Occurrence of Coxsackievirus Hepatitis in Baby Rabbits and Protection by a Formalin-Inactivated Polyvalent Vaccine

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DARRYL M. SEE AND JEREMIAH G. TILLES[1]

Department of Medicine, University of California Irvine Medical Center, Orange, California 92668

Abstract. **It was observed that 23-day-old New Zealand white rabbits came down with acute hepatitis demonstrable 3 days after intraperitoneal injection with 12 coxsackievirus group B strains. The model was used to evaluate a polyvalent, formalin-inactivated virus vaccine prepared with prototype strains of coxsackievirus groups B1–6. Seven-day-old animals received one intraperitoneal and two subcutaneous injections containing the vaccine or placebo. The regimen was repeated at 15 days of age. At 23 days of age, groups of rabbits were challenged with 1×10^5 plaque-forming units of a clinical strain of group B coxsackievirus and sacrificed 3 days later. The mean neutralizing antibody titer for the 12 strains tested ($\log_2$) was 4.5 ± 1.0 eight days after the second dose of vaccine. In vaccinated animals, elevated liver function tests in the serum, and titer of virus and histopathologic abnormalities in the liver were significantly reduced for each strain tested compared with infected, unvaccinated controls. Cultures of the heart, skeletal muscle, pancreas, blood, and spleen were all negative. Thus, clinical strains of coxsackie group B viruses produced isolated hepatitis in baby rabbits. Prophylaxis with a polyvalent, inactivated-virus vaccine significantly reduced the severity of liver involvement for all 12 clinical strains tested.**

[P.S.E.B.M. 1997 Vol 216]

The coxsackievirus group B (CVB) serotypes account for up to 3 million clinically evident infections annually in the United States (1). While the majority of these infections are benign and self-limited, current evidence suggests an association of CVB infections with some cases of insulin-dependent diabetes mellitus (IDDM), idiopathic dilated cardiomyopathy, and dermatomyositis in genetically predisposed individuals (2–4). Furthermore, newborn children are susceptible to disseminated infections which occasionally result in death, and aseptic meningitis or acute myocarditis can occur at all ages (5).

Although isolated hepatitis from a CVB infection has not been previously reported in humans or animals, hepatitis has been described as an occasional complication of disseminated CVB infections (6).

There is currently no treatment approved for use of CVB infections in humans, although efficacy has been shown in animals using the WIN class of antiviral agents (7, 8), ribavirin (9), and α-interferon (10). Studies of both univalent (11) and polyvalent (12) vaccines in mice have proven to be efficacious in preventing infections with CVB strains.

The current study reports hepatitis without involvement of other organs in baby New Zealand White rabbits infected with each of 12 clinical CVB strains. Liver samples were positive for viral nucleic acid after polymerase chain reaction (PCR) amplification up to 6 months after challenge with virus. Both titers of virus and histopathologic changes in the liver were significantly reduced in vaccinated, virus-challenged animals 3 days postinoculation (p.i.).

[1] To whom requests for reprints should be addressed at Department of Medicine, University of California at Irvine, Building 53, Route 81, 101 S. City Drive, Orange, CA 92668.

Received November 26, 1996. [P.S.E.B.M. 1997, Vol 216]
Accepted May 6, 1997.
This study was supported by a grant from Biosphere Technology.

0037-9727/97/2161-0052$10.50/0

Materials and Methods

Animals. Pregnant New Zealand White rabbits were obtained from Charles River Farms (Wilmington, MA).

Virus. The source and maintenance of the library of clinical isolates and the six prototype strains have been previously described (12). For each serotype, two clinical strains were arbitrarily chosen for the present study. Briefly, 25 clinical isolates of CVB groups 1–5 were obtained from the California Department of Health Sciences. Three isolates of CVB6 were obtained from the Centers for Disease Control (CDC). Prototype viruses of CVB1 (Conn), B2 (Ohio), B3 (Nancy), B4 (JVB), B5 (Faulkner), and B6 (Schmidt) were purchased from the American Type Culture Collection (Rockville, MD). Confluent monolayers of monkey kidney (MK) cells were inoculated with virus at a multiplicity of infection (MOI) of 1 plaque-forming unit (pfu) (13) per cell in Leibovitz's L15 medium supplemented with antibiotics, dextrose, glutamine, and arginine. The viruses were adsorbed for 60 min at 37°C, fetal bovine serum (FBS) was added to a final concentration of 10%, and incubation was continued until significant cytopathic effect (cpe) was observed, after which the virus-containing medium was harvested and frozen at −80°C prior to use. Titers of virus were assayed by the plaque technique (13).

