Rabbit Cardiomyopathy

Associated With a Virus Antigenically Related to Human Coronavirus Strain 229E

James D. Small, DVM, MPH, Laure Aurelian, PhD, Robert A. Squire, DVM, PhD, John D. Strandberg, DVM, PhD, Edward C. Melby, Jr., DVM, Thomas B. Turner, MD, and Burlina Newman, BS

A new disease of rabbits is described. Following an acute febrile course, animals die or recover by the 11th day postinoculation. The characteristic pathologic finding is multifocal myocardial degeneration and necrosis. The disease can be transmitted by various routes with tissue filtrates or with infectious sera diluted to 10^{-6} and passed through 0.1 μm filters. Virus particles with morphologic features characteristic of a coronavirus are present in infectious but not in normal rabbit serums. The antigen(s) in the infectious serums cross-reacts with the 229E and the OC43 strains of human coronavirus. Antigen cross-reacting with the 229E virus is detectable by immunofluorescent staining in frozen sections of heart tissue from sick but not from healthy animals. Animals surviving infection seroconvert to coronavirus specificity, as demonstrated by the presence in convalescent serums of antibody capable of reacting with the 229E virus. Susceptibility to infection has not been demonstrated in mice, hamsters, or guinea pigs, and the virus was not adapted for growth in tissue culture. It is uncertain whether the agent is a natural pathogen of rabbits or a coronavirus contaminant from another species, possibly human. The name rabbit infectious cardiomyopathy is suggested for this disease. (Am J Pathol 95:709–730, 1979)

In 1968, Scandinavian workers reported an acute febrile disease of rabbits following intratesticular inoculation of the Nichols strain of *Treponema pallidum*.[1,2] This strain was maintained in Scandinavia since 1953 by passage in rabbit testes. In 1961 sporadic deaths began in the inoculated animals. Mortality rates reached 50% by 1968 and 75% by 1970.[3] Rabbits died within a week of inoculation with an acute, febrile illness characterized by rectal temperatures in excess of 40 C, severe pulmonary edema, and congestion of the superficial lymph nodes. Heat-

From the Veterinary Resources Branch, Division of Research Services, National Institutes of Health, Bethesda, Maryland, and The Johns Hopkins University School of Medicine, Division of Comparative Medicine and Department of Microbiology, Baltimore, Maryland.

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Mr. Melby's present address is New York State College of Veterinary Medicine, Cornell University, Ithaca, NY. Mr. Turner's present address is The Johns Hopkins University School of Medicine, Baltimore, MD 21205. Ms. Newman's present address is Department of Environmental Health, Division of Radiation Health Sciences, The Johns Hopkins School of Hygiene and Public Health, Baltimore, MD 21205.

Address reprint requests to J. D. Small, DVM, Veterinary Resources Branch, Building 14A, Room 102, National Institutes of Health, Bethesda, MD 20205.

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ing the testicular emulsions to a temperature that killed the treponemes failed to inactivate infectivity, thereby excluding treponomes from the etiology of the disease.[3]

In 1970, the late Dr. H. Gudjónsson, a dermatologist at the Karolinska Hospital, Stockholm, Sweden, brought samples of testicular emulsion containing contaminated Nichols strain of *T pallidum* to the Johns Hopkins University School of Medicine in Baltimore. In this communication we report the results of our studies on the etiology and pathologic manifestations of this apparently new disease of rabbits. Evidence is provided that the agent designated "Stockholm Agent" by Gudjónsson et al [4] may be a coronavirus.

Materials and Methods

Animals

Randomly bred New Zealand white rabbits, of both sexes, weighing 1.2 to 4.9 kg, were purchased from commercial suppliers or obtained from the Veterinary Resources Branch, National Institutes of Health. They were housed individually and fed antibiotic-free rabbit pellets (Purina or NIH Animal Feed A) and water *ad libitum*. Depending on the laboratory, the room temperature was maintained at 15 to 18 or 21 to 24 C.

Passage of Infectious Material

Testicular emulsions (5 to 10%) in 0.2 M phosphate-buffered saline (PBS), pH 7.2, were centrifuged at 3000*g* for 5 minutes of 4 C. The supernatant fluid was removed, frozen, thawed eight times, and filtered through a 1.2-μm Millipore filter to remove large particulate matter, followed by successive filtrations through filters to decreasing porosity. Before final filtration (0.1-μm pore size), coliform bacteria were added and the filtrates were cultured to determine the efficacy of filtration. Infectious serums were centrifuged at 3000*g* for 5 minutes at 4 C and filtered through 0.45-μm filters. In some cases, serums were filtered through filters of a smaller pore size (0.22 to 0.1 μm). Bacteria were added before final filtration as described above. Rabbits were inoculated with either 0.5 to 2.0 ml infectious serums intravenously (IV), 0.3 to 0.5 ml filtrates of testicular emulsions intratesticularly (IT), or 0.5 to 2.0 ml testicular emulsions (IV).

Temperature Measurements

Rectal temperatures were recorded every 24 hours using a glass mercury thermometer inserted 30 mm. Prior to inoculation the temperatures were lower than 39.5 C.

Pathology

Complete necropsies were done on all controls and on 31 inoculated animals that died or that were euthanatized with CO_2, IV sodium pentobarbital, or IV ketamine HCl and exsanguinated 48 hours to 10 months after infection. Tissues routinely taken included brain, cervical spinal cord, eye, trachea, lung, tongue, stomach, duodenum, jejunum, ileum, pancreas, cecum, colon, liver, gallbladder, kidney, urinary bladder, heart, diaphragm, gluteal muscle, adrenal gland, gonad, salivary glands, cervical, axillary, popliteal and mesenteric lymph nodes, spleen, and thymus. Spinal cords and ganglia were removed intact from three animals showing paralysis, and several sections were taken at the cervical, thoracic, and lumbar levels. Tissues, except eyes, were fixed in 10% formalin

buffered with 2% sodium acetate or Bouin's solution. Selected tissues were fixed with formol–Zenker's. Eyes were fixed in Bouin's solution. Lungs were distended with fixative. Paraffin-imbedded sections were cut at 6 μm and stained with hematoxylin-eosin. Selected tissues were stained with periodic acid–Schiff stain (PAS), Lendrum's inclusion stain, Taylor's gram stain, Gomori's methenamine silver stain, Masson's trichrome stain, azure–eosin, alizarin red, phosphotungstic acid–hematoxylin (PTAH), and cresyl echt violet–luxol blue.[5] Selected kidney sections were cut at 3 μm and stained with PAS–methenamine silver.

