Archiv für die gesamte Virusforschung 43, 34—42 (1973)

Nelson Bay Virus

A Novel Reovirus

By

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With 4 Figures

Received March 27, 1973

Summary

Biological and serological procedures have established that Nelson Bay virus (NB847), which was recovered from the blood of a fruit bat or "flying fox" *(Pteropus poliocephalus)*, is a member of the reovirus group and that it differs from previously described reoviruses.

1. Introduction

A virus (NB847) recovered from the heart blood of a grey-headed flying fox *(Pteropus poliocephalus)* in the Nelson Bay area of New South Wales has been given the provisional name of Nelson Bay Virus (NBV). An earlier communication (4) described the morphology of NBV as being typical of reoviruses but also indicated that the virus has important differences from the accepted members of the reovirus group. NBV causes cell fusion and an unusual nuclear degeneration, while immunofluorescence indicates that viral antigen is distributed through both nucleus and cytoplasm.

Aspects of the isolation and murine pathology of NBV, the formation of pocks in eggs by the virus, and its serological characterization as a reovirus will be described here.

2. Materials and Methods

2.1. Viruses

NBV was used as a 10 per cent suspension of infant mouse brain after 10 i.c. passages in infant mice.

Reovirus type 1 (Lang strain), reovirus type 2 (Jones strain), and reovirus type 3 (Abney strain) were kindly supplied by Dr. Mary McClain, Division of Animal Genetics, Commonwealth Scientific and Industrial Research Organization Ryde, N.S.W., and

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Fahey-Crawley virus by Professor N. F. Stanley, Department of Microbiology, University of Western Australia Medical School, Perth, W.A. Stocks of these viruses were prepared by clarifying suspensions of disrupted infected BHK_{21} cells.

Japanaut virus (MK 6357) was used as a 10 per cent suspension of infant mouse brain after 5 i.c. passages. It is an orbivirus recovered from a pool of New Guinea mosquitoes (Marshall, unpublished).

2.2. Antisera

Immune rabbit serum (IRS) and immune mouse serum (IMS) were prepared from blood taken one week after a course of 4 weekly inoculations of 1:2 dilution of stock NBV, and mouse immune ascitic fluid (IAF) by the method of TIKASINGH *et al.* (14).

Immune goat serum (IGS) against each of the three reoviruses, and polyvalent IGS reactive against all three were provided by Dr. Mary McClain and IRS against the Fahey-Crawley virus by Professor N. F. Stanley.

2.3. Immunofluorescence Reagents

Fluorescein conjugated polyvalent goat anti-reovirus globulin was provided by Dr. Mary McClain. Fluorescein conjugated rabbit anti-mouse and goat anti-rabbit globulins, and rhodamine-conjugated bovine albumin were obtained from Microbiological Associates, Bethesda, Maryland.

Immunofluorescence procedures were as described by GARD and COMPANS (4).

2.4. Embryonated Eggs

Eleven-day-old chicken embryos were inoculated on the chorioallantoic membrane (CAM) and the excised membranes examined for pocks after a further three days incubation at 36° C.

2.5. Cell Cultures

The stable lines of African green monkey (Vero), and Syrian Hamster kidney (BHK_{21}) cells were originally obtained from the American Type Culture Collection, Rockville, Maryland, U.S.A.

Cells were grown in rolling Winchester bottles; Vero cells in medium 199 with 5 per cent calf serum, 0.056 per cent sodium bicarbonate and antibiotics, and BHK_{21} cells in the medium of STOKER and MACPHERSON (12). Stable pig kidney (PS) cells were prepared as in GARD and COMPANS (4).

Cell monolayers for plaque assays were prepared by heavily seeding 60 mm diameter glass Petri dishes (1 or 2×10^6/dish) to produce confluent monolayers in 24 hours, and were overlaid with Tris buffered agar. Cells for immunofluorescence studies were seeded onto 22 mm diameter coverslips in Petri dishes (4).

2.6. Mice

Multicoloured outbred mice from a low pathogen colony derived by caesarian section from Walter & Eliza Hall Institute mice were used in litters of 8 when approximately 24 hours old.

