RELATEDNESS OF RABBIT CORONAVIRUS TO OTHER CORONAVIRUSES

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ABSTRACT

Rabbit coronavirus (RbCV) was apparently first encountered in 1961 when Scandinavian investigators observed occasional mortality in rabbits used to propagate the Nichols strain of Treponema pallidum. Mortality rates reached 50 percent by 1968 and 75 percent by 1970. Contaminated samples of T. pallidum were brought to the Johns Hopkins University School of Medicine, a World Health Organization center for the study of treponematoses. There it was established that the causative agent was filterable, the heart was the target organ, and the agent was determined by electron microscopy to be a coronavirus. Also, complement fixing antibodies to the human coronaviruses 229E (two way cross) and OC43 (one way cross) were demonstrated in surviving rabbits. Immunofluorescent staining with anti-229E serum localized fluorescence in the interstitial tissue of the myocardium.

Antiserum to RbCV cross reacted with coronaviruses of three other diseases, feline infectious peritonitis (FIPV), canine coronavirus diarrhea (CCV), and transmissible gastroenteritis (TGEV) by radioimmunoassay. In plaque neutralization tests, a slight reduction was observed against TGE and CCV but not against FIP. Antiserum to 229EV, CCV, FIPV and to a lesser degree TGEV partially blocked the clinical course of the disease and reduced mortality. Slight protection was afforded rabbits by vaccination with, in descending order of survivors, CCV, FIPV, and TGEV. Vaccination with calf diarrhea coronavirus (CDCV) provided no protection.

INTRODUCTION

Rabbit coronavirus (RbCV) was first encountered in 1961 when Scandinavian investigators observed occasional mortality in rabbits used to propagate the Nichols strain of Treponema pallidum (Gudjonsson and Skog, 1968, and Jorgensen, 1968). The late Dr. H. Gudjonsson, a dermatologist at the Karolinska Hospital, Stockholm, Sweden, brought contaminated samples of testicular emulsions of T. pallidum to the Johns Hopkins University School of Medicine, a World Health Organization center for the study of treponematoses. It was established that the responsible agent was filterable and

that the heart was the apparent target organ. Coronavirus-like particles were visualized by electron microscopy in infectious serum. Additional support for the causative agent being a coronavirus was the finding of complement fixing antibodies to the human coronaviruses 229E (two way cross) and OC43 (one way cross) in surviving rabbits. Indirect immunofluorescent staining with anti-229E sera localized fluorescence in the interstitial tissue of the myocardium. Consistent with a coronaviral etiology of disease, infectivity of serum was lost following treatment with ether, indicating that an enveloped virion was present (Small, et al., 1979).

The clinical course and pathologic findings following inoculation of infectious serum or testicular emulsions have been described previously (Small, et al., 1979). Briefly, the rectal temperature increases to $>40^{\circ}\text{C}$ within 24 to 48 hrs and peaks by 72 hrs. Temperatures of 41.5°C are not unusual during this time. Fever is accompanied by a marked decrease in body weight, feed and water consumption. Mortality following iv injection of infectious serum is $>80\%$ and deaths occur between post-inoculation (PI) days 2 and 11. Coincident with the elevation in temperature is dullness of the scleras, severe congestion of the conjunctivae, irides, and, in some cases, severe chemosis and hyphema. In rabbits with hyphema that survive, the eyes clear by the 12th day. Indirect ophthalmoscopy does not reveal retinal lesions. If the rabbit survives, the ocular changes gradually resolve and the eyes appear normal 2 to 4 weeks after inoculation.

At necropsy gross lesions are confined to the heart, lungs, and lymph nodes. Right ventricular dilation with hemorrhage in the epicardium and endocardium is often seen in rabbits that die within the first 4 days PI. Pulmonary edema with 10-50 ml of clear straw-colored fluid which clots on standing is present in the thorax in early deaths. With survival beyond 4 days the volume of fluid decreases and it tends to be serosanguinous. The lungs, remain wet, but are firmer than in those dying in the first 4 days. The subendocardial and subepicardial hemorrhages decrease in severity as the time to death lengthens. Dilation of the right ventricle is still observed.

