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Immunofluorescent Studies on a New Early Antigenic Product in Reovirus Type 3 Infected Cells

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Abstract

Present studies were performed to examine whether or not the antigen(s) in infected cells with reovirus type 3, particularly which is distinct from virus capsid antigen, could be produced when the virus progeny was not detected.

In complement fixation test, the antigens of early extract prepared from infected cells 1 hour after infection and of purified virus cross-reacted with the antisera against purified virus (V antiserum) and early extract (E antiserum). It was suggested that a certain antigenic substance might be contained in both early extract and purified virus or that the capsid antigen of input virus might be contaminated in early extract. However, the fluorescent antigen which only reacted with E antiserum, not with V antiserum appeared in infected cells as early as from 1 to 4 hours after infection before the appearance of virus capsid antigen. This fluorescent antigen began to decrease after 10 hours and it was still observed at 20 hours, while, the virus capsid antigen appeared at 4 hours after infection, and developed continually during the course of infection.

The effect of actinomycin D or cytosine arabinoside on the development of this antigen was not essential, although the first appearance was postponed about 3 hours. Puromycin and 6-azauridine completely inhibited the synthesis of this antigen. This antigen might be coded for virus RNA.

Introduction

Replication of reovirus has been studied exceedingly on the basis of the genetic function of virus RNA. Ten segments of virus genome RNA are copied specif-

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fically into messenger RNA at different times after infection (Ballamy and Joklik, 1967; Watanabe et al., 1968 a). In addition, RNA polymerase which apparently transcribes messenger RNA in vitro from all segments of parent RNA has been discovered in association with virion (Borsa and Shatkin, 1968; Shatkin and Sipe, 1968; Skehel and Joklik, 1969; Banerjee and Shatkin, 1970). It would be possible that miscellaneous proteins other than virus-associated RNA polymerase might be synthesized early in the infectious cycle for further replication of virus. Actually, synthesis of 3 species of virus structural proteins and virusinduced RNA polymerase have been demonstrated individually early in the infectious cycle (Loh and Shatkin, 1968; Loh and Oie, 1969; Watanabe et al., 1968 b). However, early events of infectious cycle have not been examined closely by immunological method.

In this paper, it will be presented that a new antigenic product in reovirus type 3 infected cells is demonstrated within 1 hour after infection by using indirect immunofluorescent technique.

Materials and Methods

Cell culture and virus: All cell cultures, stable line of porcine kidney (PS) cells, HeLa cells and monkey kidney(MK) cells, were grown in Eagle's basal medium containing 10 % bovine serum, 200 unites/ml of penicillin and 120 γ/ml of streptomycin. In maintainance medium, proportion of serum was decreased to 0.5 persent and calf serum, free from antibody against reovirus, was substituted for bovine serum.

Dearing strain of reovirus type 3 kindly supplied from Japanese National Institute of Health. It was propagated four times in MK cells. The stock virus was prepared from infected PS or HeLa cells. After removal of culture fluids, infected cells were scrapped off by rubberpoliceman, resuspended in maintainance medium in proportion of 10^7 cells per 1 ml, and centrifuged at low speed after 3 cycles of freezing and thawing. The infectivity of the stock virus was given $10^{6.5}/TCD_{50}$ per ml in MK cell monolayers.

Infection of cells and the methods for infectivity, complement fixation and hemagglutination test: Well-dispersed PS cells were infected with the stock virus at the rate of 3×10^6 cells per ml of the virus. The cells were allowed to adsorb the virus at 37℃ for 30 minutes by continuous gentle shaking. The cells were centrifuged, resuspended in maintainance medium containing 8 unites of reovirus type 3 antiserum, kept at 37℃ for 30 minutes to neutralize unadsorbed virus. After washihg the infected cells three times with Dulbecco's phosphate buffer saline (PBS pH 7.2), they were refed with the same volume of maintainance medium, and incubated at 37℃. At intervals after infection, the cell-associated virus yielded from 3×10^6 of cells per ml of PBS was obtained by the method described above and assayed for infectious, complement fixing and hemagglutiating activities.

