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Regional Differences in the Cellular Immune Response to Experimental Cutaneous or Visceral Infection with *Leishmania donovani*

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Infection with the protozoan *Leishmania donovani* can cause serious visceral disease or subclinical infection in humans. To better understand the pathogenesis of this dichotomy, we have investigated the host cellular immune response to cutaneous or visceral infection in a murine model. Mice infected in the skin developed no detectable visceral parasitism, whereas intravenous inoculation resulted in hepatosplenomegaly and an increasing visceral parasite burden. Spleen cells from mice with locally controlled cutaneous infection showed strong parasite-specific proliferative and gamma interferon (IFN-γ) responses, but spleen cells from systemically infected mice were unresponsive to parasite antigens. The in situ expression of IFN-γ, interleukin-4 (IL-4), IL-10, IL-12, and inducible nitric oxide synthase (iNOS) mRNAs was determined in the spleen, draining lymph node (LN), and cutaneous site of inoculation. There was considerably greater expression of IFN-γ and IL-12 p40 mRNAs in the LN draining a locally controlled cutaneous infection than in the spleen following systemic infection. Similarly, there was a high level of IFN-γ production by LN cells following subcutaneous infection but no IFN-γ production by spleen cells following systemic infection. Splenic IL-4 expression was transiently increased early after systemic infection, but splenic IL-10 transcripts increased throughout the course of visceral infection. IL-4 and IL-10 mRNAs were also increased in the LN following cutaneous infection. iNOS mRNA was detected earlier in the LN draining a cutaneous site of infection compared to the spleen following systemic challenge. Thus, locally controlled cutaneous infection was associated with antigen-specific spleen cell responsiveness and markedly increased levels of IFN-γ, IL-12, and iNOS mRNA in the draining LN. Progressive splenic parasitism was associated with an early IL-4 response, markedly increased IL-10 but minimal IL-12 expression, and delayed expression of iNOS.

Leishmaniasis is caused by infection with trypanosomatid protozoa of the genus *Leishmania*, which are transmitted to humans by the bite of an infected phlebotomine sand fly. In the mammalian host, the organism multiplies within mononuclear phagocytic cells, resulting in the clinical manifestations of the disease. Active visceral leishmaniasis (VL) caused by members of the *Leishmania donovani* complex (*L. donovani*, *L. infantum*, *L. chagasi*) is characterized by fever, cachexia, hepatosplenomegaly, and blood cytopenias and is usually fatal without the institution of antileishmanial chemotherapy. Active VL is associated with the absence of parasite-specific cell-mediated immune responses (9). A significant number of individuals who are infected with *L. donovani* have a subclinical infection which is associated with (i) the development of antigen-specific T-cell responsiveness and lymphokine (gamma interferon [IFN-γ]) production and (ii) resistance to visceral disease (3, 9, 34). The factors that contribute to the development of subclinical infection or active disease are unknown.

There is extensive evidence from experimental models that cellular immune mechanisms mediate resistance to *Leishmania* infection. Resistance in the murine model of *L. major* infection, which has been extensively studied, is associated with the capacity of $CD4^+$ T cells (Th1 subset) to generate IFN-γ which activates the parasitized macrophage to kill the intracellular *Leishmania* (15, 16). Tumor necrosis factor alpha and interleukin-7 (IL-7) may also contribute to parasite killing by augmenting the production of reactive nitrogen intermediates by IFN-γ-primed macrophages (13, 39). Recently, administration of IL-12, which stimulates T cells and NK cells to produce IFN-γ (40), has been shown to induce protection in *L. major*-infected mice (17). Progression of disease in this model has been correlated with the production of IL-4, IL-5, and IL-10 by another distinct subset of T cells (Th2) (15, 16) and transforming growth factor β (TGF-β) by infected macrophages (4).

Fewer studies on immunity of experimental VL have been undertaken. Resistance against murine *L. donovani* infection is associated with the development of parasite-specific cell-mediated immune responses involving both $CD4^+$ and $CD8^+$ T cells (38). Endogenous IFN-γ and tumor necrosis factor alpha production (19, 30, 41), the formation of hepatic granulomas (23, 35), and the administration of exogenous IL-12 (27) are associated with a reduction in parasite burden. The inability to control acute visceral *L. donovani* infection in the susceptible mouse is associated with the loss of capacity of spleen cells to produce IFN-γ in vitro but not the production of the Th2 cytokines IL-4 and IL-5 (19).

The purpose of this study was to characterize differences in the cellular immune response associated with visceral or locally controlled cutaneous infection with *L. donovani*. Cutaneous, but not visceral, infection induced a strong splenic Th1 cell response to recall stimulation with soluble *L. donovani* antigens. Mixed expression of protective and counterprotective cytokines was observed at the site of infection, but cutaneous infection resulted in prominent expression of IFN-γ and IL-12

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in the draining LN, whereas visceral infection was associated with transient early IL-4, sustained IL-10, but little IL-12 expression in the spleen.

MATERIALS AND METHODS

Experimental infection. *L. donovani* 1S was used for these studies. Promastigotes were cultured axenically in Grace's insect medium and used to prepare soluble *L. donovani* antigen (SLDA) as previously described (24). The virulence of the strain was continuously maintained by repeated passage through Syrian golden hamsters. Purified amastigotes were obtained as previously described (27). Spleens from infected hamsters were homogenized in sterile phosphate-buffered saline (without calcium or magnesium) with 50 mM glucose–1 mM EDTA (pH 6.5) on ice, and the splenic debris and intact spleen cells were removed by multiple centrifugations at 70 × *g*. The amastigote suspension was then passed through a 26-gauge needle and layered over a discontinuous Percoll (Pharmacia) gradient consisting of layers of 90, 45, and 22.5% (diluted in Hanks balanced salt solution [HBSS]) and centrifuged at 1,400 × *g* in a swinging-bucket rotor for 20 min at room temperature. The amastigotes were collected from the 22.5%-45% interface, washed in HBSS, and used immediately for the mouse infections.

Six-week-old male BALB/c mice were infected with 5×10^6 amastigotes in HBSS by either the intravenous (i.v.; 100 μl via tail vein) or subcutaneous (s.c.; 25 μl in the hind footpad) route. Age-matched control mice received the same volume of HBSS by the same route of inoculation. At 2, 7, 14, 28, 42, and 56 days, the mice were euthanized by cervical dislocation and exsanguination, and the liver, spleen, skin, and lymph node (LN) tissues were harvested.

Quantitation of parasite burden. The parasite burden was quantified in spleen, LN, and skin tissue by limiting dilution culture, using a modification of the method of Buffet et al. (6). The organ was harvested, and the total weight was determined. In the case of the skin, the infected foot was cleansed by soaking in 20% iodine disinfectant (Wescodyne) for several minutes followed by washing in 70% ethanol. The cutaneous and subcutaneous tissue of the infected footpad was harvested with a scalpel and weighed. The isolated whole LN, footpad skin, or weighed piece of spleen (approximately 20 mg) was then homogenized between the frosted ends of two sterile glass slides in 1 ml of complete culture medium (Grace's insect medium containing 15% heat-inactivated fetal bovine serum) and diluted with the same medium to a final concentration of 1 mg/ml. Fourfold serial dilutions of the homogenized tissue suspension were then plated in a 96-well tissue culture plate and cultured at 26°C for 3 weeks. The wells were examined for viable promastigotes at 3-day intervals, and the reciprocal of the highest dilution which was positive for parasites was considered to be the concentration of parasites per milligram of tissue. The total organ burden was calculated by reference to the weight of the whole organ.

