Coxsackievirus B-3–Induced Myocarditis

Effect of Sex Steroids on Viremia and Infectivity of Cardiocytes

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Male and female BALB/c mice were inoculated with various concentrations of coxsackievirus, group B, type 3 (CVB3), ranging from 10 to 10^7 plaque-forming units (PFU). Lower viral doses (>10^2 PFU) induced severe myocarditis in male mice but caused little injury in females. With 10^7 PFU, females also developed severe disease. Females may be relatively resistant to CVB3-induced myocarditis because virus entry into the blood and heart is less effective. Males given ^{125}I-CVB3 show approximately 2–4 and 20-fold more radioactivity in the peripheral blood and heart, respectively, than females. No differences were observed between the sexes in ^{125}I-bovine serum albumin penetration. Sex steroid hormones influence viremia and virus localization; females given exogenous testosterone and progesterone demonstrate ten times more virus in their hearts than animals given estradiol. The hormones may act by increasing virus receptor expression on endothelial cells and myocytes. (Am J Pathol 1987, 126:432–438)

HISTORICALLY, myocarditis has been linked to virus infections in the heart. Although numerous agents may cause this disease, group B coxsackieviruses and ECHO viruses of the picornavirus family predominate.[1] Virus is only rarely isolated from patients with active myocarditis; thus the association of the disease with infection depends largely on serologic and epidemiologic studies. Despite circumstantial evidence for virus involvement in myocarditis, the precise role for the infectious agent in cardiac injury remains unclear. Virus replication in the myocytes may cause some damage, but present research indicates autoimmunity to heart antigens is more important.[2–5]

Sex-related differences in myocarditis susceptibility are quite obvious in adults. Men are twice as likely to contract severe myocarditis as women[6], except during pregnancy, when women can also develop severe and extensive heart damage.[7–9] We use a murine model of coxsackievirus B-3 (CVB3)-induced myocarditis in inbred BALB/c mice to study how sex hormones influence this disease.[1,10,11] As in humans, male and pregnant female mice develop significantly more cardiac injury and have greater concentrations of virus in their hearts than virgin females.[1,12,13] Castration of males reduces myocarditis susceptibility to levels normally observed in virgin females, and administration of either exogenous testosterone or progesterone to castrated males and females enhances both virus concentrations and inflammation in the heart.[12,13] Although virus concentrations are consistently elevated in mice in which myocarditis develops, the virus does not directly cause cardiac damage. T-lymphocyte depletion of both male and pregnant female animals effectively prevents inflammation and necrosis in the heart but does not affect virus concentrations.[14] Thus, immunity plays the predominant role in myocyte injury.

Clearly, sex-associated hormones could alter the

Supported by PHS Grants-in-Aid HL 28833 and HL 33256 from the National Institutes of Health and an American Heart Association Established Investigatorship awarded to Dr. Huber.

Accepted for publication October 2, 1986.

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pathogenesis of CVB3 infections, but the mechanism of hormonal action remained unknown. Two possibilities seemed likely. Testosterone and progesterone might either decrease suppressor cell activity or increase autoimmune lymphocyte blastogenesis, thus directly enhancing immune-mediated cardiac injury. Alternatively, the hormones may aggravate myocarditis indirectly by increasing the number of virus infected myocytes, thus augmenting antigenic stimulation. In this report, we demonstrate that the latter possibility indeed occurs.

Materials and Methods

Animals

Inbred BALB/c mice were originally purchased from Cumberland Farms (Clinton, TN). A breeding colony of these animals is presently maintained at the University of Vermont. Animals were fed Purina Mouse Chow #5015 (Ralston Purina Company, St. Louis, Mo) *ad libitum*.

Virus

A cardiotropic strain of CVB3 (Nancy) was grown in HeLa cells[12,15,16] (Flow Laboratories, McLean, Va). Confluent monolayers of HeLa cells in 75-sq cm tissue culture flasks (Becton Dickinson, Cockeysville, Md) were inoculated with approximately 2×10^6 plaque forming units PFU virus and incubated at 37 C for 45 minutes. Twenty-five milliliters minimal essential medium (MEM) (GIBCO, Grand Island, NY) containing 5% fetal calf serum (FCS) (GIBCO) and 0.5% gentamycin (GIBCO) were added. The flask was cultured overnight in a 5% CO_2 incubator at 37 C until more than 80% of the HeLa cells detached. The supernatant and cells were removed and alternately frozen (−70 C) and thawed three times. The cell debris was removed by centrifugation, and the supernatant containing the virus was titrated.