Microbiology

Blood, pleural exudate, lung, spleen, liver, and testicular emulsions were cultured for bacteria using fluid thioglycolate without indicator and brain–heart infusion broth with 0.1% agar. Cultures were incubated at 37 C for at least 14 days before being discarded as negative. Beef heart infusion agar supplemented with 1% peptone, 0.5% NaCl, 215% baker's yeast, and 20% horse serum was used for the isolation of mycoplasma.[6] Yolk sac inoculation of 6-day-old chick embryos was used for growth of rickettsiae.[7]

Antiserums

Hyperimmune serums were prepared in rabbits surviving inoculation with infectious material by giving four or more IV challenges with 0.5 to 1.0 ml of infectious serums (stocks 73-015, 73-022, and 34) at biweekly intervals. Animals were bled 10 to 14 days following the last injection. Pooled guinea pig antiserum to the 229E strain of human coronavirus and mouse ascitic fluid containing antibody to the OC43 strain of human coronavirus were supplied through the courtesy of Dr. A. Kapikian, NIAID, NIH. They titered ≥1:64 in the microquantitative complement fixation (CF) assay [8] using homologous antigens.

Neutralization

Equal volumes of hyperimmune serums and infectious rabbit serums were incubated at 37 C for 2 hours and inoculated IV (2 ml) into each of 3 normal rabbits. Control rabbits received 2 ml of similarly treated mixtures of virus and PBS, virus and preimmune rabbit serum, of hyperimmune rabbit serum and PBS. Morbidity and/or mortality were monitored.

Immunofluorescence

Frozen sections of heart tissue obtained from moribund or normal animals were stained by indirect immunofluorescence [9] with fluorescein-conjugated goat antirabbit (Cappel Laboratories) or fluorescein-conjugated rabbit antiguinea pig (Miles Laboratories) γ-globulin (γ-chain–specific). They were read blindly by two investigators on a Zeiss UV photomicroscope.

Complement Fixation

The microquantitative assay of Wasserman and Levine [8] was used. Its sensitivity and specificity are well established.[10,11] Infectious serums served as antigens. Dr. A. Kapikian supplied 229E virus grown in WI-38 cells and OC43 virus grown in suckling mouse brain. The reaction was considered positive if more than 20% of the complement was consumed.

Tissue Culture

The following cell lines were used: HEp-2, Vero, BSC-1, L, BHK, WI-38, and J-111. Primary rabbit kidney and MA-111 derived from testicular tissues of newborn rabbits were obtained from Microbiological Associates (Bethesda, MD) and MA-177 cells derived from

newborn human intestine were obtained through the courtesy of Dr. A. Kapikian. All were maintained in minimum essential medium with 10% fetal calf serum.

Electron Microscopy.

Virus was concentrated 60-fold from 3 ml of infectious serums by centrifugation at 100,000*g* for 2 hours. The pellets were resuspended in 50 μl of distilled water, mixed with equal volumes of 1.2% aqueous solution of sodium phosphotungstate (pH 5.8), and transferred to a lightly carbonized Formvar coated grid. Controls consisted of pellets obtained in identical fashion from normal rabbit serums. Air-dried grids were examined in an AEl-801 electron microscope at instrument magnifications of × 10–100,000. In addition, 1-mm cubes of cardiac muscle from infected rabbits were fixed in 3% glutaraldehyde in phosphate buffer (pH 7.2) and subsequently treated with 1% osmium tetroxide for 1 hour. Following dehydration the blocks were embedded in Spurr low-viscosity embedding medium. One micrometer sections were cut with glass knives and stained with toluidine blue. Selected blocks were sectioned at 60 to 90 nm thickness, stained with lead citrate and uranyl acetate, and examined at instrument magnification of 4000 to 25,000 ×.

Results

Clinical Course

Rabbits were inoculated with infectious material as summarized in Table 1. The clinical course was characterized by a fever spike of 40 to 41.5 C at 24 to 48 hours after inoculation. Animals receiving filtrates of testicular emulsions IT had a single initial fever spike followed by a gradual return to normal with recovery or, alternatively, the rectal temperatures became subnormal (<34 C) and death ensued. In survivors, body temperature returned to below 40 C at about 72 hours after inoculation, and no sequellae were observed.

Rabbits receiving infectious serum IV had an initial spike of fever (24 hours after infection) and tended to maintain elevated (40+) temperatures until 24 hours prior to recovery or death. Those receiving filtrates of testicular emulsions IV usually remained afebrile until 48 hours. In both groups, death occurred between the 2nd and 11th postinoculation day.

Rabbits that were not inoculated or that received normal serums or filtrates of normal testicular emulsions had temperatures of 38.2 to 39.5 C as previously described.[1]

Coincident with the elevation in temperature were dullness of the scleras, severe congestion of the conjunctivae, irides, and, in some cases, severe chemosis and hyphema. Hyphema occurred unilaterally and bilaterally and was not seen until the sixth day. In those rabbits with hyphema that survived, the eyes cleared by the 12th day. Indirect ophthalmoscopy did not reveal retinal lesions. Varying degrees of hindquarter muscular weakness were noted in several rabbits given testicular emulsions but not in those receiving infectious serums. Animals that recovered regained complete muscular control.

Table 1—Clinical Course Following Inoculation of Rabbits With Infectious Material

Source of infectious material	Route	Day of death										Survive	Dead/total
		2	3	4	5	6	7	8	9	10	11		
Serum	IV	3*		4			2	3	2		1	1	15/16
Testis	IV			3					1			3	4/7
Testis	IT		1	2	4	2			1		1	12	12/14
Total												16	31/47

IV = intravenous; IT = intratesticular.
* Number of rabbits

Pathologic Findings

Rabbits were divided into three groups that correlated to some degree with grossly visible lesions. Those receiving testicular emulsions IT and dying in less than 96 hours were considered acute deaths. Those receiving the same inoculums and dying after 96 hours were considered the chronic group. The third group consisted of rabbits receiving testicular filtrates IV or infectious serums IV.

