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Differential Th_1 and Th_2 Cell Responses in Male and Female BALB/c Mice Infected with Coxsackievirus Group B Type 3

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Male and female BALB/c mice differ dramatically in susceptibility to myocarditis subsequent to coxsackievirus B3 (CVB3) infection. CVB3 infection of male mice results in substantial inflammatory cell infiltration of the myocardium, and virus-immune lymphocytes from these animals give predominantly a Th_1 cell phenotypic response, as determined by predominant immunoglobulin G2a isotypic antibody production and elevated numbers of gamma interferon and interleukin-2 (IL-2)-producing $CD4^+$ T lymphocytes. Females infected with the same virus give predominantly a Th_2 cell phenotypic response, as determined by preferential immunoglobulin G1 antibody isotypic responses and increased precursor frequencies of IL-4- and IL-5-producing $CD4^+$ T cells. Treatment of females with testosterone or males with estradiol prior to infection alters subsequent Th subset differentiation, suggesting that the sex-associated hormones have either a direct or indirect effect on $CD4^+$ lymphocyte responses in this model. Treatment of females with 0.1 mg of monoclonal antibody to IL-4 reduces precursor frequencies of IL-4-producing $CD4^+$ T cells and increases frequencies of gamma interferon-producing cells. This treatment also enhances myocardial inflammation, indicating a correlation between Th_1-like cell responses and pathogenicity in CVB3 infection. The Th_2-like cell may regulate Th_1 cell activation. Adoptive transfer of T lymphocytes from CVB3-infected female mice into male animals suppresses the development of myocarditis in the recipients. Treatment of the female donors with monoclonal antibodies to either CD3, CD4, or IL-4 molecules abrogates suppression.

Myocarditis in adolescent and adult patients predominates in males. In a survey of 164 cases, approximately two-thirds of the patients were men (50). The majority of females affected by the disease developed cardiac symptoms during the third trimester of pregnancy or in the postpartum period. Epidemiologically, the murine disease induced with coxsackievirus B3 (CVB3) resembles the human disease with surprising accuracy (10, 19, 24–26, 33). Animal studies indicate that female resistance is complex but depends primarily on the protective effects of estrogens, while susceptibility in males and pregnant females results from elevated androgen (testosterone and progesterone) levels (19, 25). The latter hormones enhance CVB3 receptor expression on cardiocytes nearly threefold (26). Thus, virus localizes and replicates more rapidly in the hearts of androgen-treated than estrogen-treated animals. Although this correlation between enhanced virus replication in male and pregnant female mice and increased myocardial injury implies that the virus is directly damaging to the heart cells, this may not be the case. T-lymphocyte depletion of either the male or pregnant female substantially reduces myocarditis and cardiac necrosis even though the virus concentrations in the hearts of these animals remain elevated (21, 24, 25, 39). Thus, the immune system remains the pivotal factor in cardiac pathology.

Differences in male and female immune responses have been recognized for some time. Females generally mount better humoral immunity than males, while males usually give enhanced cellular immune responses compared with females (6, 16, 30, 38, 43). This differential immune pattern may, in part, explain the predominant incidence of autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus in females, since these diseases are usually diagnosed and correlate with the appearance of autoantibodies (2, 40). The pathogenicity may reflect differential activation of T-lymphocyte subsets in vivo. T helper cells can be divided into phenotypic subsets based on the requirements needed for their stimulation, their biological activities, and the cytokines which they produce (3, 42, 44, 47). Two major subsets are designated Th_1 and Th_2 cells. Th_1 cells are major producers of gamma interferon (IFN-γ) and interleukin-2 (IL-2), function predominantly in delayed hypersensitivity (inflammatory) reactions, and promote immunoglobulin G2a (IgG2a) antibody isotype responses (8, 13, 28). Th_2 cells produce high levels of IL-4 and IL-5 and promote IgG1, IgA, and IgE antibody isotype responses. Most likely, the Th_1 and Th_2 lymphocyte subsets differentiate from a common precursor (Th_0) cell, and factors present during the activation and differentiation of the precursor population determine the phenotype of the Th cell response (34, 36). These factors include the nature of the antigen and antigen-presenting cell (APC), how the antigen is taken up by the APC, and either the presence or absence of specific cells or cytokines early in the response (13, 14).

In this report, we demonstrate that male mice infected with CVB3 infections generate a predominant Th_1 $CD4^+$ cell response, while females give a predominant Th_2 cell response. Treatment of the males with estradiol or the females with testosterone can modulate Th subset differentiation. The type of Th cell activated during CVB3 infections may play a crucial role in determining pathogenicity.

