Reactivation of Newcastle Disease Virus Neutralized by Antibody

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In an effort to improve current technology for detection of Newcastle disease virus in convalescent birds, a procedure has been developed for efficient reactivation of virus that has been neutralized by antibody. The reactivation capabilities of fluorocarbon treatment, ultrasonic treatment, pH extremes, and proteolytic digestion were evaluated using the LaSota strain of virus. Reactivation was maximum after proteolytic digestion with either trypsin or papain, and reactivation efficiency was up to 100%, depending on the enzyme used for digestion and the amount of antibody in the neutralization mixture. Reactivation at pH extremes was considerably less efficient than reactivation by proteolytic digestion, and neither fluorocarbon nor ultrasonic treatments effectively recovered antibody-neutralized Newcastle disease virus.

For many years, Newcastle disease has caused serious economic loss to the world poultry industry. Highly pathogenic strains of Newcastle disease virus (NDV) which are currently exotic to the United States are found in domestic and wild-bird populations of many areas of the world. Constant efforts are necessary to avoid introduction of these exotic NDV strains into United States poultry flocks (10). In this regard, one area of major concern is the paucity of data describing the epizootological significance of inapparent NDV infections. Such infections have been reported in convalescent birds (6, 11), but the frequency of their occurrence and the role they play in viral dissemination are yet to be defined.

Collection of data on the significance of inapparent NDV infections has been hampered by the difficulty of identifying the infected, though clinically normal, birds. Routine virus isolation procedures generally fail to detect these infections, but NDV has been isolated from tracheal tissues of convalescent birds by prolonged *in vitro* culture of tissue explants (6, 11). This difficulty of virus isolation may be the result of neutralization of virus, either before or after sample collection, by homologous antibody in the body fluids. If antibody does interfere with virus isolation, we should be able to recover the virus from clinical materials simply by treatment to dissociate virus-antibody complexes. Other investigators have established that the infectivity of NDV (7) and other viruses (3, 13) can be retrieved after neutralization by antibody.

The purpose of this study was to evaluate the NDV reactivation capabilities of several meth-

ods known to dissociate virus-antibody complexes, and thereby to define conditions for a simple and efficient procedure that can be used in virus isolation attempts. The reactivation methods tested were fluorocarbon (FC) treatment (3, 13, 19), sonic treatment (14, 16), dissociation by pH extremes (7, 18), and digestion of antibody with proteolytic enzymes (15).

MATERIALS AND METHODS

Virus and assay. The LaSota strain of NDV grown in the allantoic sac of embryonated chicken eggs was used in most experiments. Virus-containing allantoic fluid was diluted 1:100 in pH 7.2 Earle balanced salt solution (BSS) containing 50 mM tris(hydroxymethyl)aminomethane and no NaHCO_3 , then passed through a 300- μm membrane filter and stored at -70°C in 1.0-ml volumes. The Texas GB, Rakin, and B1 strains of NDV similarly grown in eggs were used in some experiments.

Viral infectivity was assayed by the plaque method in primary monolayer cultures of chicken kidney cells prepared as described by Hopkins (12). Confluent cultures were fed with serum-free medium 199 and used within 48 h of maintenance at 37°C in an atmosphere containing 5% CO_2 . Test materials were inoculated on the bared surface of four cultures and, after 1 h of adsorption at 37°C , were overlaid with medium 199 containing a final concentration of 1:20,000 neutral red and 1% purified agar. Plaques were counted after 48 to 72 h of incubation.

Hemagglutination titers were determined at 25°C from twofold dilutions of test materials in pH 7.2 phosphate-buffered saline (PBS). Equal volumes of 0.5% chicken erythrocytes in PBS were added to the dilutions, and end points were read when the control cells had completely sedimented.

Virus neutralization. A virus suspension containing 500 to 1,000 plaque-forming units (PFU)/ml was

used in most experiments, and test and control samples were inoculated on cell cultures without further dilution. Viral-neutralizing antibody was added to a part of this suspension in concentrations indicated below and incubated for 1 h at 37°C. Another part served as virus control and was incubated similarly with normal chicken serum.

