± 0.1)
		<i>S. dublin</i> EL23	8.4 (± 3.4)
20	Peyer's patches	Saline (control)	1.0 (± 0.1)
		LT-B	1.6 (± 0.3)
		<i>S. dublin</i> EL23	2.5 (± 0.1)
	Mesenteric lymph nodes	Saline (control)	1.0 (± 0.2)
		LT-B	5.6 (± 2.2)
		<i>S. dublin</i> EL23	17.6 (± 1.9)

^a Two mice per group received saline, 200 μ g of LT-B, or 10⁸ *S. dublin* EL23 orally. At 6 or 20 h postadministration, poly(A)⁺ RNA was extracted from the Peyer's patches and mesenteric lymph nodes of each animal. QC-RT-PCR was performed to quantify increases in IL-12 p40 mRNA expression.

^b Portions of the cDNA remaining after RT-PCR had been performed for Fig. 2 and 3 were also analyzed by QC-RT-PCR to quantify IL-12 p40 mRNA expression. PCR was performed on limiting amounts of cDNA in the presence of a constant amount of competitor and radiolabeled primers. Incorporation of radiolabel into the 266-bp IL-12 p40 fragment was determined and compared with that for control animals given saline alone. The level of incorporation of radiolabel in control animals for each group was arbitrarily set at 1.0, and comparisons were made relative to this number. Results are presented as means of duplicate determinations ($\pm$ standard error).

By 20 h, IL-12 p40 mRNA expression in the Peyer's patches of mice given either antigen decreased by almost 80% compared with that observed at 6 h (Table 1). This result suggests that the response within the Peyer's patches was not a prolonged one and is consistent with our understanding of clearance of antigen from the gastrointestinal tract. This further suggests that once initiation occurs in the Peyer's patches, activated leukocytes migrate to the lymphatics to continue the developing immune response.

In keeping with this possibility, the highest level of IL-12 p40 mRNA expression was observed in the mesenteric lymph nodes of treated animals 20 h after receiving antigen (Table 1). Mice given LT-B or *S. dublin* EL23 showed increases in IL-12 p40 mRNA expression 5.6 and 17.6 times above the levels for saline controls and 5.1 and 2.1 times above those levels observed in the lymph nodes at 6 h, respectively. Taken together, the results in Table 1 indicate not only rapid but also sequential IL-12 p40 mRNA upregulation in enteric lymphoid organs following oral administration of antigen, with the *Salmonella* carrier being the most effective inducer of IL-12 p40 mRNA.

Induction of IL-12 p40 mRNA expression in the mesenteric lymph nodes of mice orally immunized with different *Salmonella* strains. While the attenuated *S. dublin* strain EL23 induced rapid and dramatic IL-12 p40 mRNA expression in mucosal lymphoid organs, it was of great interest to determine whether the parent, wild-type *S. dublin* strain from which EL23 was derived could also induce IL-12 p40 mRNA expression and whether such mRNA expression was of a similar magnitude. It is not clear whether (i) attenuated *Salmonella* strains would be as effective as the wild type in inducing IL-12 p40

FIG. 6. Induction of IL-12 p40 mRNA expression in mesenteric lymph nodes 20 h following oral immunization with *S. dublin* EL23, SL1438, or SL1363. Two mice per group were given sterile saline, 10⁸ *S. dublin* EL23, 10⁸ *S. dublin* SL1438, or 10⁸ *S. dublin* SL1363. At 20 h following administration, RNA was extracted from the mesenteric lymph nodes of each animal, 200 ng of poly(A)⁺ RNA was reverse transcribed, and then 20% of the total cDNA reaction mix was subjected to PCR amplification for the expression of IL-12 p40 mRNA. Following amplification, 10% of each PCR mix was electrophoresed on ethidium bromide-stained agarose gels. The presence of a 266-bp fragment of IL-12 p40 mRNA in the mesenteric lymph nodes was compared for control mice administered saline (lanes 1 and 2), and mice immunized with *S. dublin* EL23 (lanes 3 and 4), SL1438 (lanes 5 and 6), or SL1363 (lanes 7 and 8). The migration of DNA standards electrophoresed on the same gel is shown to the left (in base pairs). This experiment was performed twice with similar results.

mRNA expression and (ii) expression of LT-B had any effect on induction of IL-12 p40.