In vitro spleen cell responses. Spleens or LNs from control and infected mice were harvested, and a single-cell suspension was obtained by homogenization of the tissue between the frosted ends of two glass microscope slides. The erythrocytes were lysed with ammonium chloride lysis buffer (Sigma); the splenocytes were washed and cultured in complete medium (RPMI 1640 with 10% heat-inactivated fetal bovine serum, 100 mM glutamine, penicillin, streptomycin, and 5×10^{-5} M 2-mercaptoethanol) at 2×10^5 cells per well in a 96-well round-bottom culture plate. Cells were cultured in medium alone (control) or stimulated with concanavalin A (ConA; 5 μg/ml) for 3 days or SLDA (25 μg/ml) for 5 days. One microcurie of [^{3}H]thymidine was added for the final 16 to 18 h of the culture, and the cells were harvested on to glass fiber filters for scintillation counting. Prior to cell harvest, the supernatants were collected for analysis of IFN-γ concentration by sandwich enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies (capture and detection) from Pharmingen (San Diego, Calif.). The lower limit of detection of IFN-γ was 120 pg/ml.

Oligonucleotides and cDNA templates. Oligonucleotide primers specific to the murine hypoxanthine phosphoribosyltransferase (HPRT), IL-4, IL-7, IL-10, IL-12 p40, IFN-γ, and inducible nitric oxide synthase (iNOS) cDNAs were designed for use in a semiquantitative reverse transcription (RT)-PCR assay in a manner similar to that used in our previous studies of humans (25). The forward primer (sense orientation) was biotinylated at the 5′ end. An internal oligonucleotide probe (antisense orientation) was used in Southern blot analysis of the PCR product and for quantitative hybridization in the PCR ELISA. The internal probe was 3′-end labeled with digoxigenin-dUTP as instructed by the manufacturers (Boehringer Mannheim). Each of the primer pairs and internal oligonucleotides were tested for specificity by amplification of the purified cDNA template and Southern blotting prior to use in the RT-PCR studies. The primer and probe sequences (in parentheses) are as follows: for HPRT, forward (GA CAGGACTGAAAGACTTGC), reverse (GTTGAGAGATCATCTCCACC) and internal (GTCATAGGAATGGACCTATCAC); for IFN-γ, forward (GGA TATCTGGAGGAACTGGC), reverse (CGACTCCTTTTCCGCTTCCT), and internal (CAAGACTTCAAAGAGTCTGAGG); for IL-4, forward (CAGAGC TATTGATGGGTCTC), reverse (TTCCAGGAAGTCTTTCAGTG), and internal (AAATGCCGATGATCTCTCTC); for IL-7, forward (TGGAATTCCTCC ACTGATCC), reverse (TTCACCAGTGTTTGTGAGCC), and internal (GGG CAATTACTATCAGTTCC); for IL-10, forward (TACTTGGGTTGCCAAGC CTT), reverse (TTCTTCACCTGCTCCACTGC), and internal (GCAGGGAA TTCAAATGCTCC); for IL-12 p40, forward (CAACATCAAGAGCAGTAGC AG), reverse (TACTCCCAGCTGACCTCCAC), and internal (TCTCATAGT CCCTTTGGTCC); for iNOS, forward (TCACGCTTGGGTCTTGTTCAC), reverse (TTGTCTCTGGGTCCTCTGGTC), and internal (TCTGTGCTGTCC CAGTGAGGAG).

The purified murine cytokine cDNA templates were obtained as follows. The HPRT cDNA template was a 241-bp fragment generated by PCR amplification of reverse-transcribed mouse RNA, using the HPRT forward and reverse primers described above. The IFN-γ cDNA template was a 433-bp fragment generated by PCR using the forward primer TTGCAGCTCTTCCTCATGGCT and the IFN-γ reverse primer described above. The IL-7 cDNA template was a 395-bp fragment generated by PCR using the IL-7 forward and reverse primers described above. Each of these PCR products was cloned directly into the pCRII plasmid (Invitrogen). The cloned HPRT and IFN-γ cDNA inserts were excised with *Eco*RI, and the IL-7 insert was excised with *Hin*dIII and *Xho*I. The IL-4 cDNA template was a 760-bp fragment excised from pBR322 (ATCC 37561) with *Bam*HI. The IL-10 cDNA template was a 1,500-bp insert excised from pCDSRα (ATCC 68027) with *Bam*HI. The IL-12 p40 cDNA template was a PCR-generated 799-bp *Xba*I fragment which had been cloned into pBluescript SK+ and generously provided by Ueli Gubler, Hoffman-La Roche, Nutley, N.J. It was excised with *Sac*I-*Eco*RI. The murine macrophage iNOS cDNA template was a 773-bp *Bam*HI fragment excised from the full-length cDNA which had been cloned into pGEM (a generous gift from James Cunningham, Brigham and Women's Hospital, Boston, Mass.). In each case, the excised cDNA template was purified by agarose electrophoresis and elution.

Isolation of RNA. Total RNA was extracted from the frozen tissue (whole LN or approximately 50 to 80 mg of spleen tissue) following homogenization in 1 ml of Ultraspec RNA reagent (containing guanidinium isothiocyanate [Biotecx, Houston, Tex.]) with a TissueMite homogenizer (Tekmar). The RNA was isolated according to the manufacturer's instructions by precipitation with isopropanol, washing with 70% ethanol, and solubilization in water. Any possible genomic DNA contamination was eliminated by treatment of the RNA with RNase-free DNase. One microgram of RNA was incubated at ambient room temperature for 15 min in a total volume of 10 μl of DNase reaction mix consisting of 20 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 10 U of RNAsin (Promega, Madison, Wis.), and 2 U of RNase-free DNase (Boehringer Mannheim). The DNase was then inactivated by addition of 1 μl of 25 mM EDTA and incubation at 65°C for 10 min.

RT-PCR. The tissue cytokine (IL-4, IL-7, IL-10, IL-12, and IFN-γ) response to *L. donovani* infection was analyzed by a semiquantitative RT-PCR methodology (25). cDNA was reverse transcribed from the purified RNA (1 μg) in 10 μl of DNase reaction mix (see above) following addition of RT buffer (final concentration, 50 mM Tris-HCl [pH 8.3], 75 mM KCl, 5 mM $MgCl_2$, 15 mM dithiothreitol) containing 60 U of RNAsin (Promega), 5 mM each deoxynucleoside triphosphate, 50 μg of random hexamers (Promega) per ml, and 600 U of murine Moloney leukemia virus reverse transcriptase (GIBCO-BRL, Gaithersburg, Md.) to a final volume of 20 μl. The RT reaction mix was incubated at room temperature for 10 min followed by 37°C for 1 h, and the enzyme was inactivated by heating at 95°C for 10 min. The cDNA was then stored at −70°C until use. PCR amplification of 1 to 4 μl of the cDNA or 1 μl of purified cloned cDNA template at various concentrations was carried out in a 25-μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM each deoxynucleoside triphosphate, 0.625 U of *Taq* DNA polymerase (Boehringer Mannheim), and appropriate primers at a final concentration of 0.1 mM. The reaction mixture was amplified with an Ericomp thermal cycler set at 95°C for 3 min, 50°C for 2 min, and 70°C for 2 min for one cycle and then 95°C for 1 min, 50°C for 2 min, and 70°C for 2 min for a total of 30 cycles. All PCR products were analyzed by agarose gel electrophoresis prior to quantitation by ELISA to ensure that a single amplification product was obtained (i.e., there was no detectable genomic DNA contamination).

