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Characterization of Reference Strains of Newcastle Disease Virus (NDV) and NDV-like Isolates by Monoclonal Antibodies to HN Subunits

By

M. Ishida[1], K. Nerome[1], M. Matsumoto[1], T. Mikami[2], and A. Oya[1]

[1] Department of Virology and Rickettsiology, National Institute of Health, Tokyo, Japan

[2] Department of Epizootiology, Faculty of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido, Japan

With 3 Figures

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Summary

The hemagglutinin-neuraminidase (HN) subunits of NDV and NDV-like isolates were analyzed antigenically by monoclonal antibodies to the HN of Miyadera and Taka viruses. In immuno-double-diffusion (IDD) tests, all NDVs examined gave clear lines of precipitation with some of the potent monoclonal antibodies, but it was difficult to determine with certainty the immunological properties of HN subunits due to a rare disagreement with the results obtained in other immunological tests. Monoclonal antibodies used in the tests were found to show different immunological reactivities with the viruses.

Monoclonal antibodies belonging to the 1st group (1/29) inhibited the hemagglutinating (HA) activity of all strains but not the neuraminidase (NA) activity. The second monoclonal antibody (5/205) inhibited both the HA and NA activities of the restrictive NDV strains, indicating antigenic changes in HN molecules. However, the inhibitory activity of this monoclone to neuraminidase appeared to be greatly diminished when neuraminyl lactose was used as substrate. Although the 3rd type of monoclonal antibody (5/220) showed HI activity against several strains, this antibody did not inhibit NA activity of any viruses.

The remaining monoclone to the HN of Taka virus inhibited the HA activity of all reference strains of NDV and many NDV-like isolates but did not affect NA activity. Two inhibitory activities of four monoclonal

antibodies against different viruses, HI and hemolysis-inhibition, were not always consistent with inhibition of virus growth. HI and NI tests with the above four monoclonal antibodies showed that the strains tested fell into five antigenic groups according to their reaction patterns with mouse hybridoma antibodies.

Introduction

Recent virological surveillance has revealed that numerous paramyxoviruses are widely distributed in nature. They have been isolated from wild and caged birds in many areas, and they form a large and antigenically heterogeneous group (2, 3, 4, 22).

Although the viruses isolated up to 1980 were tentatively divided into 6 groups by their cross-reactivities in HI tests with antisera to whole virus particles (2), the antigenic structure of the hemagglutinin-neuraminidase (HN) antigens of avian paramyxoviruses was incompletely understood. However, there have been many reports of antigenic variation within the same subtype (2). The investigation of antigenic structure may have some bearing on antigenic drift and help to understand the natural history of avian paramyxoviruses. The recent developments of hybridoma technology have made it possible to study antigenic variation and functional significance of the structural proteins of a wide range of viruses at the molecular level (5, 10, 11, 17, 25, 27). Russell and Alexander (19) first reported the detection of antigenic drift in the HN molecules of NDV using monoclonal antibodies. In the present paper we describe antigenic variation of HN proteins amongst numerous Newcastle disease viruses (NDVs).

In addition, we report that monoclonal antibodies specific for different antigenic sites differ markedly in their inhibitory effect on HA, NA, hemolysis and virus multiplication.

Materials and Methods

Viruses

The following reference strains of NDV isolated between 1930 and 1967 were used in this study: Sato, Narashino, Miyadera, Ishii, B-1. In addition to the above reference strains, 19 NDV-like isolates from caged birds and wild ducks were also examined (see Table 1).

Serological Assays

Hemagglutination-inhibition (HI) tests were done in microplates using 0.5 per cent chicken red blood cells and the highest dilution of antiserum or monoclonal antibodies completely inhibiting agglutination of 4 HA units was determined. IDD tests were done as described previously (9, 28). Neuraminidase titrations and neuraminidase-inhibition (NI) tests were done as described previously (26) and two kinds of substrate, fetuin and N-acetyl neuramin-lactose [N-acetyl neuraminyl (2→3) and (2→6)] were used.