In the acute group, the eyes were extremely congested, and many animals had clear to serosanguinous fluid issuing from the nostrils. Frequently, the heart had red streaks on the epicardial and endocardial surfaces. Right ventricular dilatation was common. Pulmonary edema and reddening of the tracheal mucosa were severe. Usually at least 10 ml, and in 1 case 50 ml, of clear straw-colored fluid was present in the thorax. The fluid clotted on standing. Fibrin tags were present in the fluid but pleural adhesions were not seen. The submandibular, cervical, axillary, inguinal, and popliteal lymph nodes were dark red. The bladder was frequently distended with deep amber to brown urine. Other organs were considered to be within normal limits.

Rabbits in the chronic group showed greater variation in lesions. The pleural cavity contained less than 5 ml of serosanguinous fluid. Areas of the lungs were firm and wet. The heart, in most cases, had extensive red streaks on both epicardial and endocardial surfaces, and right ventricular dilatation was present. The peripheral lymph nodes were either slightly reddened and enlarged or unremarkable. Rabbits in the third group differed from the previous groups in that, regardless of when they died, pleural fluid was present but in lesser amounts. Right ventricular dilatation was common, although red streaks on the epicardial and endocardial surfaces were less frequent and of lesser degree than in the other groups. In contrast to the previous groups, several animals receiving infectious serums IV also had 10 to 20 ml of clear or serosanguinous fluid in the abdomen.

Histologic lesions specifically associated with experimental exposure to the agent were confined to the heart, diaphragm, lungs, thymus, lymph nodes, and spleen. Minimal alterations in other tissues were noted in both control and treated animals to the degree usually seen in adult rabbits. No specific intercurrent infectious diseases were observed.

The most striking and consistent finding was multifocal to diffuse myocardial degeneration and necrosis (Figures 1 to 5). The changes ranged from increased eosinophilia of scattered fibers to marked hyaline necrosis, with fiber shrinkage and fragmentation in large areas of ventricular muscle. Scattered foci of necrosis were present in which the sarcoplasm

had undergone a deeply eosinophilic, granular to floccular change, and fragmented masses were surrounded by large, basophilic macrophages. Occasionally, large and multinucleate macrophages contained fragments of necrotic sarcoplasm.

Degenerated fibers were often separated by accumulations of pink, proteinaceous material. Histiocytes and Anitchkow cells were present and often numerous in interstitial spaces, but lymphocytes and plasma cells were absent and only a few heterophils were seen.

There appeared to be no predilection for any part of the myocardium, and atrial and ventricular musculature was variably affected in most animals. No valvular or blood vessel lesions were noted.

Similar changes were observed in the diaphragmatic muscle in approximately one third of inoculated animals, but other skeletal muscles were unaffected at the light microscopic level.

The affected fibers were less basophilic or deeply eosinophilic with azure–eosin stains and were positive with periodic acid–Schiff stains. The PAS was not diastase-digestible. Necrotic fibers stained deep red with Masson's trichrome, and some were slightly positive with alizarin red. Phosphotungstic acid–hematoxylin demonstrated changes which were not easily detected with hematoxylin and eosin. There was increased prominence, through swelling and/or coalescence, of myofibrils, which stained deep purple.

The lungs consistently had intra-alveolar accumulations of pink, proteinaceous fluid. Alveolar epithelial cells were swollen, and alveolar macrophages were numerous.

Depletion of lymphocytes was observed in most thymuses. This was most striking in the periphery of the cortices. Some of the glands contained scattered heterophils, plasma cells, and large histiocytes, particularly in the depleted areas.

Lymph node changes ranged from depletion with dilated sinuses containing proteinaceous fluid and erythrocytes to diffuse lymphoid hyperplasia. The latter was characterized by an increase in medium and large basophilic lymphocytes throughout the cortical pulp. A few nodes had small follicles, but secondary follicles (germinal centers) were not generally present. Several lymph nodes had prominent swelling of venous endothelial cells.

Most of the spleens contained moderate extramedullary hematopoiesis. The most characteristic change was an increased size and cellularity of the marginal zones which surround the lymphoid sheets. The zones were characteristically sheets of large basophilic mononuclear cells extending well into the surrounding pulp. Some marginal zones showed striking

congestion and edema. Lymphoid follicles were not generally present, and the periarterial lymphoid sheets were relatively small and did not differ from those in the control animals.

Special stains did not reveal inclusion bodies, bacteria, or fungi. No abnormalities were demonstrated in the central or peripheral nervous system. Hematoxylin and eosin stains suggested basement membrane thickening in several kidneys, but this was not confirmed in 3-μm sections stained with PAS–methenamine silver.

Microbiology

Attempts to isolate bacteria, mycoplasma, or rickettsiae were unsuccessful. Three serum stocks (73-015, 73-022, and 34) were used as infectious material in all subsequent experiments. They caused characteristic fever and death at dilutions of 10^{-6} in rabbits inoculated IV.

Ether Sensitivity

Equal volumes of infectious serum 73-015 and ethyl ether CP were incubated for 2 hours at 37 C in a water bath in screw-capped tubes. The serum phase was separated, and 1 ml was injected IV into each of 2 rabbits. Infectious serum 73-015 incubated as above but not exposed to ether, normal rabbit serum similarly treated with ether, and untreated normal rabbit serum incubated as above served as controls. Fever or other clinical signs were not seen in the 2 rabbits inoculated with ether-treated infectious serum. Rabbits receiving either ether-treated or untreated normal serum (1 ether treated, 1 untreated) did not develop fever or clinical signs. The 2 rabbits inoculated with infectious serum not treated with ether developed characteristic fever and died. Subsequent IV challenge with infectious serum 73-015 of those rabbits surviving inoculation with ether-treated serum caused typical disease. The data indicate that infectivity and antigenicity are ether-sensitive.

Immune Responsiveness

Presence of specific antibody in hyperimmune serums was demonstrated by complement fixation, neutralization, and immunofluorescence. Hyperimmune but not preimmune rabbit serums fixed complement with infectious serums 73-022 and 34 used as the sources of antigen. Hyperimmune serums did not react with normal rabbit serums (Table 2). Hyperimmune serums provided a measure of protection in that 3 rabbits inoculated with mixtures of infectious serum 34 and hyperimmune antiserums survived the infection after developing characteristic disease and became resistant to IV challenge with 1 ml of infectious serum 73-022.