Infectivity was examined by inoculating of 0.2 ml of serial dilutions of virus into each 3 test tube of MK cell cultures. Cytopathic effect was observed daily for 6 days and the titer of infectivity was calculated by the method of Reed and Muench (1938).

Hemagglutination and complement fixation tests were performed as described by Rosen, D. (1960) and Pereira, H. G., et al. (1959) respectively.

Virus purification: Cell associated virus prepared from infected MK cells was treated twice with an equal volume of trichlorotrifluoroethane at room temperature for 10 minutes. To obtain purified virus, 1 ml of liquid phase was laid on the top of CsCl discontinuous gradient consisted of each 0.8 ml of buoyant density of 1.580 and 1.430, and 0.5 ml of buoyant density 1.315 in cellulose nitrate tube, then centrifuged at 39,000 rpm for 4 hours at 4℃ in Hitachi SPR-40 swing rotor. Eight tenth ml of this cushioned and concentrated virus (buoyalnt density 1.30 −1.38 g/cm) was recentrifuged in CsCl linear density gradient at 30,000 rpm for 18 hours at 4℃ in the same rotor. Linear density gradient was prepared by overlaying each 0.7 ml of CsCl at buoyant density of from 1.60 to 1.10 in increments of 0.1. CsCl was dissolved in 0.01 M Tris-HCl (pH 7.2). Fractions (15 drops each) were obtained by piercing the bottom of the tube and dialysed against PBS at 4℃ overnight. The fractions which showed maximum CF activity were used as the purified virus.

Preparation of early extracts: MK and HeLe cells were infected with the stock virus as described before. One hour after infection, cells were suspended in PBS at the rate of 1×10^7 cells per ml, disrupted by 3 cycles of freezing and thawing, and centrifuged at 30,000 rpm for 30 minutes at 4℃ in Hitachi SPR-40 swing rotor. The supernatant was used as the early extract of indected cells and stored at −75℃ until tested. Early extract of non-infected cells was prepared by the same method.

Preparation of antisera: Four kinds of antisera of guinea pigs were prepared against crude virus, purified virus, and early extracts of infected and non-infected MK cells. One tenth ml of antigen was emulsified in an equal volume of Freund's complete adjuvant and inoculated into food-pads of guinea pigs at intervals of 7−10 days three times. Guinea pigs were bled about 1 week after last inoculation of the vaccine.

Electronmicroscopic examination: PS cell monolayers in 600 ml culture bottles were infected with the stock virus to yield the same infection rate as in the method described above. At intervals after infection, infected cells were trypsinized, collected by low centrifugation, fixed with glutaldehyde, postfixed with osmium tetroxide, dehydrated by ethanol and aceton, and enbeded in epoxin-resin mixture as the method described by Vasquez, C. and Tournier, P. (1962). Ultra-thin sections of this preparations were stained by ulanyl asetate and lead nitrate.

Fluorescent antibody technique: One day monolayers of HeLa cells grown on coverslips were infected with the stock virus to yield the same infection rate as in the

methods described before. At intervals after infection, 5 replicate coverslips were sampled and washed three times with PBS. After air-drying, the samples were stored at −20℃ before tested. The stain was carried out by using indirect immunofluorescent technique described by Hayashi, K. and Russeil, W. C. (1968).

Antimetabolites: Actinomycin D (Merk, Shap and Duhme, Res., Lab, Rahway, N. J.), cytoshine arabinoside (Nut. Bio. Crop,. Cleveland, Ohio), puromycin (Nut. Bio. Corp., Cleveland, Ohio), and 6-azauridine were selected. All drugs were dissolved in distilled water.

Result

Sequential development of reovirus type 3 antigens in infected PS cells: PS cells infected with reovirus type 3 was harvested at intervals after infection and assayed for infectious, complement fixing (CF) and hemagglutinating (HA) activities. Electronmicroscopical studies were also performed to observe the appearance of progeny viruses in the section of PS infected cells.

Until 4 hours after infection, any products and antigenical substances were not detected by both thin section of cells and CF test. As shown in Fig. 1, CF antigen first appeared at 5 hours and reached its maximum at 8 hours. The appearance of HA and infectivity was occured at 10 hours, and reached its maximum at 12 hours.