Southern blotting. Qualitative analysis of the cytokine expression was performed by Southern blotting of the PCR product with detection by hybridization with the digoxigenin (Boehringer Mannheim)-labeled internal probe. Following agarose electrophoresis of the PCR products and denaturation and neutralization of the gels, the DNA was transferred to a nylon membrane (Nytran; Schleicher & Schuell) according to the manufacturer's instructions. After drying and UV cross-linking, the membranes were prehybridized, hybridized with the labeled probe at 55°C overnight, and washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and twice in 0.5× SSC, and hybridization was detected by chemiluminescence and autoradiography as specified by the manufacturer (Boehringer Mannheim).

Quantitation of PCR products by ELISA. All PCRs were performed with a biotinylated sense primer which enabled quantification of the PCR product by ELISA, using a modification of the method of Alard et al. (1). Maxisorp 96-well plates (Nunc) were coated overnight at 4°C with streptavidin (10 mg/ml; GIBCO-BRL) in 10 mM sodium acetate buffer (pH 5.0). The wells were blocked for 1 h at 37°C, using 300 μl of 150 mM NaCl–20 mM Tris-HCl (TBS; pH 7.4) containing 1% bovine serum albumin (Sigma) per well. After three washes with TBS containing 0.1% Tween 20 (TBS-T), 5 μl of PCR mixture or 5 μl of fivefold dilutions of the purified cDNA template (100, 20, 4, 0.8, 0.16, and 0.032 fg), each diluted with 45 μl of PCR buffer, was added to the plate and incubated for 1 h at room temperature. The bound DNA was then denaturated by incubation with

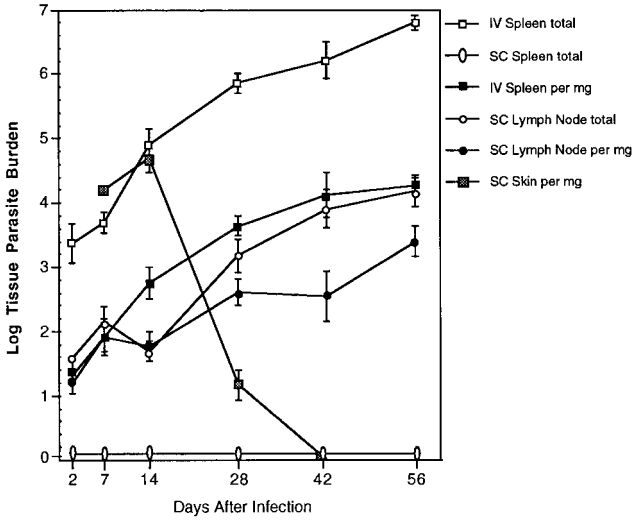

FIG. 1. Kinetics of tissue parasite burden in *L. donovani*-infected mice. Mice were infected with 5×10^6 *L. donovani* amastigotes by either s.c. or i.v. inoculation. Spleens and/or lymph nodes were harvested at 2, 7, 14, 28, 42, and 56 days after infection, footpad skin was harvested at 7, 14, 28, and 42 days after infection, and the total organ parasite burden was quantified by limiting dilution culture. The tissue parasite burden is expressed as the log of the reciprocal of the highest culture-positive dilution (mean of five samples and standard error [bars]). Results are shown for the parasite burden per milligram of tissue or for the total organ. The parasite burden in the skin was determined only per milligram of tissue because excision of the entire footpad was difficult. The spleens of the s.c.-infected mice were culture negative (<1 parasite/mg of tissue) at all time points. The differences between the parasite burden per milligram of spleen and LN tissue was statistically significant at days 14, 28, and 56 ($P = 0.03$, $P = 0.006$, and $P = 0.01$, respectively).

0.125 N NaOH (100 μl/well) for 10 min at room temperature. After six washes, plates were hybridized with the digoxigenin-labeled internal antisense probe at 0.2 pmol/well diluted in 100 μl of 0.5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]) by incubation for 2 h at 42°C. The plates were then washed six times and incubated with alkaline phosphatase-labeled anti-digoxigenin antibody (Boehringer Mannheim) diluted 1:5,000 in TBS-T for 1 h at room temperature. After six washes, the plates were developed with *p*-nitrophenyl phosphate diluted in diethanolamine buffer (Pierce) (100 μl/well) by incubation 1 to 2 h at 37°C. The absorbances were recorded at 405 nm in an automated ELISA reader (Titertek Multiscan). For each sample, the quantity of input tissue-derived cDNA was determined by interpolation from the standard curve of fivefold dilutions of the purified cytokine cDNA templates. Minor variations in the amount of input RNA among the samples were corrected by normalization of each quantified cytokine cDNA to the quantified HPRT product from the same sample.

RESULTS

Course of experimental infection. BALB/c mice were infected by s.c. or i.v. inoculation of *L. donovani* amastigotes, and the course of infection was followed for 8 weeks. Mice infected by the i.v. route showed progressive hepatosplenomegaly, whereas mice infected s.c. in the hind footpad developed minimal swelling of the footpad (barely detectable in some mice and absent in others), enlargement of the popliteal LN, but no hepatosplenomegaly. The spleen weights (median [range]) for the s.c.-infected mice compared to the i.v.-infected mice were as follows: 94 [81 to 101] mg versus 132 [119 to 160] mg (day 14), 95 [73 to 121] mg versus 161 [138 to 187] mg (day 28), and 87 [77 to 98] mg versus 324 [310 to 347] mg (day 56). At each time point, the spleen weights of i.v.-infected mice were significantly greater than the spleen weights of the s.c.-infected mice ($P \leq 0.009$ by nonparametric Mann-Whitney *U* test for each comparison).

The parasite burden of infected mice was determined by quantitative limiting dilution culture of homogenized spleen, LN, and skin (Fig. 1). The lower limit of culture quantification was <1 parasite per mg of tissue. Mice infected s.c. showed an increase in the number of viable parasites in the draining popliteal LN over the 8-week observation period, but there were no detectable viable parasites in the liver, spleen, or external sacral LNs (into which the popliteal nodes drain). The parasite burden in skin tissue from mice infected in the footpad was high at 7 and 14 days postinfection but then declined rapidly to an undetectable level by 6 weeks postinfection. The splenic parasite burden of i.v.-infected mice increased progressively over the 8-week observation period. The total splenic parasite burden of i.v.-infected mice was approximately 2 to 3 logs greater than the total popliteal LN burden resulting from s.c. infection. Starting at day 14 after infection, the splenic tissue of i.v.-infected mice also had a significantly greater parasite burden per milligram of tissue than did the LN tissue of s.c.-infected mice. The hepatic parasite burden peaked at 28 to 42 days after infection and then declined gradually over the remaining period of observation (data not shown).