MATERIALS AND METHODS

Mice. Inbred BALB/c Cum mice were originally purchased from Cumberland Farms, Clinton, Tenn. Breeding colonies of these animals are maintained at the University of Vermont. Adult mice 6 to 8 weeks of age were used in these experiments. Generally, experimental groups consisted of a minimum of five mice and experiments were repeated at least twice.

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Virus and immunization protocol. The original stock of CVB3 was obtained from J. F. Woodruff and is maintained by passage through HeLa cells. Mice were infected by an intraperitoneal (i.p.) injection of 0.5 ml of phosphate-buffered saline (PBS) containing approximately 10^4 PFU of the virus.

Organ virus titer. Hearts were aseptically removed, homogenized with Dulbecco's modified essential medium (DMEM; GIBCO, Grand Island, N.Y.) containing 100 U of penicillin and 100 μg of streptomycin ml^{-1} and 2% fetal bovine serum (FBS; GIBCO). The cellular debris was removed by centrifugation at 300 × *g* for 10 min, the supernatant was subjected to sequential 10-fold dilutions in DMEM–2% FBS, and titers were determined by the plaque-forming assay on HeLa cell monolayers.

Lymphocyte preparation. Spleens or mesenteric lymph nodes were aseptically removed and pressed through fine-mesh screens to produce single-cell suspensions. The lymphocyte populations were centrifuged on Histopaque (Sigma Chemical Co., St. Louis, Mo.) to remove erythrocytes and enriched for T cells by incubating the cells on nylon wool columns at 37°C for 30 min and retrieving the nonadherent population. The cells were washed in DMEM–5% FBS, and viability was determined by trypan blue exclusion. Enriched populations of $CD4^+$ T cells were obtained by treating the T lymphocytes with 10 μg of anti-CD8 monoclonal antibody per ml and 20% rabbit complement for 30 min at 37°C. Purity of the $CD4^+$ T cells was determined by flow cytometry.

Limiting dilution analysis. Limiting dilution analysis was performed as described by Morris et al. (27). Briefly, $CD4^+$ lymphocytes were suspended in RPMI 1640 medium containing 10% FBS, 1 mM nonessential amino acids, 1 mM sodium pyruvate, penicillin, streptomycin, L-glutamine, and 2×10^{-5} M 2-mercaptoethanol. Between 0 and 1,000 $CD4^+$ T cells were dispensed into each well of 96-well tissue culture plates together with 5×10^5 irradiated (2,000 R) syngeneic adherent splenocytes as APC and 10 μg of CVB3 per ml. The wells were cultured for 14 days, washed three times, and restimulated with 0.2 ml of medium containing 5 μg of concanavalin A (GIBCO) per ml. After 24 h of incubation at 37°C, the supernatants were retrieved and assayed for cytokines by using the appropriate enzyme-linked immunosorbent assay (ELISA). Groups consisted of 44 wells for each concentration of $CD4^+$ cells cultured with antigen and 8 wells of cells cultured without antigen as controls. Estimates of precursor frequency were obtained from the maximum-likelihood method of Good et al. (15).

Cytokines and reagents. Recombinant murine IL-2 and IL-4 were obtained from Pharmingen, San Diego, Calif. Recombinant murine IFN-γ, IL-5, and monoclonal antibody to IL-5 were obtained from Genzyme Inc. (Cambridge, Mass.). Sheep antibodies to mouse IgG1 and IgG2a were purchased from ICN Immunobiologics Inc. (Irvine, Calif.). Biotinylated rabbit anti-sheep IgG was also obtained from ICN. The hybridoma clones making antibody to CD3 (clone 500A2), CD4 (clone GK 1.5), CD8 (clone 2.43), IL-2 (clone S4B6), IL-4 (clone 11B11), and IFN-γ (clone R4-6A2) were purchased from the American Type Culture Collection (Rockville, Md.) and were grown as described previously (48). The antibody was purified from tissue culture supernatants by adsorption to affinity columns. Biotinylated monoclonal antibodies to IL-2 and IL-4 were purchased from Pharmingen. Antibody to IFN-γ and IL-5 was biotinylated by the method of Kendall et al. (23).