Hemolysis Inhibition Test

Hemolytic activity was done as described previously (15) and hemolysis inhibition tests were performed by the method of Saburi and Matsumoto (21).

Table 1. *Antigenic characterization and grouping of reference strains of NDV and NDV-like isolates based on hemagglutination- and neuraminidase-inhibition reaction with four monoclonal antibodies to HN protein*

Groupings	Test viruses	Years of isolation	Natural hosts	HI titers				NI titers			
				1/29	5/205	5/220	3/Taka	1/29	5/205	5/220	3/Taka
1	*Miyadera*	1951	Chicken	10,240	20,480	20,480	512	—[b]	20,480	—	—
	37	1976	Parakeet	20,480	20,480	256	256	—	2,560	—	—
2	*B-1*	1947	Chicken	81,920	20,480	256	1,024	—	—	—	—
	107	1976	Parakeet	20,480	1,280	5,120	512	—	—	—	—
3	H-13	1978	Parrot	10,240	2,560	128	—[a]	—	—	—	—
	H-14	1978	Parrot	10,240	2,560	20,480	—	—	—	—	—
	H-20	1978	Parrot	81,920	10,240	5,120	—	—	—	—	—
4	*Sato*	1930	Chicken	40,960	—	—	1,024	—	—	—	—
	Ishii	1962	Chicken	20,480	—	—	256	—	—	—	—
	Narashino	1967	Chicken	640	—	—	128	—	—	—	—
	472	1977	Parrot	20,480	—	—	512	—	—	—	—
	482	1977	Parakeet	20,480	—	—	512	—	—	—	—
	485	1977	Parakeet	10,240	—	—	256	—	—	—	—
	487	1977	Parakeet	20,480	—	—	256	—	—	—	—
	79-244	1979	Mynah	10,240	—	—	256	—	—	—	—
	79-260	1979	Parakeet	10,240	—	—	256	—	—	—	—
	Niigata-423	1980	Duck	40,960	—	—	256	—	—	—	—
5	H-31	1977	Mynah	20,480	—	—	—	—	—	—	—
	H-17	1978	Parrot	10,240	—	—	—	—	—	—	—
	H-122	1978	Parakeet	2,560	—	—	—	—	—	—	—
	H-123	1978	Parakeet	2,560	—	—	—	—	—	—	—
	74	1978	Parakeet	20,480	—	—	—	—	—	—	—
	B-4	1981	Purple Gallinule	10,240	—	—	—	—	—	—	—
	B-9	1981	Purple Gallinule	10,240	—	—	—	—	—	—	—

All monoclonal antibodies used in HI tests were treated with RDE. Values given represent HI titers based on reciprocals of terminal antibody dilutions inhibiting 4 hemagglutinating units of virus antigens. Reference strains were indicated by italics.

[a] Less than 32. Monoclonal antibodies used in NI tests were not treated with RDE. NI titers given represent the reciprocals of the highest ascitic fluid dilutions inhibiting 50 per cent of neuraminidase activity of test viruses

[b] Less than 10

Preparation of Hybridomas

For hybridoma cell lines, SP 2/O/Ag 14 myeloma cells were used with spleen cells from BALB/c mice, which were boosted intravenously 7 days before fusion with 5×10^3 HA units of virus. Fusion with polyethylene glycol was carried out as described by Webster and Berton (24). Hybridomas producing antibodies were screened by ELISA, HI and NI tests using a virus insensitive to non-specific inhibitors in horse and calf sera. Antibody-secreting hybridomas were cloned in soft agar as described previously (23). In order to produce potent antibodies cloned hybridoma cell lines were grown in the peritoneal cavity of Pristan-treated BALB/c mice.

