Naturally Occurring Canine Herpesvirus Infection in Japan

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Abstract. Clinical, pathological and virological examinations were carried out on naturally occurring cases of canine herpesvirus (CHV) infection. The first clinical sign observed was diarrhea, followed by anorexia, vomiting, serous nasal discharge and dyspnea. Some puppies had continual crying, abdominal pain and incoordination. The characteristic lesions of CHV infection consisted of disseminated focal necrosis, hemorrhages and the appearance of intranuclear and cytoplasmic inclusion bodies in many organs and tissues. Furthermore, characteristic herpesvirus particles were detected by electron microscopy in the typical intranuclear and cytoplasmic inclusions found in occasional hepatic cells.

Herpesvirus was isolated from the various organs of eight affected puppies. The isolate (GCH-1), which was propagated in DK cells, was similar to other herpesviruses in physico-chemical and morphological characteristics, and was immunologically related to the known CHV strains. The GCH-1 strain, following inoculation into susceptible puppies, produced the typical clinical signs and lesions that were found in the natural case. In a serological survey, neutralizing antibodies against the virus were not found in 130 sera obtained from local mongrel dogs in the Aichi and Gifu prefectures.

This paper reports the first naturally-occurring outbreak of CHV infection of neonatal puppies in Japan.

Generalized, hemorrhagic and necrotizing infection of newborn puppies due to canine herpesvirus (CHV) was first described by Carmichael et al. in America in 1965 [7]. Thereafter, the disease was reported in England [10], France [25], West Germany [3], Australia [13] and in South Africa [2]. The mortality in neonatal or infant puppies was virtually 100% in most

outbreaks [2, 7, 14]. However, puppies more than 3 weeks of age had only mild respiratory disease, or inapparent infections [1, 5].

In Japan, Motohashi and Tajima [19] isolated CHV from the lungs of a diseased adult dog, but, as yet, no reports on CHV infection in neonatal puppies have been published in Japan. Throughout the au-

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tumn of 1975, the authors encountered an outbreak of fatal infections of Afghan hound puppies and performed clinical, pathological and virological examinations. This paper deals with the first description of naturally occurring cases of CHV in neonatal puppies in Japan.

Materials and Methods

Pups: Eight of 15 affected puppies from two litters were examined (Table 1). There were two affected pups out of four in one litter and in a second litter, six out of 11 puppies became ill or died.

Pathology: After necropsy and macroscopic observations had been made, tissue specimens were collected from the puppies. The materials fixed in 10% phosphate-buffered formalin were embedded in paraffin. After sectioning specimens were stained with hematoxylin and eosin (H-E). Some pieces of the liver, lungs, kidneys and spleen were fixed with Carnoy's solution and stained with methylgreen-pyronine instead. These sections were also stained with acridine orange and examined by fluorescent microscopy according to the method of Pollard and Starr [23].

Cell cultures: Primary dog kidney (DK) cell monolayers were prepared from the kidneys of 1- to 3-month-old dogs. DK cells were grown in Hanks' balanced salt solution containing 0.5% lactalbumin hydrolysate and 10% calf serum, and were maintained in Eagle's minimum essential medium (MEM).

Viruses: The isolate, designated GCH-1, was obtained from the kidney of a puppy of natural cases. Before the examination 20 serial passages were performed in DK cell cultures. The F-205V strain and the Glasgow CHV-2 strain of CHV were kidney supplied by Dr. Carmichael of Cornell University and Dr. Thompson of the University of Glasgow, respectively. These strains were propagated in primary DK cell cultures.

Cytopathology in DK cells: Normal and infected DK cell cultures were grown on coverslips in flat-bottom tubes and fixed in 10% alcoholic formalin when cytopathic effect (CPE) was evident. The coverslips then were rinsed in distilled water, stained with H-E, and examined with a light microscope.

Virus titrations: Virus titrations were performed by the inoculation of 0.2 ml amounts of each serial 10-fold dilutions of the virus into 4 DK cell culture

tubes. End points were determined by the appearance of the typical CPE 7 days after inoculation, and 50% tissue culture infective doses (TCID₅₀) were calculated.

Physicochemical characteristics: The physicochemical properties were carried out in the same manner as described in previous reports [11, 12].