Cytokine ELISAs. Monoclonal antibody to a specific cytokine was diluted to 3 μg/ml in 0.1 M sodium carbonate buffer (pH 9.6), and 50 μl was incubated in wells of 96-well Immunon II ELISA plates (Dynatech Laboratories, Chantilly, Va.) overnight at 4°C. The wells were washed with PBS containing 0.05% Tween 20 (Sigma), and then 50 μl of the tissue culture supernatant was added to the well and incubated for 90 min at room temperature. The wells were washed with PBS-Tween, and 50 μl of the biotinylated anticytokine antibody (3 μg/ml) was added and incubated for 90 min as before. The wells were again washed, and a 1:2,000 dilution of alkaline phosphatase-conjugated ExtrAvidin (Sigma) was added for 90 min. The wells were washed, and 50 μl of *p*-nitrophenyl phosphate substrate (Sigma FAST pNPP) was added. The reaction was stopped with 3 N NaOH, and the reaction was read at 405 nm with a Bioteck ELISA reader (Winooski, Vt.).

Anti-CVB3 antibody isotype. Determination of antibody isotype was performed by ELISA. Briefly, 10 μg of UV-irradiated, sucrose-purified CVB3 per ml was adsorbed to ELISA plates in sodium carbonate buffer. After removal of unbound virus with PBS-Tween, 50 μl of twofold dilutions of sera from either normal or CVB3-infected mice was added to the wells as primary antibody. The unbound antibody was removed, and a 1:100 dilution of sheep anti-IgG1 or anti-IgG2a antibody was added for 90 min. Following washing, 50 μl of biotinylated rabbit anti-sheep IgG was added to each well. Determination of bound biotinylated antibody was performed using alkaline phosphatase-conjugated ExtrAvidin and pNPP as described above. The endpoint of the titer is the last serum dilution giving an adsorption value 0.1 units above control values (wells without either the mouse sera or sheep antibodies).

Anti-CVB3 titers. Sera obtained from infected and normal mice were subjected to twofold serial dilutions in PBS-Tween, and 50 μl was added to wells of ELISA plates coated with CVB3 as described above. The plates were incubated for 30 min at 37°C, washed three times, and incubated with 50 μl of a 1:1,000 dilution of urease-conjugated goat anti-mouse IgG (Sigma) for 30 min at 37°C. The secondary antibody was removed by washing with PBS-Tween followed by washing with distilled water. The wells were incubated with 100 μl of urease substrate (0.008 g of bromcresol purple, 0.1 g of urea, and 0.074 g of EDTA in 100 ml of distilled water [pH 4.8]) for 2 h at 37°C, and the optical density was read at 599 nm.

Flow cytometry. Lymphocytes were resuspended to 10^6 cells per ml in PBS containing 1% bovine serum albumin and 0.01% sodium azide and then stained with a 1:100 dilution of phycoerythrin-conjugated anti-CD4 antibody (Coulter Co., Hialeah, Fla.). After incubation at 0 to 4°C for 30 min, the cells were washed three times with PBS and evaluated on an Ortho Diagnostics System 50-H cytometer for percentage of positive cells.

Hormones. Dihydrotestosterone and β-estradiol (Sigma) were initially dissolved in 100% ethanol and then diluted to their final concentrations in PBS. Animals were injected with 1 mg of hormone subcutaneously 7 days before virus injection.

Histology. Hearts were fixed in 10% buffered formalin, sectioned, and stained with hematoxylin and eosin. Sections were blindly evaluated by one of us on a scale of 0 to 4, in which 0 represents no inflammation, 1 represents 1 to 10 lesions per section, 2 represents 11 to 20 lesions per section, 3 represents 21 to 40 lesions per section, and 4 represents widespread and confluent lesions.

Statistical analysis. Data obtained from individual animals such as histology and organ virus titers were analyzed by the Wilcoxon ranked-score test. Mortality was measured by χ^2 analysis using two-by-two tables. All other data was evaluated by the Student *t* test.