To address these questions, mice were orally immunized with wild-type *S. dublin* SL1363 or attenuated *S. dublin* SL1438, which is the parent strain of EL23 and contains no plasmid encoding LT-B. At 20 h after inoculation, IL-12 p40 mRNA expression in the mesenteric lymph nodes was determined by RT-PCR. As shown in Fig. 6, all three *Salmonella* strains (Fig. 6, lanes 3 through 8) were capable of inducing significant IL-12 p40 mRNA expression compared with expression in control animals receiving only saline (Fig. 6, lanes 1 and 2). In fact, when samples were subjected to QC-RT-PCR, there were only minor differences in the expression of IL-12 p40 mRNA between the groups of immunized mice (data not shown). These results demonstrate that both wild-type and attenuated *Salmonella* strains have similar abilities to induce rapid and dramatic increases in IL-12 p40 mRNA expression. Furthermore, the expression of a foreign antigen (i.e., LT-B) did not significantly alter the expression of this message at mucosal sites.

DISCUSSION

In the study reported here, we focused on the ability of an aromatic-dependent *S. dublin* expressing recombinant antigen and of the soluble recombinant antigen alone to elicit expression of IL-12 mRNA in the Peyer's patches and mesenteric lymph nodes of mice within hours of oral immunization. While IL-12 p35 mRNA expression was constitutive, IL-12 p40 mRNA expression was rapidly and dramatically upregulated in enteric lymphoid organs following oral immunization. Either antigen was capable of initiating rapid increases in IL-12 p40 mRNA expression, but *S. dublin* EL23 was dramatically effective at such induction. Furthermore, both wild-type and attenuated *Salmonella* strains were similarly effective in inducing IL-12 p40 mRNA expression in mucosal tissues.

Recently it was reported that macrophages infected with *Listeria monocytogenes* produce IL-12, which can induce development of T_H1 cells from T_H0 cells (16). Moreover, T_H1 cells are important in protective immunity against *Listeria* infection (33). Since *Salmonella* spp. are also intracellular pathogens, it is believed that protection also requires development of a T_H1 response. Our laboratories have investigated the influence of

strain viability and antigen dose on the humoral immune response against orally administered *Salmonella* carriers (5, 6). Unfortunately, little is known about the cell-mediated immune response following oral administration of attenuated *Salmonella* spp., especially in their capacity as vaccine carriers. Two principal studies measuring cellular immunity have involved *Plasmodium* antigens as models for vaccines against human malaria. Sadoff and colleagues (25) and Flynn et al. (11) have reported the construction of potential antimalaria vaccines using nonvirulent *Salmonella* strains as carriers for *Plasmodium* circumsporozoite proteins. In both of these studies, serum antibody responses were not detected even in the presence of demonstrable cell-mediated responses.

The present study may begin to explain at a molecular level how orally administered *Salmonella* carriers such as *S. dublin* EL23 might stimulate cell-mediated immunity to a greater extent than orally administered protein antigens such as LT-B. Oral administration of both wild-type and attenuated *Salmonella* strains significantly increased IL-12 p40 mRNA expression compared with oral administration of LT-B. Since IL-12 p35 mRNA is constitutively expressed in the Peyer's patches and mesenteric lymph nodes, stimulated upregulation of IL-12 p40 mRNA may represent the limiting factor required for subsequent secretion of bioactive IL-12 and therefore optimal cell-mediated immune responses.