Table 2—Complement Fixation by Hyperimmune Rabbit Serum and Human Coronaviruses (Indicated in Percentage of Complement Consumption)

	Antigen					
	Infectious rabbit serums				Normal rabbit serums	
Antibody (rabbit serums)	OC43 (1/4)*	229E (1/4)	73-022 (1/32)	34 (1/32)	16 (1/32)	A-1 (1/32)
Hyperimmune						
73-022 (1/32)†	10‡	30	58	50	0	0
73-015 (1/16)	15	25	51	49.5	0	0
Preimmune						
73-022 (1/32)	15	0	0	2.2	0	0
73-015 (1/16)	18	0	0	0	1.1	0

* Optimal antigen dilution
† Optimal dilution of antiserums
‡ Reaction considered positive if at least 20% of the complement is consumed

Three rabbits receiving mixtures of infectious serum 34 and preimmune rabbit serum or mixtures of infectious serum 34 and PBS developed the disease and died. Animals inoculated with mixtures of hyperimmune antiserums instead of infectious serum did not show signs of infection. Finally, frozen sections of heart tissue from moribund animals inoculated with infectious serum 73-022 were stained with hyperimmune serum by indirect immunofluorescence. Staining localized in the interstitial tissue of the myocardium (Figure 6A). It was not observed in similarly prepared and stained sections of normal rabbit heart (Figure 6B). Preimmune rabbit serums were nonreactive.

Filterability and Heat Resistance

Infectious serum 73-022, routinely filtered through a 0.45 μm filter was further filtered through filters of 0.22 μm and 0.1 μm porosity. All rabbits developed characteristic clinical signs and died, suggesting that the infectious agent is smaller than 100 nm. Samples of infectious serum stock 73-022 exposed to 56 C for 2 hours caused characteristic fever and death in 2 rabbits on the ninth day postinoculation, whereas one rabbit infected with the same serum exposed to 80 C for 1 hour did not develop clinical signs. Subsequent challenge of this latter rabbit with untreated serum caused death. The data agree with the results of Gudjónsson et al.[13]

Electron Microscopy

Pleomorphic virus particles, round or elliptic, 75 to 100 nm in diameter, bearing club-shaped 15-to-20-nm–long projections characteristic of coronaviruses were observed in infectious serum 73-022 (Figure 7). Similar or

different viral particles were not observed in normal rabbit serums. Significantly, serums collected at the peak of fever (2 days) and at the time of death (4 days) from 2 rabbits inoculated with infectious serum 73-022 were themselves infectious and contained morphologically identical viral particles. Serums similarly collected from 2 animals inoculated with normal rabbit serum were not infectious, as evidenced by lack of fever and clinical signs, and did not contain viral particles. The data correlate serum infectivity with the presence of viral particles having the morphologic features of a coronavirus.

Electron microscopic examination of heart tissue stained by indirect immunofluorescence did not reveal viral particles in myocardial cells, vascular cells, or other interstitial cellular elements. The myocardial changes were nonspecific and consisted principally of mitochondrial swelling with disorganization of christae. There were occasional cytoplasmic lipid droplets and mitochondrial dense granules as well. Occasionally, cytoplasmic annulate lamellae were found in capillary endothelium. The subunits composing these arrays were round or cylindric and measured 250 nm in diameter, with a center-to-center spacing of 375 nm (Figure 8).

Antigenic Relationships

Hyperimmune serums were assayed by Microbiological Associates (Bethesda, MD) for the presence of antibody to mouse hepatitis (polyvalent) and rat coronavirus using the microtiter CF assay [12] with negative results. On the other hand, in the microquantitative CF assay, guinea pig antiserum to 229E virus or mouse ascitic fluid containing antibody to the OC43 virus reproducibly fixed complement with infectious serum stocks 34, 73-022, and 73-015. Preimmmune guinea pig serums used as antibody were nonreactive, and normal rabbit serums used as antigen did not fix complement (Table 3, Text-figure 1B). When assayed against the same concentration of infectious serums (73-022 and 73-015 at 1/8) the respective titers of the anti-229E and OC43 antiserums were 32 and 16. These data indicate that infectious serums contain an antigen(s) that cross-reacts with two established coronaviruses, both of human origin.

Seroconversion to Coronavirus Specificity

To determine whether rabbits surviving infection with serum stocks 73-022 and 73-015 seroconvert to coronavirus specificity, complement fixation assays were performed on paired rabbit serums using the 229E virus. Seroconversion was demonstrated by the observation (Table 2, Text-figure 1A) that hyperimmune but not preimmune rabbit serums fix complement with the 229E virus.

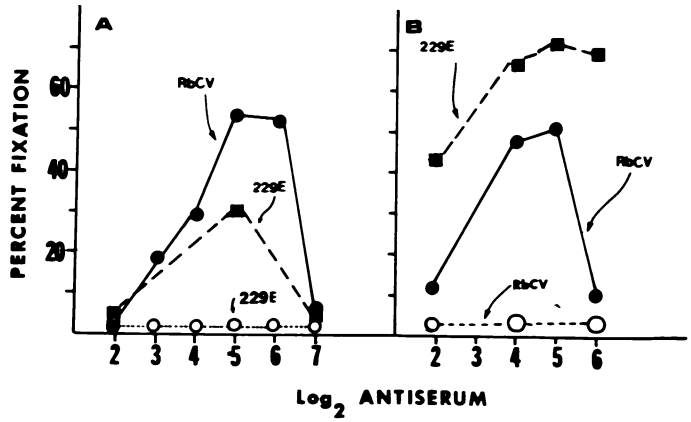
Table 3—Complement Fixation by Rabbit Serums Used as Antigen and Antibody to Human Coronaviruses (Indicated in Percentage of Complement Consumption)

Antigen (rabbit serums) (1/8)*	Antibody		
	Anti-229E (1/16)†	Anti-OC43 (1/16)	Preimmune (1/16)
Infectious serums			
73-022	48‡	41.3	6
73-015	53	46.7	0
34	40	37	4.3
Normal serums			
16	0	7	0
17	0	8.3	0
A-1	0	2.0	0

* Optimal antigen dilution
† Optimal antibody dilution
‡ Reaction considered positive if at least 20% of the complement is consumed

Localization of the Cross-Reactive Antigen(s)

Frozen sections of heart tissue from moribund animals inoculated with infectious serums 73-022, 73-105, and 34 were stained by indirect immunofluorescence with anti-229E or preimmune guinea pig serums. Fluores-