RESULTS

Comparison of Th cell responses in CVB3-infected male and female mice. Male and female BALB/c mice were injected i.p. with 10^4 PFU of CVB3 and killed 7 days later. Cumulative mortality was substantially higher over the 7-day observation period in males compared with females (60% mortality for males, compared with 25% mortality for females; $P < 0.01$). Males also showed greater myocardial inflammation (myocarditis score of 2.7 ± 0.7 for males and 0.5 ± 0.1 for females; significant at $P < 0.01$) and cardiac virus titers (maximum of approximately 10^6 PFU per heart for males, compared with approximately 10^4 PFU per heart for females; significant at $P < 0.05$). Sera obtained from the animals were assayed for IgG anti-CVB3 antibody by ELISA (Fig. 1). Females gave better humoral immune responses than males. The isotypes of the anti-CVB3 IgG responses differed between the sexes (Table 1). Males showed a predominant IgG2a isotype, while females had primarily IgG1 rather than IgG2a antibodies. These results suggest a differential activation of Th_1 and Th_2 phenotypic cell responses in these animals. To evaluate this possibility, precursor frequency analysis was performed by the method of Good et al. (15). Spleen cells, which were demonstrated by flow cytometry to contain greater than 94% $CD4^+$ T cells, were obtained from male and female mice 7 days after infection. Between 0 and 1,000 $CD4^+$ T cells were incubated in tissue culture wells for 14 days with 10 μg of virus per ml. The cells were washed and restimulated with concanavalin A for 1 day. Supernatants were retrieved and assayed by capture ELISAs for IFN-γ, IL-2, IL-4, and IL-5. Specificity of each cytokine ELISA was established by using recombinant IL-2, IFN-γ, IL-4, and IL-5. Limiting dilution analysis of cytokine-producing cells for an individual female and male animal is given in Fig. 2. The calculated percent precursor frequencies of cytokine-producing lymphocytes are given adjacent to the respective lines. This study indicated that 7 days after CVB3 infection, the male had more IFN-γ- and IL-2-producing $CD4^+$ cells than females, while the female had substantially greater numbers of IL-4- and IL-5-producing lymphocytes. Table 2 shows the mean precursor frequencies of cytokine-producing $CD4^+$ cells from three individual mice per group. While individual variability was noted in the groups individually, each male consistently showed greater numbers of IFN-γ- and IL-2-producing T cells than IL-4- and IL-5-producing cells, while in each female, more IL-4- and IL-5-producing cells than IFN-γ- and IL-2-producing cells were detected.

The next question was whether the sex-associated hormones affect relative Th_1 and Th_2 phenotypic cell responses in vivo. Male and female mice were given 1 mg of either testosterone or estradiol subcutaneously 7 days prior to infection. Seven days after infection, the animals were killed and the spleen cells were retrieved. Purified preparations of $CD4^+$ T cells were evaluated by precursor frequency analysis for cytokine-producing lymphocytes (Table 2). As stated above, IL-2-, IFN-γ-, IL-4-, and IL-5-producing cells were evaluated in individual animals by limiting dilution analysis. CVB3-infected males showed significantly greater numbers of IL-2- and IFN-γ-producing cells than cells producing IL-4 or IL-5. Treating the males with added testosterone still resulted in a predominant Th_1-like cell response. However, in males given estradiol prior to infection, the ratio of IL-2/IFN-γ- to IL-4-producing cells is more nearly equal. Interestingly, more IL-5- than IL-4-producing cells were observed in this group. Why different numbers of $CD4^+$ T cells producing IL-4 and IL-5 were observed is not known. Untreated and estradiol-treated females gave better Th_2-like than Th_1-like responses. However, the frequencies of IL-4- and IL-5-producing cells were decreased subsequent to testosterone treatment. These results demonstrate that the sex-associated hormones affect phenotypic Th_1 and Th_2 cell expression during CVB3 infection in vivo.

TABLE 1. Isotype of anti-CVB3 IgG antibodies in male and female mice[a]

Mice	Antibody titer[b]	
	IgG1	IgG2a
Male	27 ± 5[c,d]	149 ± 56[c]
Female	427 ± 85[d]	19 ± 7

[a] BALB/c mice were infected with 10^4 PFU of CVB3. Sera were obtained 7 days later and assayed for antibody to CVB3 by ELISA. Isotype of the antiviral antibody was determined by incubation with either sheep anti-mouse IgG1 or sheep anti-mouse IgG2a antisera and biotinylated rabbit anti-sheep IgG antibody.

[b] The titer is reported as the inverse of the last serum dilution giving 0.1 adsorption units above control values. Results are reported as mean ± standard error of the mean of sera from three individual mice per group.

[c] Significantly different from that for sera from female mice at $P \leq 0.05$.

[d] IgG1 isotype titer is significantly different from IgG2a isotype titer at $P \leq 0.05$.