2.7. Physico-Chemical Tests

The sensitivity of NBV to ether, heating at 56° C in 2 M $MgCl_2$, chymotrypsin, and to acid were carried out in accordance with the methods of SUNAGA *et al.* (13), SPENDLOVE and SCHAFFER (8), and BORDEN *et al.* (1), respectively.

3. Results

3.1. Isolation

NBV was recovered from the heart blood of one of 243 *Pteropus poliocephalus* collected at Nelson Bay in 1968. The blood was inoculated intracerebrally into two litters of baby mice and one mouse was dead and another was sluggish 8 days

after inoculation. The virus was slow to adapt and did not affect all inoculated mice until the third infant mouse brain passage, sick mice showing encephalitic signs of circling and spasticity. The average survival time of mice inoculated with third passage brain suspension was 14 days while mice inoculated with twelfth passage material survived an average of 5 days. Attempted re-isolation from 2 aliquots of the flying fox blood was unsuccessful.

3.2. Pathology

Discrete, necrotic nodules, up to 1 mm diameter, were observed in the hearts and interscapular brown fat of all moribund mice, and occasionally similar lesions were observed in the liver. The only other pathological feature was a softening of the brain in all mice, unaccompanied by macroscopic evidence of inflammation. These lesions were only observed in the organs of clinically affected mice.

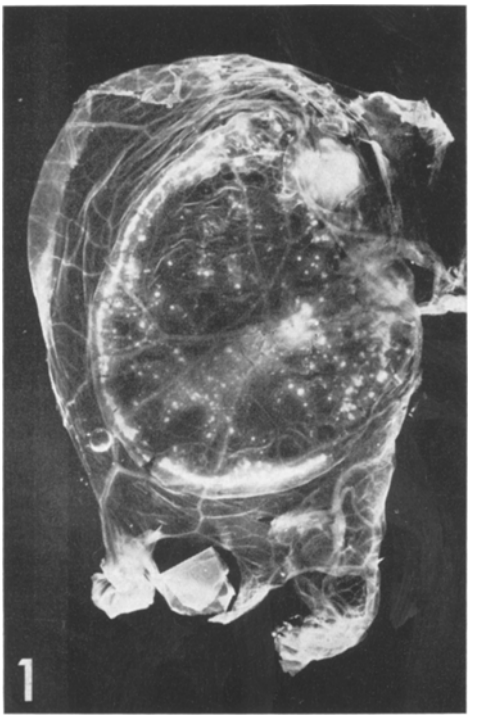

Fig. 1. Pocks on the chorioallantoic membrane of a developing chicken embryo 3 days after inoculation with NBV when 11 days old

NBV produced small, discrete pocks when inoculated onto the CAM of 11 day embryos (Fig. 1). Similar pocks were produced by both third and twentieth infant mouse brain passage material and in each case the chick embryo appeared normal.

3.3. Histopathology

Organs taken from moribund mice after intracerebral inoculation of NBV were examined histologically (Fig. 2).

