Induction of 2',5'-Oligoadenylate Synthetase and Interferon in Mouse Trigeminal Ganglia Infected with Herpes Simplex Virus

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The activity of 2',5'-oligoadenylate synthetase, which is induced by interferon action, was detected in mouse trigeminal ganglia infected with herpes simplex virus (HSV) type 1. When HSV was inoculated on the left cheek of mice, the virus began to appear in the ipsilateral trigeminal ganglia on day 2, reached its maximum accumulation on day 4, declined thereafter, and was no longer detected in the tissue homogenate after 11 days. After this short acute productive phase, the virus entered into the latent phase of infection. 2',5'-Oligoadenylate synthetase appeared in the trigeminal ganglia shortly after the beginning of virus multiplication; the synthetase activity began to rise on day 3, reached a maximum level on day 4, and then declined. Interferon activity (type I) also appeared in the infected ganglia, but diminished more rapidly than the synthetase. The antibody against HSV in the sera began to appear at the time when the virus and synthetase were declining. From these results it may be hypothesized, although not concluded, that in the acute productive phase of HSV infection in mouse trigeminal ganglia, virus multiplication is suppressed by interferon action, including 2',5'-oligoadenylate synthetase induction.

An experimental latent infection of the sensory and autonomic ganglia with herpes simplex virus (HSV) has been established in mice and rabbits (12, 15, 16, 20). HSV inoculated at various epithelial surfaces is thought to travel over neural pathways to the corresponding ganglia (12). After a short acute productive phase, the virus disappears from the ganglia, and in the latent phase of infection it can be detected only by the explant culture of the ganglionic tissue. The restriction of viral multiplication without complete destruction of the ganglion is a decisive step for the conversion from the acute phase to the latent phase of infection. However, little is known about its mechanism; the importance of an antiviral substance such as interferon induced in the infected nerve cells has been suggested, but the evidence has been lacking thus far.

It has been demonstrated that interferon induces cells to produce at least two enzymes (1): one is 2',5'-oligoadenylate synthetase, which catalyzes the formation of an unusual oligoadenylate with 2'-5' linkage from adenosine triphosphate (ATP) in the presence of double-stranded ribonucleic acid (RNA), and the other is a double-stranded RNA-dependent protein kinase, which can inactivate the initiation factor of eucaryotic protein synthesis through phosphorylation. The 2',5'-oligoadenylate synthesized activates a latent endonuclease which is

probably a constitutive element of the cell. Thus, if 2',5'-oligoadenylate synthetase activity, protein kinase activity, or both are present in cells, though no interferon activity is detected in these cells, it could be an indication that they are in the antiviral state induced by interferon. Recently, we have reported an assay method for 2',5'-oligoadenylate synthetase in cells (14), which had been originally devised by Hovanessian et al. (6). The present report describes the appearance of 2',5'-oligoadenylate synthetase and interferon activities in the trigeminal ganglia during the acute phase of infection with HSV, and their disappearance when the latent infection was established.

MATERIALS AND METHODS

Virus. HSV type 1, strain VR-3, was donated by S. Uchida, Institute of Medical Science, The University of Tokyo. The virus was passaged three times in Vero cells to prepare a stock which contained 1.2×10^8 to 2.0×10^8 plaque-forming units per ml.

Animals. Four-week-old ddY male mice (Shizuoka Laboratory Animal Center, Hamamatsu, Shizuoka, Japan) were used for all studies.

Virus inoculation. Intradermal inoculation of the left cheek of each mouse was carried out as follows. A drop of undiluted virus (0.05 ml) was placed on the cheek of the mouse, which was under anaesthesia with Nembutal, and the area of the epithelial surface innervated with trigeminal nerves was pricked with a

bundle of 20 hypodermic 27-gauge needles. In our experiments, about 80% of the mice became paralyzed in 7 to 10 days after inoculation. Of the paralyzed mice, about 25% died in 10 days, but others underwent complete clinical recovery in 2 more weeks.