Neutralization Tests

For virus growth-inhibition tests, plaque neutralization and Disk methods were employed. Primary chick embryo (CE) fibroblast cells were prepared by trypsinization of 7-day-old chick embryo and used for the former tests and an established cell line of monkey ($LLCMK_2$) cells on the latter. Plaque neutralization tests were done as described previously (12, 13) and challenge virus was diluted to produce about 100 plaques. For Disk tests, $LLCMK_2$ cell monolayers on 60 mm-plastic dishes were inoculated with the diluted virus with $1—3 \times 10^3$ $TCID_{50}$ and allowed to absorb at 35° C. After 30 minutes 4 ml of the enriched maintenance medium containing 0.8 per cent purified agar and crystallized trypsin (16) was added to infected cells on dishes. After the agar solidified, filter-paper discs 8 mm in diameter and 1.5 mm in thickness, which contained 40 µl of the different concentration of antibodies, were placed on the surface of the agar. After 3 days a second agar overlay containing neutral red (1:15,000) was added to each culture and on the following day the diameters of the zones of surviving cells were measured.

Results

Characterization of Monoclonal Antibodies by Different Tests

Immuno-double diffusion (IDD) tests. Three different monoclonal antibodies to the HN of the Miyadera strain were used in the IDD tests. The results are shown in Fig. 1. In order to detect faint reaction lines all the immunoplates were press-dried and stained with Coomassie Brilliant Blue (CBB) as described by Wood *et al.* (28). One monoclonal antibody (1/29) reacted in these tests with all viruses examined, and gave a definite single line of precipitation with each virus (Fig. 1A). Although a definite single precipitin line was seen in the unstained immunoplate (1A), the stained one revealed two lines of precipitation with Miyadera and Narashino strains (1B). The reactions of this monoclonal antibody with different viruses were consistent with the results obtained in HI tests. However, IDD testing of 5 strains with monoclone (5/205) represented the different patterns of reactivity with the above clone (Fig. 1C). This monoclonal antibody did not produce a precipitin line with Narashino or B-1 viruses, although the HA activity of the former was strongly inhibited by it (Table 1). In contrast, clear lines of precipitation were seen between 5/205 antibody and two strains, Ishii and Niigata 423, while the HA activity of the former virus was not inhibited. The monoclone 5/220 gave a broad line of precipitation only with

