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Participation of Cytophilic Antibody in Enhancement of Interferon Production in Macrophages by Under-Neutralized NDV

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With 1 Figure

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Summary

Enhancement of interferon production in mouse peritoneal macrophages by Newcastle disease virus (NDV) under-neutralized with whole antiserum or its IgG fraction was inhibited in cells pretreated with iodoacetamide, sodium nitrite or formaldehyde, which blocked adsorption of cytophilic antibody to macrophages. Mouse embryo primary culture cells had no receptor for cytophilic antibody, and in such a cell culture, under-neutralized NDV did not enhance interferon production. Antiserum enhanced interferon production by NDV which had adsorbed to cell surfaces, but did not affect NDV that had penetrated the cells.

1. Introduction

Previous experiments [Azuma *et al.* (2)] demonstrated that the production of interferon in macrophage cultures by Newcastle disease virus (NDV) was enhanced by under-neutralization of the virus with homologous antiserum, whereas interferon production was not increased when the virus was completely neutralized. Under-neutralized NDV adsorbed on normal macrophages at a higher rate than did untreated NDV.

Recent reports have described cytophilic antibody, as a 7S γ-globulin in certain immune sera which has the property of attaching to macrophages [Berken and Benacerraf (4), Boyden (5, 6), Sorkin (15)]. Howard and Benacerraf (9) and Davey and Asherson (7) showed that the macrophage receptor for cytophilic antibody was destroyed by many reagents including iodine, periodic acid, sodium nitrite, iodoacetamide and phospholipase A. It therefore became desirable to determine the effect of these reagents on interferon production in macrophages by under-neutralized NDV. The results indicate that the enhancement of interferon production can be attributed to a cytophilic antibody.

2. Materials and Methods

2.1. Viruses

The Miyadera strain of NDV was grown in embryonated eggs, and the infectious allantoic fluid was used as source of virus. The hemagglutinating activity (HAU) was 2,560 HAU/ml and the infectivity was $10^{9.3}$ plaque forming units (PFU)/ml. The Indiana strain of vesicular stomatitis virus (VSV) was inoculated into primary cultures of chick embryo cells. After incubation at 37° C for 24 hours, culture fluids was harvested and used as stock virus. The infectivity was $10^{8.0}$ $TCID_{50}$/ml.

2.2. Macrophage

Peritoneal macrophages were collected from dd/MK mice 4 days after intraperitoneal injection of 1 ml of 2% (w/v) glycogen. The cells were washed once with Hanks' buffered saline solution (Hanks' BSS), suspended at a concentration of 2×10^6 macrophages/ml in Eagle's minimal essential medium supplemented with 5% bovine serum (MEM-5%BS).

2.3. Antisera

Hyperimmune anti-NDV rabbit serum (NDV-AS) and hyperimmune anti-sheep red blood cell rabbit serum (RBC-AS) were prepared in rabbits by four intramuscular injections at 10 day intervals with 2 ml of mixture of antigen and an equal volume of Freund's complete adjuvant. In the case of NDV-AS, an additional 4 ml of NDV was then injected intravenously. Rabbits were killed 7 days after the last injection and their blood collected. The hemagglutination inhibiting activity (HIU) of NDV-AS was 25,600 HIU/ml; the hemolytic activity of RBC-AS was 4,000 units/0.5 ml.

2.4. Fractionation of Antiserum

The γ-globulin of NDV-AS was precipitated by adding saturated ammonium sulphate to give a final concentration of one third saturation. Pellets were dissolved in 0.01 M phosphate buffered saline solution (PBS, pH 7.4), and precipitation was repeated 5 times after elimination of fibrinogen by 20% saturation with ammonium sulphate. The final pellets were dissolved in PBS and dialyzed against PBS. Purified γ-globulin of NDV-AS was fractionated on a Sephadex G-200 column (2.5×90 cm) with PBS. Fractions of 5 ml were sampled and peak fractions pooled on the bases of OD_{280} measurements. Pooled samples were concentrated by dialysis against 25% (w/v) Carbowax #6000 in PBS. The IgG fraction showed 51,200 HIU/ml and 37 mg protein/ml; the IgM fraction showed no hemagglutination inhibiting activity.

2.5. Neutralization of Virus

NDV was neutralized by incubating the virus with an equal volume of serial dilutions of antiserum at 37° C for 1 hour.

Kinetics of neutralization of NDV were shown in Figure 1, in which 256 HAU/ml ($10^{8.3}$ PFU/ml) of NDV were mixed with 2,560 HIU/ml or 25 HIU/ml of NDV-AS. No reduction of infectivity of NDV was observed in mixture at 1:0.1 ratio of HAU of NDV to HIU of antiserum.