Histologic lesions are observed in the heart, diaphragm, lungs, thymus, peripheral lymph nodes, and spleen. The most prominent and consistent lesion is multifocal to diffuse myocardial degeneration and necrosis. Occasionally large multinucleate macrophages containing fragments of necrotic sarcoplasm are seen. Foci of coagulative necrosis are surrounded by macrophages or heterophils but lymphocytes and plasma cells are absent. Macrophages and Anitschkow myocytes are numerous in interstitial spaces. Both the atria and ventricles are affected but no lesions are noted in the blood vessels or cardiac valves. Similar lesions are noted in diaphragmatic muscle in approximately 33% of the rabbits. Other skeletal muscles are not affected. Pulmonary lesions consist of intra-alveolar accumulations of pink proteinaceous fluid and numerous alveolar macrophages. Salient features of lesions in the thymus, peripheral lymph nodes, and spleen are lymphoid depletion, vascular congestion with sinus ectasia and edema, or lymphoid hyperplasia. Enlargement of venous endothelial cells and extramedullary hematopoiesis is occasionally observed.

The current study presents additional data supporting the contention that the putative agent of rabbit cardiomyopathy is a coronavirus related to the human coronavirus, 229E.

MATERIALS AND METHODS

Viruses. RbCV from a pool of rabbit serum originally containing 10^7 rabbit infectious units (RIU) per ml was used throughout these studies.

Viral titer was determined in rabbits following filtration through a 0.22um MF-Millipore filter. Canine coronavirus (CCV) was the UCD-1 strain, transmissible gastroenteritis virus (TGEV) was the Miller-3 strain and feline infectious peritonitis virus (FIPV) was the UCD-1 AC/3 strain. Their origins and growth in a feline cell line have been previously described (Woods, 1982).

Hyperimmune serums. Hyperimmune serum to RbCV was prepared as previously described (Small, et al., 1979). Hyperimmune serums to TGEV, CCV, and FIPV were collected from the rabbits used in the cross protection tests described below. Rabbit origin antiserum to 229EV was kindly supplied by Dr. Kathryn Holmes.

In vitro assays. Virus neutralization was determined by a plaque reduction test using a feline cell line as previously described (Woods, 1982). The radioimmunoassay was performed as described by Woods and Wesley (In press). Double (Ouchterloney) immunodiffusion tests were done with antibody to RbCV with TGEV, CCV, and FIPV as the antigens (Johnson, 1986).

Cross protection tests. Groups of 3 male New Zealand White rabbits (Hazleton Research Animals, Denver, PA), 2.0-2.5kg, were vaccinated 4 times at weekly intervals with 2 ml of tissue culture fluid containing live CCV, TGEV, or FIPV. A fourth group was vaccinated 6 times against calf diarrhea coronavirus (CDCV), using a commercial vaccine (CALF GUARD, Norden Laboratories, Lincoln, NE). Each dose was 3 ml. This vaccine also contains a bovine rotavirus. Both are modified live viruses. All inoculations of virus were made intramuscularly into the thigh or lumbar muscles. Rabbits were weighed and rectal temperatures determined daily Monday through Friday during the vaccination period. Two weeks following the last vaccination, blood was collected from the central auricular artery for serum and each rabbit was challenged with rabbit serum containing 10^6 RIU of RbCV in the marginal ear vein. Rectal temperature, body weight, clinical signs, and mortality were recorded 7 days a week until death or until the rectal temperature stabilized in the preinoculation range (39 - 39.9°C).

Cross neutralization. Equal volumes of rabbit origin hyperimmune serums to 229EV, CCV, FIPV, TGEV, and RbCV plus rabbit serum containing 10^6 RIU of RbCV were incubated for 2 hours at 37°C as previously described (Small, et al., 1979). Two ml was inoculated into the marginal ear vein. Control rabbits received serum containing 10^6 RIU of virus diluted 1:1 with saline and incubated as above. In previous experiments controls also included similarly treated mixtures of virus and preimmune rabbit serum and hyperimmune rabbit serum with PBS (Small, et al., 1979).

RESULTS

Radioimmunoassays. Bound counts of ^{125}I viral protein are shown in Table 1. RbCV antiserum produced counts above those present in normal rabbit serum but less than those of serum from a rabbit hyperimmunized against TGEV. However, cross reactivity between RbCV antiserum and TGEV, CCV, and FIPV is evident. This indicates that RbCV is distantly related to TGEV, CCV, and FIPV.