Virus Titration

Serial tenfold dilutions of the virus suspension were made in MEM–2% FCS, and 0.2 ml of the dilutions was added to confluent HeLa cell monolayers grown in 60-sq cm plastic Petri dishes (Fisher Scientific Products, Medford, Mass). The plates were incubated at 37 C in a 5% CO_2 incubator for 1.5 hours. After the incubation period, 5 ml of a 0.6% agar solution in MEM–2% FCS was added to each plate. The plates were returned to the incubator for 48 hours. Three milliliters of 10% buffered formalin was added to each plate to inactivate the virus, and the agar was removed. The remaining cells were stained with 0.5% crystal violet. The cleared areas or plaques were counted. Virus preparations usually had between 10^7 and 10^9 PFU/ml. The virus suspensions were aliquoted and stored at −70 C in phosphate-buffered saline (PBS) solution.

Hormone

Testosterone, progesterone, and estradiol (Sigma Chemical Co., St. Louis, Mo) were dissolved in 10 ml of 95% alcohol and subsequently diluted in medium to a final concentration of 50 ng/ml. This was the maximum physiologic level in circulatory blood in mice. The final concentration of ethanol (0.0001%) had no detectable deleterious effect in tissue culture.

Mice were also given dorsal implants of 8 mg of either estradiol, progesterone, or testosterone in Silastic tubing. The animals were given rest for 25 days to allow for physiologic release of the hormones.

Preparation of Myocytes and Cardiac Endothelial Cells

The procedure for the preparation of myocytes and cardiac endothelial cells has been extensively reported earlier.[17] Briefly, the sexes of neonatal mice were determined within 24 hours of birth, and their hearts were asceptically removed, minced, and subjected to stepwise digestion with 0.4% collagenase (Worthington Biochemical Co., Freehold, NJ). Endothelial cells were removed from the dissociated myocytes by absorbing to plastic tissue culture flasks (Falcon Plastics Inc., Oxnard, Calif). The remaining cells were washed once, resuspended in Dulbecco's MEM (DMEM) containing 100 U/ml streptomycin, 5% FCS, and 10% horse serum (which had been passed through activated charcoal for removal of endogenous hormones) and cultured (3×10^4) in 96-well tissue culture plates (Falcon) for 48 hrs before use. In some groups, 50 ng/ml hormone was dissolved in the medium prior to suspending the cells. The endothelial cells were recovered by trypsinizing the cultures, suspended in media as above, and plated in 96-well tissue culture plates.

Preparation of Neonatal Skin Fibroblasts

The skin of 24-hour-old male and female mice was thoroughly washed, disinfected with 70% alcohol, removed and finely minced. The tissue was enzymatically dissociated using 0.25% trypsin (GIBCO) at 37 C for 15 minutes, and the single cells were washed three times with medium, resuspended in DMEM–5%-

dialyzed FCS containing either no or 50 ng/ml hormone and dispensed (3×10^4) into 96-well plates.

Infectious Center Assay

Cells were infected for 1 hour with 150 PFU CVB3/cell. Free virus was eliminated by incubating the cells for 30 minute with a 1 : 100 dilution of hyperimmune rabbit anti-CVB3 antiserum (initial titer, 1 : 10000). The cells were washed, cultured an additional 10 hours at 37 C in medium with or without hormones, trypsinized, counted in trypan blue for determination of viability and demonstrate that cells existed as single cells, rather than clumps, serially diluted in MEM–2% FCS, and added to HeLa cell monolayers in the plaque assay. Each plaque should represent one infected cell.[15,16]

^{125}I Labeling Virus

Virus was purified by cesium chloride gradient as described previously and iodinated with the use of the Bolton–Hunter kit from New England Nuclear (Boston, Mass).[18] Virus was separated from free label by passing it through a Sephadex G-10 column (Pharmacia Co., Piscataway, NJ) and retaining the void volume fraction; 10^4 PFU virus contained 20,853 cpm.