At 5 hours after infection, matrix areas containing a few shell forms presumed as inner capsid were first observed in cytoplasm in thin sections of infected cells. At 6 hours, empty shell viruses and maturing viruses whose center consisted of highly dense materials increased in

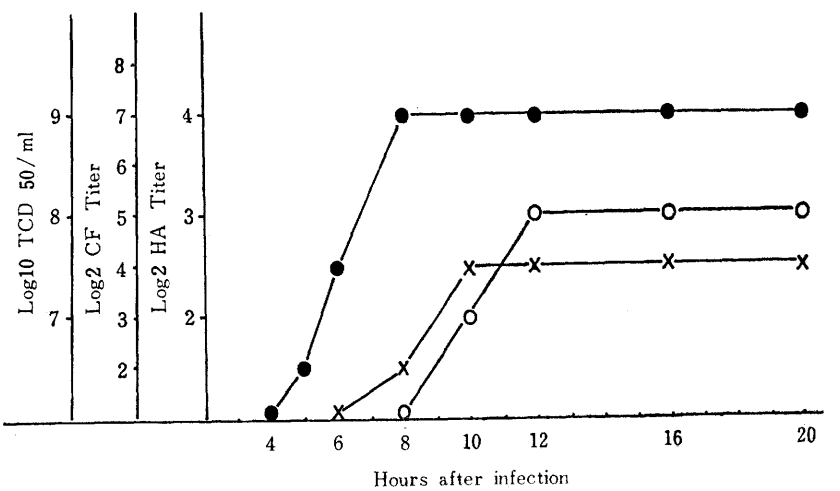

Fig. 1. Development of antigens in infected PS cells with reovirus type 3.
●——● Complement fixation activity
×——× Infectivity
○——○ Hemagglutination activity

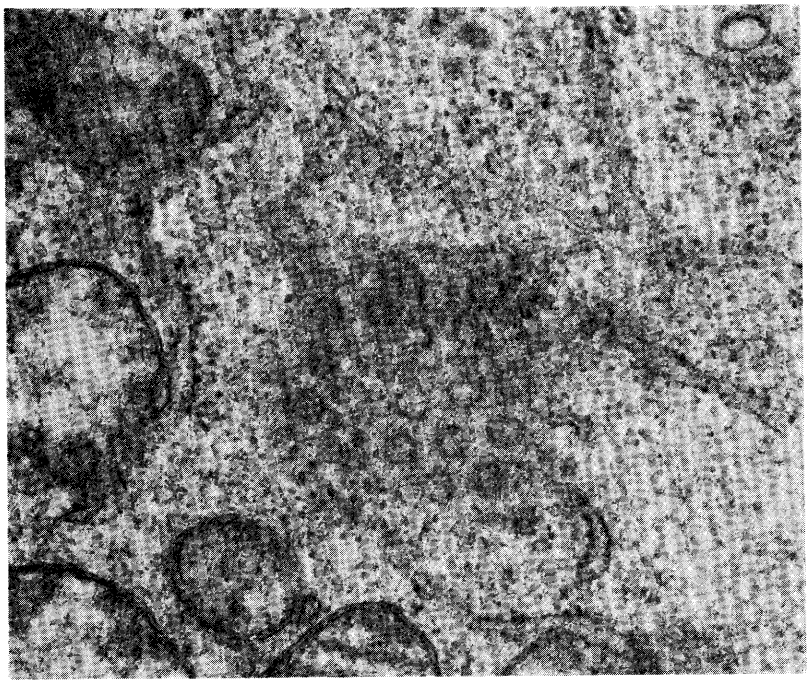

Photo 1. Matrix area produced in cytoplasm of infected PS cells with reovirus type 3 at 5 hours after infection.
43,200× magnification.