The source of viral-neutralizing antibody was a non-heat-inactivated pool of immune serum collected 4 weeks postchallenge from vaccinated chickens that had survived challenge infection with the virulent Texas GB strain of NDV. When diluted 1:10,000, this serum neutralized 50% of 1,000 PFU of NDV LaSota in 1.0 ml of BSS. Thus, the 1:10,000 dilution of serum is considered to contain 1 U of antibody activity; likewise, the undiluted serum contains 10,000 U of activity.

Fluorocarbon treatment. Two volumes of virus in BSS were added to 1 volume of the FC trichlorotrifluoroethane. The prechilled mixture was homogenized for 2 min at 16,000 rpm in a Sorvall Omni-mixer with the reaction vessel immersed in ice. Virus in the aqueous layer (top) was assayed immediately after centrifugation of the homogenate for 15 min at 1,000 $\times g$. For multiple FC treatments, the aqueous phase was removed and again treated as above. Fetal calf serum (FCS) (liquid form, GIBCO) and bovine serum albumen (BSA) (fraction V powder, Sigma Chemical Co.) were used as described.

Ultrasonic treatment. Neutralized virus was treated with a sonic frequency of 20 kc/s and a power setting of 60 W on a Bronwill Biosonik model BP-1 sonic vibrator equipped with the large 1.0-cm-diameter probe. A 20-ml sample in a 50-ml beaker was immersed in ice for treatment, and at indicated times, samples were removed and immediately inoculated on cell culture.

Low and high pH treatment. The pH of samples was adjusted at 25°C by adding 1 N NaOH or HCl with a micropipetter to a constantly stirring sample with continuous pH metering. The treatment was ended by neutralizing the pH with 1 N HCl or NaOH, followed immediately by inoculation of cells.

Proteolytic digestion. Papain (2 $\times$ crystallized lyophilized powder, Sigma Chemical Co.) was dissolved in PBS and stored at -20°C (5 mg/ml). Ten grams of tissue culture-grade trypsin (1:250 powder, ICN Pharmaceuticals) was dissolved in 90 ml of PBS, then filtered and stored at -20°C. Although tissue debris was removed by filtration, the quantity of trypsin in this concentrate will be expressed as 100 mg/ml. A 0.1 M stock of the reducing agent dithiothreitol (DTT) was prepared in PBS and stored at -20°C. Enzymes and DTT were added from the frozen concentrates (usually 50 μ l/5 ml) to give the final concentration indicated, and the digestion mixtures then were incubated at 37°C for 30 min. Before virus assay, 1 volume of FCS was added to 9 volumes of the treated sample in an effort to provide excess substrate for the proteolytic enzyme and thereby limit further digestion.

RESULTS

Reactivation with FC. Homogenization of 2 volumes of NDV with 1 volume of FC gave a

viscous opaque emulsion that readily separated upon centrifugation. Preliminary experiments showed that a marked loss of hemagglutination activity and infectivity resulted from the FC treatment of four strains of NDV not neutralized by antibody. In an effort to avoid this detrimental effect, certain supplements to the virus-suspending media were tested for their ability to stabilize NDV infectivity during FC treatment. Bovine serum proteins were effective in this regard. There was no measurable loss of infectivity of NDV strain LaSota after one FC extraction with either 10% FCS or 0.1% BSA in the virus-suspending media (Table 1). Further tests showed that, generally, 0.5% BSA protected the infectivity of this virus during one and sometimes two FC treatments; thus, the ability of FC to reactivate neutralized NDV was tested in the presence of 0.5% BSA.

The LaSota strain of NDV ($10^{7.5}$ PFU/ml) was reacted with varying concentrations of neutralizing antibody (10 to 1,000 U) and then FC treated with two successive extractions. Virus infectivity was measured after each FC extraction, and evidence of reactivation was obtained with only two of seven neutralized samples. These two samples were neutralized (>99% titer reduction) by 100 U of antibody, and the maximum reactivation observed was equivalent to 3% of the untreated control virus. Thus, FC reactivation was of a low order and not reproducible.