2.6. Interferon Production and Titration

Macrophage suspensions (2×10^6 macrophages/ml) were inoculated with 100 PFU of NDV per cell. Aliquots of the culture fluid were harvested 6 hours later and subjected to two cycles of centrifugation at 105,000*g* for 1 hour. The final supernatant fluids were used as interferon samples. In preparing the interferon samples, treatment of interferon materials at pH 2 was renounced, since the interferon produced in macrophage cultures consists of several kinds of molecular weight and the high molecular weight interferons are relatively labile to heat at 56° C and pH 2 treatment [AZUMA *et al.* (1), SMITH and WAGNER (14)]. Interferon assay was carried out by the method of CPE reduction in secondary cultures of mouse embryo (ME) cell infected with VSV as follows: Interferon samples were diluted serially four-fold-wise with maintenance

medium and 1 ml of each dilution was added to two tubes of ME cells. After incubation at 37° C for 24 hours, these cultures were replaced with $10^{1.5}$ $TCID_{50}$/ml of VSV. The interferon titers were expressed as the reciprocal of the maximum dilution showing 50% reduction of CPE, which was interpolated by REED and MUENCH's method (13).

2.7. Reagents.

GR grade of 2-mercaptoethanol, iodoacetamide, sodium nitrite, and formaldehyde were purchased from the Nakarai Chemicals Ltd., Kyoto. These chemicals were dissolved in Hanks' BSS.

2.8. Rosette Formation Test for Cytophilic Antibody

Rosette formation test was carried out according to the indirect method of HOWARD and BENACERRAF (9). Macrophage suspension (5×10^5 cells/ml) was seeded on cover glasses in Petri dishes and incubated for 12 hours at 37° C in a CO_2-incubator for attachment to occur. The cover glasses were then washed with PBS, dipped into reagent solutions of various concentrations, and incubated for 1 hour at 37° C. These pretreated cover glasses were covered with 1% sheep red blood cells sensitized with RBC-AS diluted 1:500, incubated for 30 minutes at room-temperature in a moist chamber, and then washed gently with PBS. Rosette formation was observed microscopically.

The sensitized sheep red blood cells were adheared on surface of macrophages but not on lymphocytes and polymorphonuclear leukocytes. These results show that the indirect method reveals the characteristics of cytophilic antibody but not opsonin [PARISH (12)].

3. Results

3.1. Effect of Multiplicity of Inducer on Interferon Production

Macrophage suspensions were inoculated with NDV at 100, 50 or 5 PFU/cell, and incubated at 37° C. After 6 hours, the culture fluids were sampled and assayed for interferon activity. Table 1 shows that interferon production increased with the dose of inducer.

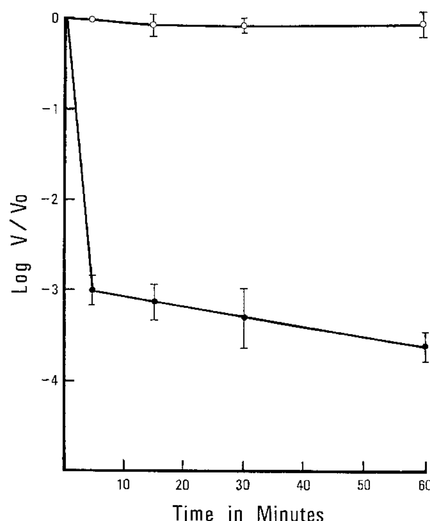

Fig. 1. Kinetics of NDV neutralization by immune serum. The open circles represent virus survivors in virus-antiserum mixture at 1:0.1 ratio of HAU of NDV to HIU of antiserum. The closed circles represent virus survivors in mixture at 1:10 ratio of HAU of NDV to HIU of antiserum

3.2. Effect on Interferon Production of Treatment of Macrophages with Various Reagents

In preliminary experiments, the treatment of macrophages with sodium nitrite, iodoacetamide, or formaldehyde at a concentration of 100 mM, 50 to 10 mM, or

Table 1. *Effect of Multiplicity of Inducer on Interferon Production in Macrophages*

Inducer		Interferon activity at 6 hours	
Virus	PFU/cell	Exp. 1	Exp. 2
NDV	100	640	320
NDV	50	160	160
NDV	5	20	<20

125 mM, respectively, inhibited rosette formation, but no inhibition was caused by treatment with these reagents at lower concentration (Table 2). Treatment with 2-mercaptoethanol at a concentration of 10 mM that did not damage the cells did not inhibit rosette formation. With these pretreatment conditions, interferon production by macrophages was not affected, except for slight reduction by macrophages pretreated with 100 mM 2-mercaptoethanol or 50 mM iodoacetamide (Table 3).