Antiserum: Antiserum against the isolate GCH-1 was prepared in young dogs by the subcutaneous injection of 20 ml of clarified (low-speed centrifugation) infected cell culture fluids that had been repeatedly frozen and thawed. Antisera against DFD-6 and F-205V strains for cross-neutralization tests were kindly supplied by Dr. Motohashi of the Nippon Institute for Biological Science, and by Dr. Carmichael of the Cornell University.

Cross-neutralization tests: The sera were diluted in Eagle's MEM in twofold steps. The virus, diluted to contain approximately 100 TCID₅₀/0.2 ml, was mixed with equal amount of each serum dilution and incubated at room temperature for 2 hours. The virus-serum mixtures then were inoculated in 0.4 ml amounts into each of 4 tubes of DK cell cultures. Neutralizing titers of the sera were recorded as the highest serum dilutions which inhibited the CPE.

Electron microscopy: Infected DK cell cultures with advanced CPE were harvested, centrifuged at 1,800 rpm for 10 minutes, washed twice with phosphate-buffered saline (PBS), and pre-fixed with 4% glutaraldehyde in 0.05 M phosphate buffer (PB, pH 7.2). The cells were then fixed with 1% osmium tetroxide in PB, dehydrated in graded ethylalcohol, and embedded in Epon 812. Thin sections were cut with glass knives, and stained with uranyl acetate and lead citrate. Furthermore, some specimens taken from the paraffin blocks were examined to investigate the structure of intranuclear and cytoplasmic inclusions seen in the H-E stained sections, according to the method of Okada and Fujimoto [20].

For negative staining, cultures with advanced CPE were harvested, and ultrasonicated for 30 seconds at maximum frequency. The resulting suspension was clarified by centrifugation at 10,000 rpm for 30 minutes. The supernatant was centrifuged at 30,000 rpm for 2 hours. The pellets obtained were resuspended in PBS. A drop of the partially purified preparation was mounted on a collodion-coated mesh, dried, and negatively stained with 2% phosphotungstic acid at pH 7.2. Samples then were examined in a Hitachi HU-12 electron microscope, operating at 75 KV.

Serological survey: A total of 130 adult mongrel dog sera was collected randomly from Aichi and

Table 1. Macroscopic findings

Case No.	Age (days)	Sex	Termination	Lesions*					
				Liver		Kidney		Lung	
				Petechial hemorrhages	Greyish-white foci	Petechial hemorrhages	Greyish-white foci	Petechial hemorrhages	Greyish-white foci
1	23	Male	Died	+++	+++	+++	++	++	+++
2	24	Female	Killed	+	+	+++	++	+	+++
3	24	Female	Killed	+	++	—	—	++	+
4	25	Female	Killed	+	++	+	+	+	++
5	25	Female	Killed	+	—	+	—	+	++
6	26	Female	Killed	—	+	—	—	—	+
7	27	Male	Killed	+	++	+	—	++	++
8	28	Male	Killed	+	+	—	—	—	+

Remarks.

*: +; Mild, ++; Moderate, +++; Severe.

Gifu prefectures during the whole period of winter in 1975. Fifteen other serum samples were collected from the breeder with the naturally occurring cases.

Experimental infection of infant puppies: Three puppies within a week of birth were inoculated intraperitoneally with 3 ml of a virus suspension that contained 10^4 TCID₅₀/0.2 ml of the isolate GCH-1. When the animals died, they were examined pathologically and virologically.

Results

Clinical findings: In October 1975, the disease occurred in a purebred Afghan hound breeding kennel where 20 adult dogs were kept. One bitch produced four puppies and the other 11. One puppy was born dead, and six were dead consecutively between 1 to 3 weeks after birth. The remaining eight puppies developed clinical illness between 2 and 3 weeks after birth. Two bitches were diagnosed as clinically normal, and were kept in the same kennel.

The first clinical sign in infected pups was a greyish-yellow or green diarrhea that progressed to a watery diarrhea shortly before death. Anorexia was observed at early stage, and all puppies rejected their food at final stage. Nausea, vomiting and salivation also were observed. Respiratory

signs that consisted of serous nasal discharge, rapid breathing, and dyspnea also were noted. The onset of continual crying was accompanied by complete loss of appetite. Some puppies had abdominal pain and incoordination. Elevated body temperatures were not detectable. Most of the puppies died 3 to 7 days after onset of the first signs of disease.