^{125}I Labeling Bovine Serum Albumin (BSA)

BSA (Sigma) was radiolabeled by the chloramine T method.[19] Approximately 20 μg protein in 20 μl of 0.1 M phosphate buffer was added to 1 mCi ^{125}I (Amersham Co., Arlington Heights, IL), mixed, added to 10 μl of 0.5 mg/ml chloramine T (Sigma), mixed and incubated for 30 seconds. Rapidly, 10 μl of 0.5 mg/ml metabisulfite was added, followed by 100 μl elution buffer (0.01 M PBS, 0.1% BSA, pH 7.4). The labeled material was then chromatographed on Sephadex G-10 (Pharmacia) which had been preequilibrated in elution buffer, for separation of bound from free iodine. This procedure results in nearly 60% of label incorporated into the protein. Approximately 1 μg ^{125}I-BSA was mixed with 20 mg unlabeled BSA, and 50,000 cpm (20 μg BSA) was injected intraperitoneally into recipient mice.

Virus Binding Studies

Approximately 5×10^2 myocytes were cultured in 96-well tissue culture plate wells (Falcon) for 48 hours with DMEM containing 5% FCS and 10% horse serum. The monolayers were washed and overlaid with 50 μl DMEM–1% FCS containing 2.5×10^4 PFU (50 PFU/cell) radiolabeled virus. Nonspecific binding of radioisotope was determined with cultures containing the above-labeled virus and 10^4-fold excess of unlabeled virus, which should competitively prevent specific virus binding. The cultures were incubated at 37 C for 2 hours and washed six times for removal of unbound label. The cells were trypsinized and the entire well contents counted for cell number by the use of trypan blue exclusion and for ^{125}I in an Intertechnique CG-4000 gamma counter. Specific virus binding represents the mean counts per minute bound in three replicate cultures containing ^{125}I-CVB3 alone minus the mean counts per minute bound in an equivalent number of cultures containing both labeled and unlabeled virus.

Localization of Radiolabeled BSA and CVB3 in the Blood and Heart

Mice were inoculated with either 1×10^4 PFU ^{125}I-CVB3 (20,000 cpm) or 20 μg ^{125}I-BSA/BSA (50,000 cpm) in 0.5 ml PBS. Animals were anesthetized with sodium pentobarbital and bled from the retroorbital plexus with heparinized capillary tubes. The thoracic cavity was opened, the aorta nicked, and the heart perfused with 10 ml PBS. The heart was cut in half and thoroughly blotted with adsorbent paper for removal of excess fluid. The heart and 0.1 ml blood were counted for radioisotope in an Intertechnique CG-4000 gamma counter.

Histology

The hearts were fixed in 10% buffered formalin and sectioned laterally approximately midway between the apex and atria, which resulted in cross-sections of both ventricles. The sections were stained with hematoxylin and eosin and projected onto paper, where the total area of the myocardium and the areas of inflammation were outlined. The percent area of the myocardium undergoing inflammation and necrosis was determined with the use of an Apple computer and the formula (total area inflamed)/(total myocardial area) $\times$ 100. To ensure impartiality, sections were usually coded and evaluated blindly.

Statistics

The Wilcoxon ranked score test was used to analyze significance of differences where multiple comparisons were made. The Student t test was used to compare the significance of a single comparison of a particular group with its appropriate control.

Results

Myocarditis Resistance of Female Mice Can be Partially Overcome by Increasing the Virus Inoculum

Previous studies demonstrated that virus concentrations in the hearts of CVB3-inoculated mice generally correlate with subsequent myocardial inflammation even though the virus is not directly responsible for tissue damage.[12,13] Thus, threshold levels of virus may be required in this organ to stimulate pathologic immune responses. Female BALB/c mice are normally resistant to CVB3-induced myocarditis, but increasing the initial virus inoculum can largely overcome this resistance. Eight to sixteen male and female mice received intraperitoneal injections of between 10 and 10^7 PFU CVB3 and were sacrificed 7 days later (Figure 1). Minimal cardiac lesions (<1% of the myocardium, usually one to three lesions with less than 20 mononuclear cells each) occurred in either sex inoculated with 10^3 or less PFU virus. At larger inocula, males developed severe myocardial inflammation, with peak damage resulting from 10^4 PFU CVB3. Myocarditis increased more gradually in females and never reached maximal levels obtained with 10^4 PFU virus in males. However, with 10^7 PFU virus, animals of both sexes developed equivalent cardiac injury.

