Immunity to *Trichinella spiralis* Infection in Vitamin A-deficient Mice

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Summary

Vitamin A–deficient (A^-) mice make strikingly poor IgG responses when they are immunized with purified protein antigens. Previously, we showed that A^- T cells overproduce interferon γ (IFN-γ), which then could inhibit interleukin 4 (IL-4)-stimulated B cell IgG responses. To determine if the altered IFN-γ regulation pattern and its immunological consequences would extend to a natural infection, we studied mice infected with the parasitic helminth *Trichinella spiralis*. The course of the infection was similar in A^- and A-sufficient (A^+) mice. These mice did not differ with respect to newborn larvae/female/hour produced in the intestine, or muscle larvae burden 5 wk postinfection. They also did not differ in the intestinal worm expulsion rate until day 15, when A^- mice still harbored parasites, whereas A^+ mice had cleared intestinal worms. Vitamin A deficiency reduced both the frequency of B lymphocytes secreting IgG1 antibodies to parasite antigens, and the bone marrow eosinophilia associated with helminth infection. The cytokine secretion patterns in infected mice were consistent with these observations and with previous studies. Mesenteric lymph node cells from infected A^- mice secreted significantly more IFN-γ, and significantly less IL-2, IL-4, and IL-5 than infected A^+ controls. A^- splenocytes secreted significantly more IFN-γ, and equivalent amounts of IL-2, IL-4, and IL-5 compared with A^+ controls. Interestingly, $CD4^-CD8^-$ cells secreted the majority of the IL-4 produced in the spleen. The IL-2, IL-4, and IL-5 steady-state transcript levels correlated with secreted protein levels, but IFN-γ transcripts did not. Although they secreted more protein, A^- cells contained fewer IFN-γ transcripts than A^+ cells. These results suggest two vitamin A–mediated regulation steps in IFN-γ gene expression: positive regulation of IFN-γ transcript levels, and negative regulation posttranscriptionally. The essentially unaltered outcome of *T. spiralis* infection in vitamin A–deficient mice probably reflects a balance between cellular and humoral responses. The IFN-γ overproduction might have a positive effect on the gut inflammatory response, but the decreased eosinophilia, cytokine production in mesenteric lymph node, and IgG1-secreting cell frequency might have a negative effect on *T. spiralis* immunity.

To study the molecular mechanism by which vitamin A sustains immune function, we produced vitamin A–deficient (A^-)[1] mice and analyzed their immune responses to a model protein antigen. The IgG1 antibody response is profoundly diminished in A^- mice, due to a decreased frequency of IgG1-secreting B lymphocyte clones (1). Helper T lymphocytes from A^- mice fail to provide the B cell stimulus for antigen-specific IgG1 responses (2). The molecular basis for this failure may be elevated IFN-γ production by A^- T lymphocytes (3). Secretion of IL-2 and IL-4 by A^- T cells is equivalent to vitamin A–sufficient (A^+) controls. Either neutralizing the excess IFN-γ or supplementing with vitamin A in vitro restores IgG1 responses to control levels. Excess IFN-γ could inhibit IL-4-stimulated B cell class switching and clonal expansion, accounting for reduced IgG-secreting cell frequencies in A^- mice. Vitamin A evidently negatively modulates IFN-γ secretion.

Regulation of cytokine transcription, translation, and secretion is complex, and not well understood (reviewed in reference 4). Studies in vitro suggest antigen-stimulated mouse T cells may differentiate to secrete particular groups of cytokines coordinately (5). Type 1 T cell clones (Th1) secrete IL-2, IFN-γ, and lymphotoxin, but not IL-4, IL-5, IL-6, or IL-10; type 2 clones (Th2) exhibit the reciprocal pattern (5). Each clonotype apparently regulates the growth and activity of the other clonotype; IFN-γ inhibits Th2 cell growth in vitro (6) and several IL-4 activities (7, 8), whereas IL-10 in-

[1] *Abbreviations used in this paper:* A^-, vitamin A deficient; A^+, vitamin A sufficient; MLNC, mesenteric lymph node cells.

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hibits Th1 cell cytokine secretion (9). In some parasitic infections, the course of the disease correlates with the apparent in vivo stimulation of either Th1- or Th2-type responses (10).

We sought to examine the immunological consequences of vitamin A deficiency during a natural infection with particular emphasis on Th cell function. We chose the parasitic helminth, *Trichinella spiralis*, since our laboratory has characterized the immune response of inbred mouse strains to this parasite (11). Infection induces elevated IgG1 and IgE levels in the susceptible B10.BR strain compared with a resistant strain. The B10.BR T cells secrete abundant IL-4 and IL-5, but very little IFN-γ and IL-2 (11). In experiments reported here, we studied parasite resistance, antibody responses, and cytokine production in A^- and A^+ B10.BR mice infected with *T. spiralis*.

Materials and Methods

Mice. Strain B10.BR breeding pairs were obtained from The Jackson Laboratory (Bar Harbor, ME). A^- and A^+ mice were produced at The University of Wisconsin as described (1, 12), and the deficiency was confirmed by serum retinol analysis (12).

Parasitology. For all experiments, mice were infected by mouth at 6–7 wk of age with 150–200 *T. spiralis* L1 larvae as described (11). Adult worm expulsion rates, female fecundity, and muscle larvae burden were analyzed exactly as described (11).

Eosinophils. We used a small brush wet with HBSS to pick up bone marrow cells from longitudinally sliced femurs and streaked them onto glass slides. Slides were fixed in methanol for 15 min and stained in Luxol fast blue MBS (0.1% wt/vol; Sigma Chemical Co., St. Louis, MO) dissolved in urea-saturated ethanol (70% vol/vol in water) (13). The stained slides were washed 2 h in running tap water, counterstained 2 min in haemotoxylin solution (Sigma Chemical Co.), rinsed, and air dried. Eosinophils were counted in the light microscope at 40× using an oil emersion objective.

