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ATTEMPTS AT THE DETECTION OF ANTIBODIES TO NEWCASTLE DISEASE VIRUS BY HAEMOFUSION-INHIBITION TEST

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Summary. — Two modifications of a haemofusion-inhibition test (HFI-1 and HFI-2) were applied for the titration of antibodies to Newcastle disease virus (NDV) in chicken sera. Statistical analysis revealed a positive correlation of the HFI-1 antibody titres with those measured by the standard haemagglutination-inhibition (HI), virus neutralization (VN) and haemolysis-inhibition (HLI) tests. The same appeared true when the HFI-2 antibody titres were compared with the HI, VN, and HLI tests. Except for the several sera collected from birds immunized with formalin-inactivated vaccine, the HFI-2 antibody titres of individual serum samples were usually lower than those determined by HFI-1. The interpretation of these differences as well as some advantages and disadvantages of the proposed test are discussed.

Key words: Newcastle disease virus; antibody; haemofusion-inhibition test

Introduction

Newcastle disease virus (NDV) is a prototype of genus Paramyxovirus. It is well known that the envelope of NDV, like that of many paramyxoviruses, contains two kinds of surface-projecting spikes. The HN glycoprotein possesses both haemagglutinin and neuraminidase activities and is involved in virus attachment (Scheid and Choppin, 1973; Seto *et al.*, 1973) while the F glycoprotein is responsible for virus penetration (Scheid and Choppin, 1974; Knutton, 1978). The latter glycoprotein has been also shown to mediate virus-induced haemolysis and cell fusion. In contrast to extensive studies reported on haemolysis (Bratt and Clavell, 1972; Clavell and Bratt, 1972; Apostolov and Waterson, 1975) and fusion of the cells in monolayer cultures (Kohn, 1965; Reeve and Poste, 1971; Reeve *et al.*, 1972; Poste and Waterson, 1975). NDV-induced fusion of erythrocytes has so far received only limited attention (Terry and Ho-Terry, 1976; Knutton *et al.*, 1977). In our preliminary observation (Trybala, 1987) extensive NDV-induced fusion of hen and chick embryo red blood cells was found at pH 5.5 but at neutral pH there was little or no fusion.

Conventional methods for assessing the immune response of fowls to NDV rely mainly on the demonstration of humoral immunoglobulins to virus envelope glycoproteins by HI, HLI, and VN tests. In this study attempts were made to establish a haemofusion-inhibiton (HFI) test for the detection of antibodies against NDV.

Materials and Methods

Virus. The lentogenic LaSota, B1 and mesogenic Roakin strains of NDV were used. For propagation they were inoculated into the allantoic cavity of 10-day-old embryonated eggs at an input multiplicity of 0.0001 egg infections dose (EID_{50}) per cell, calculated on the basis of data provided by Cuadra (1975). Infectious allantoic fluid was harvested after 48 hr and unless otherwise stated, it was stored at 4 °C and used within one week.

Red blood cells. Hen and chick embryo red blood cells were used. The latter were obtained by bleeding the 13 to 14-day-old embryo into the allantoic cavity. Both kinds of cells were triple washed with PBS-A (without calcium and magnesium ions) and used in the fusion experiments within 4 hr after collection.

Erythrocyte fusion test. The method of Kitame *et al.* (1982) with some modifications (Trybala, 1987) was used. The intensity of observed fusion was expressed in two ways: by the method of Väänänen and Kääriäinen (1980) but in five-degree graduation (method I), and as a fusion index (method II) according to the formula described by Homma and Tamagawa (1973).

Haemagglutination and haemagglutination-inhibition (HI) tests were carried out by the standard macromethods.

Haemolysis-inhibition (HLI) test. LaSota strain which had been subjected to ten cycles of freezing and thawing and then diluted in PBS-A (to achieve four-fold lower haemolysis) was used as antigen. The HLI test was performed as follows: to 0.25 ml of two-fold serial dilutions of serum in PBS-A equal volumes of virus antigen were added. The mixture of virus and serum was kept at room temperature for 30 min, after which 0.1 ml of 0.01 mol/l EDTA and 1.4 ml of 2% hen erythrocyte suspension in PBS-A were added. After incubation for 1 hr at 37 °C, the red cells were sedimented by centrifugation and the optical density (OD) of the supernatant was determined at 540 nm. The reciprocal of the final dilution of serum causing 50% reduction of haemolysis was considered as HLI titre.