Vaccine. The preparation of the polyvalent, formalin-inactivated vaccine using the six prototype strains has been described previously (12). Briefly, each prototype strain was prepared in MK monolayers and harvested as described above. The 6 prototype lots were combined in equal proportions, formalin-inactivated, and centrifuged at 1000*g* for 15 min. After ultracentrifugation of the supernatants at 105,000*g* for 7 hr, the pellets were resuspended in 10 ml of L15 diluent, vortexed for 5 min and stored at −80°C. Each lot of vaccine contained a live virus equivalent of 1×10^6 to 1×10^7 pfu/ml of each prototype virus.

Development of the Animal Model. The spectrum of rabbit ages susceptible to hepatic infection was investigated. Groups of five animals at 7, 23, 28, and 42 days of age were inoculated with 1×10^5 pfu of CVB4 strain E2. Each rabbit 28 days of age or less had a titer $>10^3$ pfu/mg of hepatic tissue 3 days after infection. One out of five animals at 42 days of age had detectable virus in the liver (sensitivity 100 pfu/mg). An additional 5 rabbits at 42 days of age which were inoculated with 1×10^7 pfu of virus also failed to have detectable virus in the liver.

To determine the earliest age of reproducible neutralizing antibody (NA) response, groups of five animals were inoculated with the vaccine at 3, 7, or 14 days. Seven days p.i. NA titers >3 ($\log_2$) for each of the six CVB serogroups were recorded in four or five out of five animals inoculated at 7 or 14 days of age, respectively, but only in one out of five rabbits inoculated at 3 days of age.

Vaccine Experiment. On Day 1, baseline blood was first collected from 7-day-old test rabbits by ear artery puncture and the serum stored at −80°C. The rabbits were then inoculated with an intraperitoneal injection of 0.3 ml and two subcutaneous injections of 0.1 ml of either placebo (Leibovitz's L15 diluent; vaccine control) or vaccine. Each rabbit inoculated with vaccine received a live virus equivalent of $5–6 \times 10^5$ of each of the six prototype strains. Eight days later (Day 15) the animals received an identical regimen of vaccine or placebo. On Day 23, after a second blood sample was collected by ear artery puncture for serum storage, each rabbit was challenged intraperitoneally with 0.3 ml containing either 1×10^5 pfu of one of the 12 test virus strains or placebo (L15 diluent; virus control). Thus, two groups of rabbits served as uninfected controls: (i) unvaccinated, uninfected controls received two doses of vaccine placebo and one dose of virus placebo; (ii) vaccinated, uninfected controls received the two doses of vaccine and virus placebo. Three days p.i. of active virus or virus placebo, the rabbits were anesthetized by intramuscular administration of ketamine (30 mg/kg) with zylozine (5 mg/kg), blood was taken by cardiac puncture, and tissues were removed for later study. Groups of vaccinated, uninfected control rabbits were sacrificed 12 weeks after the second vaccine dose. In addition, one group of rabbits received two initial doses of vaccine, followed in 2 weeks by a third dose, and sacrifice 8 days later. Finally, groups of unvaccinated animals received CVB5/C59, the strain producing the highest virus titer in the liver, on Day 23 and subsets were sacrificed 1, 3, 6, and 24 weeks p.i. and assessed for persistent infection.

Specimen Processing.

Neutralizing Antibody Detection. Titers of infectivity neutralizing antibody in the serum (from 1:8 to 1:4112) were assessed by microtiter titration as previously described (12).

Virus Assay. For each rabbit, a 0.2-ml aliquot of blood and portions of brain, heart, liver, pancreas, spleen, and anterior thigh skeletal muscle were harvested and assayed for virus by the plaque technique (13).