Table 1. In Vitro Cross Reactions to Rabbit
Coronavirus: Radioimmunoassay

ANTISERUM RABBIT NO.	VIRAL ANTIGEN		
	TGEV	CCV	FIPV
2 (RbCV)	6300 ¹	5200	5400
36 & 37 Pool (RbCV)	4900	6500	7000
91 (Normal)	900	2000	3300
95 (Normal)	900	2100	2000
TGEV + Control	27000	26000	12000
TGEV - Control	500	1300	2100

¹Protein ¹²⁵I bound counts

Virus neutralization. (Table 2) In the plaque reduction assay no activity against FIPV was observed. A slight reduction in the number of TGEV and CCV plaques was observed following treatment with RbCV antiserum. This suggests the presence of a small amount of neutralizing antibody.

Table 2. In Vitro Cross Reactions to Rabbit
Coronavirus: Virus Neutralization

ANTISERUM RABBIT NO.	VIRAL ANTIGEN		
	TGEV	CCV	FIPV
2 (RbCV)	35, 37 ¹	41, 50	52, 38
36 & 37 Pool (RbCV)	41, 42	33, 40	50, 49
91 Normal	50, 51	49, 55	46, 56
92 Normal	38, 40	47, 48	42, 45
TGE + Control	0, 1	1, 4	8, 7
TGE - Control	52, 48	49, 56	44, 38
Virus Control	48, 54	54, 60	45, 50

¹No. of plaques with serum diluted 1:4.

Double immunodiffusion. RbCV antiserum against TGEV, CCV, and FIPV produced no lines of precipitation. Antiserum to TGEV, used as a positive control, resulted in distinct lines of precipitation with TGEV and CCV but only a faint line with FIPV.

Serum neutralization. (Table 3) The incubation of 10^6 RIU of RbCV with antiserum to all of the coronaviruses listed resulted in the survival of 1 or more of 3 rabbits. No rabbit receiving incubated serum containing only RbCV survived. In all cases the rabbits became ill and developed the typical clinical picture of fever, congested eyes, and loss of weight. However, in some instances recovery with return of a normal body temperature ($<40^{\circ}\text{C}$) and resumption of eating and drinking was accelerated.

Table 3. Neutralization of Rabbit Coronavirus with Heterologous Antiserum

CORONAVIRUS ANTISERUM	RABBITS (SURVIVORS/INOCULATED)
Human (229EV)	2/3
Canine (CCV)	2/3
Feline (FIPV)	2/3
Swine (TGEV)	1/3
Rabbit (RbCV) + Control	3/3
None - Control	0/3

Cross protection. Results of vaccinating rabbits with tissue cultures of TGEV, CCV, or FIPV or a commercial vaccine to CDCV are shown in Table 4. The clinical course of the disease, even in survivors, did not appear to be altered due to vaccination. No signs of illness due to the vaccinations were noted.

Table 4. Cross Protection Against Rabbit Coronavirus Infection with Heterologous Vaccines

CORONAVIRUS VACCINE	RABBITS (SURVIVORS/INOCULATED)
CCV	2/3
FIPV	1/3
TGEV	0/3
CDCV	0/3
None	1/3

DISCUSSION

The results of the radioimmunoassay suggest that RbCV, TGEV, CCV, and FIPV are antigenically related. Hyperimmune serum to RbCV was produced in recovered rabbits by giving repeated iv injections of rabbit serum containing 10^5 - 10^7 RIU of RbCV. If adjuvants had been used, perhaps the level of antibody may have been higher. Despite evidence of cross reactivity with FIPV in the radioimmunoassay, none was seen in the plaque reduction assay with FIPV. Limited neutralization of TGEV and CCV was suggested however, by a slight reduction in the number of plaques. The reduction in plaques of TGEV and CCV lends additional support to the contention that RbCV is related to the 229E group of coronaviruses. Thus far RbCV has not been adapted to tissue culture. This has limited its study. The value of being able to use other coronaviruses in an established tissue culture system for a plaque reduction assay of RbCV is evident.