Ultrasonic reactivation. Reactivation by sonic treatment for periods up to 8 min was attempted using NDV strain LaSota (500 to 1,000 PFU/ml) neutralized by 10 U of antibody. In one trial, from 4 to 11% of the virus neutralized was reactivated in each of four samples treated by sonication, and the titer of non-neutralized virus controls increased approximately

TABLE 1. *Stabilizing effect of serum proteins on NDV treated with fluorocarbon^a*

Protein	Concn (%)	Viral infectivity (log ₁₀ , PFU/ml) ^b	
		Untreated	Fluorocarbon treated
FCS	0 (vol/vol)	7.6	2.0
	0.1	7.7	3.4
	1.0	7.6	5.9
	10.0	7.6	7.6
		7.6	2.0
BSA	0 (wt/vol)	7.6	2.0
	0.1	7.5	7.4
	0.5	7.5	7.7
	1.0	7.5	7.7

^a Virus strain LaSota in BSS supplemented as indicated was homogenized in an ice bath for 2 min at 16,000 rpm.

^b Values represent a single determination.

10% when treated similarly. In another trial, control titers were not boosted by sonication, and no infectious virus was recovered from three of four neutralized samples. The fourth sample in this series yielded a 1% recovery after sonic treatment. Sonic reactivation efficiency was not time dependent in the range (1 to 8 min) tested.

Reactivation by low and high pH. Preliminary tests of the effect of high and low pH on four strains of NDV revealed a considerable degree of instability of viral infectivity. The infectivity titers of three of the four strains tested at pH 2.0 were reduced more than 99% in 15 min at 25°C. Further tests with NDV strain LaSota revealed similar instability at pH 12.0. This instability suggested the need for precise control of conditions for pH reactivation.

Treatment of neutralized virus at pH above 3.5 or below 10.5 for periods up to 10 min did not consistently restore viral infectivity. Table 2 shows the relative effects of pH 2.0 to 3.5 and pH 10.5 to 12.0 on NDV strain LaSota before and after neutralization by antibody. Of the treatment times tested, reactivation at 1 min was optimum and there was no apparent difference in the amount of virus reactivated at low and high pH. The efficiency of reactivation at pH 11.0 was further assessed by measuring virus recovery after neutralization by various amounts of antibody (Table 3). When virus was completely neutralized by 8 or 10 U of antibody, the recovery was 18 and 14%, respectively, but recovery decreased to 3% for virus neutralized by 32 U of antibody.

Reactivation by proteolytic enzymes. Digestion with papain in concentrations ranging from 10 to 500 $\mu\text{g/ml}$ in the presence of 1.0 mM DTT was tested for ability to reactivate NDV neutralized by 10 U of antibody. Concentrations above 100 $\mu\text{g/ml}$ damaged the cell cultures used for assay, and reactivation with papain was maximum with concentrations of 50 and 100 $\mu\text{g/ml}$. Digestion with 1.0 mg of trypsin per ml failed to reactivate virus neutralized by 10 U of antibody; however, quite unexpectedly, trypsin was effective in the presence of the reducing agent DTT (Table 4).

The efficiency of reactivation by both papain and trypsin was greatly influenced by digestion pH (Table 5). Virus recovery was maximum at pH 8.5 with both enzymes, and interestingly, more virus was recovered from the neutralized mixtures than from their controls in seven of the eight samples digested at pH 8.5 and 9.0.

The addition of up to 10% normal chicken serum to suspensions of neutralized virus did not block reactivation by proteolytic digestion. In the presence of 10% chicken serum, trypsin reactivated 78% of virus neutralized by 10 U of

TABLE 2. Low and high pH treatment of NDV strain LaSota before and after neutralization by antibody

pH ^a	Viral infectivity (PFU) at minutes of treatment ^b							
	Non-neutralized virus				Neutralized virus ^c			
	0	1	5	10	0	1	5	10
2.0	102	53	19	19	0	27	5	8
2.5	100	56	48	38	0	19	8	14
3.0	107	66	60	46	0	26	9	6
3.5	106	99	80	85	0	26	6	3
10.5	92	89	55	57	0	30	15	15
11.0	98	74	70	45	0	22	22	19
11.5	96	74	38	7	0	32	6	6
12.0	64	13	1	0	0	0	0	0

^a Treatment at 25°C; pH adjusted with 1 N HCl or NaOH and similarly neutralized before inoculation.

^b PFU expressed as percentage of pH 7.0 virus control. Values represent a single determination.

^c Reacted with 5 U of antibody, 37°C, 60 min.