Macroscopic findings: The principal lesions were disseminated petechiae and greyish-white patches of pin-point to milium-size necrosis in many organs throughout the body. The present cases can be divided into severe (Case No. 1), moderate (Case Nos. 2, 4 and 7) or mild forms (Case Nos. 3, 5, 6 and 8) according to the degree of pathologic changes (Table 1).

In the severe case, the liver had a large number of petechiae and focal greyish-white foci, surrounded by hemorrhagic rings on the external and cut surfaces. The hepatic parenchyma was fragile and yellowish in color. The kidneys were characterized by mottling and multiple subcapsular hemorrhages. The sagittally-cut surface revealed wedge or irregular shaped areas of hyperemia and hemorrhages in the

cortex (Fig. 1). The lungs were markedly congested and edematous, with frothy fluids in the bronchioles, and were mottled in appearance. Multiple hemorrhages and greyish-white foci were scattered throughout the lobes. These lesions also were seen in the adrenal glands, spleen, lymphonods, urinary bladder and the subserosa of the intestinal tract.

In the moderate and mild cases, the lesions varied from case to case. Generally, greyish-white foci were prominent in the various organs and tissues. In some of the lungs, a small number of foci with white consolidation, from 3 to 5 mm in diameter, were found with hemorrhages and greyish-white foci.

In all cases, splenomegaly, focal hyperemia and hemorrhages of the turbinate mucosa. Swelling and hyperemia of the tonsils, and hyperemia of the meninges were observed.

Microscopic findings: Disseminated focal necrosis and hemorrhages in the various organs and tissues were the fundamental lesions. In the severe case, hepatic necrosis with multiple hemorrhages was present, chiefly in the areas of portal triads, and, in a random fashion, throughout the lobules (Fig. 2). The renal lesions usually occurred in the cortex, involving both the glomeruli and the tubules. In these lesions, the tubular epithelial cells were completely necrotic, and the glomeruli also had degenerative changes (Fig. 3), while some areas were characterized by marked hemorrhages. In the severe case, there was no inflammatory reaction in the necrotic areas of the liver, but, in the moderate and mild cases, there were cellular reactions around the necrotic areas that consisted of perivascular accumulation of histiocytes and mononuclear cells. Perivascular accumulations of these cells also were seen in the

cortex of the kidneys (Fig. 4). In the severe case, multiple necrotic foci were present in the alveolar walls. Fibrinous exudation into the lumens, with desquamation of septal cells and congestion and hemorrhages into the alveoli, also were prominent lesions (Fig. 5). Generally, there was close association between characteristic fibrino-necrotic lesions and blood vessels or bronchioles. Pulmonary lesions of the moderate and mild cases, however, consisted mainly of interstitial pneumonitis, with or without focal necrosis, and foci where polymorphonuclear cells predominated. The lymphatic organs, had lesions characterized by marked karyorrhexis and depletion of lymphocytes (severe case), with reticular cell hyperplasia (moderate and mild cases). In all cases, degeneration and necrosis of epithelial cells were observed in the tonsils. Necrosis of the epithelium of the tongue (severe case), and necrosis and hemorrhages of the turbinate bones (one severe and some of the moderate cases) (Fig. 6) also were present. In addition, degenerative changes were found in Auerbach's and Meißner's plexus of the intestine, with focal necrosis in the basal layers of the mucosa (Fig. 7).

In the central nervous system, hyperemia, meningeal cell proliferation, and focal microgliosis, with or without degenerative nerve cells, chiefly in perivascular areas, were noted in the severe and moderate cases (Fig. 8).

In the vicinity of the necrotic areas, acidophilic or basophilic intranuclear inclusion bodies were seen in the parenchymal, epithelial and nerve cells of several organs, such as the liver, kidneys, lungs, adrenal glands, small intestine, nasal turbinates and lymphatic organs (one severe and some of moderate cases) and in the liver cells (mild cases). They were homogeneous

Table 2. Titer of virus following chemical and physical treatments

Treatment	Titer of treated virus (TCID ₅₀ /ml)	Titer of untreated virus (TCID ₅₀ /ml)
BUDR (20 µg/ml)	<10 ⁰	10 ^{5.0}
20% ether, 4°C, 18 hours	<10 ⁰	10 ^{4.5}
10% chloroform, 4°C, 15 minutes	<10 ⁰	10 ^{4.5}
pH 3.0, 4°C, 30 minutes	<10 ⁰	10 ^{4.5} *
56°C, 5 minutes	<10 ⁰	10 ^{5.0}
37°C, 24 hours	10 ^{3.3}	10 ^{5.0}
37°C, 48 hours	<10 ⁰	10 ^{5.0}
Filtration (nm)		10 ^{4.5} **
220	10 ^{2.5}	
100	<10 ⁰	

Remarks.