In vitro lymphocyte responses. Throughout the 8-week course of infection, LN and spleen cells from control and infected mice were tested for the capacity to respond to in vitro stimulation with SLDA or the mitogen ConA. Mice infected i.v. showed no detectable antigen-specific lymphoproliferative responses and showed a blunted response to ConA (Fig. 2). In contrast, the spleen cell responses of s.c.-infected mice showed intact responses to ConA, and a strong antigen-specific recall proliferative response was observed at days 28, 42, and 56. The magnitude (counts per minute) of ConA-induced LN cell responses was no different between control and s.c.-infected animals, but the high background proliferation of LN cells from s.c.-infected animals lowered the stimulation index to 20 to 50% of the response of LN cells from uninfected animals (data not shown). This was in contrast to the blunted ConA-induced spleen cell responses seen in i.v.-infected mice, where the loss of the response was primarily due to a lowering of the magnitude of the stimulated response. The antigen-induced proliferative response of LN cells from s.c.-infected mice was not significantly greater than the response of cells from uninfected mice at any time point (stimulation index of <2 [data not shown]). This was primarily due to a high level of background proliferation (5,000 to 30,000 cpm) in the medium controls (which contained no exogenous antigen), presumably due to endogenous antigenic stimulation related to the parasites present in the LN.

Significant antigen-induced IFN-γ production was absent in spleen cells from systemically infected mice, whereas mice infected in the skin had strong antigen-specific IFN-γ production by spleen cells (Fig. 3). There was no significant increase in antigen-induced IFN-γ production from LN cells from s.c.-infected compared to uninfected mice. This was primarily related to the high level of background IFN-γ secretion in the cultures of LN cells from the infected mice, presumably due to the endogenous stimulation by amastigote antigens. In contrast, cells from the external sacral LNs (into which the popliteal nodes drain), which had no detectable parasite burden, showed strong IFN-γ production when stimulated with exogenous *Leishmania* antigens (Fig. 3). A high level of parasite-induced IFN-γ secretion by LN cells of s.c.-infected mice was evident by comparison of the ex vivo IFN-γ secretion (concentration of IFN-γ in cell culture supernatants in the absence of exogenous stimulation) by cells isolated from uninfected and infected mice (Fig. 4). In contrast, there was no ex vivo IFN-γ secretion by the spleen cells of i.v.-infected mice.

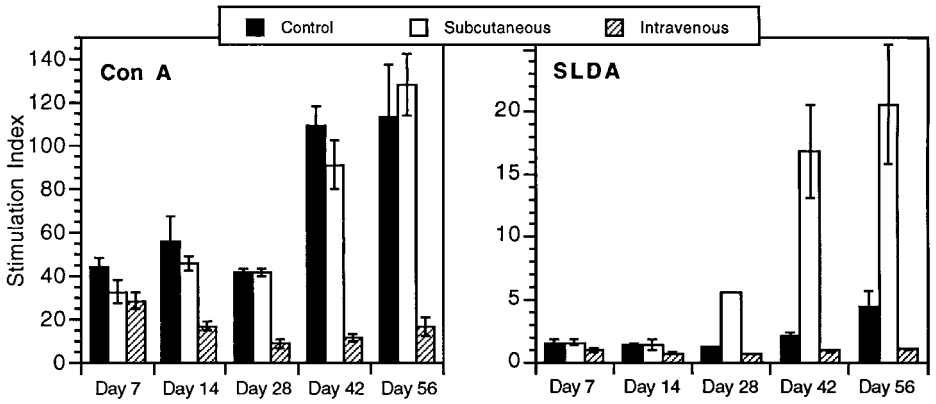

FIG. 2. Proliferative response of spleen cells from *L. donovani*-infected mice. Spleen cells were obtained from s.c.- and i.v.-infected mice and uninfected age-matched controls on days 7, 14, 28, 42, and 56 after infection. Cells were cultured in quadruplicate wells and stimulated for 3 days with ConA (5 μg/ml) or medium alone (left) or for 5 days with SLDA (25 μg/ml) or medium alone (right). One microcurie of [^{3}H]thymidine was added to the cultures for the last 16 to 18 h, and incorporation was determined by scintillation counting. Results are expressed as the mean and standard error (bars) of the stimulation indices (mean counts per minute of stimulated wells/mean counts per minute of unstimulated wells) of groups of four mice except for the antigen-induced proliferation of day 28 s.c.-infected mice, where data from only one mouse were available.

In situ cytokine and iNOS responses. Qualitative analysis of IL-4, IL-7, IL-10, IL-12 p40, IFN-γ, and iNOS expression in spleen and/or LN tissue was determined by RT-PCR and Southern blotting using a specific internal oligonucleotide probe (data not shown). In all cases, the Southern blot hybridization signal was restricted to a single amplification product of the appropriate size (there were no genomic DNA contaminants), and results were always consistent with the results of the quantitation by fluid-phase hybridization detailed below.

Semiquantitative analysis of cytokine and iNOS expression was determined by RT-PCR followed by quantification of the PCR product using fluid-phase hybridization with a specific internal oligonucleotide and an enzymatic detection system. Quantification of the amplification product was accomplished by interpolation from the linear portion of a standard curve generated by amplification of fivefold dilutions of the corresponding purified cytokine cDNA template. The standard curves were consistently linear in the range of 0.16 fg to 20 to 100 fg of input cDNA, and replicates of the standard curve dilutions always had a standard deviation of less than 10%. Representative standard curves are shown in Fig. 5. Tissue mRNA expression was always interpolated from the linear portion of the curve. The consistency and reproducibility of the RNA isolation and RT-PCR was supported by finding minimal variation (almost always less than twofold) in the quantification of *HPRT* (a constitutively expressed housekeeping gene) among equivalent amounts of total RNA isolated from the several hundred tissue samples studied.

Expression of cytokine mRNA in the spleens of infected and uninfected mice was determined at days 2, 7, 14, 28, and 56 after infection and is shown in Fig. 6. In these studies, tissues from five uninfected or infected mice were pooled prior to RT-PCR analysis. In a few experiments, individual mice (five per group) were analyzed; similar results obtained, with a standard deviation around the mean of 5 to 20% (data not shown). The level of splenic cytokine expression in s.c.-infected mice was consistently less than that of the i.v.-infected group and in most instances was not substantially greater than the levels expressed in the age-matched uninfected control mice. Statistical analysis of differences in mRNA expression was not de-