TABLE 3. Reactivation of NDV strain LaSota by high pH after virus neutralization by various amounts of antibody

Antibody activity (U) reacted with virus at 37°C for 60 min	Viral infectivity (PFU) ^a	
	Before treatment	After treatment (pH 11.0, 1 min, 25°C)
0	100	90
2	16	44
8	0	18
32	0	3
128	0	0
0	100	65
1	49	70
10	0	14
100	0	0

^a PFU expressed as percentage of untreated control. Values represent a single determination.

TABLE 4. DTT requirement for reactivation of neutralized NDV strain LaSota by proteolytic digestion with trypsin

Digestion pH (37°C, 30 min) ^a	Viral infectivity (PFU) after digestion ^b			
	Non-neutralized virus		Neutralized virus ^c	
	Without DTT	1.0 mM DTT	Without DTT	1.0 mM DTT
5	78	87	0	0
6	164	153	0	0
7	117	167	0	9
8	125	111	0	56
9	92	56	0	75

^a Adjusted with 1 N HCl or NaOH after virus neutralization.

^b PFU expressed as percentage of pH 7.0 untreated virus control. Trypsin added to final concentration of 1.0 mg/ml. Values represent a single determination.

^c Reacted with 10 U of antibody, 37°C, 60 min.

TABLE 5. Influence of pH on reactivation of neutralized NDV strain LaSota by proteolytic digestion with papain and trypsin

Digestion pH (37°C, 30 min) ^a	Viral infectivity (PFU) after digestion in presence of 1.0 mM DTT ^b			
	Papain (50 µg/ml)		Trypsin (1.0 mg/ml)	
	Non-neutralized virus	Neutralized virus ^c	Non-neutralized virus	Neutralized virus ^c
7.0	100	2	100	14
7.5	93	7	94	19
8.0	88	36	98	72
8.5	52	95	55	76
9.0	36	46	39	59
7.0	100	4	100	8
7.5	67	7	107	15
8.0	64	27	87	47
8.5	45	54	34	76
9.0	27	39	59	59

^a Adjusted with 1 N HCl or NaOH after virus neutralization.

^b PFU expressed as percentage of pH 7.0 virus control. Values represent a single determination.

^c Reacted with 10 U of antibody, 37°C, 60 min.

antibody and papain reactivated 27%. These levels of virus recovery were lower than those from digestion mixtures with no serum or with 0.2 to 4% serum.

Reactivation of NDV by proteolytic enzymes was further evaluated in tests with virus neutralized by varying amounts of antibody (Table 6). Papain and trypsin consistently restored the infectivity of virus neutralized by up to 512 U of antibody, and virus neutralized by 1,024 units of antibody was reactivated in two of three trials. Levels of virus recovered with trypsin were generally higher than those recovered with papain, and in the presence of 2 to 64 U of antibody, trypsin effected the recovery of higher levels of virus than were detected in untreated virus controls. Trypsin (10 µg/ml) added to the plaque overlay medium did not alter the titer of untreated control virus.

DISCUSSION

More sensitive methods are needed for detection of NDV infections in clinically normal convalescent birds. Neutralizing antibody is present in the serum and secretions of these birds and may interfere with virus isolation; therefore, the present study was designed to develop a procedure for isolation of NDV from samples containing small amounts of virus neutralized by antibody. The approach was to evaluate quantitatively the NDV-reactivating capability of several methods known to dissociate virus-antibody complexes.

The prerequisites for selection of reactivation methods were the following: (i) the treatment should require only minimal dilution of the test sample; (ii) the method should be readily applicable under diagnostic laboratory conditions; and finally, (iii) the treatment should not render the sample toxic or otherwise unacceptable for inoculation in a virus assay system such as cell culture.

The most efficient of the four reactivation methods tested was digestion with proteolytic enzymes. Although this procedure is not as simply performed as others tested, it is quite applicable for diagnostic purposes because it fulfills the criteria outlined above. Further, of practical significance is the fact that with this procedure, reactivation efficiency was generally independent of antibody concentration in the range of 2 to 128 antibody U, and reactivation was not blocked by 10% normal chicken serum.