Antibodies. Hybridoma cell lines secreting mouse IgG2a mAb to rat κ chains (Mar18.5) (14), mouse IgG2b mAb to $I\text{-}A^k$ (10-2.16) (15), rat IgG2a mAb to mouse CD8 (53-6.72) (16), rat IgG2b mAb to mouse CD4 (GK1.5) (17), and rat IgG1 mAb to mouse IFN-γ (R4-6A2) (18) were purchased from the American Type Culture Collection (Rockville, MD). Dr. W. E. Paul (National Institutes of Health, Bethesda, MD) provided the 11B11 hybridoma cells secreting rat IgG1 mAb to mouse IL-4 (19). Dr. R. L. Coffman (DNAX Research Institute, Palo Alto, CA) kindly gave the following hybridoma cell lines: S4B6-secreting rat IgG2a mAb to mouse IL-2, TRFK4- and TRFK5-secreting rat IgG2a and rat IgG1 mAbs, respectively, to mouse IL-5 (20). Dr. P. Scott (University of Pennsylvania, Philadelphia, PA) and Genetech (San Francisco, CA) generously donated rabbit antiserum to mouse IFN-γ.

The mAb 10-2.16 and Mar18.5 were chromatographed on protein A–Sepharose as described (21). The mAbs 11B11, TRFK4, and TRFK5 were chromatographed on a Mar18.5-Sepharose column. Ascites fluid for R4-6A2 and S4B6 was produced in pristane-primed, irradiated BALB/c mice. The mAb 10-2.16 and TRFK5 were conjugated to biotinyl-ε-amino caproic acid *N*-hydroxy succinimide ester as described (22). Isotype-specific goat antibodies to mouse IgG1 and IgG2a were from Southern Biotechnology (Birmingham, AL). Goat antibodies to mouse IgA were from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). Rabbit antiserum to mouse IgE was from Bethyl Laboratories (Montgomery, TX). These isotype-specific reagents exhibited no crossreactivity and were used at saturating concentrations. Streptavidin-β-galactosidase was from Bethesda Research Laboratories (Gaithersburg, MD).

Antibody Responses. An isotype-specific ELISA quantitated serum IgG1, IgG2a, IgA, and IgE antibodies to parasite antigens (11). Serum antibody concentrations were calculated from standard curves generated using purified mouse myeloma proteins or mAb. The detection limits of the isotype-specific ELISAs were 20–40 ng/ml. We used a two-site ELISA to quantitate total serum IgE. Wells were coated with goat antibodies to mouse heavy and light chains (10 μg/ml in PBS; Jackson Immunoresearch, West Grove, PA). Wells were then blocked, washed, reacted with serum antibodies, and the assay was completed as before (11). The detection limit for the capture IgE ELISA was 3 ng/ml.

A filter-immunoplaque assay quantitated B cells secreting IgG1 antibodies to parasite antigens exactly as described (11). Briefly, wetted nitrocellulose filters were coated with parasite proteins, blocked, and used as a substrate for cell culture. Cells were washed away with Tween 20 in buffer, and filter plaques were developed by addition of antibodies to mouse Ig coupled to horseradish peroxidase, followed by substrates peroxide and 4-chloro-1-napthol.

Cell Cultures. All cell cultures were established in HL-1 serum-free medium (Ventrex, Portland, ME) with 2-ME (50 μM), 2 mM glutamine, penicillin (10 U/ml), and streptomycin (10 μg/ml). Cultures (1 ml) were in 24-well plates (Falcon Labware, Oxnard, CA) maintained at 37°C in humidified 5% CO_2.

Various days after infection, mesenteric lymph nodes (MLN) and spleens were collected. Mesenteric lymphocytes (10^6/well) and splenocytes (10^6/well) were cultured for 48 h with medium or dialyzed L1 larval protein extract (25 μg/ml) prepared exactly as described (11). Sterile-filtered supernatants were aliquoted and stored at −20°C until analysis.

In some experiments, mesenteric lymph node cells (MLNC) and splenocytes were collected 9 d after infection and depleted of $CD4^+$ and $CD8^+$ cells. Cells (2×10^7/ml) were incubated for 45 min on ice with either medium, GK1.5 antibodies to CD4 (20% vol/vol culture supernatant in HL-1), or 53-6.72 antibodies to CD8 (10% vol/vol culture supernatant in HL-1). Washed cells were incubated for 45 min at 37°C in rabbit C (7% vol/vol in HL-1; Pelfreez, Brown Deer, WI) supplemented with mAb Mar18.5 (10 μg/ml). Depleted populations, confirmed by flow cytometry, contained <1% residual $CD4^+$ and $CD8^+$ cells.

Cytokine Analysis. IL-4 and IFN-γ were measured as described (11) with reference to standard curves of mouse rIL-4 (Genzyme, Boston, MA) and mouse rIFN-γ (Amgen Biologicals, Thousand Oaks, CA). The IL-4 detection limit was 0.01 ng/ml; the class II molecule expression increased up to 55 channels on a three-decade, 250 channel scale. The IFN-γ detection limit was 2 ng/ml.

IL-5 was measured by ELISA as described with wash steps between additions (20). The capture antibody was TRFK4 (4 μg/ml, 50 μl/well). Wells were blocked with 2.5% BSA (150 μl/well, 1 h, 37°C). Cell culture supernatants were added (50 μl/well) and incubated for 3 h at 37°C. Captured IL-5 was detected by biotinylated TRFK5 (1 μg/ml, 50 μl/well, 2 h, 37°C), followed by avidin-β-galactosidase (Bethesda Research Laboratories). The signal was amplified using biotinylated antibodies to avidin (1 μg/ml, 50 μl/well, 1 h, 22°C; Vector Laboratories, Burlingame, CA), followed by avidin-β-galactosidase, and then substrate.