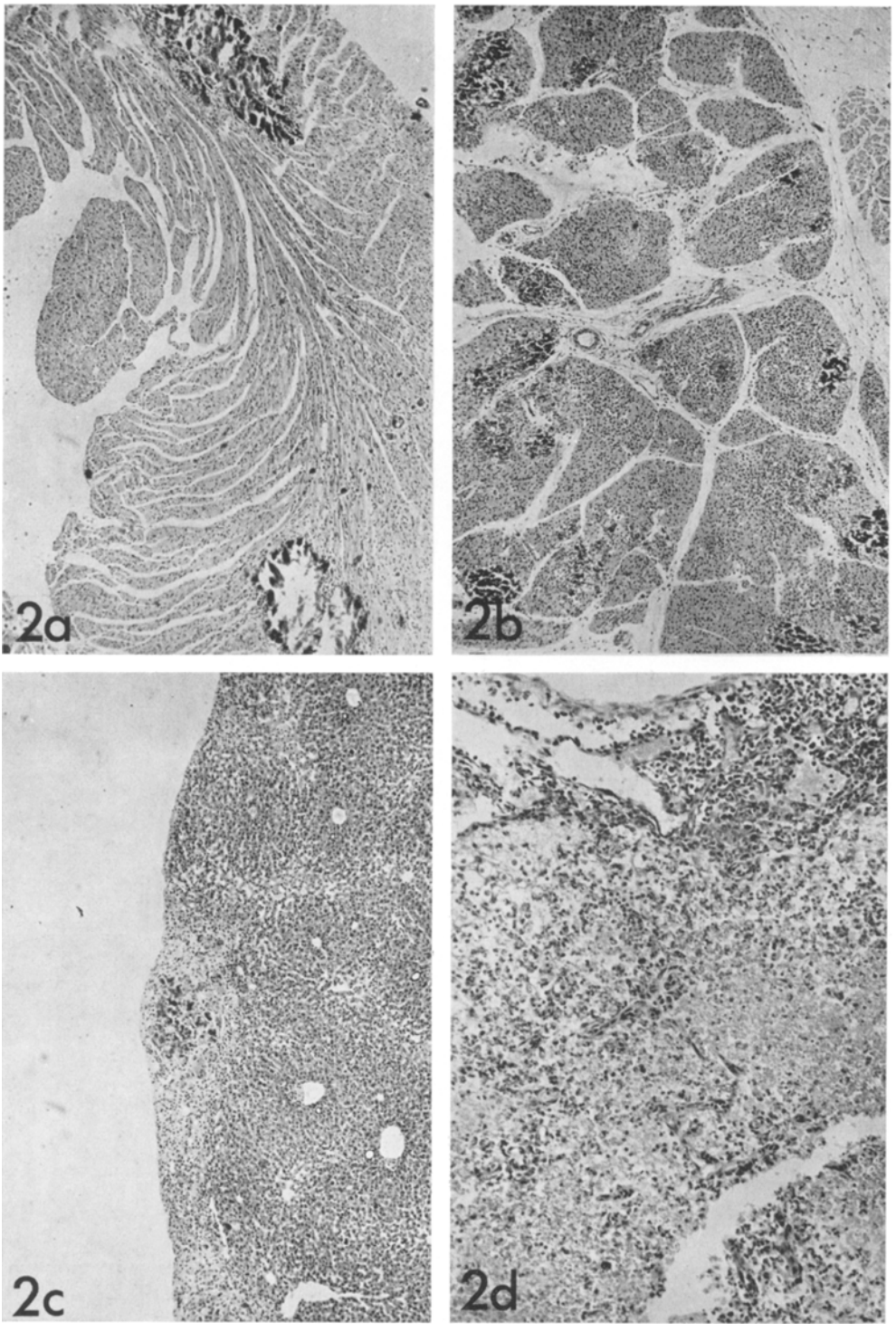

Fig. 2. Organs of newborn mice reaped when moribund five days after i.c. inoculation of NBV
a) Necrotic lesions in cardiac muscle
b) Numerous lesions in brown fat
c) Cloudy centrilobular swelling around lesions in the liver
d) Brain showing meningitis, glial cell proliferation and malacia

Cardiac lesions had centres of granular, necrotic material around which mononuclear cells were evident. Muscle cells about these abscesses showed loss of striation and degeneration (Fig. 2 a).

Lesions in interscapular brown fat were similar to those in the heart, with necrotic centres and mononuclear cell infiltration (Fig. 2 b).

Affected livers had centrilobular cloudy swelling while parenchymal degeneration and mononuclear cell infiltration were evident about the occasional necrotic lesions (Fig. 2 c).

Meningitis and neuronal degeneration were evident. Most inflammatory cells in the brain were mononuclear and the inflammatory response was manifested by glial cell proliferation and perivascular cuffing (Fig. 2 d).

3.4. Serological Characterization of NBV

Attempts to produce haemagglutinin using extracts of infected cells or mouse brain and human group 0 and bovine erythrocytes were not successful.

Cross reactions in complement fixation tests between NBV and mammalian reoviruses were not invariably obtained with all preparations of antiserum and antigen. However, 6/10 reovirus IRS and 2/6 reovirus IMS reacted to at least as high a titre with NBV antigen as with heterologous reovirus antigens. NBV IAF cross-reacted very weakly with reovirus antigens. The results with selected antisera in Table 1 suggest that NBV shares a common complement fixation antigen with mammalian reoviruses.