While this scenario is an attractive one and is consistent with what is known concerning the effects of IL-12, there are other possibilities. Since we have not yet developed specific antibodies for murine IL-12 p35 and p40 at the time of this study, an important question concerning secretion of bioactive IL-12 at mucosal sites needs to be addressed. It is possible that the coexpression of IL-12 p35 and p40 mRNA in the Peyer's patches and mesenteric lymph nodes demonstrated in these studies does not translate into secreted protein. Such a finding would be very important and surprising, since the most significant regulation of the majority of inducible cytokines does not occur posttranscriptionally. Another possibility is that the dramatic increases in IL-12 p40 mRNA expression induced by *S. dublin* EL23 translate into excess secretion of IL-12 p40 monomers, which may then function as an antagonist of IL-12 (20). Again, such a finding would be important, since it may represent a mechanism by which *Salmonella* spp. might limit IL-12-induced initiation of cell-mediated immunity.

A second limitation of the present work is the realization that IL-12 is only one of the cytokines that will be important in the initiation of immune responses in the gut. From recent studies by a number of investigators, an understanding of initiation of mucosal immune responses will most certainly entail an understanding of the expression of such cytokines as IL-4 (15, 29, 31, 34), IL-10 (23, 29), IL-13 (22), and possibly IL-15 (26). Since expression of these cytokines favors the development of optimal humoral immune responses, understanding the kinetics and magnitude of their expression in situ following oral immunizations should greatly increase our understanding of the inductive phase of the mucosal immune response.

Perhaps the most important contribution of this work is that the data are derived from real-time events occurring in vivo. It is clear that the sensitivity of RT-PCR is extreme, and yet no detectable expression of IL-12 p40 mRNA occurred in the Peyer's patches and mesenteric lymph nodes of normal mice which had limited exposure to environmental antigens. Thus, it appears that some stimulation is necessary for expression of this inducible message at mucosal sites. Analyses of this type are difficult to perform without the sensitivity of RT-PCR. Unfortunately, because of the semiquantitative nature of such analyses, QC-RT-PCR is required to obtain an accurate mea-

sure of differences in mRNA expression. The development of a competitor for IL-12 p40 cDNA allows very precise quantification of mRNA expression from small amounts of tissue taken from mucosal sites. Clearly such technology will be an important tool for future dissections of the initiation and effector phases of mucosal immune responses as they occur in vivo.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI28835 and AI32976 from the National Institute of Allergy and Infectious Diseases, by USAID grant HRN 6001A00302000, and by a grant from the Tulane Arthritis Related Research Program.