The IL-2 was measured by a growth assay using CTLL-2 cells (provided by Dr. D. Paulnock, University of Wisconsin, Madison, WI); human rIL-2 (provided by Dr. B. Baranski, University of Wisconsin) served as the standard. CTLL-2 cells (5×10^3/well) were cultured (0.2 ml, 96-well plate) with supernatants for 48 h in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with

1 mM sodium pyruvate, 2 mM glutamine, 10% FCS, 10 μg/ml streptomycin, 10 U/ml penicillin, and antibodies to IL-4 (1 μg/ml). Control cultures included antibodies to IL-2 (0.5% vol/vol ascites fluid in medium), which completely blocked CTLL proliferation in response to the supernatants. [^{3}H]Thymidine (1 μCi/well; DuPont Co., Wilmington, DE) was included for the final 12–18 h of culture. Cells were harvested onto glass fiber filters, and radioactivity was determined by liquid scintillation counting.

Northern Blot Analysis. MLNC and splenocytes were collected 6 d after infection and cultured (10^7/well) for 3, 6, or 12 h with L1 larval proteins (25 μg/ml). At each time point, supernatants were collected for cytokine analysis, and cells were collected for RNA extraction.

Total cellular RNA was isolated essentially as described (23). For Northern blot analysis, 5 μg of each RNA sample was subjected to electrophoresis through a 1% agarose-formaldehyde gel (24) and transferred to Genescreen Plus (DuPont Co.) by capillary blot in 10× SSC (24). The RNA was ultravioletly crosslinked to the filter (1 min) and baked at 65°C for 1–2 h. Filters were prehybridized (15 min, 65°C) in Pipes buffer (50 mM Pipes, pH 6.8, 50 mM sodium phosphate, 100 mM sodium chloride, 5% SDS, 1 mM EDTA). The labeled probe was added (10^8–10^9 cpm/μg) and hybridization was done for 18 h at 65°C. The IFN-γ, IL-4, IL-5, and β-actin blots were washed at 65°C to a final stringency of 0.5× SSC with 0.1% SDS. The IL-2 blots were washed at 65°C to a final stringency of 0.2× SSC with 0.1% SDS. The blots were autoradiographed (XAR5; Kodak) at −70°C with one intensifying screen (Lightning Plus, DuPont Co.).

Probes were labeled by random priming (25). Dr. F. Lee (DNAX Research Institute) provided the plasmids containing coding sequences for IL-2, IL-4, and IFN-γ. The plasmid containing the IL-5 coding sequence was purchased from the American Type Culture Collection. Dr. B. Olwin (University of Wisconsin) provided the chicken β-actin probe. The IL-2 probe was a 1.1-kb BamHI fragment of the plasmid pcD.IL2 (26). The IL-4 probe was a 726-bp BamHI fragment of the plasmid pcD.IL4 (27). The IFN-γ probe was a 1.5-kb BamHI fragment of the plasmid pcD.IFN (28). The IL-5 probe was a 1.6-kb BamHI fragment of the plasmid pmIL5-4G (29).

Statistics. Four to six mice comprised each experimental group. An individual mean and SD was calculated from replicate measurements for each animal. The group means for A^+ and A^- mice were compared using the Wilcoxon W test for nonparametric data (30).

Results

The results reported here are attributable to a single nutrient deficiency in vitamin A. Weights of A^- (21.0 ± 2.5 g) and A^+ (21.2 ± 2.5 g) mice were not different. The A^- serum retinol concentration was <20% of A^+ controls, and the A^- mice exhibited no other symptoms of vitamin A deficiency.

Parasite Resistance. We assayed three parameters of resistance to primary *T. spiralis* infection and they were essentially similar in A^- and A^+ mice. Similar numbers of worms established themselves in the small intestines of A^- and A^+ mice on day 6 postinfection (Fig. 1). Expulsion rates were identical until day 12, but differed on day 15 ($p < 0.008$); at this time, the A^+ mice had completely cleared worms from the gut, whereas the A^- mice still harbored some

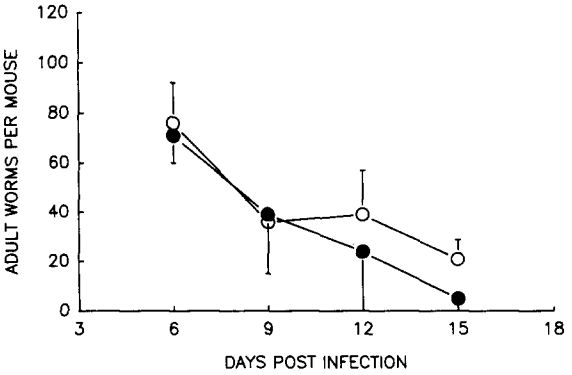

Figure 1. Intestinal worm expulsion from A^- and A^+ *T. spiralis*–infected mice. (○) A^- mice; (●) A^+ mice. Intestinal worms were quantified as described in Materials and Methods. Values represent mean and SD for five to six mice per group; one representative experiment of two.

worms. Adult female worms obtained from A^- and A^+ mice did not differ in fecundity (one to three newborn larvae/female/h) on days 6–15. Finally, A^- (20,000 ± 8,000) and A^+ mice (16,000 ± 4,000) did not differ significantly in muscle larval burdens (10 mice/group; $p > 0.05$). Thus, the course or primary *T. spiralis* infection is quite similar in A^- and control mice, with the exception of a prolonged intestinal phase in the deficient mice.

Antibody Responses. Previous experiments showed a dramatic decline in the IgG-secreting B lymphocyte frequency in A^- mice (1). Our results in *T. spiralis*–infected animals confirmed this observation (Fig. 2). At the response peak, the A^- mice had ~11 B cells per million MLNC secreting parasite-specific IgG1, whereas A^+ mice had ~29 per million. In the spleen, A^- mice had about three B cells per million secreting parasite-specific IgG1, whereas A^+ mice had ~15 such cells per million. Therefore, vitamin A deficiency diminished the memory IgG1-secreting B lymphocyte frequency about three- to fivefold during *T. spiralis* infection.