Histopathology. Portions of harvested tissues were fixed, sectioned, mounted, and stained with hematoxylin and eosin utilizing standard methods (12). The severity of liver pathology was scored blindly by light microscopy on a scale of 1+ to 4+ in terms of severity of liver necrosis. A 1+ score reflected 1–10 necrotic hepatocytes per medium-power field (×250), a 2+ score indicated 11–50 dead cells, a 3+ score denoted 51–100 necrotic cells, and a 4+ score indicated >100 necrotic hepatocytes.

Hepatocyte Autoantibody Titers. Sera obtained from vaccinated, uninfected controls 12 weeks after the second dose of vaccine were tested for the presence of autoantibody against normal hepatocytes of 23-day-old unvaccinated, uninfected rabbits. Fluorescein-labeled goat anti-rabbit IgG was utilized as the second antibody in an indirect immunofluorescence test as described previously (14).

Presence of Viral RNA. Each tissue harvested 3 days p.i. was evaluated for the presence of enteroviral nucleic acid segments using PCR amplification as previ-

ously described (14). Briefly, RNA was extracted, and complementary-strand DNA (cDNA) was synthesized and amplified by PCR using primers specific to a conserved region of the 5′ noncoding region of enteroviruses (upstream [sense] primer sequence: 5′-CCTCCGGCCCCT-GAATGCGGCTAAT-3′; downstream [antisense] primer: 5′-ACCGACGAATACCACTGTTA-3′) yielding at 154-bp product visualized by electrophoresis. The efficacy of the PCR process was monitored by amplification of rabbit β-actin mRNA in all negative samples. Furthermore, the presence of amplification inhibitors was evaluated by spiking all negative samples with 2 μl of a stock CVB strain followed by amplification and electrophoresis. The presence of viral RNA in various tissues was sought at later time points in rabbits infected with CVB5/C59. Finally, PCR was applied to sections of liver from vaccinated, uninfected controls 12 weeks after the second dose of vaccine.

Liver Function Tests. Serum was assayed for alanine transferase (ALT), lactate dehydrogenase (LDH), and alkaline phosphatase (AP) levels at various time points by the UCI Medical Center clinical laboratory service using standard methods.

Statistics. The differences in histology scores were evaluated by the Kruskal-Wallis test. Arithmetic means were calculated for liver function tests and geometric means were calculated for viral and neutralizing antibody titers. The significance of differences between arithmetic or geometric means were calculated by the Student's *t* test or the Wilcoxon ranked sum test, respectively.

Table I. Neutralizing Antibody Titers for Clinical and Prototype Strains of Coxsackie Group B Viruses 8 Days after a Second Dose of Vaccine

Strain	*n*	Mean neutralizing antibody titer ($log_2 \pm SD$)	
		Range	Mean
Clinical			
CVB1/C22	10	<3–4.9	4.2 ± 1.1
CVB1/C23	10	3.3–5.8	4.9 ± 1.3
CVB2/C8	10	3.2–6.9	5.3 ± 0.7
CVB2/Krahl	10	<3–4.8	4.1 ± 0.4
CVB3/C4	10	3.6–7.0	5.6 ± 1.1
CVB3/Cole	10	<3–4.6	3.6 ± 1.0
CVB4/E2	10	<3–4.9	3.8 ± 0.9
CVB4/Mejia	10	<3–6.5	4.6 ± 1.3
CVB5/C59	10	3.8–7.9	5.9 ± 2.0
CVB5/Free	10	<3–5.4	4.3 ± 0.7
CVB6/O17	10	3.5–5.5	4.6 ± 0.6
CVB6/139	10	<3–4.2	3.3 ± 0.7
Mean			4.5 ± 1.0
Prototype			
CVB1/Conn	10	4.9–8.0	6.8 ± 1.6
CVB2/Ohio	10	5.6–9.7	7.1 ± 1.8
CVB3/Nancy	10	4.3–7.6	5.8 ± 1.4
CVB4/JVB	10	5.1–9.4	8.2 ± 2.3
CVB5/Faulk	10	4.5–8.2	6.4 ± 1.7
CVB6/Schmidt	10	4.6–8.7	6.8 ± 1.9
Mean			6.8 ± 1.8

Note. Titers <3 were assigned a value of 2 for purposes of calculating the mean. Values represent the mean ± SD for 10 mice. Titers were determined 8 days after the second dose of vaccine. Baseline titers were <3 for all strains.