Virus titration. Mice were decapitated, and whole blood or serum was collected. The trigeminal ganglia were surgically removed and washed three times with ice-cold Hanks balanced salt solution buffered with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.2, and frozen at -70°C until use. Tissue homogenate was obtained after the disruption of the tissue with a glass microhomogenizer in 0.3 ml of Eagle minimal essential medium supplemented with 10% heat-inactivated fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.), and then centrifuged at $1,000 \times g$ for 15 min at 4°C . Serial 10-fold dilutions of the sample were prepared in ice-cold Eagle minimal essential medium containing 5% heat-inactivated fetal calf serum, and 0.05 ml of each dilution was placed in six replicate wells of 96-well microtiter plates (Nunc Products, Denmark). Then, 0.05 ml of the suspension of Vero cells (2×10^5 cells per ml) was immediately added, and the cultures were maintained at 37°C in an atmosphere containing 5% CO_2 . The cytopathic effect induced by HSV was monitored over 5 days, and viral titers were expressed as 50% tissue culture infectious doses (TCID_{50}).

Serum antibody titer. Serum was obtained by keeping the blood at 4°C overnight followed by two cycles of centrifugation and holding it at -70°C until use. Titers of complement-requiring neutralizing antibody were determined by the microassay method described by Tada and Yoshino (18) and expressed as the reciprocal of the highest dilution of serum inducing positive neutralization.

2',5'-Oligoadenylate synthetase activity. For the assays of 2',5'-oligoadenylate synthetase and interferon in the trigeminal ganglia, a supernatant fluid of tissue homogenate was prepared. The tissues were removed, washed, and chopped with scissors in 0.2 ml of buffer (HGB buffer: 10 mM HEPES, pH 7.5; 50 mM KCl; 3 mM magnesium acetate; 0.3 mM ethylenediaminetetraacetic acid; 7 mM 2-mercaptoethanol; and 20% glycerol, vol/vol). They were then sonically disrupted by three 10-s bursts of a Branson Sonifier (special microtip; output setting of 2) and centrifuged at $17,000 \times g$ for 15 min. The protein content in the tissue extract was estimated by the method of Lowry et al. (9). The synthetase activity was measured by the column method as described previously (14). The tissue extract was applied to a column of polyinosinic acid [poly(I)]-polycytidylic acid [poly(C)]-Sephacrose (50 μl), washed with 5 ml of HGB buffer at room temperature, and then incubated at 33°C in the presence of [^3H]ATP (25 μCi ; 1 mM; 0.2 mCi/ml). The eluate (1 ml) from the column was collected and applied to a diethylaminoethyl-cellulose column (0.1 ml of gel) equilibrated with HGB buffer. After the column was washed with 10 ml of HGB buffer containing 50 mM KCl to remove unreacted ATP and its derivatives, the concentration of KCl was increased to 0.3 M and the eluate (1 ml) containing oligoadenylate was collected. The radioactivities of the oligoadenylate were measured by a liquid scintillation counter by

using an ACS II (Amersham) scintillator.

Interferon activity. Interferon activity in the tissue extract was assayed by the microassay method described by Kawade et al. (8). Reduction of incorporation of [^3H]uridine into RNA of vesicular stomatitis virus in actinomycin D-treated mouse L cells was estimated. The titers were expressed in international mouse reference units in terms of the National Institutes of Health Research Reference Mouse Interferon Preparation, catalog no. G002-904-511.

RESULTS

Virus production in the trigeminal ganglia and appearance of serum antibody against HSV. Figure 1 shows the kinetics of viral production in the ipsilateral trigeminal ganglia after inoculation with the VR-3 strain of HSV-1 on the left cheek of the mouse. HSV began to appear on day 2, reached maximum titer on day 4, and declined thereafter. The virus was no longer detected in tissue homogenates after 11 days, but we have confirmed that it could be recovered by an explant culture of trigeminal ganglia which were removed from mice inoculated 2 months before, indicating that the latent infection was established in our experimental conditions. Figure 1 also shows the kinetics of appearance of the complement-requiring neutralizing antibody against HSV in sera. It was first detected on day 5 when viral titers in the trigeminal ganglia were declining, and it continued to increase when the virus disappeared completely.

Presence of 2',5'-oligoadenylate synthetase in HSV-infected trigeminal ganglia. 2',5'-Oligoadenylate synthetase activity was detected in the trigeminal ganglia from infected mice; that is, ^3H -labeled oligoadenylate was formed when the tissue extract that had been incubated with [^3H]ATP at 33°C was added to a column of poly(I)-poly(C)-Sephacrose. The oligoadenylate thus formed was resistant to ribonuclease T_2 and was composed mainly of trimer, containing small amounts of both dimer and tetramer (Fig. 2). The inhibitory activity of the oligoadenylates in protein synthesis was also confirmed by using a rabbit reticulocyte lysate system (data not shown).