We analyzed parasite-specific serum antibody concentrations as a function of time (days 12–32) postinfection, and they were similar in A^- and A^+ mice. The peak IgG1 responses occurred on day 21 (A^-, 1.0 ± 0.8 mg/ml; A^+, 1.1 ± 0.7 mg/ml); the A^- and A^+ mice did not differ at any time postinfection. The *T. spiralis*–reactive serum IgA and IgE antibody concentrations were low, variable, and not

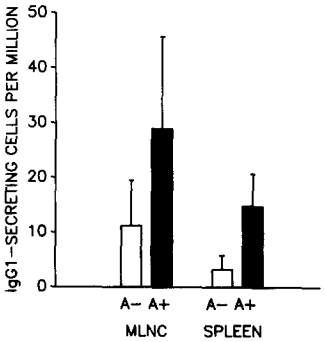

Figure 2. Lymphocytes secreting parasite-specific IgG1 antibodies in A^- and A^+ *T. spiralis*–infected mice. (□) A^- mice; (■) A^+ mice. The filterimmunoplaque assay was performed on day 21 as described in Materials and Methods. Values represent mean and SD for six mice per group; one representative experiment of three.

Table 1. *Antigen-nonspecific IgE Responses in T. spiralis-infected A⁻ and A⁺ Mice*

Day postinfection	Serum IgE antibodies	
	A⁻	A⁺
	mg/ml	
12	2.21 ± 1.13	1.77 ± 0.56
15	1.92 ± 0.46	2.28 ± 0.78
21	2.36 ± 0.82	2.49 ± 0.61
28	2.33 ± 1.60	2.61 ± 0.76

different between the groups. Serum IgG2a responses were undetectable. We also analyzed nonspecific serum IgE concentrations on days 12–28 postinfection (Table 1). IgE was not detectable in serum from uninfected mice (data not shown). The A⁻ and A⁺ mice showed the elevated serum IgE levels that are characteristic of helminth infections, and they did not differ at any time postinfection.

Eosinophils. Eosinophilia is a hallmark of the immune response to parasitic helminth infection. We measured bone marrow eosinophil percentage as a function of time during *T. spiralis* infection (Fig. 3). Eosinophilia peaked at day 21 in A⁺ mice, with 22 ± 3% bone marrow eosinophils. In contrast, A⁻ mice had just 7–8% bone marrow eosinophils throughout the infection. Eosinophils in peripheral blood and spleens of both A⁺ and A⁻ mice were low and variable. Thus, vitamin A deficiency dramatically reduced bone marrow eosinophil synthesis during *T. spiralis* infection.

Parasite-stimulated Cytokine Secretion. Previous experiments showed excess IFN-γ production from A⁻ T lymphocytes (3). Our results in *T. spiralis*–infected mice confirmed and extended this observation. Freshly explanted lymphocytes cultured in medium secreted insufficient protein for direct cytokine assays. Therefore, we collected cells 3–15 d postinfection and restimulated them in vitro with parasite extract. Culture supernatants collected 48 h later contained sufficient

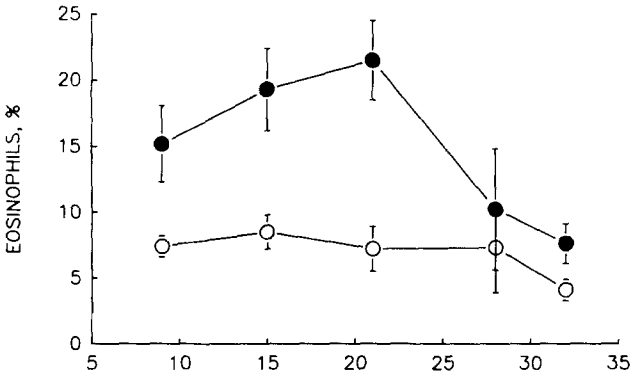

Figure 3. Bone marrow eosinophils in A⁻ and A⁺ *T. spiralis*–infected mice. (○) A⁻ mice; (●) A⁺ mice. Eosinophils were determined as described in Materials and Methods. Values represent mean and SD for 18 mice per group; composite of three experiments.

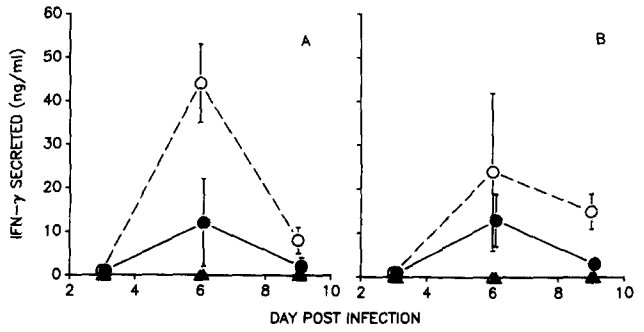

Figure 4. Development of IFN-γ-secreting cells in A⁻ and A⁺ *T. spiralis*–infected mice. (*A*) MLNC; (*B*) spleen. (○) A⁻ cells; (●) A⁺ cells cultured with antigen; (△) A⁻ cells; (▲) A⁺ cells cultured in medium. Samples were assayed for IFN-γ using a capture ELISA as described in Materials and Methods. Values represent mean and SD of five to six mice per group; one representative experiment of three.

cytokines for assay. The percentages of B220⁺, I-A⁺, Thy-1⁺, CD4⁺, and CD8⁺ cells in the MLN and spleens of A⁺ and A⁻ mice were similar at the initiation of each culture (data not shown).

IFN-γ secretion was greatest from cells collected 6 d postinfection in the MLN (Fig. 4 *A*) and in the spleen (Fig. 4 *B*). The infected A⁺ and A⁻ mice did not differ in IFN-γ production kinetics. At the peak of the response, A⁻ MLNC and splenocytes secreted significantly more IFN-γ than A⁺ controls. These results concur with previous studies (3) and suggest that vitamin A negatively regulates IFN-γ secretion.

IL-2 secretion was greatest from cells collected 6 d postinfection in the MLN (Fig. 5 *A*), and plateaued by day 6 in the spleen (Fig. 5 *B*). The A⁺ MLNC secreted more IL-2 than A⁻ cells on day 6, but the responses were equivalent on days 3 and 9 (Fig. 5 *A*). In the spleen, IL-2 production was undetectable on day 3 and the groups did not differ at later times (Fig. 5 *B*). Therefore, splenic IL-2 secretion in infected mice closely matched previous results obtained with a model protein antigen (3).