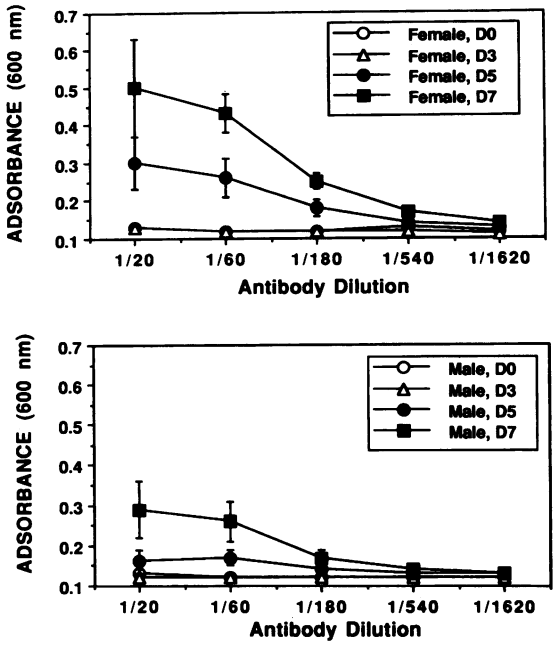

FIG. 1. IgG antibody responses in males and females. Serum was obtained from male and female mice on days (D) 0, 3, 5, and 7 after i.p. injection of 10^4 PFU of CVB3. Serum was serially diluted and assayed for binding to immobilized virus on ELISA plate flat-bottom wells, using urease-conjugated anti-mouse IgG (heavy-chain-specific) antibody and urease substrate.

Potential role of Th_2 cells in myocarditis resistance. Previous studies demonstrated the presence of a suppressor T lymphocyte in CVB3-infected female mice which actively abrogated the pathogenic immune responses causing myocardial injury in this disease (22). On the basis of experiments described above and the known ability of Th_2 cells to inhibit Th_1 cell responses, we hypothesized that the suppressor cell might belong to the Th_2 lymphocyte subset. In the first experiment, female BALB/c mice were injected i.p. with CVB3 and divided into three groups. One group received no further

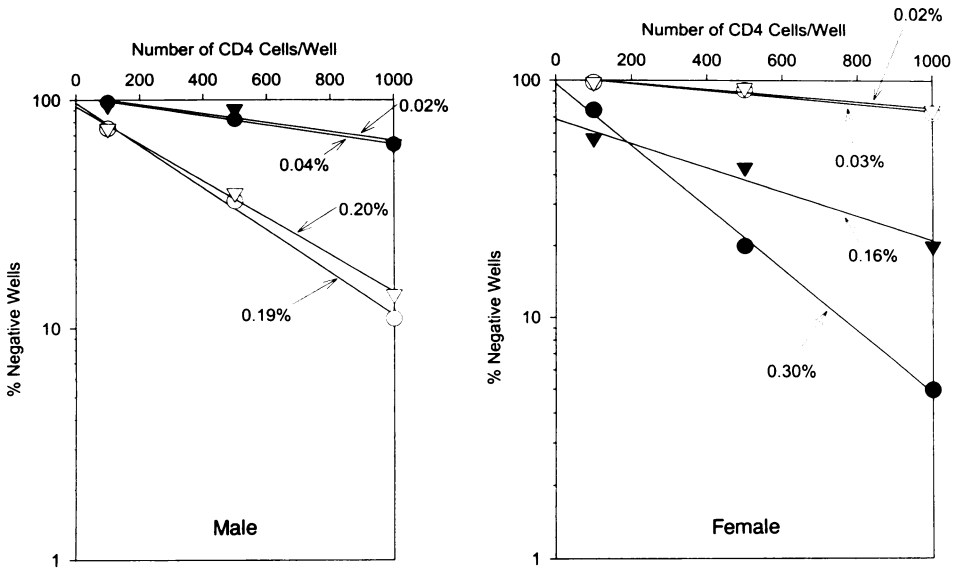

FIG. 2. Purified CD4$^+$ T cells were obtained from male and female BALB/c mice 7 days after infection with CVB3. Various concentrations of CD4$^+$ T cells were cocultured with APC and virus for 14 days, washed, and restimulated with 5 μg of concanavalin A for 1 day. Supernatants were assayed for IL-2 (▽), IFN-γ (○), IL-4 (●), and IL-5 (▼). Precursor frequencies are given by each line and were determined for each lymphocyte population, using Poisson statistics.