Table 2. *Effect of Treatment with Various Reagents on Macrophage Receptor for Cytophilic Antibody*

Macrophage-settled cover glasses were washed with PBS, soaked in reagents of desired concentration, and incubated for 1 hour at 37° C. These cells were covered with 1% sheep red blood cell suspension sensitized with anti-sheep red blood cell-rabbit serum, incubated for 30 minutes at room temperature, and then gently washed with PBS. Rosette formation was observed microscopically

Treatment of cells with		Rosette formation
2-Mercaptoethanol	100 mM	± [a]
2-Mercaptoethanol	10 mM	+
2-Mercaptoethanol	1 mM	+
Sodium nitrite	100 mM	—
Sodium nitrite	10 mM	+
Sodium nitrite	1 mM	+
Iodoacetamide	50 mM	—
Iodoacetamide	10 mM	∓
Iodoacetamide	1 mM	+
Formaldehyde	125 mM	—
Formaldehyde	12.5 mM	∓
Formaldehyde	1.25 mM	+
Hanks' BSS		+

\+ 81 to 100% of the cells show complete rosettes
± 51 to 80% of the cells show complete or incomplete rosettes
∓ less than 50% of the cells show incomplete rosettes
— no rosette formation is observed

[a] Almost half of the cells are damaged.

Macrophages pretreated with various reagents were inoculated with infective NDV or under-neutralized NDV at a multiplicity of 100 PFU/cell, and the culture fluids were harvested 6 hours later. The results, summarized in Table 4, showed no enhancement of interferon production in macrophages pretreated with iodoacetamide, sodium nitrite and formaldehyde at the concentration which inhibited rosette formation.

Table 3. *Effect of Treatment of Macrophages with Various Reagents on Interferon Production*

Macrophage suspensions were centrifuged and the cells were resuspended into reagents of required concentration. After incubation at 37° C for 1 hour, cells were washed twice with Hanks' BSS, resuspended into MEM-5%BS at a concentration of 2×10^6 cells/ml, inoculated with infective NDV at 100 PFU/cell, and incubated at 37° C for 6 hours. Interferon activity of culture fluid was assayed

Pretreatment of cells with		Inducer	Interferon activity	
			Exp. 1	Exp. 2
2-Mercaptoethanol	100 mM	NDV	160	ND[a]
2-Mercaptoethanol	10 mM	NDV	320	ND
2-Mercaptoethanol	1 mM	NDV	320	ND
Sodium nitrite	100 mM	NDV	320	320
Sodium nitrite	10 mM	NDV	320	320
Sodium nitrite	1 mM	NDV	320	320
Iodoacetamide	50 mM	NDV	80	160
Iodoacetamide	10 mM	NDV	160	160
Iodoacetamide	1 mM	NDV	320	320
Formaldehyde	125 mM	NDV	320	320
Formaldehyde	12.5 mM	NDV	320	320
Formaldehyde	1.25 mM	NDV	320	640
Hanks' BSS		NDV	320	320

[a] not done

3.3. Effect of Isolated IgG and IgM Fractions on Interferon Production

An experiment was made to determine the extent to which antiserum fractions (IgG or IgM) enhanced interferon production when under-neutralized NDV was used as inducer. Table 5 shows that when NDV was under-neutralized with IgG, its ability to induce interferon was markedly increased; this enhancement of interferon induction was inhibited by pretreatment of macrophages with iodoacetamide or formaldehyde. IgM did not enhance interferon induction, when NDV was treated with IgM at a concentration equivalent to the protein content of an effective dose of IgG.