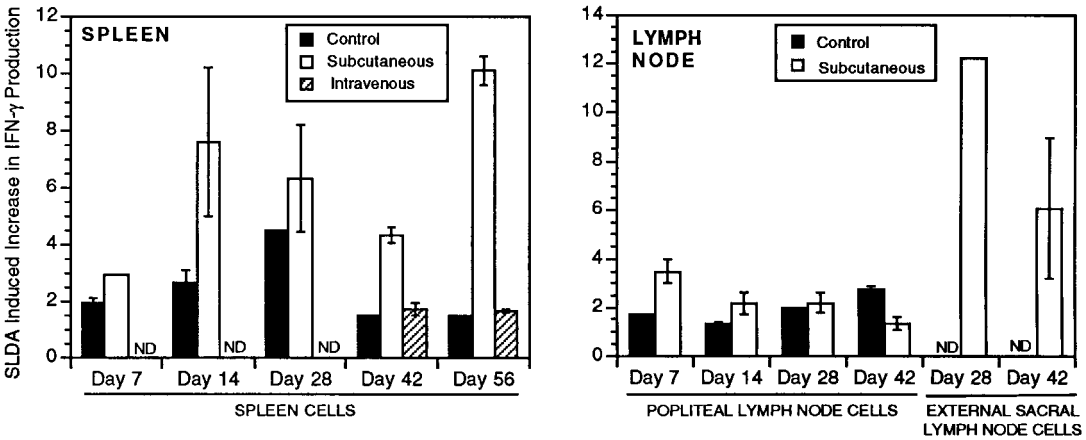

FIG. 3. IFN-γ production by spleen (left) and LN (right) cells from *L. donovani*-infected mice. Cell cultures were set up as described for Fig. 2. Supernatants from SLDA-stimulated and unstimulated (medium control) spleen cells were collected after 5 days culture and assayed for IFN-γ by sandwich ELISA. Results shown are from uninfected controls and from s.c.- and i.v.-infected mice and are expressed as the mean and standard error (bars) of the fold increase in IFN-γ production of SLDA-induced over unstimulated cells. Results are from quadruplicate cultures from one to four mice per group. External sacral LN cells from uninfected mice were not studied.

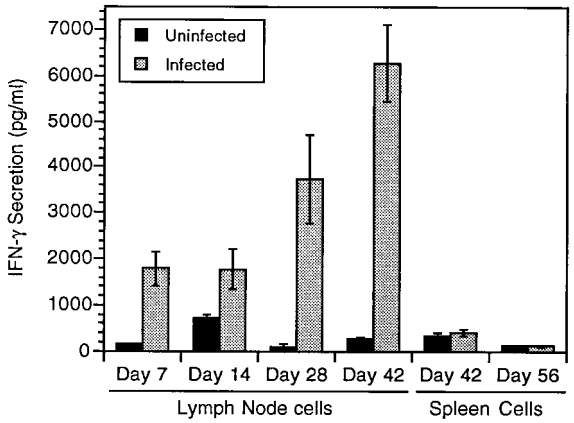

FIG. 4. Ex vivo IFN-γ secretion by LN and spleen cells isolated from *L. donovani*-infected and uninfected mice. Popliteal LN cells from s.c.-infected mice and spleen cells from i.v.-infected mice, or LN and spleen cells from control uninfected mice, were cultured as described for Fig. 2 in the absence of exogenous antigen stimulation. Supernatants were harvested on day 5 of culture and assayed for IFN-γ by sandwich ELISA. Results are expressed as the mean and standard error (bars) of quadruplicate cultures of two to four mice per group.

termined because the quantification was performed on mRNA pooled from groups of five mice.

Baseline splenic IL-7 expression was detected in all samples, but there was no increase in expression following systemic or cutaneous infection (data not shown). Increased expression of IFN-γ was first detected in the spleens of i.v.-infected mice at day 7 after infection. The level of IFN-γ mRNA increased slightly over time such that at day 28 it was twice that of uninfected controls. Subcutaneous infection resulted in a minimal (albeit consistent) increase in splenic IFN-γ mRNA at days 7, 14, and 28 after infection. The IL-4 response to infection was most evident at 2 days after infection, when two- and sixfold increases were observed in s.c.- and i.v.-infected mice, respectively. The IL-4 expression in i.v.-infected mice then dropped to approximately twice that of the uninfected controls throughout the rest of the course of infection, and the expression in s.c.-infected mice dropped to normal levels. There was no increase in splenic IL-10 mRNA synthesis at any of the time points following s.c. infection. In contrast, i.v. infection resulted in increased splenic IL-10 expression as early as 2 days after infection, and it increased gradually throughout the course of infection. IL-12 expression was modestly increased only at day 7 after infection in both the s.c.- and i.v.-infected groups and then dropped to levels equal to or less than what was observed in control mice throughout the rest of the course of infection. Thus, systemic infection with *L. donovani* resulted in a prominent early IL-4 and late IL-10 response but only slightly increased IFN-γ mRNA and transient IL-12 expression in the spleen.

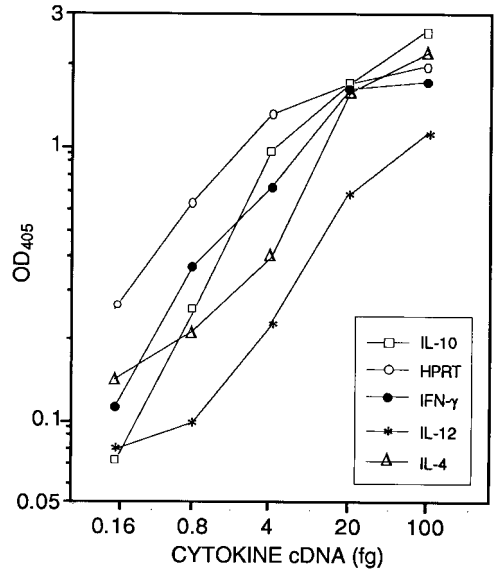

FIG. 5. Representative standard curves used for quantitation of the RT-PCR amplification products. Fivefold serial dilutions of purified cytokine and HPRT cDNA templates were amplified by using specific primers, and the product was quantified by fluid-phase hybridization with a labeled internal oligonucleotide probe and colorimetric detection. Shown are the linear portions of the curves which were used for interpolation in the determination of the quantity of RT-PCR-amplified mRNA from tissue samples. The values are graphed as the optical density at 405 nm (OD_{405}) of the colorimetric reaction versus the concentration of the input purified cDNA template.

The expression of cytokines in the LNs of mice following cutaneous infection was studied (Fig. 7). There was no increase in LN IL-7 mRNA expression in s.c.-infected compared to uninfected mice (data not shown). The LN expression of IFN-γ mRNA in response to s.c. infection was similar to what was observed in the spleen following i.v. infection, i.e., a two- to fourfold increase over control levels on days 7 through 56. LN IL-4 expression showed a dramatic (approximately 30-fold) increase at day 7 and then dropped to half-maximal levels on days 14 through 56. LN IL-10 expression was increased in infected mice over controls starting at day 7 and increasing through day 56. In striking contrast to the splenic response, the LN mRNA for IL-12 was increased at day 7 and continued to increase dramatically (>35-fold increase over controls) through day 56. Thus, the IL-4 and IL-10 responses were accompanied in the LN by a dramatic sustained increase in IL-12 expression. The expression of IL-10 and IL-12 at the cutaneous site of infection paralleled what was observed in the draining LN, despite the absence of any overt cutaneous lesion. A 10- to 20-fold increase in these cytokine transcripts was observed in the infected skin (compared to the skin of uninfected controls) at 7 and 14 days after inoculation (data not shown). The cutaneous expression of other cytokines was not studied.