TEXT-FIGURE 1—Microquantitative CF. Percent fixation is plotted as a function of antibody concentration expressed as the $\log_2$ reciprocal antiserum dilution. Antigen (designated by the *arrow* on top of each curve) is infectious serum 73-015 (rabbit coronavirus, RbCV) or 229E virus used at optimal concentrations. Hyperimmune serum was prepared with infectious serum 73-015. A—Hyperimmune serum vs RbCV (*solid circles*); preimmune rabbit serum vs 229E (*open circles*); hyperimmune serum vs 229E (*squares*). Concentration of 73-015 and 229E is 1/32 and 1/4, respectively. B—Anti-229E vs 229E (*squares*); anti-229E vs RbCV (*solid circles*); preimmune guinea pig serum vs RbCV (*open circles*). Concentration of 73-015 and 229E is 1/8 and 1/4, respectively. Normal rabbit serums used as antigen are nonreactive. Similar results were obtained with infectious serum 73-022 and with hyperimmune serums raised with infectious serum stocks 34 and 73-022. At optimal concentrations, anti-OC43 reacts with OC43 virus, giving rise to curves superimposable on those of the 229E system.

cence localized in the interstitial tissue of the myocardium was observed in sections stained with anti-229E but not in those stained with preimmune guinea pig serum (Figure 6C). Frozen sections from normal rabbit heart did not stain (Figure 6D).

Attempts to Establish *In Vitro* Growth

Infectious serums (73-022 and 73-015) were passed blindly for five consecutive passages on the various cell lines at 34 and 37 C. They were read for CPE at 2, 4, and 8 days postinoculation. CPE was observed only in MA 177 and primary rabbit kidney cells for two passages. CPE was lost thereafter. Although 2.5% of the second-passage MA 177 cells stained with hyperimmune serums in immunofluorescence, neither the cells (1×10^5) nor the supernatants were infectious to rabbits. The same infectious serums were passaged in the allantoic fluid of 12-day-old embryonated eggs. Allantoic fluid collected after the first but not the fourth passage caused characteristic disease in 2 rabbits, one of which died.

Attempts to Adapt the Infection to Other Animal Species

Disease was not caused in 6 newborn mice (N:GP SW) inoculated intracerebrally with filtrates of testicular emulsions or with serum 73-022 (0.02 ml). Illness was also not noted in 4 hamsters given 0.25 ml of infectious serum 73-015 IT or in 4 guinea pigs given 1 ml of infectious serum 73-022 subcutaneously.

Discussion

The studies reported in this communication suggest that the disease of rabbits described in part by Gudjónsson et al,[4,13] Jórgensen,[2] and Fennestadt et al [14] is characterized by myocardial degeneration and necrosis and appears to be caused by a virus antigenically and biologically similar to human coronavirus. The experimental disease was characterized by acute onset of fever, anorexia, weakness, and death or by recovery within 2 weeks. The results merit discussion from the standpoint of the pathologic manifestations and the etiology of the disease.

The most consistent and prominent pathologic change observed was multifocal myocardial degeneration and necrosis with little or no inflammatory response. (The name "rabbit infectious cardiomyopathy" is thus suggested.) The diaphragm was affected in about one third of the animals, although other striated muscles were not observed to be affected. The only other pathologic findings associated with exposure to the agent were hydrothorax, pulmonary edema, and lymphoid changes which varied from depletion to diffuse hyperplasia of basophilic lymphocytes. Deple-

tion in the thymus and the lymph nodes was occasionally accompanied by the presence of a few scattered heterophils and plasma cells. The pulmonary edema and hydrothorax were considered to be the result of acute heart failure. The rapid clotting of the fluid also suggests high protein content and capillary damage, although this is not supported by light microscopy. Differences in the day of death following inoculation appeared to be due to biologic variation rather than variability in the inoculums as determined by their similar antigenicity (Tables 2 and 3) and infectivity titers (10^{-6}).

Bacteria, mycoplasma, and rickettsiae were excluded from the etiology of this disease, as they could not be cultured from infectious materials. They were not seen in electron micrographs of infectious serums even after 60-fold concentration, and infectivity was associated with agent(s) 100 nm in diameter or smaller, as evidenced by the filtration data. Consistent [15] with this size estimate, infectivity was correlated to the presence of virus particles with the morphologic features of a coronavirus (Figure 7). Infectivity was shown to be ether-sensitive. The relatively higher heat resistance (2 hours at 56 C) than reported for coronaviruses grown in tissue culture [15] may be due to protection by serum proteins.

The concept that a coronavirus causes this clinical syndrome is supported by two observations: First, convalescent rabbit serums display seroconversion to coronavirus specificity as demonstrated by their ability to reproducibly fix complement with the 229E strain of human coronavirus (Table 2, Text-figure 1). Preimmune rabbit serums, on the other hand, do not react with the 229E virus. The reaction is specific as evidenced by the observation that rabbit serums (convalescent and preimmune) do not fix complement with extracts of uninfected human cells, including WI-38, in which the 229E virus was grown.[16] The 229E virus is the NIH reference strain, and the reaction occurs over a wide range of serum dilutions, giving rise to classic CF curves that further support the specificity of the reaction.[17] In the context of the seroconversion to coronavirus specificity, it is significant that the cross-reactivity of 229E and the coronavirus in rabbit infectious serums is a two-way reaction. This is demonstrated by the reproducible reactivity of infectious rabbit serums 73-022, 73-015, and 34 with the anti-229E serum. Preimmune guinea pig serum is nonreactive (Table 3).

The second observation that supports a coronavirus etiology is the ability of the anti-229E serum to specifically stain in immunofluorescence, the interstitial tissue of the myocardium from animals infected with serums 73-022, 73-015, and 34. The specificity of this reaction is demonstrated by the absence of fluorescence in similarly prepared sections of

normal rabbit heart stained with anti-229E serum and the lack of reactivity of preimmune guinea pig serums.

Inability to demonstrate viral particles in infected tissues may be due to a concentration effect. If large amounts of virus are being produced by only a small number of cells, it would be relatively easy to miss the cells in the productive stage of the infection. Alternatively, the fluorescence may indicate presence of viral antigens associated with cells which are not yet producing recognizable virions.

We recognize that absolute evidence for a coronavirus etiology must await the isolation of the virus in pure culture and, in the style of classic Koch postulates, its re-isolation from the heart of sick animals. However, the difficulties with the *in vitro* growth and isolation of coronaviruses are well established.[15] The possibility cannot be excluded that the clinical syndrome is caused by an unidentified virus, present in the same serum stocks in which we identify particles with the morphologic features of a coronavirus. However, this is improbable. Not only would such a virus have to resemble the coronaviruses in size and ether sensitivity, but also, to escape detection in 60-fold concentrated serums, it would have to be present in minimal amounts and still cause infection at dilutions of 10^{-6}. To explain the observed seroconversion to coronavirus specificity and the presence of a coronavirus antigen in heart tissue from sick animals, the putative virus would also have to be closely associated with the coronavirus.