Influence of Sex Hormones on Viremia and Virus Localization in the Heart

Next we questioned whether CVB3 might localize differently in the hearts of age-matched male and virgin female mice immediately after virus inoculation. Animals were given 1×10^4 PFU ^{125}I-CVB3 (20,000 cpm) intraperitoneally and sacrificed either immediately or at various times up to 12 hours. Blood and hearts were analyzed for radioactivity (Figure 2). Virus rapidly appeared in the blood of both sexes, but significantly more virus was observed in the blood of male mice (434 cpm/0.1 ml blood, $P < 0.05$) than in the blood of female (217 cpm) mice. Virus localized more slowly in the heart, compared with blood, increasing gradually throughout the 12-hour observation period in males. Little or no virus was observed in the hearts of female mice during this same period. The inability of virus to preferentially localize in female hearts immediately after inoculation may explain the requirement of larger virus inocula to induce significant myocarditis in this sex.

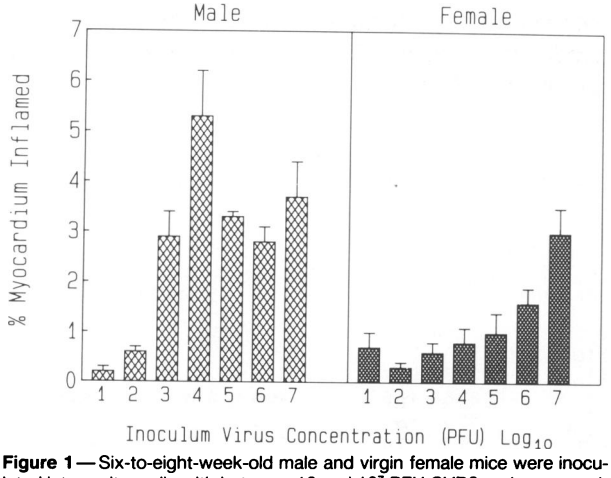

Figure 1—Six-to-eight-week-old male and virgin female mice were inoculated intraperitoneally with between 10 and 10^7 PFU CVB3 and were sacrificed 7 days later. Eight to 16 mice in each group were evaluated for cardiac inflammation. The results represent the mean ± SEM.

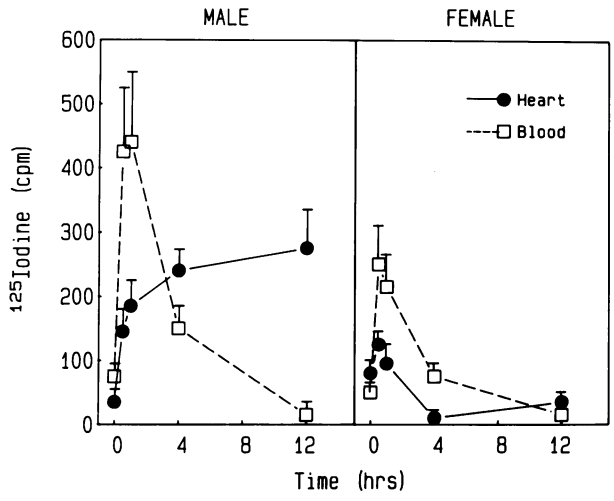

Figure 2—^{125}I-CVB3 was injected intraperitoneally into age-matched mice. At various times thereafter, heparinized blood samples and hearts were obtained from anesthetized mice and counted for radioisotope content. Results represent experiments done.

Females and males differ in numerous physiologic parameters which could affect virus entry into the blood and heart, including sex-associated steroids. To determine whether these hormones influence virus localization, age- and weight-matched virgin female mice were given testosterone, progesterone, and estradiol implants subcutaneously. Approximately 4 weeks later, the animals were infected with 6×10^4 PFU CVB3 intraperitoneally. The hearts of the treated and untreated mice were thoroughly perfused 4 hours after infection, removed, and titered for virus (Figure 3). We chose this end point because the previous experiment suggests blood virus concentrations are limited at this time. Animals given either progesterone or testosterone showed elevated virus content in their hearts, compared with females receiving no hormone or estradiol. The hormonal effect was specific for virus, as shown by injecting ^{125}I-BSA (50,000 cpm) intraperitoneally into females treated with the various hormones as before. Unlike the results with