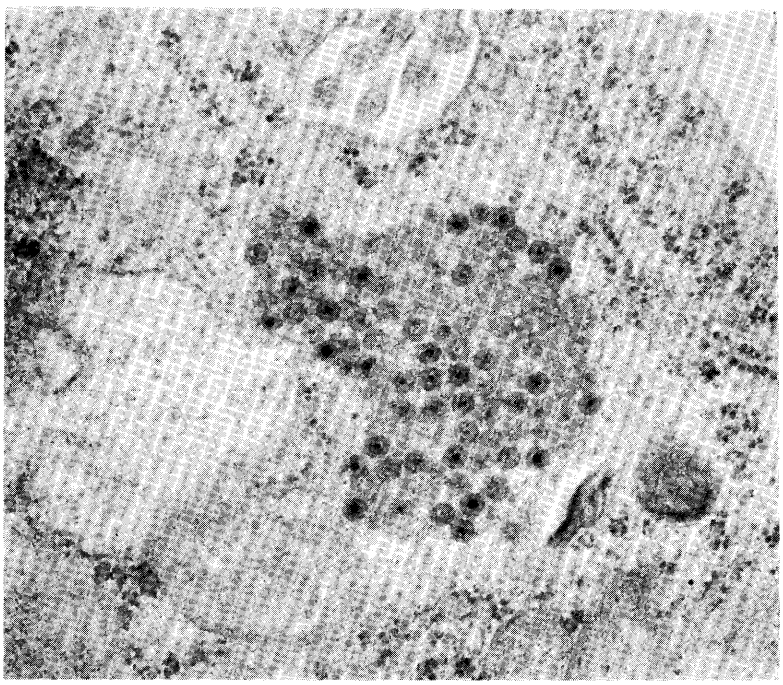

Photo 2. Matrix area observed in cytoplasm of infected PS cells at 6 hours after infection.
41,000× magnification.

number as the infection progressed (Photo. 1 and 2). The empty shell virus in the matrix areas was observed throughout the experiment.

Considering of electronmicroscopical observations, the infectivity should occure at 6 hours after infection simultaneously as well as CF antigen. Both the infectivity and HA, however, were not detected until 10 hours. This discrepancy might be due to the assay system for infectivity in this experiment.

Detection of virus induced product in early stage of infection by complement fixation test: Early extracts of infected MK and HeLa cells were prepared as the method described before. Early extracts of non-infected MK and HeLa cells and of infected HeLa cells with poliovirus type 1 (Mahoney) were used as controls of the antigen and antiserum against early extract of non-infected cells were also used in CF test for further controls.

As shown in Fig. 2, early extract of infected HeLa cells reacted with antiserum against crude virus as well as with homologous antiserum (E antiserum). While, crude virus also cross-reacted with E antiserum. These cross-reactions were demonstrated at lowest titer by using even purified virus and its antiserum (V antiserum). There were no cross-reaction between control antigens and these antisera except that between non-infected MK

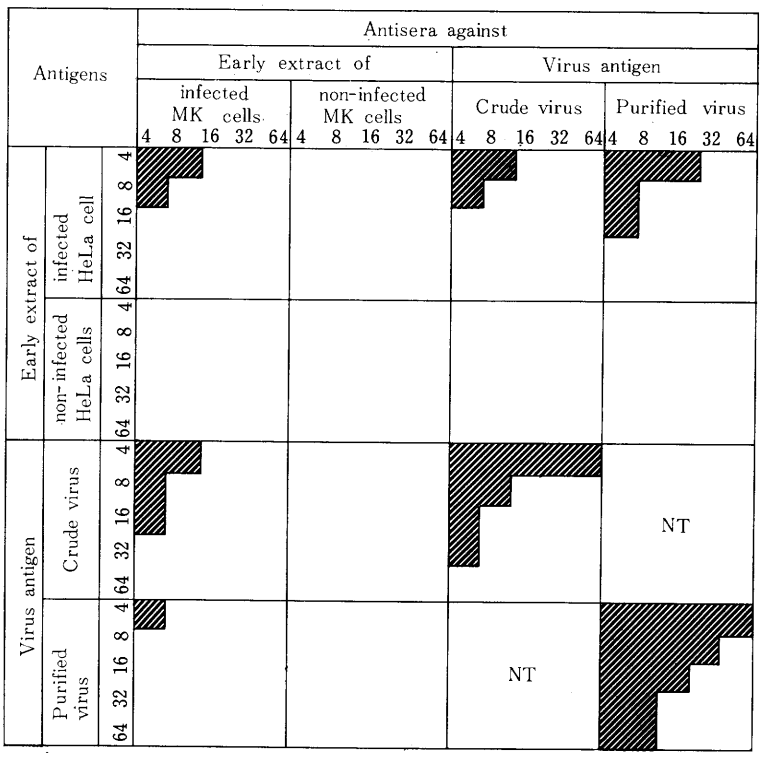

Fig. 2. Box titration of complement fixation test performed by using the early extract purified virus and their antisera.
The figures mean the titer which was expressed as the reciprocal of dilutions.
The sign of NT shows the cases not tested.

cells and E antiserum (dilution at 1:4).