REFERENCES

1. Arai, K. L., F. Miyajima, A. Miyatake, S. Arai, and T. Yokota. 1990. Cytokines: coordinators of immune and inflammatory responses. *Annu. Rev. Biochem.* **59**:783-836.
2. Bao, J. X., and J. D. Clements. 1991. Prior immunologic experience potentiates the subsequent antibody response when *Salmonella* strains are used as vaccine carriers. *Infect. Immun.* **59**:3841-3845.
3. Cárdenas, L., and J. D. Clements. 1992. Oral immunization using live attenuated *Salmonella* spp. as carriers of foreign antigens. *Clin. Microbiol. Rev.* **5**:328-342.
4. Cárdenas, L., and J. D. Clements. 1993. Development of mucosal protection against the heat-stable enterotoxin (ST) of *Escherichia coli* by oral immunization with a genetic fusion delivered by a bacterial vector. *Infect. Immun.* **61**:4629-4636.
5. Cárdenas, L., and J. D. Clements. 1993. Stability, immunogenicity, and expression of foreign antigens in bacterial vaccine vectors. *Vaccine* **11**:126-135.
6. Cárdenas, L., U. Dasgupta, and J. D. Clements. 1994. Influence of strain viability and antigen dose on the use of attenuated mutants of *Salmonella* as vaccine carriers. *Vaccine* **12**:833-840.
7. Cherwinski, H. M., J. H. Schumacher, K. D. Brown, and T. R. Mosmann. 1987. Two types of mouse helper T cell clones. *J. Exp. Med.* **166**:1229-1244.
8. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acidic guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156-159.
9. Clements, J. D., and S. El-Morshidy. 1984. Construction of a potential live oral bivalent vaccine for typhoid fever and cholera-*Escherichia coli*-related diarrheas. *Infect. Immun.* **46**:564-569.
10. Clements, J. D., F. L. Lyon, K. L. Lowe, A. L. Farrand, and S. El-Morshidy. 1986. Oral immunization of mice with attenuated *Salmonella enteritidis* containing a recombinant plasmid which codes for production of the B subunit of heat-labile *Escherichia coli* enterotoxin. *Infect. Immun.* **53**:685-692.
11. Flynn, J. L., W. R. Weiss, K. A. Norris, H. S. Seifert, S. Kumar, and M. So. 1990. Generation of a cytotoxic T-lymphocyte response using *Salmonella* antigen-delivery system. *Mol. Microbiol.* **4**:2111-2118.
12. Gazzinelli, R. T., S. Hieny, T. A. Wynn, S. Wolf, and A. Sher. 1993. IL-12 is required for the T-lymphocyte-independent induction of interferon-gamma by an intracellular parasite and induces resistance in T-cell-deficient hosts. *Proc. Natl. Acad. Sci. USA* **90**:6115-6119.
13. Gubler, U., A. O. Chua, D. S. Schoenhaut, D. M. Dwyer, W. McComas, R. Motyka, N. Nabavi, A. G. Wolitzky, P. M. Quinn, P. C. Familletti, and M. K. Gately. 1991. Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. *Proc. Natl. Acad. Sci. USA* **88**:4143-4147.
14. Heinzel, F. P., D. S. Schoenhaut, R. M. Rerko, L. E. Rosser, and M. K. Gately. 1993. Recombinant IL-12 cures mice infected with *Leishmania major*. *J. Exp. Med.* **177**:1505-1509.
15. Hsieh, C. S., A. B. Heimberger, J. S. Gold, A. O'Garra, and K. M. Murphy. 1992. Differential regulation of T helper phenotype development by interleukins 4 and 10 in an alpha beta T-cell-receptor transgenic system. *Proc. Natl. Acad. Sci. USA* **89**:6065-6069.
16. Hsieh, C. S., S. E. Macatonia, C. S. Tripp, S. F. Wolf, A. O'Garra, and K. M. Murphy. 1993. Development of TH1 CD4+ T cells through IL-12 produced by *Listeria*-induced macrophages. *Science* **260**:547-549.
17. Kobayashi, M., L. Fitz, M. Ryan, R. M. Hewick, S. C. Clark, S. Chan, R. Loudon, F. Sherman, B. Perussia, and G. Trinchieri. 1989. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J. Exp. Med.* **170**:827-845.
18. Manetti, R., F. Gerosa, M. G. Giudizi, R. Biagiotti, P. Parronchi, M. P. Piccinini, S. Sampognaro, E. Maggi, S. Romagnani, and G. Trinchieri. 1994. IL-12 induces stable priming for interferon gamma production during differentiation of human T helper cells and transient interferon gamma production in established TH2 cell clones. *J. Exp. Med.* **179**:1273-1283.
19. Manetti, R., P. Parronchi, M. G. Giudizi, M. P. Piccinini, E. Maggi, G.