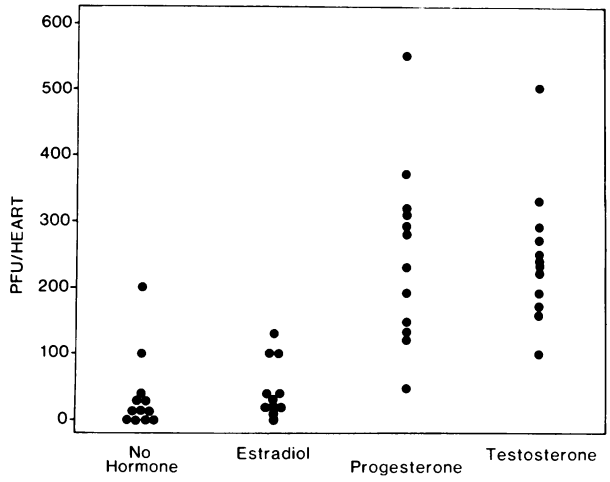

Figure 3 — Eight-week-old female mice were given subcutaneous implant of 8 mg hormone and 25 days later were given intraperitoneal injections of 6×10^4 PFU CVB3. Four hours later, the hearts were removed, perfused with PBS, and titered for virus. Each point represents an individual animal. Progesterone- and testosterone-treated groups showed significant elevation in PFU, compared with animals not receiving hormone, at $P \leq 0.05$.

CVB3, hormones had no significant effect on BSA entry into blood (maximal levels of 1000 to 1200 cpm in hormonally treated and untreated mice by 6 hours after intraperitoneal injection); and despite relatively high levels of radioactivity in the blood, no label was observed in perfused hearts (0 cpm in all groups), which indicate that ^{125}I-BSA was unable to penetrate into the myocardium.

Hormones Affect Viral Binding to Myocytes

The following experiments investigated whether hormones affect virus infection of susceptible cells. Myocytes, cardiac endothelial cells, and neonatal skin fibroblasts were grown from male and female mice and infected with CVB3 in medium containing either no hormone or 50 ng/ml testosterone, progesterone, or estradiol. The percentage of infected cells was determined by infectious center assay (Table 1). Significantly more male myocytes and cardiac endothelial cells were infected when cultured in progesterone (33.2% and 59.1%, respectively) or testosterone (47.7% and 46.5%, respectively), compared with cells cultured without hormone or with estradiol. Similar results were obtained with female-derived myocytes. All three steroids enhanced infectivity of female endothelial cells, although testosterone had the greatest effect and variability in the estradiol-treated group prevented the increase over no hormone from reaching significance at the $P < 0.05$ level. Steroid effect on fibroblast susceptibility differed from that observed with cardiocytes. Hormonal treatment of male fibroblasts with any of the three sex steroids resulted in decreased infection, while treatment of female fibroblasts slightly but nonsignificantly enhanced numbers of cells infected. These divergent hormonal effects on female and male fibroblasts are interesting but not understood. However, they do indicate that steroids can affect virus infectivity of certain cells but that the effect may not be identical on all cells in the organism.

The enhanced infectivity of myocytes noted in the above experiments might represent increased binding of the virus to the cell surface. Alternatively, because the cells in the previous experiment were cultured for 10 hours prior to the infectious center assay, the hormones might also accelerate virus replication, decreasing the time required for release of progeny virions and subsequent infection of new cells. For determining whether hormones could affect direct virus binding to myocytes, these cells were cultured in hormone-free medium and medium containing 50 ng/ml of testosterone, estradiol, or progesterone as before. The cells were incubated with ^{125}I-labeled CVB3 either alone or with a 104-fold excess of unlabeled CVB3 for identification of nonspecific radioisotope binding to the cells (Table 2). Testosterone and progesterone both enhanced virus binding, and estradiol was ineffective.

Table 1 — Demonstration of Enhanced Virus Infectivity in Cardiac Myocytes and Endothelial Cells Cultured in the Presence of Various Hormones*

	% cells infected					
	Myocytes		Endothelial cells		Skin Fibroblasts	
Hormone	Male	Female	Male	Female	Male	Female
None	17.7 ± 8.8	9.8 ± 3.1	31.6 ± 8.1	23.3 ± 9.1	87.1 ± 9.5	54.8 ± 14.3
Estradiol	13.2 ± 8.0	9.8 ± 3.1	24.5 ± 8.7	39.6 ± 11.5	46.5 ± 16.4†	88.8 ± 11.2
Progesterone	33.2 ± 14.2†	46.6 ± 13.7†	59.1 ± 10.4†	40.0 ± 8.4†	42.0 ± 8.7†	69.5 ± 16.3
Testosterone	47.7 ± 17.2†	24.3 ± 2.5†	46.5 ± 9.2†	49.7 ± 10.4†	54.2 ± 18.6	77.0 ± 14.8

*Cells cultured in 50 ng/ml hormone for 2 days, infected with CVB3, and cultured an additional 10 hours in hormone. The results represent the mean ± SEM of six replicate cultures for each group.
†Significantly different from cells without hormone at $P \leq 0.05$.