The origin of this rabbit coronavirus is unknown. It displays two-way cross-reactivity to one strain of human coronavirus (229E) and one-way cross-reactivity to another strain (OC43) of human coronavirus (Tables 2 and 3). Serologic relationships of this kind between closely related viruses are not uncommon. Measles and canine distemper are two such viruses.[18] Also, in the herpesvirus group, antiserums to herpes B virus will neutralize herpes simplex virus, but the contrary is not true. The explanation of such serologic interaction is not clear, but it has been suggested that it is due to immunodominance.[19] However, besides causing a variety of clinical syndromes, including upper respiratory disease in humans,[15,20] dogs,[21] and fowl [22,23]; hepatitis in mice [24]; gastroenteritis in cattle [25] and swine [26]; encephalomyelitis in swine [27]; and peritonitis in cats,[28] the coronaviruses are also considered to be species-specific. As such, it is difficult to conclude that the rabbit coronavirus is of different species origin. Nevertheless, it still remains to be determined whether this is a natural pathogen of rabbits or whether it is a human virus adapted to rabbits by inadvertent contamination of the *T pallidum* stocks.

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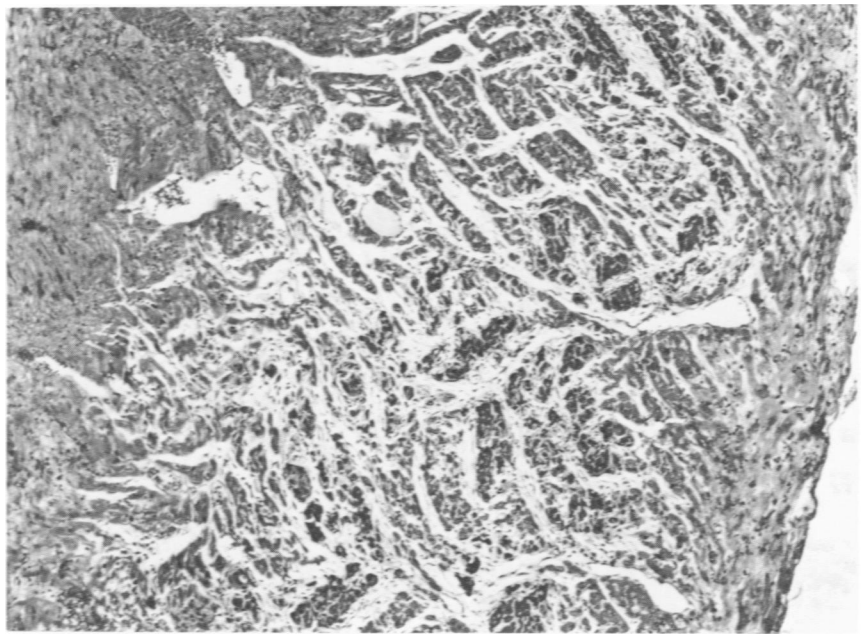

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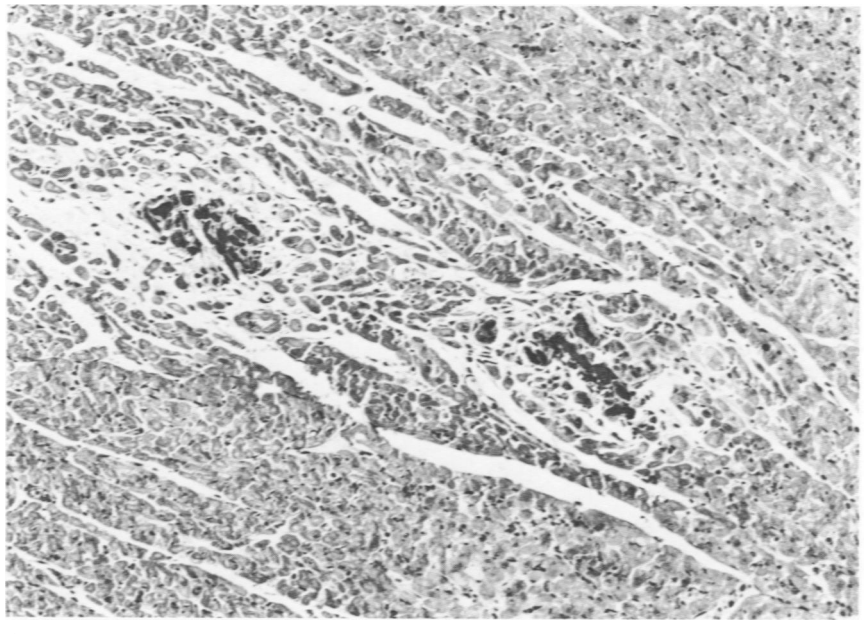

2

Figure 1—Myocardium showing widespread degeneration and necrosis. Fibers are shrunken, fragmented and deeply eosinophilic. (H&E, ×80) **Figure 2**—Myocardium with two foci of necrosis. (PAS, ×140)

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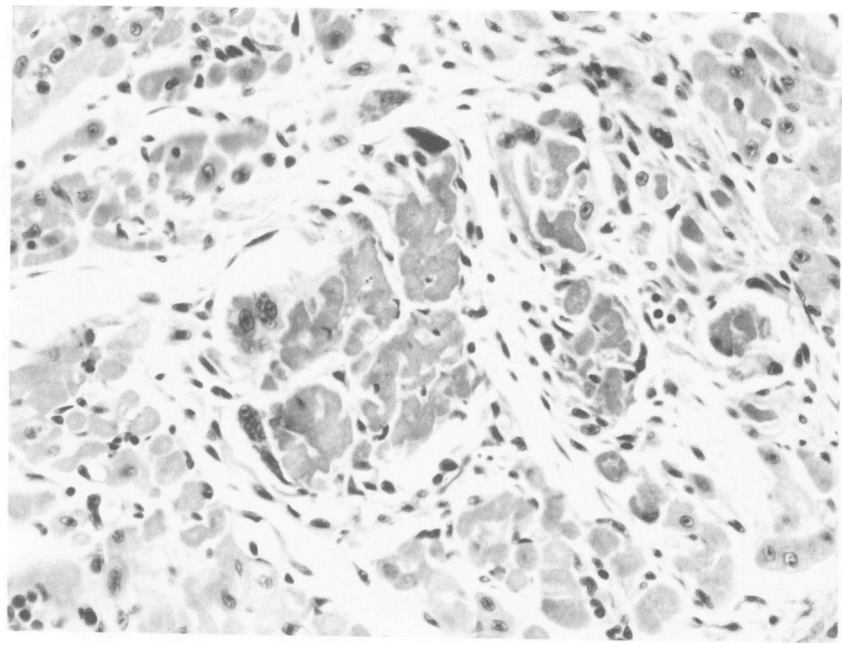