treatment, while each mouse in the remaining groups was given a daily injection of 0.1 mg of either anti-IL-4 or anti-IFN-γ beginning 2 days after infection. All animals were killed 7 days after infection, and the hearts were evaluated for cardiac inflammation and virus titers. Animals receiving virus only had a mean of 4.5 + 0.45 $\log_{10}$ PFU per heart. This compared with 4.8 ± 1.1 $\log_{10}$ PFU per heart for anti-IL-4-treated mice (not significantly different at $P < 0.05$) and 6.3 ± 1.5 $\log_{10}$ PFU per heart for anti-IFN-γ-treated animals (significantly different from the value for virus-only group at $P < 0.05$). Histologically, animals treated with anti-IL-4 developed significantly more myocarditis than the other groups (Fig. 3). Anti-IL-4 treatment reduced relative Th_2 phenotypic cell responses and increased relative Th_1 phenotypic cell responses, as determined by precursor frequency analysis (Table 3). Thus, there is a correlation between either reduced Th_2 cell activation or enhanced Th_1 cell activation and enhanced myocarditis. In the next experiment, female mice were infected with CVB3 and divided into groups. One group received no further treatment (immune females). Each mouse in the other groups of immunized females was given 2 mg of either anti-CD4 (anti-L3T4), anti-CD8 (anti-Lyt 2.2), or anti-CD3 antibody 2 days prior to infection. Two additional groups were given daily injections of anti-IL-4 or anti-IFN-γ as before. Lymphocytes enriched for T lymphocytes were obtained 7 days later. Male mice were infected with CVB3 and 3 days later were injected intravenously with 5×10^6 lymphocytes from either normal or immune females treated in the ways described above. The males were killed 7 days after infection, and their hearts were evaluated for myocarditis (Fig. 4). T cells from immune female mice abrogated myocarditis when adoptively transferred into male animals. Depletion of all T cells (anti-

TABLE 2. Precursor frequencies of Th_1 and Th_2-like cells alter with hormonal treatment

Mice	Hormone	Precursor frequency (%)[a]			
		IL-2	IFN-γ	IL-4	IL-5
Male	None	0.097[b] (0.05–0.185)	0.091[b] (0.05–0.155)	0.013 (0.01–0.015)	0.013 (0.01–0.015)
	Testosterone	0.112[b] (0.035–0.17)	0.070[b] (0.035–0.11)	0.028[c] (0.02–0.04)	0.020 (0.015–0.025)
	Estradiol	0.018[c] (0.015–0.025)	0.022[c] (0.02–0.025)	0.028[c] (0.02–0.04)	0.065[c] (0.06–0.07)
Female	None	0.058[b] (0.04–0.085)	0.060 (0.015–0.155)	0.162 (0.085–0.20)	0.143 (0.12–0.16)
	Testosterone	0.103[b] (0.065–0.150)	0.068 (0.055–0.09)	0.055[c] (0.035–0.07)	0.047[c] (0.025–0.070)
	Estradiol	0.012[b,c] (0.005–0.02)	0.027[b] (0.015–0.055)	0.077 (0.04–0.14)	0.088 (0.02–0.15)

[a] Male and female BALB/c mice were treated with 1 mg of testosterone or estradiol and then infected with 10^4 PFU of CVB3. Seven days later, spleen CD4$^+$ cells were isolated and assayed by limiting dilution analysis for precursor frequencies of various cytokine-producing cells. Results are means for three individual animals per group. Ranges are given in parentheses.

[b] Precursor frequencies of IL-2/IFN-γ-producing cells were significantly different from frequencies of IL-4/IL-5-producing cells in individual animals at $P \leq 0.05$.

[c] Results for hormone-treated animals were significantly different from those for untreated animals at $P \leq 0.05$.

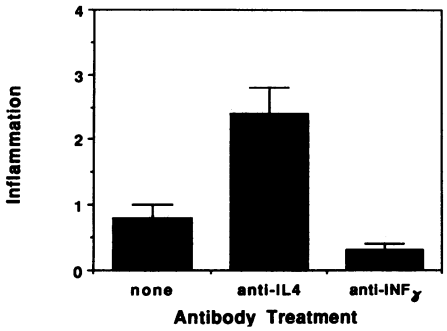

FIG. 3. Monoclonal antibody treatment of female mice. Female BALB/c mice were injected i.p. with 10^4 PFU of CVB3. Beginning 2 days later, each animal in groups of five mice each received 0.1 mg of either anti-IL-4 or anti-IFN-γ per day. All animals were killed 7 days after infection.