*: At pH 7.2. **: Not filtered.

or sometimes granular. Acidophilic cytoplasmic inclusion bodies with halos also were found in the liver cells of the severe, and some of the moderate cases (Fig. 9). Moreover, numerous virus particles of herpesvirus-type were detected by electron microscopy in the nuclei and cytoplasm of the liver cells within the inclusion bodies (Fig. 10).

Virologic findings: Virus was recovered from the tissues of eight affected puppies. All of the lung and kidney specimens produced typical CPE within 48 hours after inoculation. One isolate, designated GCH-1, was chosen as the prototype strain. Typical CPE consisted of foci of rounded cells at 12 hours after inoculation (Fig. 11). At 24 hours, the cells had detached from the glass, leaving cleared plaques surrounded by degenerating cells. Typical eosinophilic inclusion bodies were found in the nuclei stained with H-E stain.

Physicochemical properties of the virus are shown in Table 2. Nucleic acid type of the virus was examined by adding 5-bromo-2-deoxy-uridine (BUDR) to the DK cell culture medium. Viral replication was inhibited by the incorporation of 20 µg of BUDR for 48 hours. The nucleic acid of the virus then was inferred as deoxyribonu-

cleic acid. The susceptibility of the virus to lipid solvents was tested by adding diethyl ether or chloroform, 10–20% by volume, to an undiluted virus suspension and titrating the virus for infectivity. No viable virus was detectable after exposure to either ether or chloroform. To examine the approximate size of the virus, stock virus was filtered through membrane filters 100 and 200 nm in average pore diameter. Infectivity was determined before and after each filtration. The virus passed readily through the 220 nm filter, but it did not pass through the 100 nm filter. In heat sensitivity tests, the virus was rapidly inactivated at 56°C for 5 minutes. Exposure to 37°C for 24 hours decreased the viral titer about 10^{1.7} TCID₅₀. After exposure for 48 hours, no viable virus was detectable. The virus was completely inactivated at pH 3.0, whereas it was still viable after exposure to pH 7.2 for the same period of time. The DK cell culture fluids were tested for hemagglutination with a variety of erythrocytes at 4°C, room temperature and at 37°C. Hemagglutination was not observed.

Neutralization tests were carried out with an antiserum against the isolate GCH-1, and with antisera prepared against known

Table 3. Neutralization tests in dog kidney cell cultures

Virus	Antiserum		
	Anti-GCH-1	Anti-F-205V	Anti-DFD-6
GCH-1	20*	40	80
F-205V	40	40	160
CHV-2	20	40	80

Remarks.

*: Expressed as the reciprocal of the highest dilution of sera that neutralized 100 TCID₅₀ of each strain.

strains (Table 3). Antigenic differences between the GCH-1 strain and the other strains were not observed, for the GCH-1 virus not neutralized by the F-205V and the DFD-6 antisera. The F-205V and CHV-2 strains were also neutralized by antiserum against the isolate GCH-1. The titers ranged from 1:20 to 1:160.

In thin-section preparations, virus particles were observed in both the nucleus and the cytoplasm. Various forms of naked capsids were found scattered throughout the nucleus (Figs. 12 and 13). They were spherical or hexagonal in shape, and measured about 80 to 90 nm in diameter. Various types of membranous structures characteristic of herpesvirus-infected cells were frequently observed in the cytoplasm (Fig. 14). Capsids and lamellar structures also were found in the membranous structures. The large enveloped particles appeared to bud through the vesicular membrane. The enveloped particles with peripheral spikes were frequently found in the cytoplasmic vacuoles. These particles measured approximately 150 nm in diameter. In negatively stained preparation, particles with the characteristic morphology of herpesviruses were observed (Fig. 15). The capsids were hexagonal in outline and they measured about 110 nm in diameter, indicating that the virus had cubic symmetry.