Results

Neutralizing Antibody Response to Vaccination. Neutralizing antibody titers prior to immunization were <3 (log_2) for each strain tested (Table I). The mean neutralizing titer for each strain 8 days after the second vaccine dose was determined on serum samples in those 10 rabbits which were destined to be challenged with that strain. The mean for all 12 strains was 4.5 ± 1.0. Sera from 10 mice 8 days after the second vaccine dose were evaluated for neutralizing antibody response to all six of the prototype strains. The mean for the six strains was approximately 5-fold higher than for the clinical strains (6.8 ± 1.8).

Infection of Various Tissues after Challenge with Clinical CVB Strains. The capacity of the 12 clinical strains to infect various tissues was evaluated three days p.i. Challenge with each of the 12 strains produced hepatic infection in all five 28-day-old rabbits tested (mean mean [log_{10}], 4.9 ± 1.1 pfu/mg). The portions of blood, brain, heart, pancreas, and skeletal muscle tested were all culture negative and therefore had less than 100 pfu/mg (12). The majority of spleens (55/60) and a few blood samples (7/60) 3 days p.i. were positive for enteroviral nucleic acid after PCR amplification. Brain, heart, pancreas, and skeletal muscle samples were all PCR negative.

The capacity of CVB5/C59 to produce persistent infection was examined. Portions of liver from five, one, or zero out of five rabbits were culture positive at 1, 3, or 6 weeks p.i., respectively. However, viral nucleic acid was still detectable after PCR amplification in five out of five samples of liver 24 weeks p.i. At 1 week p.i., all five blood samples tested were PCR negative (sensitivity of 20 pfu/mg [14]). Similarly, by 6 weeks p.i., all five spleen samples evaluated were negative for viral nucleic acid.

Protection from Acute Infection with CVB Strains. The ability of primary and 8-day booster immunizations to protect rabbits from acute hepatitis when challenged with CVB strains was assessed by measurement of the serum enzymes that reflect liver function, histopathological examination of liver tissue, and assay of viral titers in the liver (Table II). Mean serum ALT and LDH levels were significantly elevated in infected, unvaccinated animals compared with uninfected controls for rabbits challenged with each of the 12 clinical strains ($P \leq 0.05$–0.01). AP levels were generally less significantly elevated. Mean ALT or LDH levels were significantly higher in unvaccinated, infected animals compared with vaccinated, infected animals for 12 or 11 of 12 strains tested, respectively. Similarly, mean histopathologic score (Fig. 1) and titer of virus in the liver were significantly reduced with each of the 12 strains for infected vaccinated animals compared with infected, unvaccinated controls.

Table II. The Effect of Vaccination on Various Parameters in the Livers of Rabbits 3 Days after Inoculation with Clinical Coxsackievirus Group B Strains

Strain	Mean serum liver function tests (mg/dl)			Mean histopathologic score	Mean virus titer (log_{10} pfu/mg)
	ALT	LDH	AP		
None (uninfected)	40.5 ± 7.4	189.8 ± 15.3	97.9 ± 10.5	0.0	<2
Mean for 12 clinical strains					
Vaccine	54.2 ± 13.7	234.8 ± 23.9	113.3 ± 27.2	0.9 ± 0.4	1.8 ± 0.7
No vaccine	352.7 ± 36.3	558.2 ± 91.3	168.7 ± 31.3	3.5 ± 1.1	4.9 ± 1.0
P value	<0.01	<0.01	<0.05	<0.01	<0.01

Note. Titers <2 were assigned a value of 1 for purposes of calculating the mean.