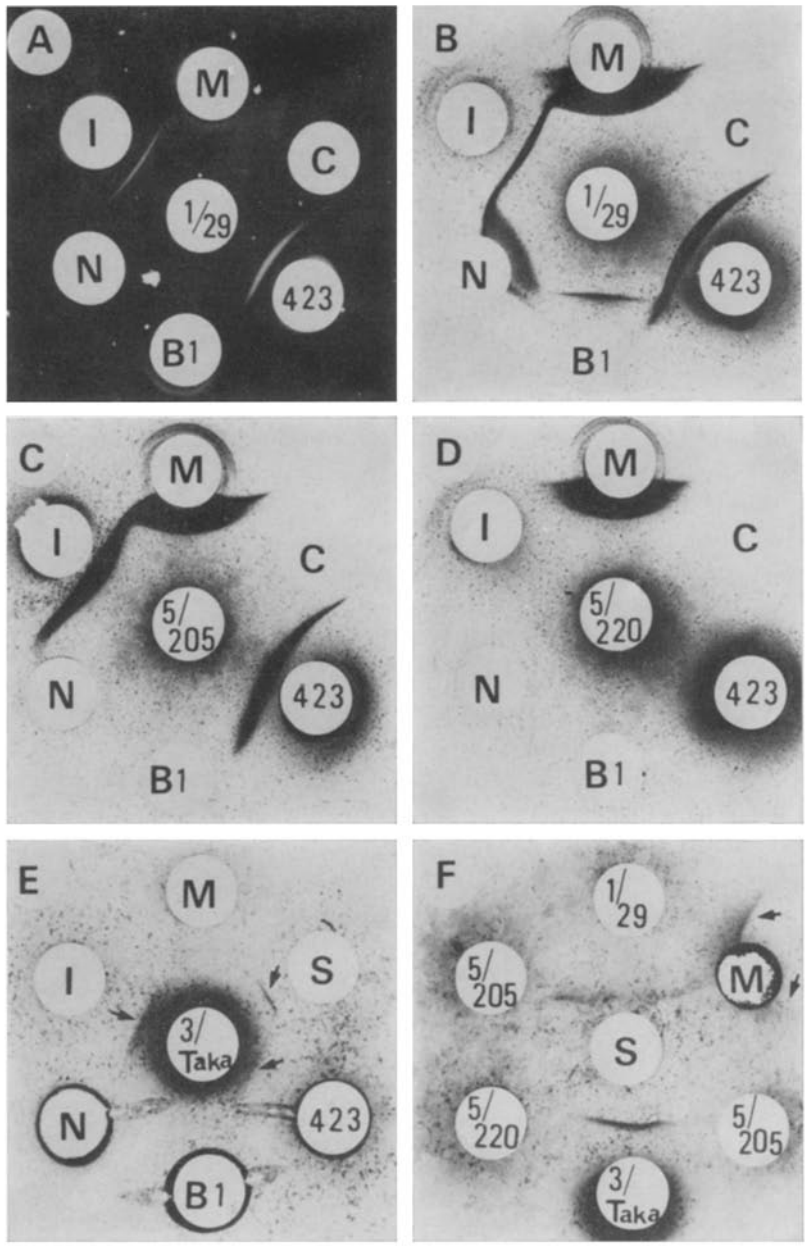

Fig. 1. Immuno-double-diffusion reactions of reference strains of NDV and the Niigata isolate with four monoclonal antibodies. Immunoplates shown in B-F were press-dried and stained according to the single-radial diffusion method described by Wood *et al.* (28). All center wells except for plate F contained monoclonal antibodies. The center well of F contained virus antigen (Sato). Antigens were placed in outer wells: *M* Miyadera; *I* Ishii; *N* Narashino; *B1* B-1; *S* Sato strain; *423* Niigata 423 isolate; *C* absence of antigen. Each arrow indicates a faint line of precipitation. For comparison, this figure also shows a pair of immunoplates before and after staining *(A, B)*

the homologous virus (Fig. 1D). The remaining monoclonal antibody to Taka virus gave a single line of precipitation with Ishii, Sato and isolate 423 (indicated by arrows) (Fig. 1E). IDD tests also showed that the Sato strain contained (Fig. 1F) epitopes recognized by two monoclones (1/29, 3/Taka).

HI and NI tests. All the monoclonal antibodies were characterized by HI and NI tests with 5 reference strains of NDV. Monoclonal antibody 1/29 reacted with all reference strains (in italics) and inhibited their HA activity (Table 1). However, NA activity was not inhibited, indicating that the region of HA activity was located away from that of NA activity. The remaining three kinds of monoclone demonstrated different patterns of reactivity with reference strains. Reaction with 5/205 monoclone showed that, of those examined, only the HA activity of Miyadera and B-1 viruses was inhibited but the NA activity of the latter strain was not influenced by this antibody, suggesting that antigenic changes in this epitope are not recent events. With the monoclone 5/220, the hemagglutination-inhibition patterns of reference strains were identical to those obtained in HI tests with the 2nd type of monoclone (5/205), whereas this clone was different from the latter clones in its neuraminidase-inhibiting activity. In NI tests with fetuin (Table 1), monoclone 5/205 strongly inhibited the neuraminidase activity of two strains, Miyadera and isolate 37. In contrast, neuraminidase activity was inhibited incompletely by this antibody when neuraminyl lactose, which has a low molecular weight, was used as the substrate; the highest levels of inhibition against isolate 37 and Miyadera strain were 70 and 48 per cent respectively (Fig. 2). Although the reaction was weak, inhibitory activity gradually decreased with antibody dilution, showing a linear relationship. Monoclonal antibody to the HN molecule of Taka virus inhibited the HA activity of all the reference strains, although some variation in the HI activities was seen among them. Despite the these HI activities, this monoclone failed to inhibit the NA activity of any viruses (Table 1).