Serological survey: Neutralizing antibodies against the virus were not found in 130 sera from local mongrel dogs. On the other hand, the parent dogs of the naturally infected puppies were found to possess neutralizing antibodies against the virus. The titers were 1:2 and 1:4, respectively.

Experimental infections of infant puppies: The puppies manifested clinical signs typical of those observed in naturally occurring cases of the disease. All died 7 to 10 days after inoculation. The puppies had typical hemorrhagic and necrotizing lesions similar to those found in the natural cases. Canine herpesvirus was recovered from the various tissues including the tongue of the puppies. Additionally, virus was recovered from the heart blood of one puppy.

Discussion

The evidence obtained from this study indicated that the clinical, macroscopic and microscopic findings are almost identical to those seen in CHV infection as described previously [2, 7, 9, 13, 26]. Also, the isolate GCH-1, capable of growing in DK cells, is similar to other canine herpesvirus isolates in physicochemical, morphological and immunological characteristics. Therefore, the present authors have shown that the disease exists also in Japan as a cause of neonatal pup death.

From the viewpoint of histopathology, it is interesting that characteristic lesions also were recognized in the tongue, turbinate bones and Auerbach's and Meißner's plexus, and that the cytoplasmic inclusion bodies with virus particles were observed in the liver. These findings have not been reported previously. In contrast to the lesions in severe case, those of moderate and mild cases were somewhat variable. Namely, in addition to the fundamental lesions, they had inflammatory reactions

around necrotic foci or in the perivascular areas in various organs and tissues, and interstitial pneumonia was often present. The former have not been observed in most instances of fatal illness of puppies [7, 9], but the latter have been described as a lesions in puppies surviving infection with CHV [21]. Watt et al. [31] reported hemosiderosis as a lesion of this infection in a 3 week-old puppy. Therefore, the lesions of fatal puppies surviving 3 weeks or longer may be modified variously.

Carmichael [6] has mentioned that the body temperature and its regulation is an important factor in the pathogenesis of CHV infection in puppies, however, it is probably too simple to view this mechanism as the only one. Appel et al. [1] have noted that the destructive effect of canine distemper virus on lymphatic tissue also may have a role in the pathogenicity of CHV. In the experimental CHV infection, Percy et al. [22] have suggested that the reticuloendothelial system is the first line of defense. Destructive or hyperplastic changes in the lymphatic organs presented here are also described by Kakuk and Conner [14]. Accordingly, these findings suggest that function of the lymphoreticular system may also have a relation to the pathogenicity of CHV infection.

The route of natural infection of the present outbreak was not established, although other investigators have suggested that infection of puppies may occur in utero via transplacental infection [29] or during passage through an infected vagina at birth [7]. Cornwell and Wright [9] have observed in-contact transmission of infection from inoculated to uninoculated littermates. Furthermore, Poste and King [24] isolated a herpesvirus from the genital tract and suggested sexual transmission. Therefore, the rôle of CHV in canine

genital disease will be an interesting and important problem for research in the future.

The age of an animal at the time of infection with many viruses often determines the outcome of disease [27]. It is well known that fatal infection, under both natural and experimental condition, has only been recorded in puppies less than 3 weeks of age [1, 2, 7, 26]. On the other hand, the experimental exposure of young dogs over 3 weeks of age to CHV has produced disease of variable severity but none of the inoculated dogs died [30, 32]. The present authors observed the death or moribund condition in puppies 23 to 28 days of age, suggesting that fatal infection might indeed occur in puppies more than 3 weeks of age.

The biophysical and biochemical characteristics of the virus presented here were investigated. The virus was shown to be of the DNA type, ether and chloroform sensitive, formed intranuclear inclusions, was labile to heat and low pH, its size ranged from 100 to 220 nm, and it did not hemagglutinate. These characteristics are in general agreement with those of CHV as described by other workers [8, 16, 19, 20].