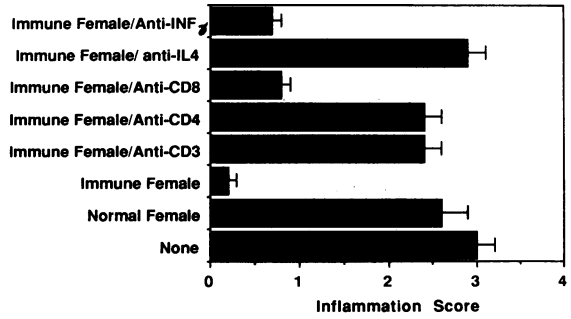

FIG. 4. Adoptive transfer of suppression with immune female T lymphocytes. Female BALB/c mice were injected with 10^4 PFU of CVB3. Some animals additionally were given either 2 mg of anti-CD4, anti-CD8, and anti-CD3 antibody 2 days prior to infection or 0.1 mg of anti-IL-4 and anti-IFN-γ daily beginning 2 days after infection; 5×10^6 lymphocytes from normal and infected female mice killed 7 days after infection subsequently were injected intravenously into male mice given CVB3 3 days earlier. The surviving recipient male animals were killed on day 7 after infection, and hearts were evaluated blindly for myocarditis on a scale of a 0 to 4, in which 0 represents no inflammation, 1 represents 1 to 10 lesions per section, 2 represents 11 to 20 lesions per section, 3 represents 21 to 40 lesions per section, and 4 represents widespread and confluent inflammation.

CD3 treatment) or $CD4^+$ T cells in the females eliminated suppression. Lymphocytes from females given either anti-CD8 or anti-IFN-γ antibodies retained the ability to inhibit myocarditis in male recipients. No significant differences were observed in virus titers in the hearts of the male recipients (data not shown). Thus, the suppression in CVB3-infected females is most likely either mediated by or dependent on a $CD4^+$ T cell which is sensitive to anti-IL-4 treatment. To determine whether the suppressor cell is actually a $CD4^+$ T cell, T cells from CVB3-infected females were treated with either anti-CD3, anti-CD4, and anti-CD8 antibody and complement or complement alone and then injected into infected male recipients (Table 4). Depletion of either all T cells (anti-CD3) or $CD4^+$ cells abrogated suppression, while depletion of $CD8^+$ T cells did not.

DISCUSSION

Differential activation of Th_1 and Th_2 cell subsets plays a crucial role in the resistance or susceptibility of animals to a variety of infectious diseases. In murine models of cutaneous leishmaniasis, resistant or healer phenotype mice generate a predominant Th_1 cell response, while the nonhealer mouse strains which develop progressive disease give predominant Th_2 cell responses (27). In this case, the IFN-γ produced by the Th_1 cells activates macrophages which eliminate the infectious organisms and limit the disease (17). Similarly, susceptibility in *Plasmodium chabaudi* (41), *Candida albicans* (36), and *Trichuris* (intestinal nematode) (7) infections have been associated with acute Th_2 cell responses and/or the lymphokines released by this Th cell population. Pathogenicity in Theiler's virus encephalomyelitis is most likely due to preferential activation of Th_1 cells (32). Mice can be protected from the pathogenic effects of Theiler's virus infection by administering UV light-treated virus conjugated to syngeneic splenocytes, a method successfully used to tolerize individuals to specific antigens. In the Theiler's virus model, tolerance is associated with a decrease in IgG2a antibody responses and an increase in IgG1 antibody responses, implying a change in Th subset activation between the pathogenic and tolerized states.

Since differential Th subset activation can determine pathogenicity in infections, an important question is why certain individuals preferentially activate one type of Th cell phenotype rather than another. In many of the infectious disease models described above, the presence of IFN-γ in the acute phase of the infection favors Th_1 cell responses (4, 9, 35, 39). In studies by Scharton and Scott, natural killer (NK) cells were

TABLE 3. Precursor frequencies of IFN-γ- and IL-4-producing cells in females treated with anti-IL-4 or anti-IFN-γ antibody

Antibody treatment	% Cumulative mortality (no. dead/total)	Precursor frequency (%; mean ± SEM)[a]	
		IFN-γ	IL-4
None	17 (1/6)	0.060 ± 0.025	0.162 ± 0.038
Anti-IL-4	50 (3/6)[b]	0.135 ± 0.075[b]	0.09 ± 0.05[b]
Anti-IFN-γ	0 (0/6)	0.028 ± 0.025	0.20 ± 0.085

[a] Female BALB/c mice were injected i.p. with 10^4 PFU of CVB3. Beginning 2 days later, each animal in groups of six mice each received 0.1 mg of monoclonal antibody to either IL-4 or IFN-γ per day. All animals were killed 7 days after infection. Spleen $CD4^+$ T cells from three individual animals per group were evaluated for precursor frequencies of IFN-γ- and IL-4-producing cells by ELISA.

[b] Significantly different from results for untreated females at $P \leq 0.05$.

