Immunochemical Study of Parainfluenza Virus (Type 2) in Amnion Cells

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Received for publication 16 January 1966

ABSTRACT

DE VAUX ST. CYR, C. Columbia University, New York, N.Y.), AND C. HOWE. Immunochemical study of parainfluenza virus (type 2) in amnion cells. *J. Bacteriol.* **91**:1911–1916. 1966.—Immunoelectrophoretic analysis of stable amnion cells in which parainfluenza virus (type 2) was being actively synthesized revealed at least three precipitating antigens not found in normal cells. These “new” antigens differed from viral neuraminidase and hemagglutinin in both specificity and electrophoretic mobility; their identity and function remain to be elucidated.

Since the initial report concerning the neuraminidase of type 2 parainfluenza virus (CA virus, 1), further work with this agent has shown that, in addition to hemagglutinin and enzyme, at least three other infection-specific antigens appear intracellularly during the course of viral development. The results of this investigation, as they relate to the growth cycle of the virus, form the basis of this report.

MATERIALS AND METHODS

Cell cultures. Stable human amnion cells (1) were propagated in Eagle's basal medium with Hanks' balanced salt solution, supplemented with 10% calf serum.

Virus. Myxovirus parainfluenzae, type 2 (CA virus), obtained from the American Type Culture Collection, had been maintained since 1963 in amnion cell passage. For propagation of virus in bulk, fully grown amnion cell bottle cultures were washed with balanced salt solution, inoculated with undiluted infective culture fluid, and incubated at 37 C for 2 hr. The inoculum was repeatedly redistributed over the cell sheet during the infection period, either by hand or by means of a rocking platform. Maintenance solution was then added in which serum was replaced by lactalbumin hydrolysate at a final concentration of 0.1%. This serum-free medium was also used routinely for maintaining replicate tube cultures used in infectivity titrations.

Virus titrations and neutralization tests. Virus hemagglutination and infectivity titrations, and hemagglutination-inhibition tests were performed as previously described (1). For hemadsorption inhibition, dilutions of serum were added to replicate cultures in which infection with parainfluenza 2 virus

had developed to a stage regularly giving confluent hemadsorption. After 30 min at room temperature (25 C), washed guinea pig erythrocytes (0.5% suspension) were introduced and the tubes were stored at 4 C for 30 to 60 min. After being washed with cold saline, they were examined grossly and microscopically for hemadsorption. The end point was taken as the highest dilution of serum completely inhibiting hemadsorption.

Cell extracts. Bottle cultures of amnion cells were inoculated with parainfluenza virus (type 2) as described above. At suitable intervals after infection, cells were scraped into maintenance solution, centrifuged at $1,000 \times g$, resuspended in $\frac{1}{50}$ the original volume of solution, and disrupted by ultrasonic vibration. After clarification by centrifugation at $1,000 \times g$, the extracts were used within 24 to 48 hr, since it was found that they could be neither stored frozen nor lyophilized without affecting the solubility characteristics of certain of the precipitating antigens. Results reported are therefore based on experiments with freshly prepared extracts. Extracts similarly prepared from uninfected cells, maintained in serum-free medium, served as control material.

Analytical methods. Electrophoretic and immunoelectrophoretic analyses were carried out as described by Grabar and associates (3). Electrophoretic separations were performed at room temperature in 0.06 M Veronal buffer (pH 8.2) with standard microscopic slides (7.7×5.2 , or 7.7×2.6 cm) at 10 ma per slide. Antisera used were in the form of globulin from pooled or individual sera of high antibody content. Viral neuraminidase activity was estimated as previously described (1), with either Collocalia mucoid (4) or *N*-acetylneuraminic acid-lactose as substrate. Agglutination of normal amnion cells was assayed as follows. The confluent cell sheet from one 32-oz prescription-bottle culture was removed from the glass by trypsinization, as for routine cell passage. The cells were washed three times in ethylenediaminetetraacetic

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acid buffer (5) and resuspended in 40 ml of the same buffer containing 0.1% neutral red. To serial twofold dilutions of antiserum, 0.1 ml of cell suspension was added. The tubes were incubated at 37 C for 1 hr and then refrigerated overnight, after which agglutination was read grossly and microscopically.