Table 2—Virus-Specific Binding of ^{125}I-CVB3 to Myocytes Cultured in the Presence of Various Hormones*

Hormone	cpm added	cpm bound: Total ^{125}I-CVB3 alone	cpm bound: Non-specific ^{125}I-CVB3 10^4 excess CVB3	cpm bound: Specific binding (total nonspecific)	Estimated bound PFU/myocyte
None	5×10^4	689 ± 75	125 ± 32	564	0.6
Estradiol	5×10^4	815 ± 46	198 ± 17	617	0.6
Progesterone	5×10^4	1917 ± 312†	233 ± 49	1684	1.7
Testosterone	5×10^4	3305 ± 810†	259 ± 117	3046	3.0

*Five hundred myocytes were cultured in media containing either no or 50 ng/ml hormones for 48 hours; 2.5×10^4 PFU ^{125}I-CVB3 (5×10^4 cpm) were added to the cultures, and some cultures also received 2.5×10^8 unlabeled virus. All cells were incubated 1 hour at 37 C and washed six times, and residual radiolabel bound to the cells was determined. The results represent the mean ± SEM of triplicate wells in one of two replicate experiments.

†The counts per minute bound exceed ($P \leq 0.05$) that in cultures without hormone and with estradiol.

Discussion

Sex-associated hormones influence myocarditis susceptibility in the murine model. Castration of male mice reduced myocarditis after CVB3 infection to levels normally observed in virgin females.[1] Administration of exogenous testosterone or progesterone in physiologic concentrations to castrated mice restored myocarditis susceptibility, whereas treatment with estrogens provided protection.[12,13] The variation in cardiac injury observed with hormone treatment was also reflected in virus concentrations in the heart. Virgin females and animals treated with estrogens had significantly less virus in their hearts than males, pregnant females, and either testosterone- or progesterone-treated mice.[12,13] This previous work involved giving both male and female mice 10^4 PFU virus, the concentration shown to be optimal in males. However, by increasing the amount of virus in the initial inoculum, female susceptibility was enhanced and male susceptibility decreased. Presumably, increasing virus concentrations in males might more effectively activate host antiviral mechanisms such as interferon and natural killer cell induction, which would decrease cardiac virus concentrations and result in less myocarditis. In females, where virus infection of the heart is less efficient, increasing the initial inoculum would be expected to enhance cardiac disease, because more virus localizes in the heart. Female myocarditis never reaches maximal levels in males, probably because exogenous administration of virus to the less susceptible females never approaches levels obtained in males.

Females are generally more resistant to infections than males, a phenomenon potentially contributing to their relatively greater life span.[20] The reasons for the observed sex differences remain elusive, but two potential explanations exist. First, infections may be more prevalent in males because androgens suppress immunity, delaying microbe elimination from the body.[21,22] Females frequently have higher specific immune responses than males, especially in terms of antibody titers.[23-30] Various reports indicate that exogenous estrogen administration enhances humoral immune responses to selected antigens, delays the normal decrease in antibody titers, and increases the resistance of mice to certain bacterial infections.[6,31-34] However, these effects are not consistent. Other investigators report entirely opposite findings.[6,21,22,31,33,34] Despite the incredible confusion surrounding sex-associated hormones and the immune response, most workers concede that these steroids can alter immunity, although the effect may largely depend upon the experimental animals, antigens, and conditions used.

A second explanation may be that hormones influence infections directly by altering either virus penetration or production in the host.[35-37] Metabolic changes in the cell might result in easier virus binding and entrance due to enhanced receptor expression. The present work indicates that enhanced susceptibility to CVB3 infection may result from increased virus binding to the cell. Over twice the amount of radiolabeled virus bound to testosterone-treated myocytes, compared with cells cultured either without hormone or with estradiol. Progesterone had a similar, but less dramatic, effect. Demonstration of increased binding might explain why more cells become infected with virus *in vitro* and also why more virus enters the blood and hearts of males immediately after intraperitoneal inoculation. Greater numbers of receptors on various susceptible cells could expedite vascular entry and dissemination of the virus throughout the body. This period of increased viremia immediately after virus inoculation is extremely short, lasting only 1 hour, which probably indicates that the greater numbers of receptors on endothelial cells also readily clear virus from the blood, localizing it in various tissues.

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