- Trinchieri, and S. Romagnani. 1993. Natural killer cell stimulatory factor (IL-12) induces T helper type 1 specific immune responses and inhibits the development of IL-4 producing TH cells. *J. Exp. Med.* **177**:1199–1204.
20. Mattner, F., S. Fischer, S. Guckes, S. Jin, H. Kaulen, D. Schmitt, E. Rude, and T. Germann. 1993. The interleukin-12 subunit p40 specifically inhibits effects of the interleukin-12 heterodimer. *Eur. J. Immunol.* **23**:2202–2208.
 21. McGhee, J. R., and H. Kiyono. 1993. New perspectives in vaccine development: mucosal immunity to infections. *Infect. Agents Dis.* **12**:55–73.
 22. McKenzie, A. N. J., J. A. Culpepper, R. W. Malefyt, F. Brière, J. Punnonen, G. Aversa, A. Sato, W. Dang, B. G. Cocks, S. Menon, J. E. deVries, J. Banchereau, and G. Zurawski. 1993. IL-13, a T-cell-derived cytokine that regulates human monocyte and B-cell function. *Proc. Natl. Acad. Sci. USA* **90**:3735–3739.
 23. Moore, K. W., A. O'Garra, R. W. Malefyt, P. Vieira, and T. R. Mosmann. 1993. Interleukin-10. *Annu. Rev. Immunol.* **11**:165–190.
 24. Mosmann, T. R., and R. L. Coffman. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* **7**:145–173.
 25. Sadoff, J. C., W. R. Ballou, L. S. Baron, W. R. Majarian, R. N. Brey, W. T. Hockmeyer, J. F. Young, S. J. Cryz, J. Ou, G. H. Lowell, and J. D. Chulay. 1988. Oral *Salmonella typhimurium* vaccine expressing circumsporozoite protein protects against malaria. *Science* **240**:336–340.
 26. Schoenhaut, D. S., A. O. Chua, A. G. Wolitzky, P. M. Quinn, C. M. Dwyer, W. McComas, P. C. Familletti, M. K. Gately, and U. Gubler. 1992. Cloning and expression of murine IL-12. *J. Immunol.* **148**:3433–3440.
 27. Scott, P. 1993. IL-12: initiation cytokine for cell mediated immunity. *Science* **260**:496–497.
 28. Seder, R. A., R. Gazzinelli, A. Sher, and W. E. Paul. 1993. IL-12 acts directly on CD4+ T cells to enhance priming for IFN-gamma production and diminishes IL-4 inhibition of such priming. *Proc. Natl. Acad. Sci. USA* **90**:10188–10192.
 29. Seder, R. A., W. E. Paul, M. M. Davis, and B. F. de St. Groth. 1992. The presence of IL-4 during in vitro priming determines the lymphokine-producing potential of CD4+ T cells from T cell receptor transgenic mice. *J. Exp. Med.* **176**:1091–1098.
 30. Stern, A. S., F. J. Podlaski, J. D. Hulmes, Y.-C. Pan, P. M. Quinn, A. G. Wolitzky, P. C. Familletti, D. L. Stremlo, T. Truitt, R. Chizzonite, and M. K. Gately. 1990. Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B lymphoblastoid cells. *Proc. Natl. Acad. Sci. USA* **87**:6808–6812.
 31. Swain, S. L., A. D. Weinberg, M. English, and G. Huston. 1990. IL-4 directs the development of TH2-like helper effectors. *J. Immunol.* **145**:3796–3806.
 32. Sypek, J. P., C. L. Chung, S. E. H. Mayor, J. M. Subramanyam, S. J. Goldman, D. S. Sieburth, S. F. Wolf, and R. G. Schaub. 1993. Resolution of cutaneous leishmaniasis: IL-12 initiates a protective T helper type 1 immune response. *J. Exp. Med.* **177**:1797–1802.
 33. Tripp, C. S., M. K. Gately, J. Hakimi, P. Ling, and E. R. Unanue. 1994. Neutralization of IL-12 decreases resistance to *Listeria* in SCID and C.B-17 mice. *J. Immunol.* **149**:1883–1887.
 34. Urban, J. F., K. B. Madden, A. Svetic, A. Cheever, P. P. Trotta, W. C. Gause, I. M. Katona, and F. D. Finkelman. 1992. The importance of TH2 cytokines in protective immunity to nematodes. *Immunol. Rev.* **127**:205–220.
 35. Wolf, S. F., P. A. Temple, M. Kobayashi, D. Young, M. Diczg, L. Lowe, R. Dzialo, L. Fitz, C. Ferenz, R. M. Hewick, K. Kelleher, S. H. Herrmann, S. C. Clark, L. Azzoni, S. H. Chan, G. Trinchieri, and B. Perussia. 1991. Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells. *J. Immunol.* **146**:3074–3081.