Table 1. *Cross Complement Fixation Tests with Reoviruses and Nelson Bay Virus*

	Antigen[a]					
Antiserum	Reo 1	Reo 2	Reo 3	NBV	BHK_{21} control	Mouse brain control
Reovirus 1 IRS	*512*[b]	16	16	16	8	<4
Reovirus 2 IRS	16	*128*	16	32	8	<4
Reovirus 3 IRS	32	16	*32*	16	8	<4
NBV IAF	4	<4	4	*512*	<4	<4
N. rabbit serum	<4	<4	<4	4	<4	<4

[a] Reovirus antigens prepared from disrupted infected BHK_{21} monolayers. NBV antigen prepared from 10 per cent mouse brain suspension.

[b] Reciprocal of antiserum dilution end point with optimal antigen dose.

An antigenic relationship between NBV and reoviruses was more convincingly demonstrated by cross immunofluorescence tests. Using indirect immunofluorescence staining techniques, NBV IAF and IRS were shown to combine with antigen in PS cells infected with reovirus 1, 2 or 3, and in the syncytia formed in PS and Vero monolayers infected with NBV (examples in Figs. 3 and 4). Fluorescence was most intense in the homologously stained systems, but no foci of fluorescence comparable with those in heterologous systems were observed in uninfected control cells.

PS and Vero cells infected with NBV, and PS cells infected with reovirus types 1, 2 or 3 were examined after direct staining with fluorescein-conjugated goat polyvalent anti-reovirus globulin, but fluorescence was observed only in reovirus infected cells.

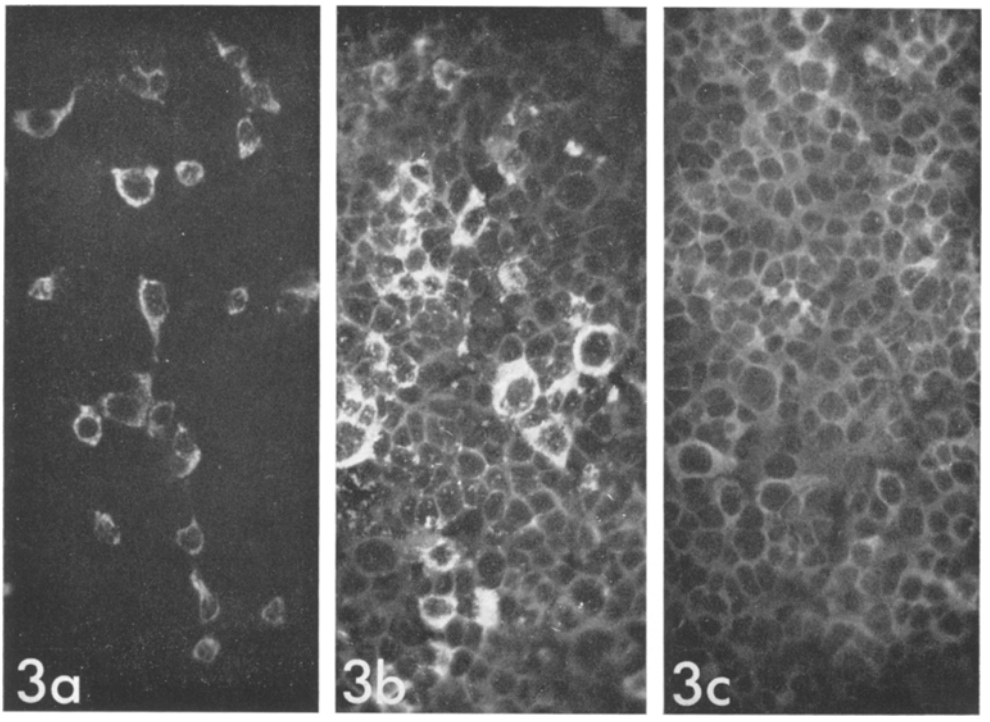

Fig. 3. Fluorescence staining of reovirus type 3 viral antigen in PS cells 24 hours after infection. ×40
a) Direct staining with polyvalent antireovirus conjugate
b) Indirect staining using NBV IRS
c) Indirect staining using Fahey-Crawley IRS