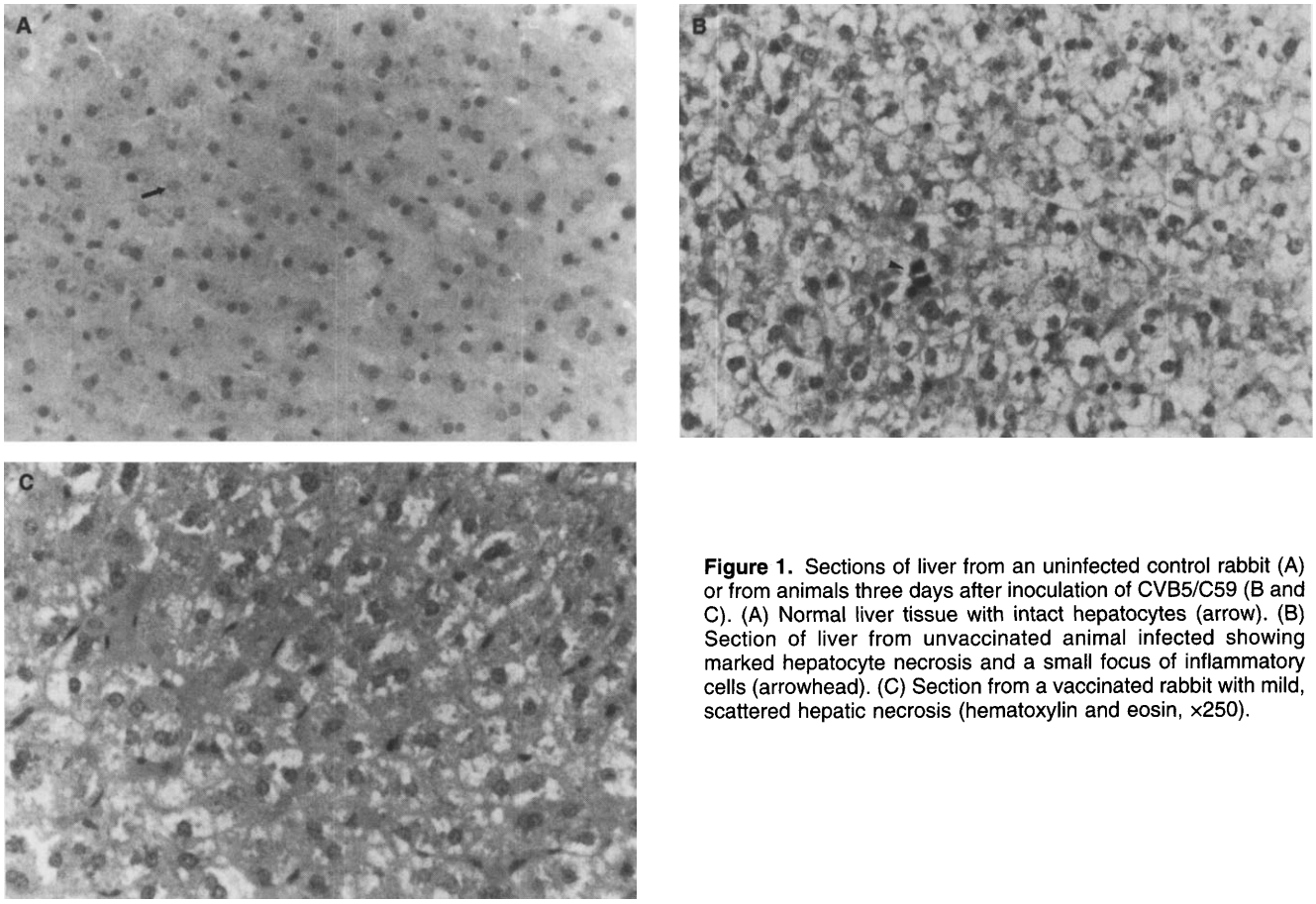

Figure 1. Sections of liver from an uninfected control rabbit (A) or from animals three days after inoculation of CVB5/C59 (B and C). (A) Normal liver tissue with intact hepatocytes (arrow). (B) Section of liver from unvaccinated animal infected showing marked hepatocyte necrosis and a small focus of inflammatory cells (arrowhead). (C) Section from a vaccinated rabbit with mild, scattered hepatic necrosis (hematoxylin and eosin, ×250).