The expression of iNOS mRNA in the spleens and LNs of infected and uninfected mice is shown in Fig. 8. The splenic expression of iNOS mRNA was increased substantially at 28 and 56 days after i.v. infection but was not detected in the spleens of s.c.-infected or uninfected mice. LN iNOS expression was observed earlier at 14 days after s.c. infection, and expression increased dramatically thereafter. Baseline iNOS mRNA expression was not detected in the LNs of uninfected mice.

DISCUSSION

Leishmania spp. are inoculated into the skin of the mammalian host during the bite of an infected sand fly. The visceralizing strain *L. donovani* does not typically cause cutaneous disease but disseminates from the site of inoculation to replicate in the liver, spleen, and bone marrow. Infection may be controlled by the host without the development of overt clinical symptoms, or active disease with the clinical features of fever, cachexia, hepatosplenomegaly, and pancytopenia may ensue. Subclinical infection is probably not associated with complete elimination of the parasite but small numbers of organisms persist in the skin or reticulendothelial system, where they are contained by the host cellular immune response

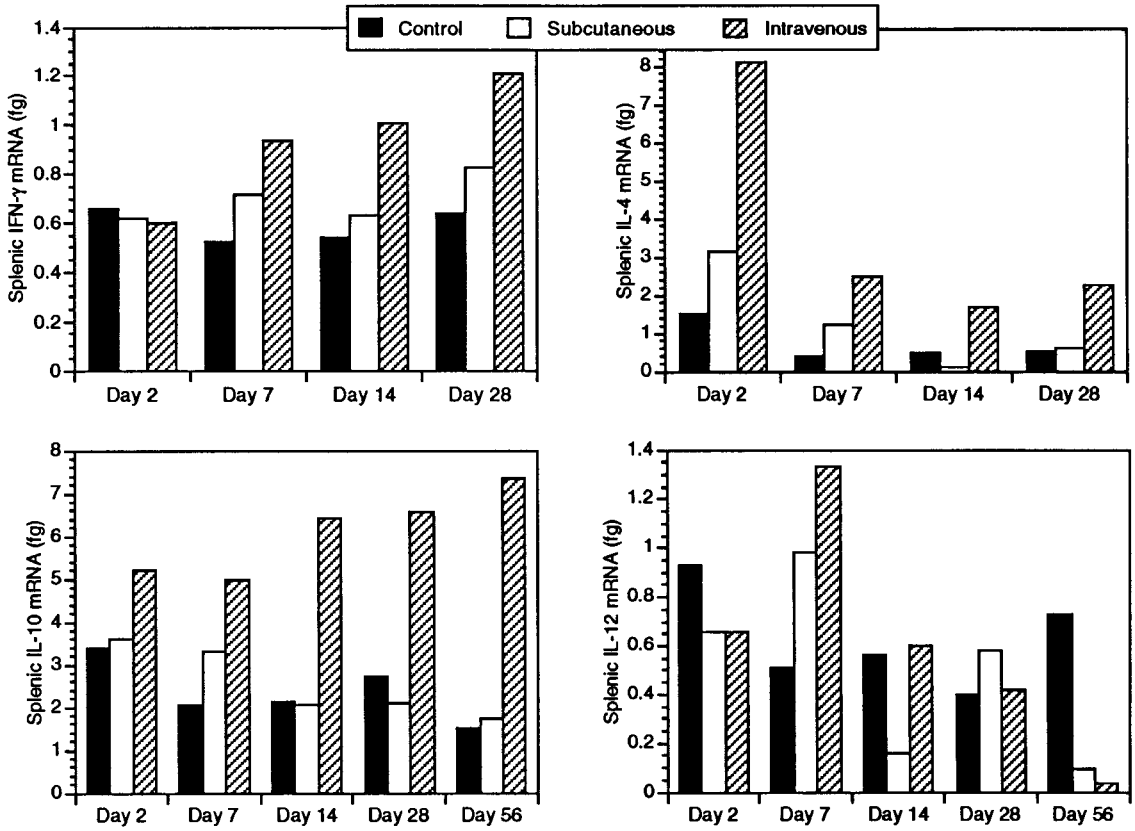

FIG. 6. Splenic cytokine expression in *L. donovani*-infected and uninfected mice. Mice were infected with 5×10^6 *L. donovani* amastigotes by either s.c. or i.v. inoculation. Age-matched uninfected mice served as controls. Spleens were harvested at 2, 7, 14, 28, and 56 days after infection, and total RNA was isolated. Then 0.5 μg of total RNA was pooled from the spleens of five mice in each group, reverse transcribed, amplified by PCR, and quantified by fluid-phase hybridization with a labeled internal oligonucleotide probe and colorimetric detection (RT-PCR ELISA). For each sample, the quantity of input cDNA was interpolated from the standard curve. Minor differences in the quantity of total RNA between samples were corrected by normalization to the quantity of HPRT in the same sample. The quantity is expressed as the femtograms of cytokine mRNA normalized to the quantity of HPRT mRNA.

(3, 32). The parasite, vector, or host factors which contribute to subclinical infection or active visceral disease have not been defined.

In this study, we examined the cellular immune response to cutaneous or systemic infection with *L. donovani* in a murine model. Cutaneous infection resulted in minimal and transient swelling at the site of inoculation, transient parasite replication in the skin, and a slowly increasing parasite burden in the draining LN. Dissemination of the parasite to visceral organs or secondary LNs was not detected by culture of the parasite. Intravenous infection, however, resulted in marked hepatosplenomegaly with a splenic parasite burden which increased throughout the 8-week study period. The splenic parasite burden following systemic infection was significantly greater than that of the LN following cutaneous infection when either the total organ parasite burden or parasite number per milligram of tissue was considered. The lower parasite burden in the skin and draining LN following s.c. infection suggests that the host immune response was more efficient in controlling the infection at these sites than in the spleen.

We characterized the cytokine response to cutaneous and visceral *L. donovani* infection at the site of the infected tissue. Splenic, LN, and cutaneous tissues were harvested at multiple time points during the course of infection, and cytokine expression was determined by RT-PCR. The most striking difference in the LN and spleen response to *L. donovani* infection was seen in the analysis of IL-12 expression and IFN-γ production. Splenic IL-12 mRNA peaked at a modest level at 7 days postinfection and then declined to barely detectable levels as the parasite burden increased. In contrast, LN IL-12 mRNA increased dramatically throughout the 8-week course of infection, and at 56 days postinfection the level (normalized to tissue HPRT expression) was approximately 300 times greater than that detected in the spleen. The LN IL-12 response is likely to be a major contributor to the strong antigen-induced spleen cell proliferative and IFN-γ responses observed in the mice following localized cutaneous infection. IL-12 has been shown in experimental *L. major* infection to play a major role in the development of the Th1 cell response (17, 33) and has been demonstrated to prevent dissemination of cutaneous *L. major* infection (21). It probably contributes in similar fashion to the prevention of visceralization following cutaneous infection with *L. donovani* in this model. The migration of cutaneous Langerhans cells with development into dendritic cells, which are highly efficient antigen presentation cells (36) and producers of IL-12 (22), may be the driving force behind the Th1 response which follows cutaneous infection. Other investigators have shown that inoculation *L. major* in the footpad of intermediately susceptible mice favored the development of a healing Th1 response, whereas inoculation at other cutaneous sites favored Th2 expansion and disease progression (20, 31). The mechanisms involved in this dichotomy have not been defined, but a difference in antigen presentation capacity (e.g., by dendritic cells) has been postulated.