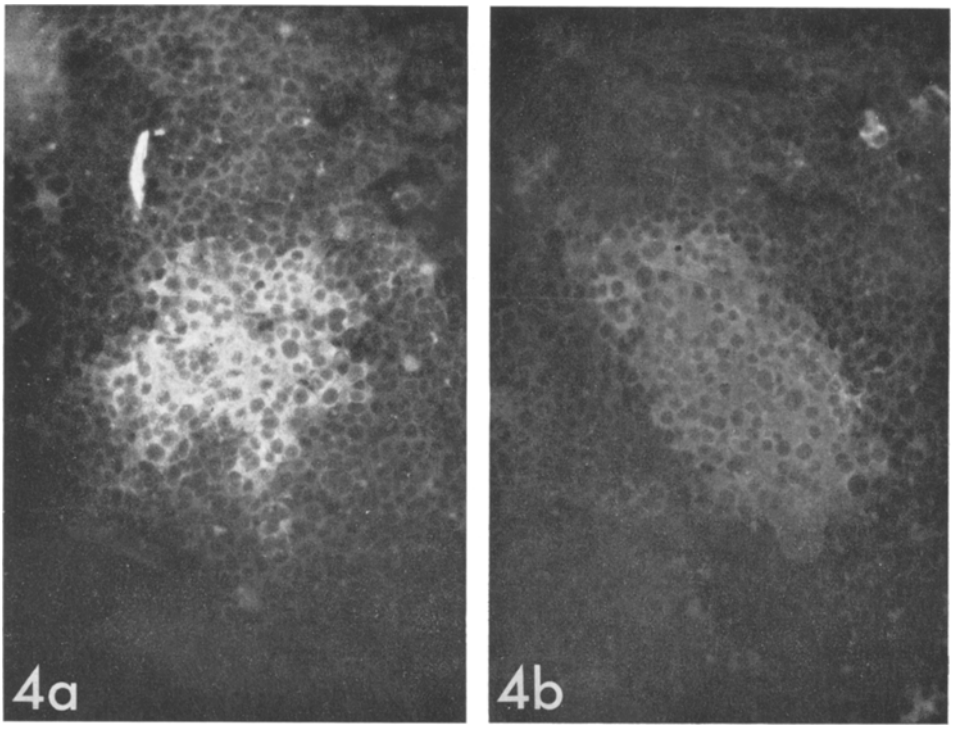

Fig. 4. Fluorescence staining of NBV viral antigen in PS cells 48 hours after infection. ×25
a) Indirect staining using NBV IRS
b) Indirect staining using Fahey-Crawley virus IRS

The distinct antigenic characteristics of NBV were confirmed by cross neutralization tests (Table 2), where no significant heterologous reactions were obtained between the NBV and reovirus reagents. During these tests it was noted that the titre of NBV was consistently reduced by normal sera and ascitic fluid. Further investigation indicated that this inhibitor is present in the presumed normal sera of a wide range of laboratory and wildlife species, is not removed by acetone extraction and is still active at a 1:10 dilution of serum. It has no demonstrable complement fixation or immunofluorescent activity, and does not react with other reoviruses.

Table 2. *Cross-Neutralization Tests with Reoviruses and Nelson Bay Virus*

	Log neutralization index					
	Plaque reduction test on PS monolayers				Plaque reduction test on Vero monolayers	
Viruses incubated with	Reo 1	Reo 2	Reo 3	NBV	Fahey-Crawley virus	NBV
Normal ascitic fluid	0[a]	0	0	0.2—0.3		
Reovirus type 1 IGS	*>3.0*	1.7	2.3	<0.3		
Reovirus type 2 IGS	1.3	*1.5*	1.1	<0.3		
Reovirus type 3 IGS	2.0	1.4	*3.0*	<0.3		
Polyvalent anti-reovirus IGS	3.0	1.1	2.0	0.5		
NBV IAF	<0.3	<0.3	<0.3	*2.0*		
Normal rabbit serum					0[a]	0.3—0.6
Fahey-Crawley IRS					*2.0*	0.6
NBV IRS					0.1	*2.1*

[a] LNI compared with titre of virus—gelatine saline control.