Development of two fluorescent antigens in infected cells: From the result shown in CF test, it was conceivable that both the antigens of early extract and purified virus might contain a certain common antigenic substance(s) or that the virus capsid antigen caused by input virus might be contaminated in early extract of infected cells. Suppose, one step curves specifically reacted with E and V antiserum were obtained respectively, an antigen which is distinct from virus capsid antigen studied up to date would be present in the early extract of infected cells.

Monolayers of HeLa cell grown on coverslips were infected with virus as described before and processed the indirect immunofluorescent method at intervals after infection. Persent of immunofluorescence positive cells was figured out by counting in total 100 cells under the fluorescent microscope (Nikon).

One hour after infection, the specific fluorescent antigen against E antiserum was observed in cytoplasm particularly in the region of cytoplasmic membrane as dots and flecks, and increased in number and intensity until 8 hours (Photo. 3). From 8 to 10 hours, the fluorescence began to decrease in number and weaken in intensity but it was still observed at 20 hours. The fluorescent antigen against V antiserum was first observed in cytoplasm at 4 hours as granules in various size, and reached its maximum from 6 to 8 hours. It was observed continually even after 20 hours without tendency to decrease as observed in infected cells stained with E antiserum (Photo. 4). These findings seemed to be compatible with appearances of CF activity and progeny viruses in thin sections of infected cells as observed before. The sequential development of the fluorescent antigen against E and V antisserum is shown in Fig. 3.

The use of E and V antiserum blocked with homologous antigen at 4℃ overnight respectively, resulted in no specific fluorescence throughout the experiment. In addition, the use of antisera blocked with heterologous antigen was found to have almost no effect on the development of the two fluorescent antigens. These results indicate the presence of an antigen which is distinct from virus capsid

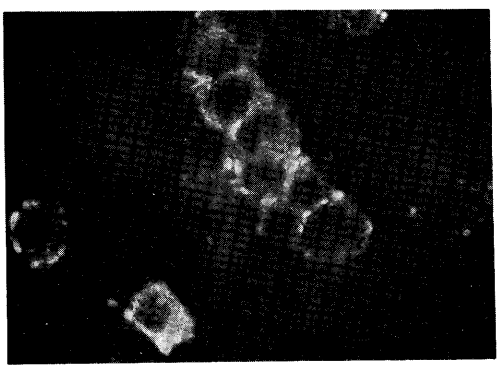

Photo 3. FA antigen in HeLa cells stained with E antiserum at 3 hours after infection

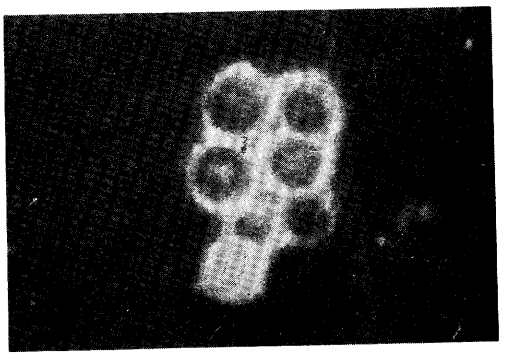

Photo. 4. FA antigen in HeLa cells stained with V antiserum at 8 hours after infection