Antisera. Rabbits were immunized with several types of antigen derived from normal and infected cells, as summarized in Table 1. The preparation of antisera to antigen 1 has already been described (1). For antigen 2, serum-free maintenance solution from bottle cultures of amnion cells infected 7 days previously with parainfluenza 2 virus was centrifuged at $40,000 \times g$ for 3 hr, a 50- to 100-fold concentration of viral hemagglutinin being thus achieved. Such freshly prepared material was used for immunization without further purification. For antigen 3, amnion cells showing confluent hemadsorption at 4 days after infection were scraped into maintenance solution, concentrated by centrifugation, and lyophilized. Normal, uninfected cells, similarly maintained and harvested, served as antigen 4. All antigens were incorporated in complete Freund adjuvant, and were given either subcutaneously (antigen 1, 3 weekly injections) or into the footpads in divided doses totaling 2 mg of antigen per animal (antigens 2, 3, and 4). From 1 to 3 months after this primary immunization, booster doses were given intravenously. All animals were bled out 7 to 10 days after the final injection. In some instances, sera with high titers were pooled and the globulins were concentrated by precipitation with sodium sulfate at 33% of saturation, pervaporation, and dialysis against phosphate-buffered saline (pH 7.4). Globulin from certain of these sera was conjugated with fluorescein or with ferritin for use in correlated fluorescence and electron microscopic studies, the results of which will be reported in a separate communication. Antibody to normal cells and calf serum constituents were removed from antiviral sera by absorption with lyophilized normal amnion cells (10 mg/ml of serum) and calf serum (20 mg/ml of serum). Completeness of absorption was determined by means of double diffusion in agar against the absorbing antigen.

RESULTS

Immunochemical analysis of normal and infected cells. The data summarized in Table 1 show that significant antibody levels against parainfluenza 2 virus were obtained by each immunization procedure, as determined by inhibition of hemagglutination and hemadsorption, and by neutralization of infectivity and neuraminidase. Somewhat more potent antisera, however, were obtained when virus concentrated from infective serum-free maintenance fluid (antigen 2) was used. Immunization with normal amnion cells elicited high titers of agglutinins for viable amnion cells in suspension. Absorption of these sera with calf serum caused no significant change in titer, thereby fortifying the conclusion that the agglutination was

due to antibody directed against cell constituents rather than to residual calf serum on the test cells.

Immunoelectrophoretic analyses were done on extracts of normal cells and on extracts of cells prepared 5 days after viral inoculation. The patterns were developed with antibody to normal and infected cells (antigens 3 and 4) and with antibody to concentrated virus (antigen 1). Each of the cell extracts contained several antigens identifiable as calf-serum components, despite the fact that after viral inoculation the cells had been maintained in serum-free medium. In Fig. 1, calf serum albumin (a) and globulin of intermediate mobility are shown to be present in different concentration in extracts of both normal and infected cells. In the latter, however, at least two antigens (v) were revealed which did not precipitate with antiserum to normal cells and which were absent in extracts of normal cells. A cell-specific antigen of slow mobility was also revealed by the antiserum to infected amnion cells (antigen 3), which was present in extracts of both normal and infected cells (N, Fig. 1 and 2).

Two of the antisera containing infection-specific antibodies were absorbed with lyophilized normal amnion cells and with whole calf serum, as described above. The absorbed sera failed to give any lines of precipitation with either absorbing antigen. Both sera, however, retained antibody for components in the extract of infected amnion cells (Fig. 2). The antiserum to concentrated virus (antigen 1) contained antibody to a third antigen, difficult to demonstrate in unstained immunoelectrophoretic plates, but readily discernible in double diffusion, as shown in the diagram included in Fig. 2. After staining with Ponceau red, the immunoelectrophoretic pattern with absorbed antibody showed all three antigens (Fig. 3). The double line of precipitate (1, Fig. 3) may represent two antigens showing a reaction of partial identity.