Figure 3 shows the kinetics of formation of 2',5'-oligoadenylate in poly(I)-poly(C)-Sephacrose columns (50 μl of gel) when the tissue extracts were applied in two different quantities, 75 and 30 μg of protein. The enzyme reaction at 33°C proceeded linearly only for 2.5 h for 75 μg of protein and for 5 h for 30 μg of protein, which indicates the presence of enzyme degrading the oligoadenylate in the tissue extract, although the amounts of the extracts applied and those of the products were roughly proportional for the ini-

FIG. 1. Kinetics of production of HSV in the trigeminal ganglia and appearance of complement-requiring neutralizing antibody against HSV in sera after inoculation of HSV. Each point denotes the datum obtained from each individual mouse. (circles and solid line) viral production; (triangles and dashed line) serum antibody. (open symbols) infected mice without clinical signs; (closed symbols) infected mice with clinical signs.

tial 2.5-h incubation. Therefore, we adopted a 2.5-h incubation for the assay of the synthetase in the following experiments. The assay method used here, however, must be regarded as semi-quantitative because of the lack of assurance to bind all of the synthetases present in the tissue extract to the poly(I):poly(C)-Sepharose and because of the contamination of an enzyme degrading the oligoadenylate in the assay system.

Kinetics of induction of 2',5'-oligoadenylate synthetase after HSV infection. Figure 4 shows two independent experiments on the induction of 2',5'-oligoadenylate synthetase in the trigeminal ganglia after the inoculation of HSV. In experiment A, the enzyme was assayed by using homogenates of 10 pooled ganglia, each removed unilaterally from the individual mouse at the indicated time; whereas in experiment B, each point in the graph denotes the datum obtained on homogenates of three pooled ganglia. Essentially the same results were obtained from both experiments. The synthetase activity in the ipsilateral (left) ganglia began to rise on day 3, reached a maximum level on day 4, and then declined. In the latent phase of infection, only very low levels of activity were detected. In the contralateral (right) ganglia, the virus was detected in some mice at low levels during the acute phase, and 2',5'-oligoadenylate synthetase was also detected; the maximum activity was observed on day 7, later than in the ipsilateral ganglia.

Induction of interferon activity. Table 1 shows the levels of interferon in the trigeminal

FIG. 2. Diethylaminoethyl-cellulose chromatography of 2',5'-oligoadenylate synthesized with the tissue extract from infected trigeminal ganglia. Ipsilateral (left) trigeminal ganglia removed from 10 different mice on day 4 after inoculation of HSV were pooled and homogenized. The $17,000 \times g$ supernatant fraction of the homogenate (10 μ l; 75 μ g of protein) was used as the enzyme source. The oligoadenylate produced was treated with ribonuclease T_2 (1.5 U/ml) at pH 4.5 for 10 min at 37°C, and then applied to a column of diethylaminoethyl-cellulose (0.5 by 17 cm) equilibrated with 50 mM tris(hydroxymethyl)amino-methane-hydrochloride (pH 8.0) containing 7 M urea. Elution (1 ml/fraction, 3.5 ml/h) was with a linear gradient of 50 to 150 mM NaCl (40 ml/40 ml). Adenosine (Ad), adenosine monophosphate (AMP), adenosine diphosphate (ADP), ATP, adenosine 5'-tetraphosphate (pA), and guanosine 3'-diphosphate 5'-diphosphate (ppGpp) were included as markers (open circles). Closed circles show 3H -labeled oligoadenylates. A_{260} , Absorbance at 260 nm.

FIG. 3. Synthesis of 2',5'-oligoadenylate. The tissue extract from infected trigeminal ganglia indicated in Fig. 2 was used. (open circles) 75 μ g of protein; (closed circles) 30 μ g of protein. AMP, Adenosine monophosphate.

ganglia, as determined with the same tissue homogenates as indicated in Fig. 4. Interferon first appeared on day 3 and increased in proportion to the activity of 2',5'-oligoadenylate synthetase

until day 4, but it could not be detected at all on day 7 when the synthetase activity was still present. No interferon activity was detected from the contralateral ganglia except for one sample of day 7 in experiment B which had a relatively high level of the synthetase activity. No sera from the infected mice contained detectable interferon. Interferon induced in the trigeminal ganglia was stable at pH 2 for 20 h,

but was unstable at 56°C for 1 h. Moreover, the activity was neutralized by the antibody to mouse L-cell interferon induced by Newcastle disease virus, supplied by Y. Yamamoto (Y. Yamamoto and Y. Kawade, *Virology*, in press).