Unexpectedly, digestion with proteolytic enzymes often resulted in recovery of more infectious virus than was found in treated (Table 5) or untreated (Table 6) control preparations. This enhanced recovery was especially obvious in trypsin-treated preparations and appears not to be related to the effect of residual trypsin on the virus assay system because trypsin added to the overlay did not increase plaque titers of NDV LaSota as it increases influenza virus titers (1). Possibly, antibody fragments remain on the virus after reactivation (15) and protect the virus from degradation by proteolytic digestion. This protection could explain the more

TABLE 6. Reactivation of NDV strain LaSota by proteolytic digestion after virus neutralization by various amounts of antibody

Antibody activity (U) reacted with virus (37°C, 60 min)	Untreated virus control (PFU)	Viral infectivity (PFU) after digestion of neutralized virus ^a	
		Papain (50 µg/ml)	Trypsin (1.0 mg/ml)
0	100	66	74
2	17	72	128
4	1	72	123
8	0	79	115
16	0	80	112
32	0	90	130
64	0	79	121
128	0	71	91
256	0	35	54
512	0	25	29
1024	0	8	8

^a After virus neutralization, pH was adjusted to 8.5 with 1 N NaOH and samples were digested at 37°C for 30 min in presence of 1.0 mM DTT. PFU expressed as percentage of untreated virus control. Values are means of three determinations.

efficient recovery of neutralized virus in Table 5 (pH 8.5 and 9.0) where control virus was enzyme treated. However, in some experiments, the control virus was not enzyme treated (Table 6), and recovery of neutralized virus after trypsin digestion still exceeded control levels. Therefore, the enhanced recovery may indicate that the neutralized virus is more thermostable than non-neutralized virus at 37°C, or may reflect the proteolytic dissociation of viral aggregates, even though virus stocks were prefiltered.

Interestingly, the optimal pH for reactivation was the same for both papain and trypsin (Table 5) and was not the same as the pH optimum reported for these enzymes with other substrates (8, 17). This pH dependency may then relate to configurational changes of bound antibody which make the molecule more accessible to enzymes. The requirement for the reducing agent DTT in trypsin reactivation (Table 4) also may be antibody dependent, but we should note that a relatively crude pancreatic extract of trypsin was used, and there is no assurance that trypsin acted alone in this reaction.

Reactivation with FC was initially considered the method of choice for recovery of neutralized NDV because of its simplicity and suitability for diagnostic applications. This procedure has been successfully used in reactivation of non-enveloped viruses neutralized by antibody (3, 13, 19). Considerable effort was made to define conditions under which FC would efficiently restore the infectivity of neutralized virus. Earlier reports (2, 5, 9) of the stability of enveloped viruses during FC treatment were encouraging. However, FC treatment did not effectively restore the infectivity of neutralized NDV, possibly because of the relative instability of enveloped viruses when treated with FC (9).

Ultrasonic treatment was not a satisfactory method for recovery of neutralized NDV. The results herein do not reflect the high sonic reactivation efficiency reported for influenza virus and poliovirus (14, 16), and this apparent discrepancy may be related to methodology because the previously reported studies used a virus excess, and samples were diluted immediately after sonication. Dilution has been reported to facilitate virus-antibody dissociation (4).

Reactivation of neutralized NDV by exposure to pH extremes were used successfully by Granoff (7) in fundamental studies of virus neutralization. In those studies, virus concentrations were high and pH was adjusted by dilution of the sample in buffer of the pH desired. This

approach is not acceptable for diagnostic purposes because of the necessity to avoid unnecessary dilution of test materials. When the pH adjustment procedure was modified to minimize sample dilution, reactivation of NDV at pH extremes still was effective (Tables 2 and 3). The strains of NDV tested were quite unstable at pH levels necessary for reactivation (Table 2 and text), and this instability diminishes net reactivation efficiency. Also, the efficiency was highly dependent on the concentration of antibody that had reacted with virus during neutralization (Table 3).

Results in the present studies show clearly that infectious NDV LaSota can be recovered very efficiently after neutralization *in vitro* by hyperimmune serum. Virus recovery is maximum when a rather simple method of proteolytic digestion with trypsin or papain is used and optimum conditions for the digestion reaction are described. Further experiments are necessary to determine whether this procedure will facilitate the detection of NDV infections in carrier birds.

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