3.4. Interferon Production in Mouse Embryo Cell Cultures

Rosette formation tests for cytophilic antibody on mouse embryo cell (ME) primary cultures were completely negative. The following experiment was made to determine whether interferon production by under-neutralized NDV was enhanced in ME cultures which had no receptor for cytophilic antibody. Four day

Table 4. *Effect of Treatment of Macrophages with Various Reagents on Interferon Enhancement by Under-Neutralized NDV*

Experimental conditions are the same as described in Table 3, except that infective NDV and under-neutralized NDV (mixture at 1:0.1 ratio of HAU of NDV to HIU of anti-NDV serum) were used as inducers

Treatment of cells with	Interferon activity 6 hours postinoculation with				Ratio of interferon activity (1)/(2)	
	Exp. 1		Exp. 2			
	(1) Under-neutralized NDV	(2) Infective NDV	(1) Under-neutralized NDV	(2) Infective NDV	Exp. 1	Exp. 2
2-Mercaptoethanol 100 mM	320	80	ND	ND	4	—
2-Mercaptoethanol 10 mM	640	160	ND	ND	4	—
2-Mercaptoethanol 1 mM	320	160	ND	ND	2	—
Iodoacetamide 10 mM	40	80	40	40	0.5	1
Iodoacetamide 1 mM	640	160	320	160	4	2
Iodoacetamide 0.1 mM	640	160	640	160	4	4
Sodium nitrite 100 mM	160	160	80	160	1	0.5
Sodium nitrite 10 mM	640	160	640	160	4	4
Formaldehyde 125 mM	80	80	160	160	1	1
Formaldehyde 12.5 mM	160	80	160	160	2	1
Hanks' BSS	640	160	640	160	4	4

Table 5. *Interferon Production by NDV Treated with IgG and IgM Fraction of NDV-Hyperimmunized Rabbit Serum*

Macrophages pretreated with 10 mM iodoacetamide or 125 mM formaldehyde as described in Table 3 and untreated macrophages were inoculated with infective NDV or NDV treated with serially diluted IgG or IgM at 100 PFU/cell. In case of IgM-treatment, NDV was mixed and incubated with IgM at a concentration equivalent to the protein content of IgG in case of IgG-treatment

Treatment of cells with	Inducer	Interferon activity at 6 hours
	NDV:IgG (1:1)[a]	80
	NDV:IgG (1:0.1)[a]	2560
	NDV:IgG (1:0.01)[a]	1280
	Infective NDV	640
Iodoacetamide 10 mM	NDV:IgG (1:1)[a]	40
Iodoacetamide 10 mM	NDV:IgG (1:0.1)[a]	640
Iodoacetamide 10 mM	Infective NDV	320
Formaldehyde 125 mM	NDV:IgG (1:1)[a]	40
Formaldehyde 125 mM	NDV:IgG (1:0.1)[a]	320
Formaldehyde 125 mM	Infective NDV	320
	NDV:IgM (0.37)[b]	640
	NDV:IgM (0.037)[b]	640
	NDV:IgM (0.0037)[b]	640
	Infective NDV	640

[a] IgG-treated NDV (HAU of NDV:HIU of IgG).

[b] mg of IgM protein.

ME primary cultures (4×10^6 cells/bottle) were inoculated with infective NDV and neutralized NDV at 100 PFU/cell. Culture fluids were harvested 8 and 24 hours after inoculation and assayed for interferon activity. As shown in Table 6, no enhancement of interferon production was observed in cultures induced with under-neutralized NDV.

These results indicate that under-neutralized NDV did not enhance interferon induction in ME culture, since ME lacked a receptor for cytophilic antibody.

Table 6. *Interferon Production in Mouse Embryo Cells by Infective NDV and Neutralized NDV*

Inducer	Interferon activity at 8 hours	Interferon activity at 24 hours
NDV:NDV-AS (1:2)[a]	<20	20
NDV:NDV-AS (1:0.2)[a]	160	160
NDV:NDV-AS (1:0.02)[a]	80	160
Infective NDV	160	160

[a] Anti-NDV serum-treated NDV (HAU of NDV:HIU of antiserum).

Table 7. *Effect of Anti-NDV Serum on Interferon Inducibility of NDV Adsorbed to and Penetrated into Macrophages*

Macrophage suspensions (3×10^6 cells/ml) were inoculated with active NDV at 100 PFU/cell, incubated, for 2 hours at 4° C, and washed once with Hanks' BSS at 4° C. In experiment 1, NDV-adsorbed macrophages were treated with serially diluted NDV-AS or normal serum (NS) at 4° C for 2 hours, and then incubated at 37° C for 6 hours. In experiment 2, NDV-adsorbed cells were incubated at 37° C for 1 hour before treatment with serially diluted NDV-AS or NS