Virus neutralization (VN) test. The VN test was performed by a micromethod on monolayers of chick embryo lung cells, using serial serum dilutions and 100 $TCID_{50}$ of the Roakin strain.

Sera. Nine-week-old chickens, divided into 3 groups of 8 birds were immunized intramuscularly with various preparations of Roakin strain. The chickens of the first group received live vaccine, based on the virus preparation harvested from eggs 10 hr after infection. The birds of the second and third group were immunized with live, commercial vaccine "R" produced by Biowet, Pulawy, Poland, and with a formalin-inactivated vaccine emulsified in an incomplete Freund's adjuvant respectively. Sera were collected at 7, 14, 35, 65 and 105 days in the first group, at 14 days in the second, and at various times from 2 to 5 weeks post-vaccination in the third group. All serum samples were inactivated at 56 °C for 30 min before use.

Results

Choice of red blood cells and virus antigen

The extent of the fusion of hen (HRBC) and chick embryo red blood cells (CERBC) induced by fresh as well as by 5 times and 10 times frozen and thawed LaSota, B1 and Roakin strains respectively, is presented in Table 1. The most efficient fusion by the fresh virus preparation was observed with the Roakin strain. Freezing and thawing of LaSota preparation enhanced its HRBC- and CERBC-fusing activity. In the case of B1 strain

Table 1. Haemofusing activity of LaSota, B1 and Roakin strain preparations subjected to various number of freezing and thawing cycles

Virus strain	Red blood cells	Cycles of freezing and thawing		
		0	5	10
LaSota	chick embryo	+++ (43.7)	++++ (n.d.)	++++ (n.d.)
	hen	++ (30.7)	+++ (n.d.)	n.d. (n.d.)
B1	chick embryo	+ (12.0)	+++ (n.d.)	n.d. (n.d.)
	hen	++ (19.8)	n.d. (n.d.)	n.d. (n.d.)
Roakin	chick embryo	++++ (77.6)	++++ (n.d.)	++++ (n.d.)
	hen	+++ (57.2)	+++ (n.d.)	+++ (n.d.)

Note: In parenthesis the fusion index values calculated by the method II; + to ++++ – the extent of fusion estimated by the method I; n.d. – not determined, due to virus-induced lysis of red blood cells. For further explanations see Materials and Methods.

similar effect could be observed with CERBC. However, erythrocyte fusion by the frozen and thawed preparations was accompanied by lysis which made impossible to determine the fusion index, or to determine the extent of fusion. Haemofusing activity of Roakin strain was fully expressed before treatment and remained unchanged throughout. Moreover, it appeared that CERBC were rather aggregated than fused when lower doses of both Roakin and LaSota strains were used (data not shown). For the above listed reasons, fresh Roakin strain preparation and HRBC were chosen for the test.

Table 2 shows the HRBC-fusing activity of Roakin strain in relation to its concentration (expressed in HAU). The smallest amount of virus necessary to induce satisfactory degree of fusion was 40 HAU for the given

Table 2. Roakin strain-induced fusion of hen erythrocytes in relation to the number of its haemagglutination units (HAU)

HAU	Degree of fusion[a]	
	Method I	Method II
640	+++	58.1
320	+++	59.4
160	+++	55.7
80	+++	52.7
40	+++	41.5
20	++	24.6
10	++	21.2
5	+	8.3

[a] Average values out of 5 determinations.

Table 3. Geometric mean HI, HLI, VN, HFI-1 and HFI-2 antibody titres in sera of chickens vaccinated with the live vaccine

Days after vaccination	Serologic test				
	HI	VN	HLI	HFI-1	HFI-2
7	78	29	16	22	19
14	1580	256	222	454	190
35	987	436	147	349	150
66	105	104	19	37	31
109	133	76	17	35	30

number of hen erythrocytes. Since haemofusing activity of Roakin strain preparations harvested from individual eggs did not always closely correspond with their haemagglutination titres (data not shown), the virus preparation with fusion index value of about 40 % was chosen as antigen.