DISCUSSION

Isaacs and Hitchcock (7) first demonstrated that interferon appeared in the lungs of mice when influenza virus multiplied and only when the virus was already declining could the first antibody be detected. Many studies on administration of exogenous interferon or interferon inducers to animals have shown that interferon effectively suppresses a variety of virus infections (17). Gresser et al. (5) have shown that treatment of virus-infected mice with antiinterferon antibody caused more severe infection. Thus, interferon is thought in general to be a key element of resistance to virus infection. However, there has been no direct evidence indicating in virus-infected animals that cells or tissues become resistant to virus through interferon action. The presence of 2',5'-oligoadenylate synthetase activity in some tissue, which is considered to be induced by interferon, could provide good evidence for the antiviral state of the tissue, but only recently an exceptional case has been reported in interferon-treated mouse embryonal carcinoma cells (21). Although these cells do not respond to interferon by becoming antiviral (2), induction of the 2',5'-oligoadenylate synthetase but not of the protein kinase was observed. No interferon production has been shown in the embryonal cells after treatment with a wide variety of inducers (2). These situations may be only for undifferentiated embryonal cells and not be true for differentiated tissue cells.

In this paper it has been shown that the activities of both 2',5'-oligoadenylate synthetase and interferon parallel viral titers during the acute phase of HSV infection in the trigeminal ganglia of mice after cheek skin inoculation. The 2',5'-oligoadenylate synthetase appeared shortly

FIG. 4. Induction of 2',5'-oligoadenylate synthetase in the trigeminal ganglia after inoculation of HSV. In experiment A the tissue extract was prepared from a pool of unilateral ganglia from 10 mice and contained 6 to 8 mg/ml of protein; in experiment B, it was prepared from each of three ganglia, separately, and contained 2 to 4 mg/ml of protein. For the assay of the synthetase, each tissue extract containing 75 μ g of protein was used and the incubation was carried out for 2.5 h at 33°C. (circles and solid line) ipsilateral ganglia; (crosses and dashed line) contralateral ganglia. AMP, Adenosine monophosphate.

TABLE 1. Induction of interferon in the trigeminal ganglia after inoculation of HSV

Expt ^a	Side of ganglia	Interferon activity (IU/mg of protein) ^b at day after inoculation:								
		0	1	2	3	4	7	12	17	25
A	Ipsilateral	<20				550	<20	<20	<20	
	Contralateral					<20	<20	<20	<20	
B	Ipsilateral	<10	<10	<10	130	240	10	<10		<10
		<10	<10	<10	90	310	<10	<10		<10
	Contralateral	<10	<10	<10	<10	<10	20	<10		<10
		<10	<10	<10	<10	<10	<10	<10		<10

^a The tissue extracts used in the experiments of Fig. 4 were assayed.

^b IU, International mouse reference units.

after the beginning of virus multiplication, and its activity declined with the disappearance of virus from the tissue. Interferon was also detected in the tissue extract, but diminished more rapidly than the synthetase. On the other hand, antibody against HSV appeared in the sera at the time when the virus and the synthetase were declining and the interferon activity had already diminished. These results let us speculate that in the conversion from the acute phase to the latent phase of infection the virus multiplying in the trigeminal ganglia induces the production of interferon, which then induces 2',5'-oligoadenylate synthetase and as a consequence impairs the multiplication of HSV.

It has been demonstrated (11) that cell-mediated immunity plays an important role in the resistance of mice to infection with HSV, but under appropriate conditions passively transferred serum anti-HSV antibody was also effective in preventing the spread of HSV (10). Immune interferon (type II) produced by stimulation of immune lymphocytes by viral antigen has been implicated as having a role in the defense of the immunized host to HSV (4). In the present study, lymphocytes infiltrating the ganglionic tissue might be partly responsible for the interferon detected, but the animals used had not been immunized and the interferon was clearly differentiated from type II interferon. Therefore, during the acute phase of HSV infection, type I interferon may play an important role in preventing viral multiplication.