To estimate the time of appearance of the precipitating antigens in relation to viral development, replicate amnion cell cultures were inoculated simultaneously with parainfluenza 2 virus, as described. At successive intervals after infection, individual cultures were examined, the fluid being titered for infective virus and hemagglutinin, and extracts of the corresponding cells being titered for infectivity, hemagglutinin, and neuraminidase. Each cell extract was also subjected to immunoelectrophoretic analysis with several antisera. With respect to the development of virus, a significant increase in free and cell-associated infective virus and hemagglutinin occurred by 24 hr, the titers reaching a maximum at 48 hr. These levels were maintained through the 5th day after

TABLE 1. *Antisera*

Antigen	Serum titer ^b			
	Inhibition of		Neutralization of	
	Hemagglutination (3 to 5 HAU)	Hemadsorption (4+)	Infectivity (10 ³ TCID)	Neuraminidase (% reduction) ^b
1. Cell-associate + concentrated virus (5 × 10 ⁶ TCID/ml) ^d	512-1024	40-80	160 ^c	40
2. Ultracentrifuge - concentrated virus (3,200 HAU/ml)	2048-6400	>40	640	60
3. Lyophilized infected amnion cells	128-256	20-80	80	25
4. Lyophilized normal amnion cells ^a	0	0	0	0

^a Agglutinins for normal amnion cells were 512 to 2,048. There was no change after absorption with normal calf serum.
^b Given as reciprocal of serum dilution. All preimmunization sera were negative.
^c Globulin from pooled high-titered sera used.
^d See Literature Cited (1).

FIG. 1. Immunoelectrophoretic analysis of extracts of normal amnion cells, and amnion cells 5 days after infection with parainfluenza virus (type 2) developed with antisera to antigens 1, 3, and 4 (Table 1). Symbols: a = calf serum albumin; N = antigen common to normal and infected cells; and v = infection-specific antigens.

FIG. 2. Immunoelectrophoretic pattern of extract of amnion cells 5 days after infection with parainfluenza virus (type 2) developed with antiserum to infected cells (antigen 3, Table 1) and antiserum to concentrated virus (antigen 1, Table 1), the latter containing antibody to a third antigen, as revealed by double diffusion in agar (right).

FIG. 3. Immunoelectrophoretic pattern of extract of amnion cells 5 days after infection with parainfluenza virus (type 2), developed with antiserum to antigen 1 (Table 1) absorbed with lyophilized normal amnion cells and calf serum. Dried and stained with Ponceau red. Numbers 2 and 3 correspond to antigens shown in Fig. 4; 1 may represent a third antigen or two antigens with partial identity.

initiation of infection. Cell-associated neuraminidase activity appeared at 2 days and, likewise, remained at a constant maximum through the 5th day. The new antigens described above were not detectable during the first 24 hr after infection, but were revealed in greatest concentration at the 3rd to 5th day in cells still attached to the glass. The precipitating antigens were not found in cells

which had become detached at later stages. These detached cells, however, still contained considerable hemagglutinin and neuraminidase activity.

Since the appearance of the new soluble antigens in infected cells coincided with the period of maximal production of virus, their identification as hemagglutinin and neuraminidase was attempted. Freshly prepared extracts were absorbed

twice with chicken erythrocytes (final concentration 25%) in the cold, and the absorbed material was reduced to its original volume by pervaporation. Little or no hemagglutinating activity was detected in the extract after absorption, 75 to 100% being recovered by elution from the erythrocytes. This eluate, after concentration by pervaporation, failed to show any precipitation with any of the virus-specific antisera. The soluble infection-specific antigens, however, were still present in the absorbed extract.