Table 3. *Nelson Bay Virus and Reoviruses: Physico-Chemical Tests*

	Ether 4° C 24 hours		2 M $MgCl_2$ 56° C 3 minutes		20 µg/ml α-Chymotrypsin 37° C 60 minutes		pH 3.0, 4° C 3 hours	
Virus	test	saline control	test	control	test	control	test	control
NBV	6.0[a]	6.2	5.9	6.1	6.1	6.0	6.3[b]	5.7[b]
Reovirus type 1	7.4	7.3	7.1	7.0	7.2	7.9		
Reovirus type 2	4.5	4.6	5.7	4.2	7.8	4.4		
Reovirus type 3	7.9	7.9	7.7	7.5	8.0	8.1		

[a] Titre in $\log_{10}$ PS PFU/ml.
[b] Titre in $\log_{10}$ infant mouse LD_{50}/ml.

3.5. Physico-Chemical Tests

Although the infectivity of NBV was not enhanced by chymotrypsin or 2 M $MgCl_2$ at 56° C, as occurs with some preparations of reoviruses, its resistance to these treatments and to ether and acid conditions (Table 3) is consistent with its classification as a reovirus. The infectivity of Japanaut virus, an orbivirus, used as a control, was completely destroyed when treated at pH 3.0 for three hours in parallel with NBV (1).

4. Discussion

There can be little doubt that the Nelson Bay virus was recovered from the heart blood of a "flying fox" *(Pteropus poliocephalus)*. Arbovirus investigations in this laboratory over the past 10 years have involved the i.c. inoculation of many thousands of litters of infant mice without the detection of any murine viruses of this type, and neither NBV nor reovirus types 1, 2 or 3 antibodies could be detected in the sera of 20 old adults from the colony. The cell cultures used in the investigations reported here were screened for NBV, reovirus types 1, 2 and 3 and the Fahey-Crawley virus and were considered to be free of these agents. There is no likelihood that NBV was recovered from some other wildlife specimen because *Pteropus poliocephalus* was the only species collected during the particular field trip that yielded the virus.

NBV has properties in common with, and at variance to, other members of the "mammalian reovirus" and "avian reovirus" groups. Some of these similarities and differences have been discussed previously (4). Mammalian reoviruses have a common CF antigen but can be readily categorized into 3 types by HI and N tests (9) and DESHMUKH, SAYED and POMEROY (3) have demonstrated antigenic relationships by all 3 methods between their suite of avian reoviruses, and mammalian reoviruses.

Although avian reoviruses have yet to be demonstrated as agents infecting mammals in nature, there is evidence that at least one mammalian reovirus can infect birds. MILES, AUSTIN, MACNAMARA and MAGUIRE (6) isolated reovirus type 3 from the blood of several birds and STANLEY and LEAK (10) demonstrated antibodies against the same type in domestic fowls. The mammalian reoviruses types 1, 2 and 3 produce a granular type of CPE in individual cells whereas avian reoviruses form syncytia in similar cultures. NBV is morphologically (4) and serologically a reovirus, it was recovered from the blood of a mammal, and it produces syncytia in cultured cells (4) similar to those produced by avian reoviruses.

Some avian reoviruses are highly pathogenic for infant mice (3) while the Fahey-Crawley virus produces only transitory symptoms in this host when inoculated intracerebrally (7). The avian reoviruses described by DESMUKH and POMEROY (2) and those described by KAWAMURA, SHIMIZU, MAEDA and TSUBAHARA (5) kill chick embryos when inoculated onto the CAM. NBV is highly pathogenic for infant mice, particularly after adaptation, but does not kill chick embryos.

Hepato-encephalomyelitis virus, a strain of reovirus 3, has been shown to produce pocks on the CAM of embryonated eggs without causing embryonic death (11) and also produces lesions similar to NBV in the hearts and interscapular brown fat of infected mice (15, 16). However, the symptoms in mice infected with the two viruses differ; hepato-encephalomyelitis virus produces jaundice and steatorrhoea with an associated "oily hair effect", encephalitis, alopecia and runting (11, 15) while the only regular symptoms with NBV infection are related to encephalitis.

Presumed normal rabbit sera and normal mouse ascitic fluid reduce the infectivity of NBV, but do not have any demonstrable CF or immunofluorescence activity. Preliminary experiments have demonstrated that this NBV inhibitor is present in the presumed nonimmune serum of a wide range of species, is insoluble

in acetone, is still active at a 1:10 dilution of serum, and has no action on other reoviruses.

NBV appears to be a previously undescribed reovirus sharing some antigenic components with other members of the reovirus group, but having biological characteristics that defy categorization within the "mammalian reovirus" or "avian reovirus" groups.

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