The peak of the IL-4 response was delayed relative to IL-2

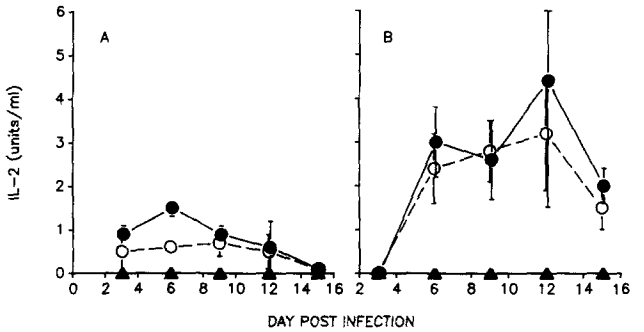

Figure 5. Development of IL-2-secreting cells in A⁻ and A⁺ *T. spiralis*–infected mice. (*A*) MLNC; (*B*) spleen. (○) A⁻ cells; (●) A⁺ cells cultured with antigen; (△) A⁻ cells; (▲) A⁺ cells cultured in medium. Samples were assayed for IL-2 by a CTLL-2 growth assay described in Materials and Methods. Values represent mean and SD of five to six mice per group; one representative experiment of three.

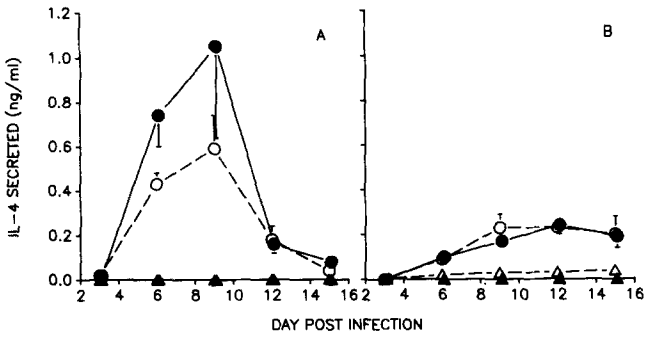

Figure 6. Development of IL-4-secreting cells in vitamin A^- and A^+ *T. spiralis*-infected mice. (*A*) MLNC; (*B*) spleen. (O) A^- cells; (●) A^+ cells cultured with antigen; (Δ) A^- cells; (▲) A^+ cells cultured in medium. Samples were assayed for IL-4 by a small B cell class II induction assay described in Materials and Methods. Values represent mean and SD of five to six mice per group; one representative experiment of three.

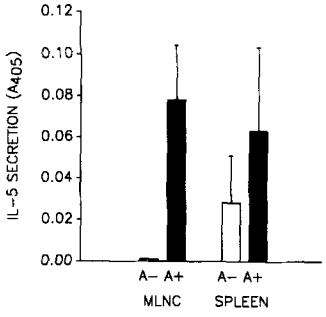

Figure 7. Development of IL-5-secreting cells in A^- and A^+ *T. spiralis*-infected mice. (□) A^- cells; (■) A^+ cells. Cells were collected day 9 postinfection and restimulated with parasite antigens. Samples were assayed for IL-5 using a capture ELISA described in Materials and Methods. Values represent mean and SD of five to six mice per group; one representative experiment of three.

and IFN-γ. IL-4 secretion was greatest from cells harvested 9 d postinfection in the MLN and spleen (Fig. 6). On days 3–9, A^+ MLNC secreted more IL-4 than A^- cells. However, A^+ and A^- splenocyte IL-4 secretion did not differ on any day, consistent with previous results (3).

We detected IL-5 secretion only from cells harvested 9 d after infection (Fig. 7). A^+ MLNC secreted significantly more IL-5 than A^- cells. Splenocyte IL-5 secretion was variable, and not significantly different.

Depletions of CD4⁺ and CD8⁺ Cells. The major producers of IFN-γ include $CD4^+$ T cells (31), $CD8^+$ T cells (32), and NK cells (33). To phenotype the cytokine-secreting cells in infected A^- and A^+ mice, we collected cells 9 d after infection and treated them with C alone, or with antibodies to CD4 or CD8 plus C. Antigen-stimulated, untreated cultures of A^- MLNC secreted 10-fold more IFN-γ than A^+ MLNC cultures (Table 2, group B). Treatment with C only did not significantly affect those responses. $CD4^+$ cell depletion completely eliminated IFN-γ secretion in both A^- and A^+ groups. $CD8^+$ cell-depleted cultures produced slightly more IFN-γ than untreated cultures, consistent with an enrichment for IFN-γ producers. $CD8^+$ cell-depleted, A^- MLNC secreted >15-fold more IFN-γ than $CD8^+$ cell-depleted A^+ MLNC.

We observed similar results in the spleen (Table 2). Untreated A^- splenocytes secreted about threefold more IFN-γ than A^+ splenocytes (group B). Treatment with C only did not significantly affect those responses. $CD4^+$ cell depletion completely removed IFN-γ secretors in both groups, whereas $CD8^+$ cell depletion enriched IFN-γ producers. Therefore, $CD4^+$ cells appear to be the primary IFN-γ secretors. We conclude that vitamin A deficiency enhances the development or function of IFN-γ secreting $CD4^+$ MLNC and splenocytes.