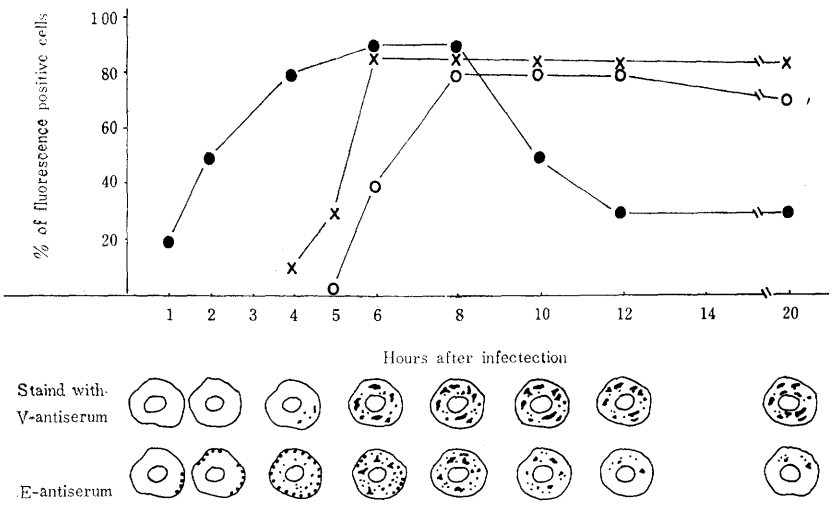

Fig. 3. Development of fluorescent antigens in reovirus type 3 infected HeLa cells.

●—● stained with E antiserum serum
×—× stained with V antiserum serum
○—○ stained with V antiserum serum absorbed with early extract.

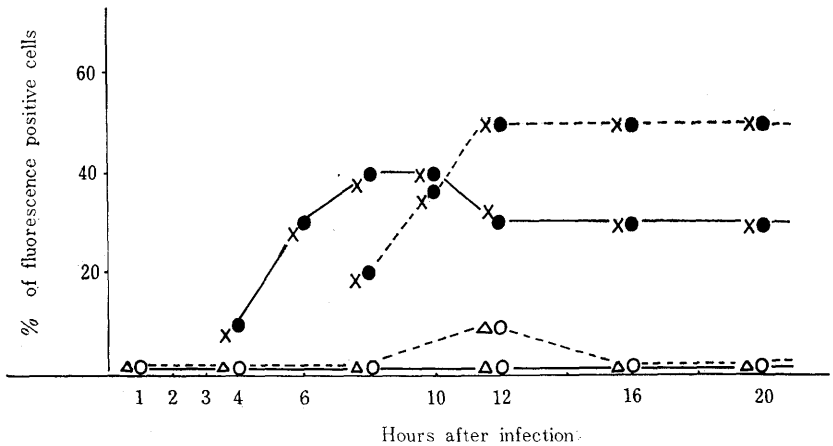

Fig. 4. Effect of antimetabolites on the development of fluorescent antigens against E and V antiserum.

Solid line indicates the development of early product (s) in the presence of actinomycine D (●), cytocine arabinoside (×), puromycine (○) and 6-azauriaine (△). Dotted line indicates that of capsid antigens.

antigen in the early extract of infected cells.

Effects of antimetabolites on the development of early product: The antigenic product detected as early as at 1 hour after infection was characterized with antimetabolites. Actinomycin D at the concentration of 20 γ/ml, cytosine arabinoside 40 γ/ml, puromycin 50 γ/ml and 6-azauridine 10 γ/ml were selected.

Monolayers of HeLa cells grown on coverslips were pretreated for 1 hour before infection, and infected with the virus. The drugs were allowed to contact with the cells during the experiment. As shown in Fig. 4, the presence of actinomycin D or cytosine arabinoside postponed the first appearance of the fluorescent antigen against E and V antiserum about 3 hours. There was, however, no significant effect on the development of these antigens was observed throughout the experiment.

Discussion

Concerning with the development of reovirus antigen in infected cells, it has been found that virus capsid antigen appeared in cytoplasm of infected cells at 6 to 8 hour after infection by immunofluorescent method (Rhim et al., 1962; Spendlove, et al. 1963). However, it was not examined whether or not an early antigenic product, particularly an antigen which is distinct from virus capsid antigens, is produced in the early stage of reovirus infection before the appearance of virus progeny.

In the present study, the matrix areas in infected cells and CF activity were first detected at 5 hour after infection. Accordingly, early extracts of infected cells during from 1 to 4 hour after infection and thier antisera were prepared.