Hemolysis inhibition (HLI) tests. To determine the interrelationships between different activity showed by monoclonal antibodies, all monoclonal antibodies were tested for hemolysis-inhibitory activity. A summary of these tests is shown in Table 2. Two undiluted monoclonal antibodies (1/29, 5/220) inhibited the hemolytic activity of five reference strains. However, the remaining two clones (5/205, 3/Taka) prevented hemolysis by both Miyadera and B-1 strain, and 3/Taka inhibited Sato virus, indicating disagreement between HI and HLI activities.

Neutralization tests. Paper-discs methods were used to screen for neutralizing activity of four monoclonal antibodies; paper discs with various 2-fold dilutions of an antiserum to the isolated HN of the Miyadera strain were placed on the agar overlay cultures infected with $10^{2.5}$—$10^{5.5}$ $TCID_{50}$ of virus. These box-titration tests with a series of antibody and virus dilutions showed that the size of the zone area free from plaques were in linear

proportion to antibody dilutions and virus concentrations (data not shown). When the virus infectivity was increased, the zone area decreased. In order to achieve the optimal defined edge and sensitivity, it was found best, to use challenge virus with $10^{3.0}$—$10^{3.5}$ $TCID_{50}$/dish in the present disk tests. Fig. 3 shows the results obtained in the disk tests with three monoclonal antibodies. Monoclone 1/29 produced a definite ring of protected area in $LLCMK_2$ cell monolayers infected with Miyadera, Ishii, B-1 and Sato viruses, showing that this antibody neutralized the multiplication of these viruses.

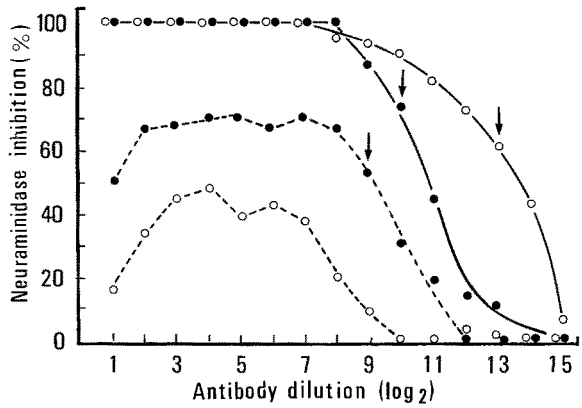

Fig. 2. Comparison of neuraminidase inhibiting activity of a monoclonal antibody using two substrates with different molecular weights. According to the WHO method (25), a series of two-fold dilutions of hybridoma-antibody-(5/205)-containing ascitic fluid were reacted with two NDVs and neuraminidase activity was measured based on the release of neuraminic acid from two different sized substrates of neuraminidase. The inhibition curves were computed from the hydrolysis of N-acetyl neuraminidase lactose (— — —) and fetuin (———). Miyadera strain (o) and isolate 37 (•) were used in the tests. Arrows show the point of antibody dilutions inhibiting over 50 per cent of neuraminidase activity of two strains tested

However, virus neutralizing activity was not detected between this monoclone and Narashino virus (Fig. 3). As can be seen in figure, the 2nd monoclone (5/205) did not produce zone areas free from plaque, indicating the lack of virus neutralizing activity. With the exception of 2 of the strains tested, the 3rd monoclone (5/220) was found to have neutralizing activity. Neutralizing activity detected by paper-disk methods was confirmed by plaque titration assays in primary CE cells, and the results obtained in both tests were consistent. The disk tests were considered to be useful for routine screening of neutralizing antibody because RDE-untreated serum or antibody can be used in these tests.