The mobility in agar of hemagglutinin and neuraminidase was estimated directly. Extract of cells infected for 5 days was subjected to electrophoretic separation under the same conditions as previously. At the end of the run, the agar was cut at right angles to the path of migration into sections of uniform size, approximately 40 sections per slide. Each section was then incubated at 37 C for 18 hr with an appropriate sample of substrate in 0.15 M phosphate buffer (pH 6.0), and the solution was subsequently analyzed for free *N*-acetylneuraminic acid. Sections from a duplicate sample on the same slide were extracted at 37 C with phosphate-buffered saline, and the extracts were subsequently tested for hemagglutinin. Figure 4 shows the distribution of hemagglutinin and neuraminidase activity in relation to the precipitating antigens previously described. It will be seen that the mobility of both hemagglutinin and neuraminidase is distinctly different from that of the soluble antigens. Whole concentrated virus, subjected to the same conditions of analysis, showed an essentially similar distribution of hemagglutinin and neuraminidase, but none of the precipitating antigens was detected.

In preliminary experiments, not otherwise given in detail, ultracentrifuge-concentrated virus was

treated with ether (2) in an attempt to release hemagglutinin and neuraminidase. In three separate preparations, ribose-free protein was obtained which showed neuraminidase and hemagglutinating activity [0.1 to 1 hemagglutinating unit (HAU) per microgram]. None of these preparations contained any antigen precipitating with the antiviral antisera.

DISCUSSION

At least three precipitating antigens have been detected in stable amnion cells maintained in serum-free medium after inoculation with parainfluenza 2 virus. These new antigens, not found in normal, uninfected cells maintained under similar conditions, were present in greatest concentration during the first 3 to 4 days after infection, during which time infected cells remained attached to the glass and virus production was maximal. The new antigens were not detectable in extracts of cells which had become detached from the glass by the 5th to 7th day after inoculation of cultures. Such cells, however, retained hemagglutinin and neuraminidase activity, probably because of unreleased viral particles. The identity of the "new" soluble antigens is still not certain. It has been shown, however, that they are neither hemagglutinin nor neuraminidase, both of which display electrophoretic mobilities strikingly different from the antigens in question. Moreover, neither hemagglutinin nor neuraminidase, separated from whole viral particles by treatment with ether, precipitated with any of the antisera, although the latter contained high titers of both anti-hemagglutinin and antineuraminidase. In addition, removal of hemagglutinin from cell extracts by absorption with chicken erythrocytes failed to diminish the concentration of the new antigens

FIG. 4. Immunoelectrophoretic pattern of extract of amnion cells 5 days after infection with parainfluenza virus (type 2) developed with antiserum to concentrated virus (2, Table 1). Areas marked HA, NA-ase correspond to position (with respect to starting well) of hemagglutinin and neuraminidase, respectively, in agar from duplicate analyses of the same material.

as revealed in immunoelectrophoretic analysis. The possibility that they might represent viral ribonucleoprotein was suggested by the fact that their appearance coincided with the accumulation, in the cytoplasm of infected cells, of masses of virus-specific antigen demonstrable by specific immunofluorescence. This cytoplasmic antigen, which was present in maximal concentration during active production of virus, may correspond to the "soluble" antigen found with other myxoviruses, such as Newcastle disease virus, the ribonucleoprotein core of which can be shown to have immunological identity with "soluble" antigen extracted from cells infected with the corresponding virus (6). Attempts to demonstrate a similar relationship with the parainfluenza-amnion cell system have been hampered by difficulties in separating viral particles from cell fragments in the culture fluids. Investigation of this aspect of the problem is continuing.

ACKNOWLEDGMENT

This investigation was supported by a Public Health Service General Research Grant and grant

AI03168 from the Institute of Allergy and Infectious Diseases, and by the Damon Runyon Memorial Fund for Cancer Research, Inc.

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