Cultures from the experiment shown in Table 2 were also assayed for IL-4 (Table 3). As before in MLN (Fig. 6 *A*), untreated, antigen-stimulated A^+ cells secreted more IL-4 than A^- cells, and C treatment alone had no effect. $CD4^+$ cell depletion completely eliminated IL-4 secretors in both MLNC groups. $CD8^+$ cell-depleted cultures produced as much IL-4 as untreated cultures. Similarly, $CD4^+$ cell depletion but not $CD8^+$ cell depletion removed IL-2- and IL-5-secreting cells

Table 2. *The CD4 and CD8 Phenotype of IFN-γ-producing Cells*

			IFN-γ secreted			
			Mesenteric LN		Spleen	
Group*	Antigen	Treatment	A−	A+	A−	A+
			ng/ml			
A	−	−	1	<1	<1	<1
B	+	−	23	2	8	2
C	+	C	31	2	5	2
D	−	αCD4 + C	<1	<1	<1	<1
E	+	αCD4 + C	<1	<1	<1	<1
F	−	αCD8 + C	<1	<1	<1	<1
G	+	αCD8 + C	37	2	16	2

* Cells pooled from five A^- mice and from five A^+ mice were treated and cultured as described in Materials and Methods. The IFN-γ was assayed by ELISA. Values represent the mean of duplicate assay wells in the linear titration range. One representative experiment of two.

Table 3. *The CD4 and CD8 Phenotype of IL-4-producing Cells*

Group*	Antigen	Treatment	IL-4 secreted			
			Mesenteric LN		Spleen	
			A−	A+	A−	A+
			ng/ml			
A	−	−	<0.01	<0.01	0.15	0.03
B	+	−	0.11	1.96	0.39	0.34
C	+	C	0.20	1.76	0.37	0.22
D	−	αCD4 + C	<0.01	<0.01	0.06	0.01
E	+	αCD4 + C	<0.01	<0.01	0.39	0.15
F	−	αCD8 + C	<0.01	<0.01	0.08	<0.01
G	+	αCD8 + C	0.15	1.97	0.39	0.23

Samples from the experiment shown in Table 1 were assayed for IL-4 as described in the Fig. 6 legend. One representative experiment of two.

(data not shown). We conclude that $CD4^+$ cells are the major IL-2, IL-4, IL-5, and IFN-γ producers in the MLN of infected mice.

The IL-4 producers in the spleen showed a different phenotype. As before (Fig. 6 *B*), splenocytes from infected A^- mice secreted a detectable amount of IL-4 without antigen restimulation (Table 3, group A). Antigen stimulation increased the IL-4 secretion to equivalent levels in the A^- and A^+ groups. $CD4^+$ cell depletion slightly decreased IL-4 secretion by unstimulated splenocytes (group D), and had little effect on the antigen-stimulated splenocyte secretion (group E). $CD8^+$ cell depletion did not significantly affect IL-4 secretion in any group. These results demonstrate that $CD4^+$ splenocytes contribute some IL-4. However, in the spleens of infected A^+ and A^- mice, most IL-4 derives from $CD4^-CD8^-$ cells.

Cytokine Transcript Analysis. Cytokine secretion is primarily regulated at the transcriptional level (4). To determine if cytokine transcription followed the observed secretion patterns, we performed Northern blot analysis (Fig. 8). MLNC and splenocytes were isolated 6 d after infection, and restimulated for 3, 6, or 12 h with parasite extract. The cells were cultured at a 10-fold higher density than was used previously. Total RNA was isolated, subjected to electrophoresis, and blotted, and sequentially probed for IFN-γ, IL-2, IL-4, IL-5, and actin transcripts. All transcripts were of the expected sizes. IFN-γ transcription in MLNC was detectable 3 h after restimulation, and declined by 12 h. In the spleen, IFN-γ transcripts peaked at 12 h. Contrary to the secreted IFN-γ levels, steady-state IFN-γ transcript levels were higher in A^+ than A^- MLNC and splenocytes at all times. We conclude that vitamin A exerts some positive effect on IFN-γ steady-state transcript levels, and some negative effect on IFN-γ secretion.

Unlike IFN-γ, other cytokine transcript levels correlated with protein secretion patterns. IL-2 transcripts were detectable 3 h after restimulation in MLNC and splenocytes, and declined by 12 h in MLNC, but remained high through 12 h in splenocytes. In MLNC, IL-2 transcripts were more abundant in A^+ than A^- cells at 3 h, but similar at later times.

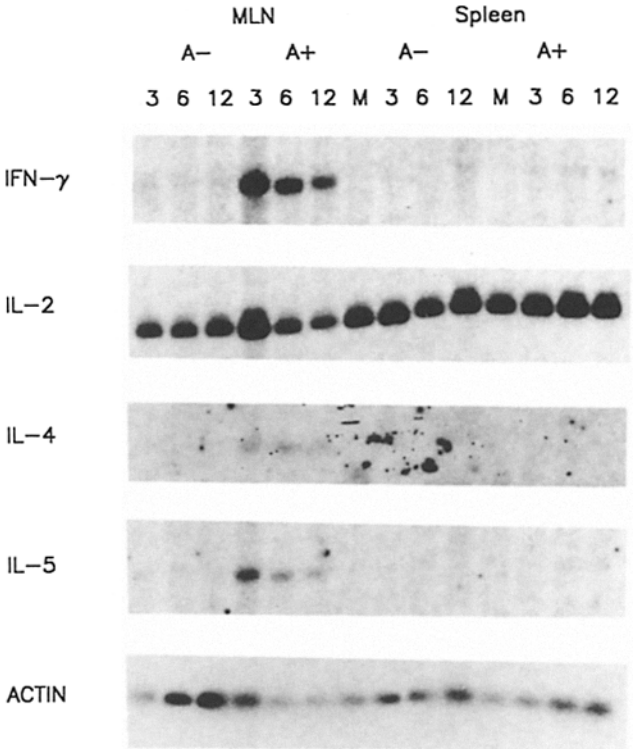

Figure 8. Cytokine transcript levels in cells from A^- and A^+ *T. spiralis*–infected mice. Cells were collected and pooled from five mice per group on day 6 postinfection, restimulated, and extracted as described in Materials and Methods. RNA (5 μg) was subjected to electrophoresis through a 1% agarose-formaldehyde gel and sequentially probed for IFN-γ, IL-2, IL-4, IL-5, and actin transcripts. The first lane in each splenocyte group represents splenocytes cultured for 6 h with medium (*M*). One representative experiment of two.