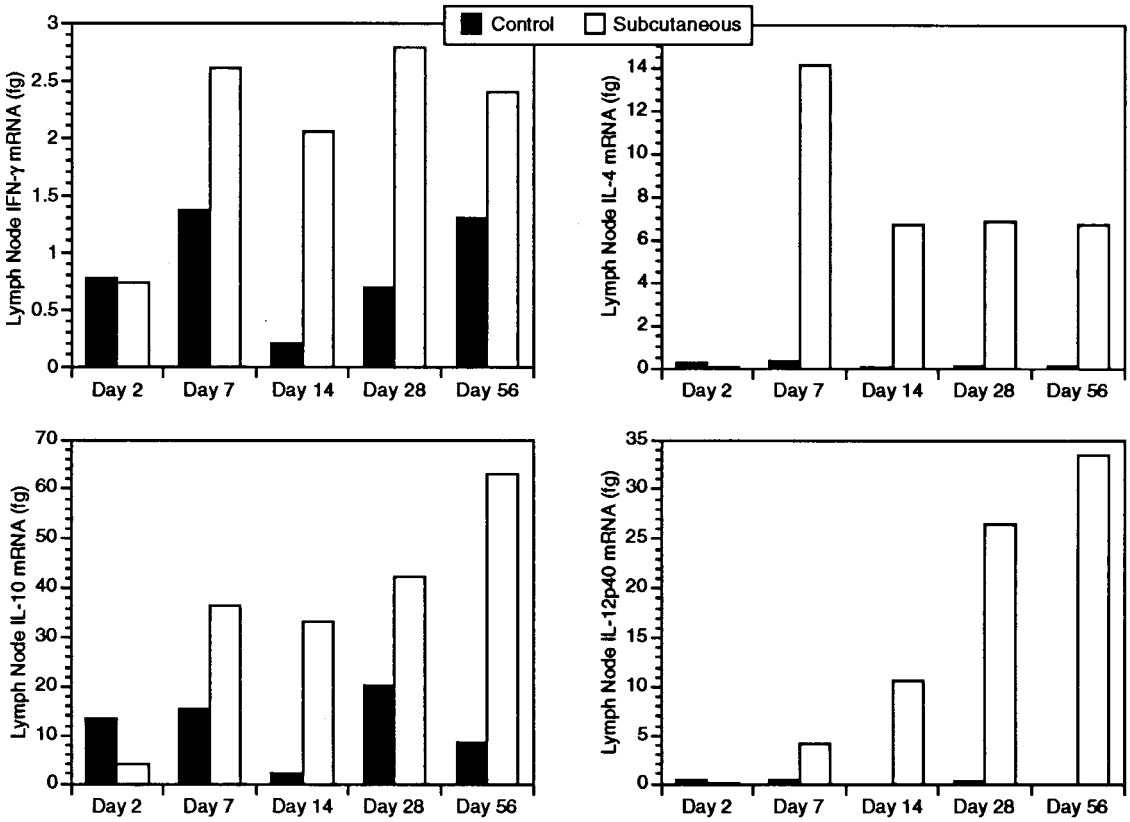

FIG. 7. Popliteal LN cytokine expression in *L. donovani*-infected mice. Mice were infected s.c. in the footpad and the draining popliteal LN harvested. Age-matched uninfected mice served as controls. Total RNA was isolated and the cytokine and HPRT mRNAs quantified by RT-PCR ELISA, and results are presented as described for Fig. 6.

It should be noted that amastigotes were used for infection in these studies, whereas natural infection in humans is initiated by promastigotes. Amastigotes were used because this form of challenge has been the standard model of experimental visceral infection in mice (19, 28, 29, 35, 38, 41), and any possibility of variation in parasite virulence related to axenic cultivation of promastigotes would be eliminated. Recently it has been noted that *Leishmania* promastigotes evade the host defenses in part by failing to induce cytokine production, and blocking IL-12 synthesis, by macrophages (7). Thus, the use of amastigotes instead of promastigotes for the cutaneous infections in these studies may have circumvented promastigote escape mechanisms and favored the development of a protective, Th1 response in the skin and draining LN. Studies comparing the cutaneous and lymph node responses to amastigote versus promastigote challenge are under way.

IFN-γ mRNA was increased in response to either splenic or LN parasitization. The increasing parasite burden in the spleen in the face of increasing IFN-γ mRNA is similar to what has been observed in human VL (18). Since infection in this model is not lethal, and neutralization of IFN-γ results in increased parasite replication (35), this modest level of IFN-γ expression would appear to be required for control of the infection but not sufficient to eliminate the parasite. Although there appeared to be only a 2- to 10-fold increase in LN IFN-γ mRNA, there was up to a 43-fold increase in the amount of IFN-γ released ex vivo from LN cells isolated from s.c.-infected compared to uninfected mice. The high level of IL-12 expression in the LN is likely to be a major stimulus to this LN IFN-γ production. In contrast, spleen cells from systemically infected mice expressed little IL-12 mRNA and did not produce IFN-γ above the level seen in uninfected controls.

IL-4 expression was also increased in the LN and spleen early after cutaneous and i.v. infection, respectively. A previous study by Miralles et al. demonstrated in situ hepatic expression of IL-4 by RT-PCR at 10 days to 8 weeks after infection, but splenic responses were not examined (28). In our study, it is striking that IL-4 was the only cytokine expressed in the spleen early (2 days) after infection. Although this early response could contribute to the establishment of the infection, treatment of mice with anti-IL-4 antibody has been shown to have no effect on *L. donovani* tissue burden (28). The failure to sustain a strong splenic IL-4 response during active infection, however, contrasts with *L. major* infection in BALB/c mice (15, 16) and may contribute to the nonlethal nature of this model. The level of IL-10 expression in both the LN and spleen increased in parallel with the increase in parasite burden observed during the course of infection. The IL-10 expression was not sufficient to inhibit expression of IFN-γ and IL-12. This has also been observed in the bone marrow of human VL (18) and the lesions of human cutaneous leishmaniasis (25, 26). Despite the inhibitory effect that IL-10 has on macrophage killing of *Leishmania* and other intracellular pathogens (12, 42), the prominent expression of IL-10 in the skin and draining LN following cutaneous infection was not sufficient to cause parasite visceralization, probably because of the coexpression of IL-12 and IFN-γ.