Haemofusion-inhibition test procedure

Two versions of the haemofusion-inhibition test are proposed, that is HFI-1 and HFI-2. The HFI-1 test was performed as follows: to 0.25 ml of serial two-fold dilutions of serum, an equal volume of virus antigen (fusion index about 40%; approximately 40 HAU) was added. The mixture was kept for 30 min at room temperature, then transferred into an ice-water bath, and then 0.5 ml of 0.025% HRBC suspension was added. After 45 min adsorption at 2—4 °C, the cells were sedimented at 250× g for 5 min, and the supernatant was carefully discarded. Subsequently, 0.2 ml of 0.02 mol/l acetate buffered saline, pH 5.5, containing Ca^{2+} ions and bovine serum albumin (Kitame *et al.*, 1982) was added to the sedimented cells. After incubation for 15 min at 37 °C, 50 µl of 5% glutaraldehyde in the

Table 4. HI, VN, HLI, HFI-1 and HFI-2 antibody titres of individual serum samples from chickens vaccinated with live virus vaccine

Bird no.	Serologic test				
	HI	VN	HLI	HFI-1	HFI-2
1	1280	531	320	480	320
2	1280	371	160	480	240
3	2560*a*	224	640	640	320
4	2560	371	640	320	160
5	1280	447	160	320	160
6	640	133	120	320	80
7	2560	531	640	320	160
8	1280	266	60	160	160

Table 5. HI, VN, HLI, HFI-1 and HFI-2 antibody titres of individual serum samples from chickens vaccinated with the formalin-inactivated vaccine

Bird no.	Serologic test				
	HI	VN	HLI	HFI-1	HFI-2
1	<10	<5	<10	<10	<10
2	40	28	30	10	20
3	160	112	80	80	80
4	<10	<5	<10	<10	<10
5	80	33	30	20	15
6	320	224	80	40	120
7	40	23	10	20	10
8	20	14	20	10	<10

same buffer was added. After shaking, 35 µl of this mixture was placed on a slide and spread over a surface of one cm^2. The results were read under the microscope at a magnification of × 150.

In HFI-2 test the virus antigen and erythrocytes were mixed at ratio of 1 : 2 (components as used in HFI-1 test), and kept for 30 min in an ice-water bath. Then 0.75 ml of this virus-erythrocyte suspension was added to 0.25 ml of previously cooled to 4 °C serial two-fold dilutions of serum. After shaking the test tubes were kept for 45 min in the ice-water bath. The cells were next sedimented at 250 × g for 5 min and the rest of the procedure was made as described for HFI-1 test.

The reading of the HFI titre should be based on a 50% reduction of the fusion index. However, to make the test less time-consuming, the highest serum dilution in which no more than 7 polyerythrocytes in ten fields of view were found, was arbitrary taken as the HFI-1 or HFI-2 titre. Figs. 1—4 show the typical pictures seen during the measurement of antibodies with HFI-2 test (in a serum sample with the HFI-2 titre of 1 : 160). In low serum dilutions individual, disaggregated erythrocytes, similarly as in the erythrocyte control were found (Fig. 1). At the serum dilutions four-fold and two-fold lower than the HFI-2 antibody titre, aggregated, swollen and occasionally fused erythrocytes (polyerythrocytes) were observed (not shown). Polyerythrocytes were seen at the serum dilution of 1 : 160 (Fig. 2) and higher (Fig. 3) as well as in the virus antigen control (Fig. 4).