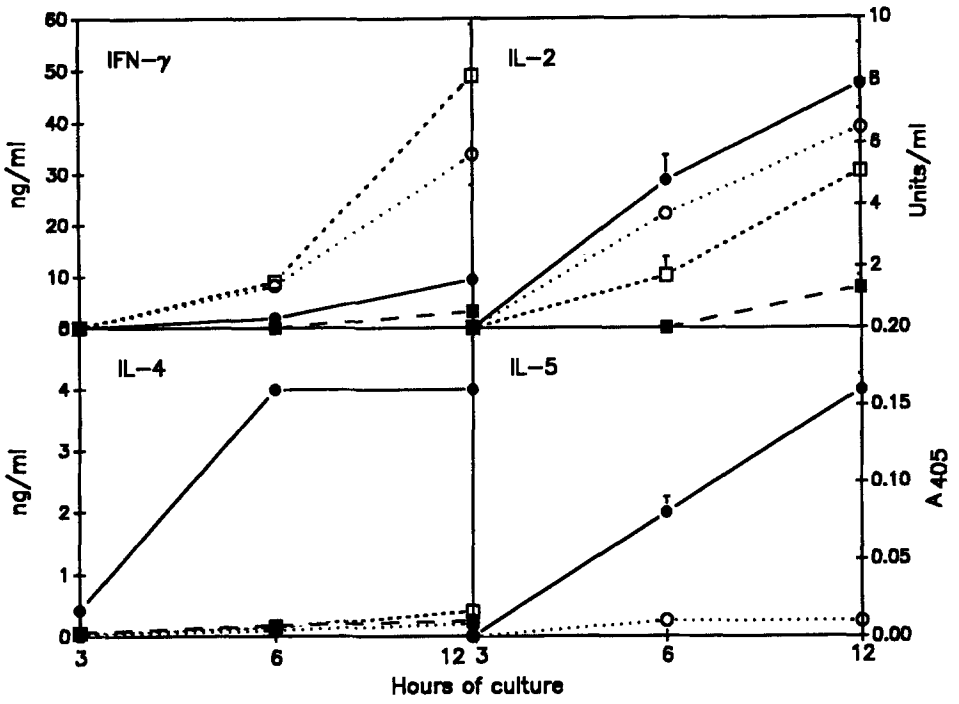

Figure 9. Time course of *T. spiralis*–stimulated cytokine secretion. Samples from the experiment shown in Fig. 8 were assayed for cytokines. (○) A^- MLNC; (●) A^+ MLNC; (□) A^- splenocytes; (■) A^+ splenocytes. IL-5 was not detected in splenocyte cultures.

IL-2 transcripts in A^+ and A^- splenocytes did not differ. Splenocytes cultured without antigen also transcribed IL-2, even though protein was not detected in these cultures.

IL-4 and IL-5 transcripts were detectable 3 h after activation in MLNC, and declined thereafter. Consistent with the protein levels, A^+ MLNC contained significantly more IL-4 and IL-5 transcripts than A^- MLNC. Neither IL-4 nor IL-5 transcripts were detectable in splenocytes.

We also isolated and analyzed transcripts from unrestimulated MLNC collected on days 0–8 postinfection (data not shown). We detected low levels of IL-2 and IL-4 transcripts, but no IL-5 or IFN-γ transcripts. The IL-2 transcript levels increased from day 0 to 6 and declined thereafter; at the peak, transcript levels were greater in A^+ cells than A^- cells. The IL-4 transcript levels showed no induction through day 8, nor were they different in A^+ and A^- cells. These results confirm in part the results presented in Fig. 8, where antigen restimulation in vitro was used to increase cytokine transcript levels.

To determine if cytokine secretion 3–12 h after restimulation in vitro followed previous results obtained 48 h after restimulation (Figs. 4–7), we analyzed supernatant samples from the Northern blotting experiment for cytokines (Fig. 9). As before, A^- MLNC and splenocyte cultures contained more IFN-γ than controls. Results in this experiment (Fig. 9) differed from previous results (Fig. 5) in that in A^- and A^+ MLNC IL-2 production was similar, whereas A^- splenocytes secreted significantly more IL-2 than A^+ controls. Previously, splenocyte IL-2 production was equivalent on day 6 (Fig. 5). This discrepancy might reflect several differences between the two experiments. The assay measures free IL-2 in the supernatant without regard to receptor-bound IL-2. At high cell concentrations (Fig. 9), more of the secreted IL-2 may be receptor bound in A^+ splenocytes, if A^- and A^+ differ in IL-2 receptors, thereby reducing the amount of IL-2 measurable in the culture fluid. IL-4 secretion was greater for A^+ than A^- MLNC and not measurable in splenocyte cultures. IL-5 secretion was only detected in MLNC cultures. Consistent with previous results, A^+ MLNC secreted significantly more IL-5 than A^- cultures. We conclude that IL-2, IL-4, and IL-5 transcript levels correlate with secreted protein levels.

Discussion

Vitamin A deficiency changes the pattern of cytokine secretion from activated $CD4^+$ T cells during primary *T. spiralis* infection, thereby altering immune responses to parasite antigens. Vitamin A deficiency decreased bone marrow eosinophil synthesis and the frequency of parasite-specific IgG1-secreting cells, consistent with the observed cytokine secretion patterns. A^- MLNC secreted more IFN-γ, and less IL-2, IL-4, and IL-5, than controls, whereas A^- splenocytes secreted more IFN-γ and equivalent IL-2, IL-4, and IL-5, compared with A^+ controls. Despite these differences, the outcome of the infection was quite similar in A^- and A^+ mice. This suggests that the outcome of primary *T. spiralis* infection depends on multiple immune resistance mechanisms.