Exp.	Treatment of NDV-adsorbed cells	HAU of adsorbed NDV HIU of NDV-AS	Interferon activity at 6 hours
1	10^{-1} diluted NDV-AS, 4° C 2 hours	1:16	<20
	10^{-2} diluted NDV-AS, 4° C 2 hours	1:1.6	20
	10^{-3} diluted NDV-AS, 4° C 2 hours	1:0.16	160
	10^{-4} diluted NDV-AS, 4° C 2 hours	1:0.016	80
	10^{-1} diluted NS, 4° C 2 hours	—	40
2	37° C 1 hour, then 10^{-1} diluted NDV-AS, 4° C 2 hours	1:16	80
	37° C 1 hour, then 10^{-2} diluted NDV-AS, 4° C 2 hours	1:1.6	80
	37° C 1 hour, then 10^{-3} diluted NDV-AS, 4° C 2 hours	1:0.16	80
	37° C 1 hour, then 10^{-4} diluted NDV-AS, 4° C 2 hours	1:0.016	80
	37° C 1 hour, then 10^{-1} diluted NS, 4° C 2 hours	—	80

3.5. Effect of Antiserum on Interferon Inducing Ability of NDV Adsorbed to or Penetrated into Macrophages

Ten macrophage suspensions were inoculated with infective NDV at 100 PFU/cell. After incubation at 4° C for 2 hours with stirring, these cells were washed once at 4° C with Hanks' BSS. Five suspensions were resuspended into serially diluted NDV-AS or normal rabbit serum (NS) at 4° C for 2 hours. The others were resus-

pended into MEM-5% BS, incubated at 37° C for 1 hour, and then resuspended into serially diluted NDV-AS or NS at 4° C for 2 hours. All of these treated cells were washed once, resuspended into MEM-5% BS at a concentration of 2×10^6 macrophages/ml, and incubated at 37° C for 6 hours.

The interferon activities of the culture fluids are shown in Table 7. Enhancement of interferon production was not observed in the cultures treated with serially diluted NDV-AS after incubation at 37° C for 1 hour, whereas an increased interferon activity was found in cultures treated with NDV-AS at 1:0.16 ratio of HAU of adsorbed NDV to HIU of NDV-AS without incubation at 37° C. Thus, the antiserum acts on NDV adsorbed to cell surface and gives enhancement of interferon production, whereas penetrated NDV is not affected by antiserum.

4. Discussion

In macrophages treated with reagents which inhibit the adsorption of cytophilic antibody by, presumably, destroying the cell receptor sites [HOWARD and BENACERRAF (9)], enhancement of interferon production by under-neutralized NDV was blocked. On the other hand, stimulation of interferon production by under-neutralized NDV did not occur in ME culture which has no receptor for cytophilic antibody. The enhancing activity of antiserum was a function of IgG. These results strongly suggest that cytophilic antibody participates in adsorption of inducer to macrophages.

MANDEL (10, 11) showed that poliovirus neutralized at a low ratio of antibody to virus adsorbed to HeLa cells better than unneutralized virus, and that the neutralized virus eluted less from the cells. AZUMA *et al.* (2) demonstrated that under-neutralized NDV adsorbed on normal macrophages at a 50 % higher rate than did untreated NDV. By this under-neutralized NDV, a four-fold enhancement of interferon production, resulting from an increased multiplicity of virus, was observed (Table 1 and 4). As shown in Table 7, treatment of adsorbed virus with antiserum enhanced interferon production, whereas no effect was observed in cultures treated with antiserum after penetration of virus. Recent investigations [AZUMA (3)] showed that infective NDV and under-neutralized NDV did not multiply in macrophages and no synthesis of double-stranded viral RNA was detected, though after adsorption, uncoating of these viruses in macrophages proceeded at the same rate. It is assumed that cytophilic antibody does not operate directly in the interferon production of macrophage as inducer, but acts to increase the adsorption of virus on macrophage and to stabilize the interaction between cell and virus, thus, causing enhancement of interferon induction. Increase of uncoating and synthesis of double-stranded viral RNA does not participate in this enhancement. Therefore, a four-fold increase of relevant interferon titers should be considered significant in this virus-cell system.

However, the relationship between cytophilic antibody and neutralizing antibody is to date not known, whereas in the previous report [AZUMA *et al.* (2)] immunological specificity of antibody action in enhancement of interferon production was determined. Recently, UHR (16) and HAMAOKA and KITAGAWA (8) showed that macrophages interacted firmly with antigen complexing with antibody which was prepared by immunization of animals without Freund's complete

adjuvant and which was 19S and 7S γ-globulin, and that the antibody did not operate as cytophilic antibody in the interaction of antigen-antibody complexes with macrophages. Experiment should be undertaken to determine whether treatment of inducers with purified cytophilic antibody enhances interferon induction, and to clarify the relationship between cytophilic antibody and neutralizing antibody.

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