The reduction in mortality (33-67%) observed in rabbits given RbCV incubated with antiserum to 229EV, CCV, FIPV, or TGEV provides additional evidence for the relatedness of RbCV to the 229E group of coronaviruses. While the numbers of rabbits per virus are small, collectively 7 of 12 (58%) survived whereas 0 of 3 receiving heated but otherwise unaltered virus containing serum lived. Historically, mortality has been 83-100% with this stock and dose of virus and route of administration. This observation has been noted with other stocks of RbCV given in a similar manner (Small, et al., 1979).

The results of vaccinating rabbits with tissue cultures of live CCV, FIPV, TGEV, or commercial CDCV vaccine suggested that CCV and possibly FIPV but not TGEV or CDCV vaccine induced limited immunity to RbCV. However, the results were less impressive than those seen with the coinubation of virus and antiserum. The small numbers of rabbits per virus and the survival of 1 RbCV control rabbit make meaningful conclusions difficult. In the present study a total of 6 rabbits received RbCV (10^6 RIU) alone. Only 1 survived. In the original study, 15 of 16 rabbits died following the iv inoculation of infectious serum (Small, 1979). However, in the original study the titer of virus injected was not always known. Evidence for the induction of immunity to TGE infection in swine with FIPV has been presented (Woods and Pedersen, 1979; Woods, 1984) as has evidence for induction of immunity to TGE infection with CCV (Woods and Wesley, 1986).

These new findings provide additional evidence that the rabbit cardiomyopathy agent is a coronavirus related to human coronavirus 229E and others in that group. While the coronaviruses are highly species specific, their tissue tropisms vary considerably. This RbCV appears to be the first coronavirus to preferentially attack the heart; however, a coronavirus has been observed in the feces of rabbits with enteritis and an antigenic relationship with 229EV has been reported (Lapierre, et al., 1980). The value of the RbCV reported here in the study of viral induced cardiomyopathies in humans remains to be elucidated. Severe perimyocarditis has been reported in a child in association with a rise in antibody titer to human coronavirus OC 43 (Riski and Hovi, 1980). Examination of the cardiovascular system in humans with respiratory disease due to a coronavirus merits consideration.

REFERENCES

- Gudjónsson, H. and E. Skog. 1968. Fever after inoculation of rabbits with Treponema pallidum - Jarisch-Herxheimer reaction? Proc. 18 Meeting Scand. Dermatol. Assoc. Turku 71-77.

- Laboratory Immunology" (N.R. Rose, H. Friedman, and J.L. Fahey, eds.), pp. 16-17. Am. Soc. Microbiol., Washington, D.C.
- Jørgensen, B.B. 1968. Spontaneous deaths among rabbits inoculated with Treponema pallidum less than 2 weeks before. Abnormal susceptibility in apparently normal laboratory rabbits indicated by serological tests for human syphilis. Z. Versuchstierkd. 10:46-54.
 - Lapierre, J., G. Marsolais, P. Pilon, and J.-P. Descôteaux. 1980. Preliminary report on the observation of a coronavirus in the intestine of the laboratory rabbit. Canad. J. Microbiol. 26: 1204-1208.
 - Riski, H. and T. Hovi. 1980. Coronavirus infections of man associated with diseases other than the common cold. J. Med. Virol. 6:259-265.
 - Small, J.D., L. Aurelian, R.A. Squire, J.D. Strandberg, E.C. Melby, Jr., T.B. Turner, and B. Newman. 1979. Rabbit cardiomyopathy associated with a virus antigenically related to human coronavirus strain 229E. Am. J. Pathol. 95:709-729.
 - Woods, R.D. 1984. Efficacy of vaccination of sows with serologically related coronaviruses for control of transmissible gastroenteritis in nursing pigs. Am. J. Vet. Res. 45:1726-1729.
 - Woods, R.D. 1982. Studies of enteric coronaviruses in a feline cell line. Vet. Microbiol. 7:427-435.
 - Woods, R.D. and N.C. Pedersen. 1979. Cross-protection studies between feline infectious peritonitis and porcine transmissible gastroenteritis viruses. Vet. Microbiol. 4:11-16.
 - Woods, R.D. and R.D. Wesley. 1986. Immune response in sows given transmissible gastroenteritis virus or canine coronavirus. Am. J. Vet. Res. 47:1239-1242.