IFN-γ overproduction in vitamin A deficiency could have profound immunological consequences. IFN-γ is a potent stimulator of cell-mediated immunity. It stimulates macrophage oxidative metabolism, antimicrobial activity, phagocytosis, and tumoricidal activity (34–36), and contributes to CTL development (37). We hypothesized that certain B10.BR

mice are more susceptible to primary *T. spiralis* infection than resistant strains, because they are less able to mount an inflammatory immune response during the intestinal phase of the infection (11). However, A^- mice produced more IFN-γ than A^+ mice, yet female worm fecundity and muscle larvae burdens were equivalent in A^- and A^+ animals and worm expulsion was slightly delayed. Inflammation mediators like IL-2, lymphotoxin, granulocyte/macrophage-CSF, and macrophage inflammatory proteins may also be quite important; A^- animals produce less IL-2 in the MLN, and may also produce less of some other inflammatory proteins.

IFN-γ also inhibits antibody-dependent immunity. IFN-γ acts directly on B cells to inhibit class II protein induction (38), which would reduce B cell antigen-presenting activity, and inhibits switching to IgG1 and IgE (7). Moreover, IFN-γ inhibits in vitro proliferation of the T cells that produce IL-4 and IL-5 (6), and blocks IL-4-induced bone marrow cell hemopoiesis (39). Thus, excess IFN-γ secretion in A^- mice is consistent with the decreased IL-4 and IL-5 production by MLNC in these animals (1). IL-4 plays a protective role in the immune response to secondardy infection with the gastrointestinal nematode, *Heligosomoides polygyrus* (40). Neutralizing IL-4 in vivo suppressed the primary and secondary IgE response to the parasite, and compromised the host animal's ability to limit worm survival and fecundity in the secondary infection. Neutralizing IL-4 did not consistently influence parasite survival and fecundity in the primary infection. In our study, the A^- but not the A^+ animals harbored larvae-producing adult worms in the gastrointestinal tract on day 15 postinfection. In concert with our IL-4 measurement, these results would be compatible with a protective role for IL-4 in *T. spiralis* infection.

Vitamin A deficiency dramatically decreased bone marrow eosinophil development in infected A^- mice. Eosinophils were once thought to be an important component of immune resistance to *T. spiralis* and other helminth infections (41). In A^- mice, eosinophil-mediated, antibody-dependent killing mechanisms would be impaired, yet the course of infection was relatively unaffected. Others have shown that depleting eosinophils by neutralizing IL-5 in vivo did not change resistance to *Schistosoma mansoni* (42), *Nippostrongylus brasilienis* (43), and *Heligosomoides polygyrus* (40). These results support the notion that eosinophils are relatively unimportant for resistance to certain trematode and nematode infections.

The decreased IL-4 and IL-5 secretion by A^- MLNC could contribute to both the decreased frequency of parasite-reactive, IgG-secreting cells, and the decreased bone marrow eosinophil percentage. IL-4 stimulates B cell class switching to IgG1 and IgE secretion (44), whereas IL-5 is necessary and sufficient for eosinophil development and activation (45). The decreased IL-4 and IL-5 secretion could be secondary to either the excess IFN-γ production, or the decreased IL-2 production, or both. Excess IFN-γ could inhibit the clonal expansion of IL-4 and IL-5 secretors (6). Alternatively, insufficient IL-2 could retard growth and differentiation of IL-4 and IL-5 secretors (46, 47). The earlier development of cells secreting IFN-γ and IL-2 in *T. spiralis*–infected animals compared with IL-4 and IL-5 secretors would support either mechanism.

The difference in IL-4 production between MLNC and splenocytes is noteworthy. The A^- MLNC secreted less IL-4 than A^+ cells, whereas A^- and A^+ splenocyte IL-4 secretion was equivalent. Distinct IL-4-secreting cell populations, $CD4^+$ MLNC, and $CD4^-CD8^-$ splenocytes apparently account for this discrepancy. These splenic $CD4^-CD8^-$ cells may be mast cells; mastocytosis characterizes helminth infections (48) and mast cells can secrete IL-4 (49, 50). *N. brasiliensis* infection stimulated a large increase in splenic non-B, non-T cells that produced abundant IL-4 (51). In contrast to its effect on Th2 cells (7), an inhibitory effect of IFN-γ on non-B, non-T cell IL-4 secretion has not been demonstrated.

Vitamin A may regulate cytokine secretion at the transcriptional level via one or more of the nuclear retinoic acid receptors (52). These receptors are members of the steroid hormone receptor family and they act as ligand-inducible transcription factors (52). The mechanism by which vitamin A regulates IFN-γ secretion is complex. The increased IFN-γ mRNA level in A^+ compared with A^- T cells suggests positive transcriptional control. Our experiments did not establish the CD4/CD8 phenotype of T cells producing this mRNA. Chrivia et al. (53) proposed a model for human IFN-γ gene regulation wherein dominant negative elements upstream of the coding sequence maintain the IFN-γ in a repressed state; selective derepression accompanying T cell activation was envisioned as the mechanism to induce IFN-γ transcription. One could imagine a role for vitamin A in the selective derepression step to account for the positive effect of retinoids on IFN-γ transcription.

The increased IFN-γ protein secreted from A^- compared with A^+ $CD4^+$ T cells, despite the abundance of IFN-γ transcripts in A^+ cells, suggests negative posttranscriptional control. Posttranscriptional regulation over IFN-γ gene expression may involve mRNA translation. Induction of IFN synthesis by synthetic polynucleotides was enhanced and prolonged by cycloheximide treatment (54). Similarly, cycloheximide blocked retinoic acid–mediated inhibition of IFN secretion from virally induced mouse L cells (55). Inhibition of protein synthesis might suppress the accumulation of a regulatory protein that interacts with IFN-γ mRNA to prevent its translation (56). Alternatively, vitamin A may regulate IFN-γ secretion posttranslationally.

Vitamin A deficiency and infectious disease have long been recognized as a deadly combination in humans (57). Cytokines are powerful regulators of immune responses, and our study shows that vitamin A deficiency dramatically affects the regulation of cytokine secretion. In particular, IFN-γ overproduction in vitamin A deficiency may account for depression of antibody-dependent immune system function. Future studies will probe the molecular basis for retinoid-dependent regulation of cytokine secretion, in order to better understand the strong correlation between vitamin A deficiency and increased mortality from infectious disease.

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