In the present survey, neutralizing antibodies against CHV were all negative. However, the parent dogs, whose puppies naturally contracted the CHV infection, had positive titers. Since the number of sera studied was limited, the authors might have failed to demonstrate neutralizing antibodies against CHV in field dogs. Failurs to detect antibody may be due to the lack of sensitivity of the SN test system used, for it has been reported [5] that low titers may be found in apparently "negative" sera when complement is added to the neutralization system. Lundgren and Clapper [18] reported that neutralizing anti-

bodies were found in 12.8% of local mongrel dogs and 1.7% of beagle dogs. Although outbreaks of disease have not yet been reported in Japan, inapparent infection of field dogs may be present. Many members of the herpesvirus groups are considered to persist in the host species as inapparent infection [1]. Karpas et al. [15] have established that CHV may cause inapparent and persistent infection of respiratory tract. Therefore, it is interesting important problem to survey the inapparent or persistent infection of CHV in dog population and elucidate whether latent infection in adult dogs correlate with neonatal mortality.

In many breeding kennels there may occur sudden unexpected deaths of neonatal or infant puppies and this is a major problem for dog breeders [4]. Bibrack and Schaudinn [3] have described that CHV infection was demonstrated in 17 of 43 kennels in Western Germany. Love and Huxtable [17] and Bartsch et al. [2] also have reported the natural occurrence of the disease in breeding kennels. Even in the present cases, the infection occurred where 20 purebred adult Afghan hounds were reared. Therefore, it appears that this infection is an especially serious threat in breeding kennels. Recently, large number of dogs are reared as animals for various experimental purpose. Infectious disease, especially fatal viral infections of dogs, are becoming recognized as serious problems to such breeding establishments. It will be the subject for future studies to develop useful polyvalent vaccines or antiserum against the disease, should it prove to be sufficiently important, especially as regards the effects of CHV on breeding programs.

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Explanation of Figures

- Fig. 1. Multiple subcapsular hemorrhages and wedge-shaped areas of hyperemia and hemorrhages are characteristic in the corticomedullary junction of the kidneys. Case No. 2.
- Fig. 2. Multiple focal necrosis and hemorrhages are scattered throughout the liver parenchyma. Case No. 1. Hematoxylin and eosin (H-E) stain, $\times 120$.
- Fig. 3. Severe necrosis in both of the tubular epithelium and the glomerulus are observed in the cortex of the kidney. Dissociation of the cortical parenchyma and hemorrhages are also seen. Case No. 2. H-E stain, $\times 235$.
- Fig. 4. Perivascular mononuclear cell accumulation is seen in the cortex of the kidney. Case No. 8. H-E stain, $\times 235$.
- Fig. 5. Focal fibrino-necrotic pneumonia is characteristic in the lung. The necrosis of the alveolar walls and fibrinous exudation into the alveolar lumens are noted. Swollen macrophages can be seen in the alveolar lumens. Case No. 1. H-E stain, $\times 125$.
- Fig. 6. Focal cellular degeneration consisted of osteoblastic cells, lymphocytic cells and necrosis of the turbinate bone including periosteal tissues are noted. The nasal epithelium (arrow) is intact. Case No. 2. H-E stain, $\times 250$.
- Fig. 7. Degenerative changes of Meißner's plexus are seen in the jejunum. Arrow shows intranuclear inclusion body in the nerve cell. Case No. 1. H-E stain, $\times 490$.
- Fig. 8. Focal microgliosis accompanied with nerve cell degeneration in the mesencephalon is seen. Case No. 1. H-E stain, $\times 235$.
- Fig. 9. Intranuclear (short arrows) and cytoplasmic inclusion bodies (long arrow) with halo are seen in the liver cells. Case No. 2. H-E stain, $\times 770$.
- Fig. 10. Many capsids in the nucleus of the liver cell are shown. Specimens for electron microscopy were taken from a paraffin block. Case No. 2. $\times 55,000$.
- Fig. 11. Cytopathic effects of the isolate GCH-1 in DK monolayer. 12 hours after inoculation. $\times 100$.
- Fig. 12. Various types of capsids are scattered in the nucleus. $\times 33,000$.
- Fig. 13. Clusters of the immature virus particles are observed in the nucleus. $\times 33,000$.
- Fig. 14. The development of membranous structure from the vesicular membrane and enveloped particles in the cytoplasmic vacuoles, which have peripheral spikes, are observed (arrows). Lamellar structures are also seen. $\times 15,400$.
- Fig. 15. Single viral particle of the isolate GCH-1 negatively stained. Capsomeres are evident. $\times 165,000$.