Antigenic Analysis of NDV-Like Isolates by HI and NI Tests

In addition to 5 reference strains, a total of 19 NDV-like viruses isolated from caged and wild birds from 1976 to 1981 were characterized antigenically

Table 2. *Effect of monoclonal antibodies on different biological activities of reference strains of Newcastle disease virus*

	Monoclonal antibodies																			
	1/29					5/205					5/220					3/Taka				
Viruses	HI	NI	NT	HLI	IDD	HI	NI	NT	HLI	IDD	HI	NI	NT	HLI	IDD	HI	NI	NT	HLI	IDD
Miyadera	+[a]	−[b]	+	+	+	+	+	−	+	+	+	−	+	+	+	+	−	+	+	−
Ishii	+	−	+	+	+	−	−	−	−	+	−	−	+	+	−	+	−	+	−	+
Narashino	+	−	−	+	+	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−
B-1	+	−	+	+	+	+	−	−	+	−	+	−	+	+	−	+	−	−	+	−
Sato	+	−	+	+	+	−	−	−	−	−	−	−	−	+	−	+	−	−	+	+

The assessment of antibiological activities of monoclonal antibodies were based on hemagglutination-(HI), neuraminidase-(NI), hemolysis-(HLI) and growth-inhibition of five reference strains. Neutralizing ability of each monoclonal antibody was detected by paper-disc and plaque methods.

[a] Failed to react in each test

[b] Absence of inhibition even at lowest dilution

+ or − in immuno-double-diffusion (IDD) tests represents absence and presence of precipitin lines respectively

with the panel of monoclonal antibodies (Table 1). Reactions with monoclonal antibody (1/29) to viruses demonstrated similar reactivity patterns with all viruses examined, indicating that these antibodies recognised an epitope, which has been conserved in all viruses for some years (Table 1). The same viruses were subsequently analyzed by NI tests with this monoclone and were found to possess NA activity non-reactive with it. Using

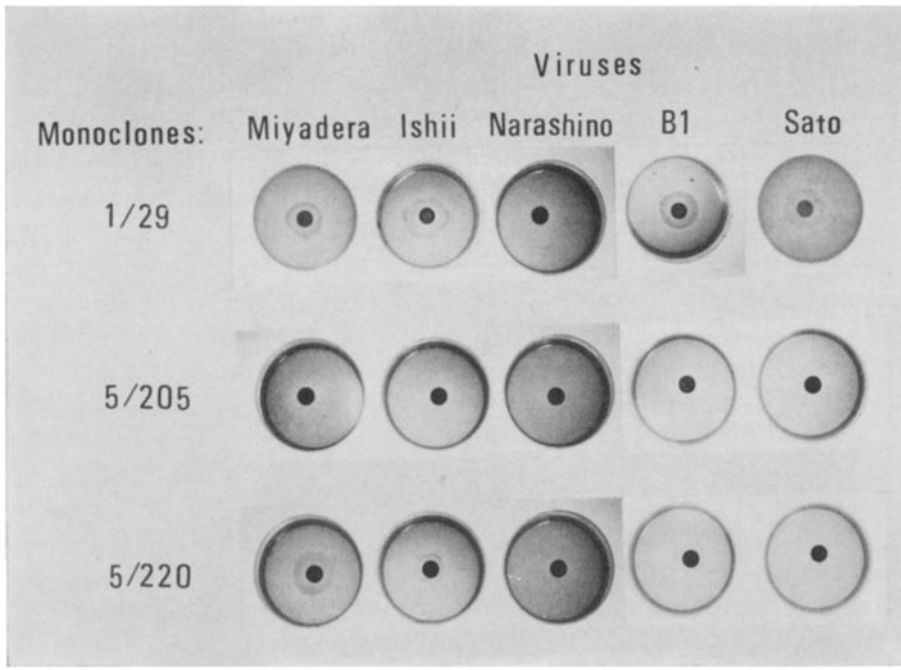

Fig. 3. Screening of neutralizing activity of three monoclonal antibodies by the paper-disc method. Protected areas can be seen around paper-discs dipped in monoclonal antibodies (1/29, 5/205, 5/220). Each filter paper contained 40 µl of RDE-untreated mouse ascitic fluid from antibody producing hybridomas

two monoclone (5/205, 5/220), 7 strains isolated between 1947 and 1978 were shown to be closely similar to each other but these viruses were divided into two groups based on results obtained in the NI tests. In addition, of 24 strains, 10 contained an HN subunit which lacked the epitope recognized by monoclone 3/Taka. Of the reference strains and many isolates tested, only the isolate 37 from 1976 and the Miyadera strain gave the same pattern of reactivity with all monoclonal antibodies. It was concluded that different antigenic variants have been co-circulating since 1930. The monoclonal antibodies proved capable of distinguishing five antigenic variants of NDV using hemagglutination- and neuraminidase-inhibition tests (Table 1).