The releiable data were obtained by using the early extract of infected cells 1 our after infection and its antiserum (E antiserum). The fluorescent antigen against E antiserum in infected cells was observed in cytoplasm as particular forms of dots and flecks as early as at 1 hour after infection and developed considerably before the appearance of the fluorescent antigen against V antiserum. From 8 to 10 hours after infection, the fluorescence began to decrease in number and in intensity but it was still observed at 20 hours after infection. These findings were not observed in poliovirus type 1 infected cells and reovirus infected cells stained with the antiserum against early extract of non-infected cells. In addition, no fluorescent antigen against E and V antiserum absorbed with homologous antigen respectively were observed in infected cells, and there was no significant difference betweenthe fluorescent antigen against V antiserum and that absorbed with early extract prepared from the infected cells 1 hour after infection. Consequently, it may be safely said that the fluorescent antigen against E antiserum was distinct from the capsid antigen.

From the fact of the appearance of

this product, at least following three possibilities can be considered. The first possibility is that the antigenicity of this product is due to the remainings of input virus capsid protein. However, this possibility seems unlikely, since the fluorescence against V antiserum did not demonstrate in early stage in infected cells but it was able to detect only against E anti serum.

Secondly, this product would be an enzyme which might be essential for further replication of virus. The last possibility is that this antigenic product may be a precursor or a component(s) of late proteins.

In the studies on early events of reovirus infectious cycle, 3 species of virus structural proteins and virus-induced RNA polymerase have been demonstrated individually as early as at 1 hour after infection by using polyacrylamide gel electrophoresis (Loh and Shatkin, 1968; Loh and Oie, 1969; Watanabe et al., 1968b). Although it is not clear at present whether or not these early proteins contain the RNA polymerase, and consist of three different enzymes, it seems possible to speculate that the early antigenic product detected in the present study might be or will be one of them.

Generally speaking, reovirus replication is not inhibited by DNA synthesis inhibitors, cytosine arabinoside etc., but by RNA synthesis inhibitors, 6-azauridine and actinomycine D (Gomatos et al., 1962; Silagi, 1965; Rada and Shatkin, 1967). The appearance of this product and virus capsid antigens were completely inhibited by 6-azauridine (at a concentration of 10γ per ml) and puromycin (50γ/ml), but not by actinomycin D (2γ/ml) and cytosine arabinoside (40γ/ml)although the first appearance was postponed about 3 hours. In addition, no different behaviors against these antimetabolites were observed between the early product and virus capsid antigens. Considering these results, it seems probable that actinomycin D or cytosine arabinoside has no essential effect on the development of the product detected in the present study and it would be coded for virus RNA.

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Reovirus 3型感染細胞内における初期抗原の研究

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摘　　要

本研究は，reovirus 3型感染細胞内において，virus capsid 抗原とは異なった抗原が，感染初期に産生されるか否かを企図して行なったものである．

補体結合反応では，感染1時間後の細胞抽出液でモルモットを免疫した抗血清（抗E血清）は，自原はもとより，精製 virus 抗原にも反応した．一方，この抽出液は抗 reovirus 3型血清（抗V血清）にも反応する事が判った．この事実は，抽出液及び精製 virus に共有される抗原物質に原因するか，或いは接種 virus の混入に帰因するかの何れかが考えられた．次に，抗E血清及び抗V血清を用い螢光抗体法の間接法によって感染細胞内に産生される特に感染初期の抗原について検討した．そして，virus capsid 抗原が未だ認められない時期，即ち，感染初期の1時間から4時間に出現する抗原のあることが判った．この抗原は，感染後 10時間目から消褪し始め，感染 20時間後もなおわずかに残存するのを観察した．他方，virus capsid 抗原による螢光は，感染後4時間目より出現し，感染 20時間後でも消褪する事はなかった．この際，抗E及び抗V血清は，実験に使用する細胞で充分吸収されたものである．即ち，感染1時間目の細胞から得た抽出抗原は virus capsid 抗原とは異なった産生過程を示し，感染の初期に出現する新しい抗原であると考えられた．この抗原の産生は，actinomycine D や，cytosine arabinoside によって阻止されず（但し，抗原の出現する時間は約3時間のずれがある．），puromycine や 6-azauridine の存在下では完全に阻止された．この事実は，上記の抗原が，virus RNA によって code されたものである事を示唆していると思う．