Puga et al. (13) detected an HSV-specific deoxyribonucleic acid sequence in the trigeminal ganglia of mice at both the acute and latent phases of the infection. On the other hand, virus-specific messenger ribonucleic acid was found at the acute phase, but was not found during the latent phase. They suggested that the viral genome may exist in a nonreplicating form during the latent phase of infection. Their considerations are compatible with the result that the activities of both 2',5'-oligoadenylate synthetase and interferon disappeared during the latent phase of the infection, since the continuous multiplication of the virus may be required for the maintenance of some levels of these activities. 2',5'-Oligoadenylate synthetase and interferon may not be involved in the maintenance of latency in the case of HSV infection.

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LITERATURE CITED

1. Baglioni, C. 1979. Interferon-induced enzymatic activities

- and their role in antiviral state. *Cell* 17:255-264.
2. Burke, D. C., C. F. Graham, and J. M. Lehman. 1978. Appearance of interferon inducibility and sensitivity during differentiation of murine teratocarcinoma cells in vitro. *Cell* 13:243-248.
3. Cook, M. L., and J. G. Stevens. 1973. Pathogenesis of herpetic neuritis and ganglionitis in mice: evidence for intra-axonal transport of infection. *Infect. Immun.* 7: 272-288.
4. Fujibayashi, T., J. J. Hooks, and A. L. Notkins. 1975. Production of interferon by immune lymphocytes exposed to herpes simplex virus-antibody complex. *J. Immunol.* 115:1191-1193.
5. Gresser, I., M. G. Tovey, C. Maury, and M. Bandu. 1976. Role of interferon in the pathogenesis of virus diseases in mice as demonstrated by the use of anti-interferon serum. II. Studies with herpes simplex, Moloney sarcoma, vesicular stomatitis, Newcastle disease virus, and influenza virus. *J. Exp. Med.* 144:1316-1323.
6. Hovanessian, A. G., R. E. Brown, and I. M. Kerr. 1977. Synthesis of low molecular weight inhibitor of protein synthesis with enzyme from interferon-treated cells. *Nature (London)* 268:537-540.
7. Isaacs, A., and G. Hitchcock. 1960. Role of interferon in recovery from virus infections. *Lancet* ii:69-71.
8. Kawade, Y., T. Matsuzawa, Y. Yamamoto, K. Tsukui, and Y. Iwakura. 1976. A rapid and precise microassay of interferon based on the inhibition of viral RNA synthesis. *Annu. Rep. Inst. Virus Res. Kyoto Univ. (Kyoto Daigaku Uirusu Kenkyusho Nankan Kiyo)* 19:52-58.
9. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
10. McKendall, R. R., T. Klassen, and J. R. Baringer. 1979. Host defenses in herpes simplex infections of the nervous system: effect of antibody on disease and virus spread. *Infect. Immun.* 23:305-311.
11. Oakes, J. E. 1975. Role of cell-mediated immunity in the resistance of mice to subcutaneous herpes simplex virus infection. *Infect. Immun.* 12:166-172.
12. Price, R. W., B. L. Katz, and A. L. Notkins. 1975. Latent infection of the peripheral ANS with herpes simplex virus. *Nature (London)* 257:686-688.
13. Puga, A., J. D. Rosenthal, H. Openshaw, and A. L. Notkins. 1978. Herpes simplex virus DNA and mRNA sequences in acutely and chronically infected trigeminal ganglia of mice. *Virology* 89:102-111.
14. Shimizu, N., and Y. Sokawa. 1979. 2',5'-Oligoadenylate synthetase activity in lymphocytes from normal mouse. *J. Biol. Chem.* 254:12034-12037.
15. Stevens, J. G., and M. L. Cook. 1971. Latent herpes simplex virus in spinal ganglia of mice. *Science* 173: 843-845.
16. Stevens, J. G., and M. L. Cook. 1973. Latent infection induced by herpes simplex virus. *Cancer Res.* 33:1399-1401.
17. Stewart II, W. E. 1979. The interferon system, p. 266-291. Springer-Verlag, New York.
18. Tada, A., and K. Yoshino. 1978. A new microplate neutralization test for typing of herpes simplex virus. *Microbiol. Immunol.* 22:415-426.
19. Youngner, J. S. 1977. Properties of interferon induced by specific antigens. *Tex. Rep. Biol. Med.* 35:17-22.
20. Walz, M. A., R. W. Price, and A. L. Notkins. 1974. Latent ganglionic infection with herpes simplex virus type 1 and 2: viral reactivation in vivo after neurectomy. *Science* 184:1185-1187.
21. Wood, J. N., and A. G. Hovanessian. 1979. Interferon enhances 2-5A synthetase in embryonal carcinoma cells. *Nature (London)* 282:74-76.