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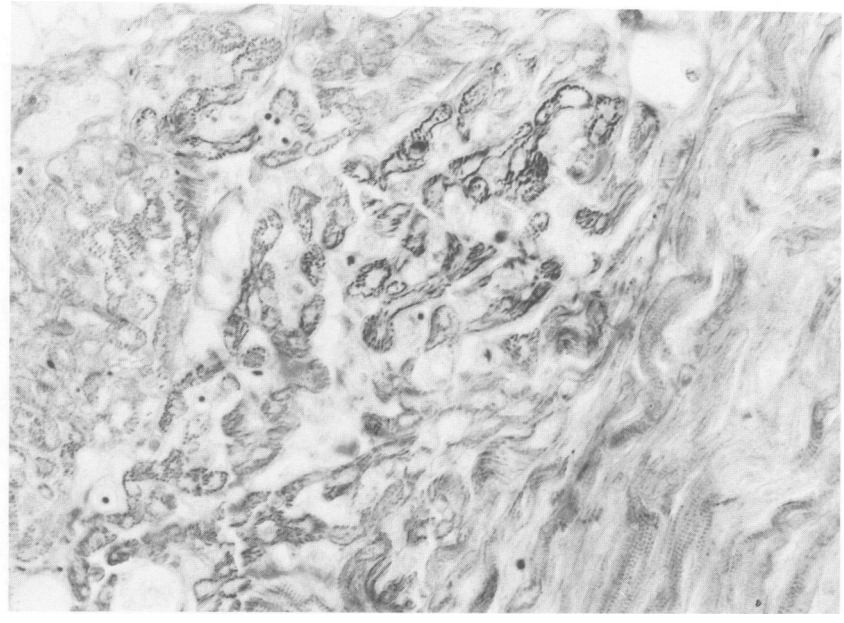
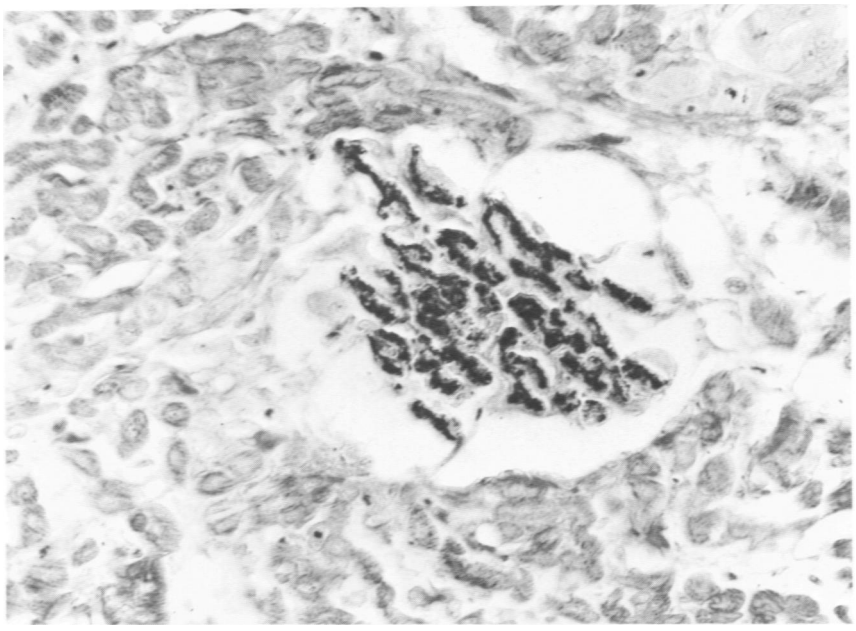


Figure 3—Focus of necrosis in myocardium. Note giant cells around necrotic fibers. (H&E, ×350) **Figure 4**—Focus of early myocardial degeneration. Note prominent swollen myofibrils. (PTAH, ×350)



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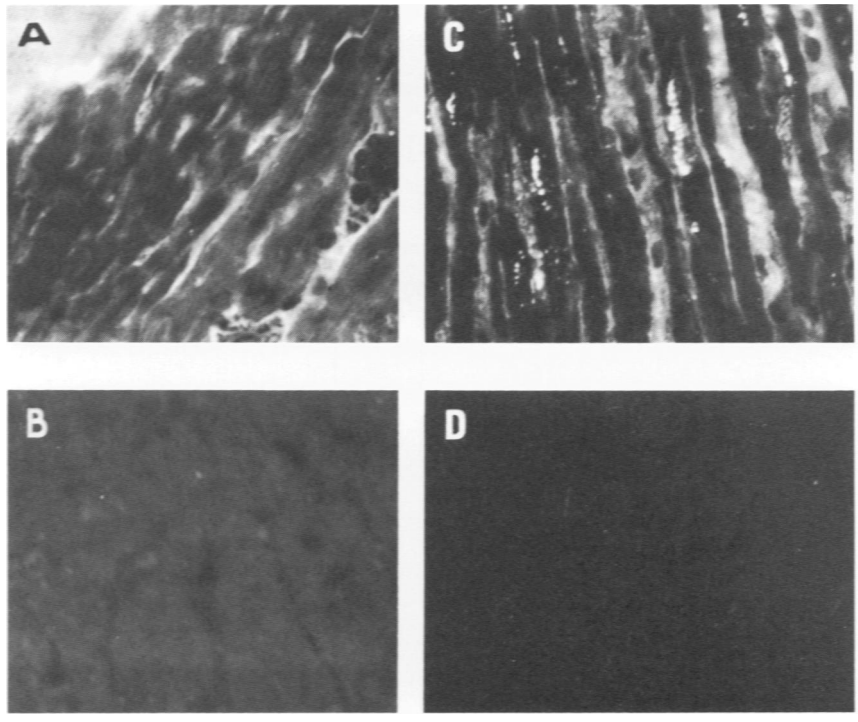