Comparison of HFI tests with the standard HI, HLI and VN tests

A total of 56 serum samples were examined in HI, HLI, VN, HFI-1 and HFI-2 tests. The geometric mean antibody titres in sera of chickens of group I are presented in Table 3. The antibody titres of individual serum samples from birds vaccinated with live (group II) and inactivated vaccine (group III) are shown in Tables 4 and 5, respectively. Except for one serum sample (Table 5) all sera positive in HI, HLI and VN tests were also positive in HFI-1 and HFI-2 tests. Linear regression analysis of the antibody titres

in birds vaccinated with live vaccine (group I and II) revealed correlation coefficients r = 0.91, 0.93 and 0.87 when the HFI-1 test was compared with HI, HLI and VN test, respectively. In comparing the HFI-2 titres with HI, HLI and VN ones the correlation coefficient values were r = 0.82, 0.81 and 0.72, respectively. Except for a few serum samples HFI-2 antibody titres were usually lower than the HFI-1 ones.

Discussion

NDV was shown to cause extensive haemofusion at pH 5.5 (Trybala, 1987). At this pH only approximately 40 HAU of Roakin strain per the amount of hen erythrocytes used in our experimental conditions appeared to be necessary to induce satisfactory degree of fusion. Based on this, two versions of the HFI test were established in this study. The term of haemofusion-inhibition was proposed on the analogy of haemagglutination-inhibition or haemolysis-inhibition. A positive correlation was found between the HFI-1 antibody titres and those measured by the standard HI, HLI and VN tests. The same appeared true when HFI-2 antibody titres were compared with HI, HLI and VN antibodies. This indicates, that both HFI-1 and HFI-2 tests may be useful for the detection of antibodies to NDV.

Except for the several serum samples, HFI-2 antibody titres were usually lower than HFI-1 ones. This probably resulted from the different procedures employed in these tests. Namely, in the HFI-1 test (similarly as in the H1 and HLI tests), the virus and antibodies were reacted first and then erthrocytes were added. Consequently, haemofusion in HFI-1 test was mainly inhibited as a secondary effect of the prevention of virus attachment to the erythrocyte. In HFI-2 test the procedure was changed. The antibodies were added to the virus already adsorbed at low temperature to erythrocytes. At this temperature, the virus particle does not penetrate into the cell, therefore, there is a theoretical possibility to inhibit the activities associated particularly with the virus F glycoprotein. The findings, that antibodies against Sendai virus (Howe and Morgan, 1969) or against the F glycoprotein of SV5 (Merz *et al.*, 1981) inhibited viral activities mediated by this glycoprotein, even when they were added after virus attachment to erythrocyte, support such a possibility. Also some HFI-2 antibody titres found in sera from birds immunized with formalin-inactivated vaccine, cannot rule out this possibility, because it was shown, that sera directed against formalin-inactivated Sendai virus contained anti-F antibodies (Örvell and Norrby, 1977). The importance of anti-F antibodies in preventing the spread of paramyxovirus (SV5) infection was emphasized by Merz *et al.* (1980). In the case of NDV, monoclonal antibodies to the F glycoprotein appeared to have significant titres of virus neutralization (Russell *et al.*, 1983; Abenes *et al.*, 1986), and were able to protect chickens better against virus challenge than anti-HN antibodies (Meulemans *et al.*, 1986).

Inhibition of the ability of NDV to cause cell fusion has so far been studied in cell cultures (Kohn, 1965; Umino *et al.*, 1984). The proposed HFI test

seems to be less time-consuming, needs no sterility and can be applied even in strains which have little cell culture-fusing capacity (Poste and Waterson, 1975). On the other hand, the HFI test as compared with the standard HI and HLI tests is more complicated and time-consuming in the reading of antibody titre (despite of the proposed simplification). Moreover it should be stressed, that NDV haemofusing activity seems to have somewhat similar nature to viral haemolytic property. Its level may depend on many environmental factors acting upon the viral envelope during the virus multiplication, storage or purification. This must be also taken into account whenever viral antigen for the test is prepared.

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Explanation of Micrographs (Plates XXIV–LXXVIII):

Figs. 1–4. Examples of the measurement of antibodies in HFI-2 test.
Disaggregated erythrocytes at a dilution of 1 : 40 (1); polyerythrocytes at serum dilutions of 1 : 160 (2) and 1 : 320 (3); virus control (4); arrows indicate polyerythrocytes (640 ×)