Discussion

In order to compare the biological and antigenic properties of many NDVs isolated between 1930 and 1981, monoclonal antibodies prepared against the Miyadera strain of NDV were examined by different tests. The characteristics of four monoclonal antibodies were summarized in Table 2. Unlike orthomyxoviruses (5, 10, 11, 25), with the paramyxovirus HN subunit the functional significance of each monoclonal antibodies is not well understood in relation to their steric structure. However, it was of interest to know that monoclonal antibody 5/205 gave a strong precipitin line with Niigata 423 virus but failed to inhibit its hemagglutinating activity. These results suggest that epitope recognized by this monoclone might be located in a peptide area separate from the HA active site of the HN molecules.

The biological activities of the NDVs analyzed in the present study suggested that active sites of hemagglutinin and neuraminidase on HN molecules were quite distinct from each other and might vary with the strain for example monoclone 5/205 inhibited the hemagglutinating activity of 7 strains tested, but, affected the neuraminidase activity of only two of them. Recent work, with paramyxovirus revealed that the inhibitory activity of monoclonal antibodies against neuraminidase of the virus varied according to the kinds of substrate used and their molecular sizes (20). The data obtained in the present study confirm this (9) and this suggests that the epitope recognized by monoclone 5/205 is located in an area near the neuraminidase activity but not in the active site. HI and NI tests with three monoclones 1/29, 5/205 and 3/Taka lacked neuraminidase inhibiting activity, although they were able to inhibit hemagglutinating activity; these results suggest that the epitopes recognized by these clones were situated in the HN molecule away from the area of the polypeptide related to neuraminidase activity. The overall results obtained with these 3 monoclones and all the viruses suggest the presence of three different antigenic determinants on the polypeptides of the HN subunits.

Although previous papers described paper-disc neutralization tests with different viruses (8) we established paper-disc neutralization methods using $LLCMK_2$ cells and a number of NDVs. One monoclone (5/205) was found to lack virus-neutralizing activity despite its high hemagglutination inhibition activity. Furthermore the HI activity of monoclones was not always consistent with neuraminidase-, hemolysis- and growth-inhibiting activities. Although it is generally thought that the fusion process is an essential event in an early stage of paramyxovirus infection, some of the monoclonal antibodies with inhibitory activity against hemolysis failed to neutralize virus multiplication. These results were also shown by plaque neutralization tests with primary CE cells, indicating that paper-disc methods can be routinely used.

Recent monoclonal work demonstrated the presence of four distinct antigenic domains on the HN molecule of human type 1 virus (29), and GOSWAMI and RUSSELL (7) reported the monoclonal antibodies to HN of SV5, which differentiate antigenic differences among simian and human parainfluenza type 2 viruses. Until 1982 antigenic variations of the HN subunits of NDV were not fully understood although a number of papers reported the antigenic characterization of numerous NDVs and isolates (1, 2, 6, 14, 18). However, RUSSELL and ALEXANDER (19) showed antigenic change in HN subunits of NDV based on the binding of 9 monoclonal antibodies to different NDVs and isolates. Our study on monoclonal antibodies defined antigenic change in HN molecules of NDVs, indicating that, these changes are not recent events. The results obtained in the present study and the recent finding by RUSSELL and ALEXANDER (19) revealed that a number of NDV variants have been circulating in a wide range of wild, caged and domesticated birds in many parts of the world since 1930.

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Authors' address: Dr. M. ISHIDA, Department of Virology and Rickettsiology, National Institute of Health, 10—35, Kamiosaki 2-Chome, Shinagawa-ku, Tokyo 141, Japan.

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