There was a striking difference in the ratio of IL-10 to IL-12 mRNA expression in the spleen and LN. At days 28 and 56 after infection, the ratio of IL-10 to IL-12 expression was 10-

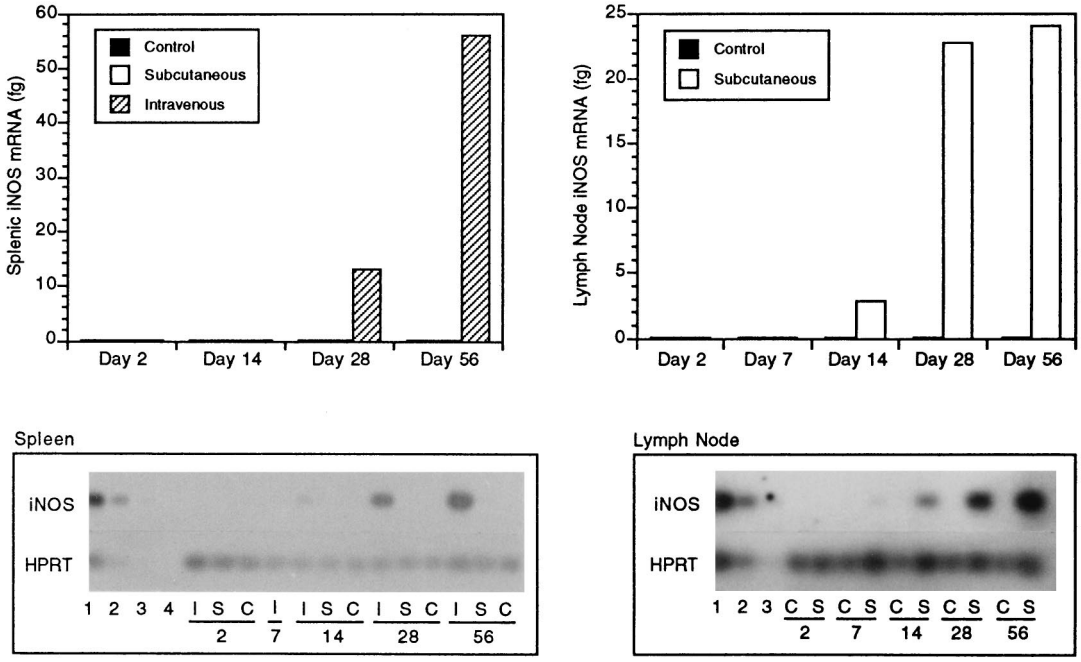

FIG. 8. Splenic and LN expression of iNOS mRNA in *L. donovani*-infected and uninfected mice. Mice were infected s.c. or i.v. or received HBSS alone by the same route. The draining popliteal LN and spleens were harvested as described in the legend to Fig. 6. Total RNA was isolated, and the iNOS and HPRT mRNAs were analyzed by Southern blotting (lower panels) and quantified by RT-PCR ELISA (top panels). Lanes 1 to 4 on the Southern blot correspond to 0.8, 0.16, 0.032, and 0 fg of input purified HPRT or iNOS cDNA template, respectively. Other lanes: I, i.v.; S, s.c.; C, uninfected control. The iNOS mRNA quantified by RT-PCR ELISA is expressed as the femtograms of iNOS mRNA normalized to the quantity of HPRT mRNA. The results are presented as described in the legend to Fig. 6.

and 70-fold higher in the spleens of systemically infected mice than in the LNs of cutaneously infected mice. In addition to the inhibitory effects of IL-10 on macrophage function, the overproduction of IL-10 relative to IL-12 within the spleen following systemic infection would also suppress splenic T-cell effector function. Studies by Wilson et al. using the *L. chagasi* murine model demonstrated that production of IL-10, a potent inhibitor of T-cell effector function and cytokine synthesis (10), by non-T-cell splenocytes contributes to the observed suppression of splenic T-cell function (43). We have also identified strong IL-10 and TGF-β production in spleen tissue by immunohistochemical staining (27a). Expression of TGF-β mRNA, another potent inhibitor of T-cell proliferation and cytokine synthesis, was not analyzed in this study because it is not transcriptionally regulated (2) and therefore quantitative measurement by RT-PCR would be difficult to interpret.

The initiation of a strong Th1-like host response in the LN and spleen following cutaneous infection, but not in the spleen following systemic infection, was also demonstrated by in vitro studies. Our study confirmed the results of other investigators who demonstrated that visceral infection resulted in a dramatic loss of spleen cell proliferative response to a mitogen and an absence of significant proliferation and IFN-γ production when stimulated with exogenous *Leishmania* antigens (19, 30). We also found that there was no increase in the ex vivo secretion of IFN-γ from spleen cells isolated from systemically infected mice compared to spleen cells from uninfected controls. In contrast, cutaneous infection resulted in (i) high levels ex vivo IFN-γ secretion from draining LN cells and (ii) strong antigen-specific spleen cell proliferative and IFN-γ recall responses. These differences parallel those seen in human *L. donovani* infection, where those individuals who have had cutaneous exposure but have no clinical evidence of visceral involvement have parasite specific Th1 responses, but those who suffer from active visceral disease are anergic to parasite antigens (3, 8, 9). In this murine model, the development of a Th1-type spleen cell response following cutaneous infection is also associated with resistance against subsequent i.v. challenge (23a). This is also analogous to the human situation where individuals in an area where disease is endemic who have evidence of past subclinical infection (positive leishmanin skin test) are protected against the subsequent development of active visceral disease (3, 34).

The expression of macrophage iNOS and generation of nitric oxide in response to IFN-γ is a critical effector mechanism in the control of murine *L. major* infection (14, 37), but its role in experimental VL has not been previously defined. We observed an increase in iNOS mRNA expression in both the LN and spleen in response to cutaneous and visceral infection, respectively. The increased expression of iNOS in response to *L. donovani* infection is in striking contrast to the minimal level of iNOS expression induced by *L. major* infection, which is progressive and lethal in this mouse strain (37). Thus, the generation of NO in response to this infection may account for the ultimate control of infection in this nonlethal model. The upregulation of iNOS expression however, was not temporally associated with a reduction in parasite burden as has been seen in resistant mice infected with *L. major* (37).

There was considerable delay between the time of inoculation, expression of IFN-γ mRNA, and the detection of iNOS mRNA. The reason for this delayed iNOS response is unknown but is similar to what was observed in a resistant mouse strain infected with *L. major* (37). The LN iNOS response following cutaneous infection was detected earlier (14 days) than that of the spleen following i.v. infection (28 days) and may contribute to the lower tissue parasite burden in the LN and the absence of visceralization following cutaneous infection. The delayed iNOS expression in the spleen may be due to

transient downregulation by the observed early splenic expression of IL-4 or IL-10 or by local production of TGF-β (5, 11, 12).

In these studies, we found that locally controlled cutaneous infection was associated with antigen-specific spleen cell responsiveness, markedly increased levels of IFN-γ, IL-12, and iNOS mRNAs in the draining LN, and a high level of parasite-induced IFN-γ secretion by LN cells. In contrast, progressive splenic parasitism was associated with an absence of antigen-specific spleen cell responsiveness, a dramatically increased ratio of splenic IL-10 to IL-12 mRNA expression, and no parasite-induced IFN-γ secretion. Additionally, in the spleen there was delayed expression of iNOS in response to visceral infection. The balance between IL-10 and IL-12 expression and the associated level of IFN-γ production and macrophage activation is likely to play a critical role in the differences in the parasite burdens observed at the different sites of infection. These findings provide insight into the host mechanisms that may contribute to the development of subclinical infection and active visceral disease in human *L. donovani* infection.

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