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Figure 5—Necrotic focus in myocardium, with clumping of myofibrils. (PTAH, ×350) **Figure 6A** and **B**—Indirect immunofluorescent staining with hyperimmune serum, induced with infectious serum stock 34, and fluorescein-conjugated goat antirabbit IgG. **A**—Frozen section of heart muscle from a sick rabbit (infected with serum from 73-022). **B**—Frozen section of heart muscle from a normal rabbit. **C** and **D**—Indirect immunofluorescence with guinea pig antiserum to 229E virus and fluorescein-conjugated rabbit antiguinea pig IgG. **C**—Frozen section of heart muscle from a sick rabbit (infected with serum from 73-022). **D**—Frozen section of heart muscle from a normal rabbit. (×160)

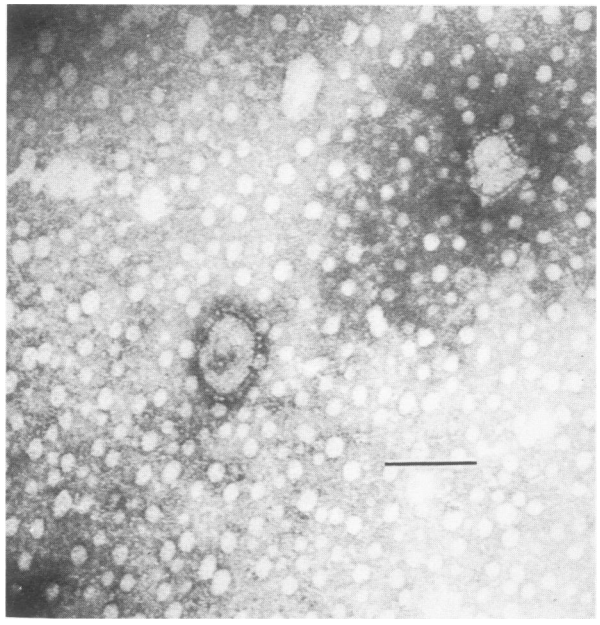

Figure 7—Electron micrograph of infectious serum stock 73-022 showing virus particles with morphologic features of coronavirus. Bar = 100 nm. (×120,000)

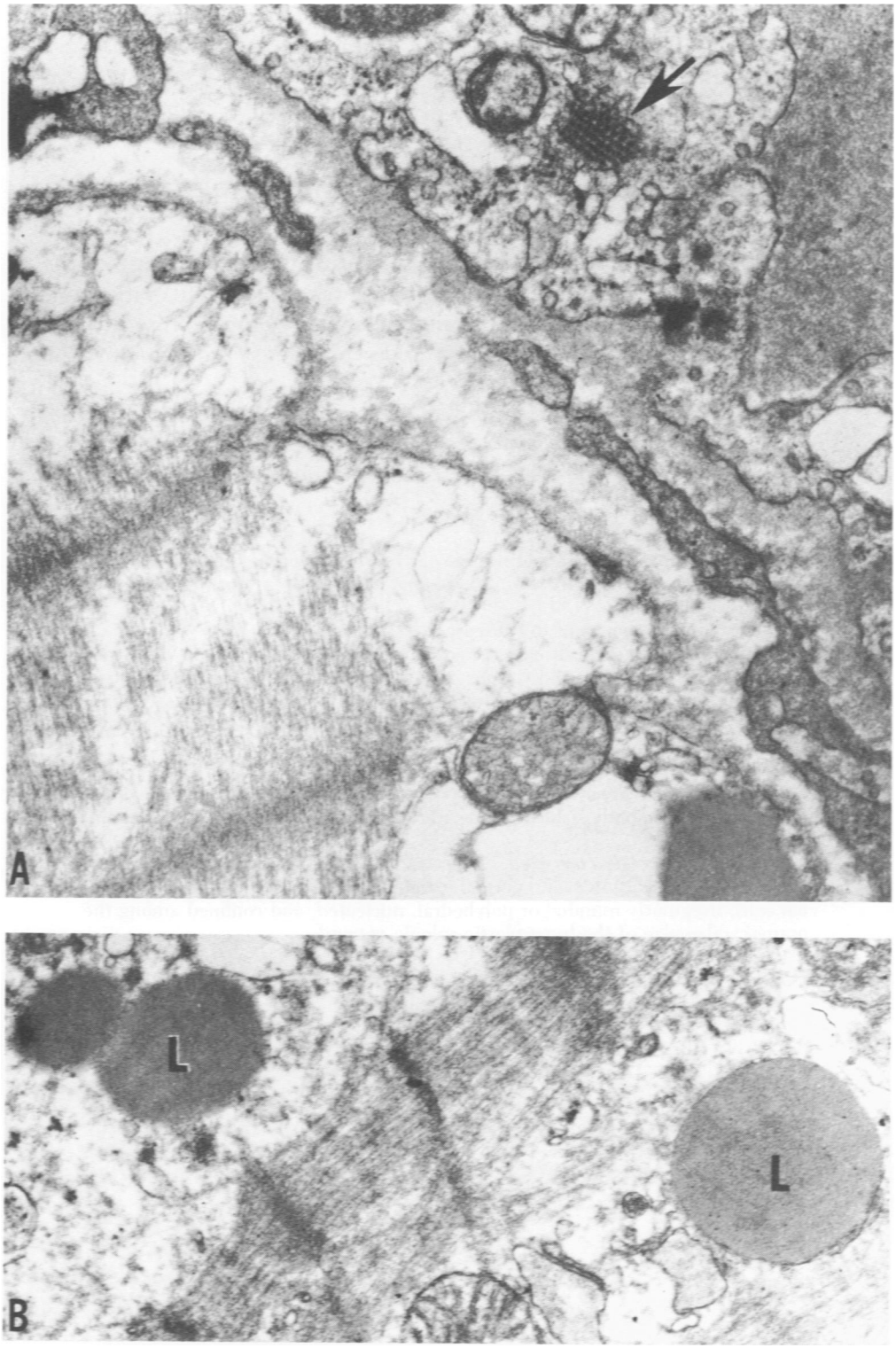

Figure 8—Sections through the myocardium of an infected rabbit showing homogeneous lipid droplets (*L*) and nonspecific myocardial cell swelling. An endothelial cell contains an annulate lamella (*arrow*). (**A**, ×40,000; **B**, ×32,000)