Long-Term Effects of Vaccination. Ten vaccinated, uninfected animals were evaluated for adverse effects 12 weeks after a second dose of vaccine. Antihepatocyte antibody titers were <1:8 in the serum of each of the 10 rabbits tested, and their livers had neither histopathologic changes nor viral nucleic acid by PCR (sensitivity 20 pfu/mg).

Discussion

Previous studies have shown the capacity of CVB inoculated into mice to infect a variety of organs, including brains, skeletal muscle, myocardium, pancreas, and liver (14). However, a model of isolated hepatitis in mice has not been developed. One study in baby rabbits challenged with CVB demonstrated myositis without other organ involvement (15). In the current study, infection of 23-day-old New Zealand White rabbits with each of 12 clinical strains of CVB produced isolated hepatitis. Muscle involvement was not demonstrated for any of the 12 strains tested, but may be explained by presence in a titer less than that detectable by the PCR assay used in the study, resistance of the rabbit strain used, or sampling errors as only portions of thigh muscle and heart were evaluated. Susceptibility of the baby rabbits to infection diminished rapidly with advancing age. By 42 days of age, hepatitis was demonstrable in only 20% of animals challenged with virus.

Although case reports have suggested that the liver is a target organ in disseminated CVB infections in humans (6),

reports of isolated hepatitis have not appeared. The current study suggests that CVB can cause acute and chronic hepatitis in rabbits. Studies to evaluate an association of CVB in otherwise unexplained cases of acute or chronic hepatitis in humans may be warranted.

Of the estimated >15,000,000 annual CVB human infections in the United States, the vast majority are asymptomatic. Most clinically evident cases are characterized by benign manifestations, especially respiratory involvement or rash. Myopericarditis is a potentially fatal acute infection from arrythmias or heart failure and may complicate up to 5% of clinically apparent CVB infections (1). Some cases may progress to dilated cardiomyopathy. Aseptic meningitis is commonly caused by CVB and may cause significant morbidity. Evidence suggests an association of CVB with some cases of insulin-dependent diabetes mellitus (IDDM), a disease with significant morbidity and mortality that affects up to 1:200 of Americans. If this association proves to be definitive, genetic screening of susceptible individuals may uncover candidates especially suited for prevention with a CVB vaccine.

Immunity to enteroviral infections is humoral. Multiple clinical trials as well as clinical experience have demonstrated the capacity of simple formalin-inactivated vaccine preparations of hepatitis A and poliomyelitis to stimulate vigorous antibody responses and provide clinical protection against wild-type strains. Previous work in mice with inactivated prototype strains of CVB has shown good cross-reactivity to clinical strains (14).

Although the antibody response to the current vaccine after two administrations was modest, it was sufficient to provide clinical protection of rabbits against infection with each of the 12 clinical strains tested. A third dose given 2 weeks after the second elicited a further rise in neutralizing antibody response of approximately 4- to 8-fold (data not shown). Because a majority of older animals do not manifest an acute hepatitis, the benefit of a third dose was not evaluated with the present model.

Coxsackieviruses have the capacity to establish latent infection in target organs which stimulates autoimmune tissue damage in the host. Therefore, the current vaccine was tested to determine if it resulted in persistence of viral RNA in the liver or stimulation of autoimmune hepatitis. Viral RNA was not detected in the livers of vaccinated, uninfected animals 12 weeks p.i. Furthermore, antihepatocyte antibody production was not stimulated by the vaccine, and livers of vaccinated, uninfected rabbits were histologically normal 3 days, 3 weeks, and 12 weeks p.i.

Thus, infection with 12 of 12 clinical strains of CVB tested caused isolated, persistent hepatitis in baby rabbits. A study is in progress to determine the mechanism of disease induction. The animals could be protected from infection after two administrations of a formalin-inactivated polyvalent vaccine produced from prototype strains of coxsackieviruses groups B1–6.

We gratefully acknowledge the technical assistance of Tonya Clark.

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