TABLE 4. Depletion of $CD4^+$ T cells in CVB3-immune lymphocyte population abrogates suppression

Transferral to male	Treatment	Myocarditis score[a]
None		2.1 ± 0.4
From:		
Normal female	None	1.8 ± 0.5
CVB3-immune female	Complement	0.4 ± 0.2[b]
	Anti-$CD3^+$ complement	1.9 ± 0.3
	Anti-$CD4^+$ complement	1.8 ± 0.3
	Anti-$CD8^+$ complement	0.6 ± 0.3[b]

[a] Female BALB/c mice were infected with 10^4 PFU of CVB3 i.p. and killed 7 days later. Mesenteric lymph node cells were isolated and treated either with 100 μg of antibody per ml and 20% rabbit complement for 45 min at 37°C or with complement alone. Male BALB/c mice were infected i.p. with 10^4 PFU of CVB3 and 3 days later were given 5×10^6 lymphocytes from normal or immune female donors (intravenously). The males were killed 7 days after infection, and the hearts were evaluated for myocarditis on a scale of 0 to 4. Results represent means ± standard errors for of four or more animals per group.

[b] Significantly less than for infected males not given lymphocytes at $P \leq 0.05$.

identified as the source of IFN-γ which drove Th_1 subset responses in leishmaniasis (39). This corroborates findings of other investigators who also implicate NK cells as a major factor in differential Th cell activation (4, 9, 35). However, in *C. albicans*-infected mice, non-NK cells are required for Th_1 cell differentiation (37). Selective depletion of NK cells failed to alter Th subset responses. In this case, non-NK cells such as T cells expressing the $\gamma\delta^+$ T-cell receptor may be the source of the IFN-γ needed for preferential Th_1 cell stimulation (52).

Generally, other investigators studying the role of Th subsets in pathogenicity of infection have used different inbred strains of animals in which the predilection toward Th_1 or Th_2 cell responses is relatively stable. In this report, we show that the physiological state of the individual at the time of infection can also affect differential Th cell activation. In this case, the sex-associated hormones are either directly or indirectly responsible for influencing Th_1 or Th_2 cell activation in a single inbred strain of mice. Araneo et al. (1) demonstrated that exposure of T lymphocytes to dihydrotestosterone in vitro caused a substantial reduction in IL-4 and IFN-γ production without affecting release of IL-2. Testosterone had no effect in this system. The data from this study are somewhat difficult to interpret but might suggest that some sex-associated hormones can have a direct effect on the types of lymphokines produced by a cell. Alternatively, we have previously demonstrated that the sex-associated hormones influence the expression of either the CVB3 receptor or accessory binding molecules which affect virus attachment to the cell surface (26). This means that CVB3 localizes more quickly in the hearts of androgen-treated than estrogen-treated animals. Also, cardiac virus titers are significantly elevated in males and testosterone-treated females compared with untreated females. These elevated virus concentrations might support a more vigorous NK cell response in the presence of androgens, and these NK cells may be the deciding factor favoring preferential Th_1 cell responses. The potential problem with this explanation is that studies by this laboratory (20) and others (49) indicate that splenic NK or NK-like cell responses are either equivalent between CVB3-infected males and females or higher in the females. Therefore, either factors other than NK cells are influencing differential Th cell responses in CVB3 infections or the observation on peripheral NK cell responses in males and females is misleading. Perhaps the important events determining Th subset responses occur in the heart and not in the peripheral lymphoid organs.

One of the interesting observations in the present model is the presence of an immunoregulatory T cell in females which actively inhibits myocarditis. Lymphokines have both stimulatory and suppressive properties. IL-10 suppresses the growth of Th_1 cells by inhibiting production of IL-2 and also synergizes with IL-4 to inhibit macrophage function (11, 12, 29, 31). This inhibition of macrophage function could include interference with the ability of macrophage to act as APC for the Th_1 cell population. IFN-γ selectively blocks growth of Th_2 cells and inhibits antibody production by B lymphocytes (5, 18). Thus, Th_2 cells can act as suppressors of Th_1 cell-dependent immune responses, while the Th_1 cells can return the favor for Th_2 cell-dependent immunity. Lymphocytes from CVB3-infected females are enriched for Th_2 cells and are highly inhibitory to myocarditis in males. When the proportion of Th_2 cells in the female is reduced by treatment of the animals with anti-IL-4 antibodies, the suppression is eliminated. The results obtained in this experiment are compatible with the Th_2 cells in the female inhibiting Th_1 cell activation in recipient males. Whether this is the correct explanation is not known. Future experiments will attempt to clone the Th_2 cells in CVB3-infected females. Adoptive transfer of the cloned Th_2 cells may provide more definitive proof of the immunoregulatory T cell in the female.

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