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EXPERIMENTS FOR THE ISOLATION OF THE HERPES ZOSTER VIRUS

Orvosi Hetilap
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The pathogenic agent of herpes zoster was first isolated by Weller, who observed, in 1953, a characteristic cytopathogenic change in human embryonic fibroblast cultures infected with herpes zoster vesicle fluid [1]. The virus cultured in vitro has the interesting property that infectious particles do not appear in the nutrient fluid of the tissue culture, and the passage of isolated virus strains can be achieved only by means of a cell suspension.

On the basis of Weller's work and making use of his methods, we attempted the isolation of the pathogenic agent from the vesicle fluid of patients infected with herpes zoster, as well as the identification of this agent by neutralization and immunofluorescence methods.

Materials and Methods

By means of a sterile glass capillary, we obtained vesicle fluid from eight patients with herpes zoster in the initial period of the eruption. 0.05 ml portions of the undiluted vesicle fluid were used to infect embryonic fibroblast cultures prepared in a Wassermann tube. Depending on the quantity of vesicle fluid, we used material from one patient to inoculate simultaneously 2-4 tissue cultures.

Preparation of Fibroblast Cultures. Two to three month old human embryos were deprived of bones, cut up and washed several times with a pH 7.2 phosphate buffer. The material was then treated overnight at +4°C with a 0.25% Difco trypsin solution. After centrifuging, the settled cells were resuspended in proliferating nutrient fluid, and after determining the cell count, an amount containing 15 million cells was transferred into a one-liter Roux bottle. The bottle cultures were incubated for 10-14 days at 37°C and then used, after separation by means of trypsin, for the preparation of the secondary tube cultures. Each Wassermann tube contained 300,000 cells in 1.5 ml proliferating solution. A cell layer suitable for infection was obtained after an incubation lasting 3-5 days.

The basic nutrient fluid used for the tissue culture and maintenance was Hanks solution containing 0.5% lactalbumin hydrolyzate (enzymatic Difco solution), to which in the case of the proliferating nutrient fluid 15% calf serum and 15% bovine amniotic fluid was added, while in the case of the maintenance nutrient fluid 20% calf serum was added. The proliferating nutrient fluid was used for the preparation of bottle- and tube cultures, while the maintenance fluid was used for the isolation of virus strains.

Method of Virus Passage. An infected cell culture containing 14-day old cytopathogenic foci was scraped off the wall of the test tube by means of a pipette and suspended in 0.8 ml of maintaining nutrient fluid. This suspension was used to infect 4-6 day old fibroblast cultures. On the seventh day the nutrient fluid of infected cultures was replaced by maintenance nutrient.

Performance of the Virus Neutralization Test. An 8-10 day old infected fibroblast culture was separated from the surface of the glass by means of 0.25% trypsin. After centrifuging, the cells were resuspended in maintenance nutrient fluid in such a way that the suspension obtained from three test tubes was always suspended in 2 ml of serum-free Hanks solution containing lactalbumin hydrolyzate. This basic suspension was diluted to 1:10, and 0.5 ml of the diluted suspension was treated with 1 ml of undiluted acute or convalescent human serum. The mixture was incubated for two hours at +37°C, then diluted with lactalbumin-containing Hanks solution in such a way that the concentration of human serum in the final mixture was 20%. Of this mixture 1 ml portions were transferred to 2-4 day old fibroblast cultures. For control, infected cell suspension incubated with calf serum was also transferred to tissue culture. The extent of neutralization was expressed by the reduction of the number of cytopathogenic foci developing in the presence of

acute or convalescent sera relative to the number of foci developing in the tubes containing control calf serum; this decrease was expressed in percent, the number of foci developing in the control cultures being taken as 100%.

Immunofluorescence Method. Human embryonic fibroblast cells cultured on a cover slip were infected with a cell suspension containing herpes zoster virus. Three days after infection the cultures were fixed and the slides were then covered with acute or convalescent sera of patients with herpes zoster, diluted 1:10. The sera were kept for a half hour on the cultures placed into a humidifying chamber. After washing, the preparations were treated, according to the above method, with fluorescein isothiocyanate conjugated with anti-human horse gamma globulin, and after renewed washing, they were subjected to fluorescence microscopy.

Preparation of Stained Products. On an 18 x 18 mm cover slip placed into small bottles having a basic surface area of 8 cm², fibroblast cultures were prepared and infected with herpes zoster virus. After three days, following fixation with Bouin solution, the cultures were stained with hematoxylin-eosine.

Results

Isolation of Cell Strains and Study of Their Cytopathogenic Effect. We were able to isolate cytopathogenic agents from six of the eight vesicle fluids collected.

Table 1.

Results of Experiments for the Isolation of Virus from Vesicle Fluids Obtained from Patients with Herpes Zoster

(1) A beteg	(2) A kiütés kezdése (napok)	(3) A kiütés kezdése (napok)	(4) A kiütés kezdése (napok)	(5) A kiütés kezdése (napok)
(2) Jelen	(3) Jelen	(4) Jelen	(5) Jelen	(6) Jelen
S. S.	56	2.	+	Z-1.
L. O.	15	7.	+	Z-2.
K. I.	22	3.	+	Z-3.
N. Gy.	45	7.	+	Z-4.
G. A.	15	2.	+	Z-5.
Sz. I.	60	2.	+	Z-6.
P. J.	56	3.	negatív szövettenyésztés spontán degenerálódot	(7) -
F. M.	62	4.	negatív bakteriológiai fertőzött halyagbrúnc	(8) -

1 -- Patient; 2 -- Name; 3 -- Age; 4 -- Number of days after start of eruption when sample was obtained; 5 -- Result of isolation experiment; 6 -- Code of strain obtained; 7 -- Negative;

tissue culture spontaneously degenerated; 8 -- Negative; vesicle fluid bacterially infected.

For the inoculation we always aspirated fluid from those vesicles which appeared to be the most recent among the skin changes in the various stages. It is thanks to this method that we were able to isolate virus -- from fresh vesicles -- even on the seventh day following the appearance of the first eruption. The reason for one of our unsuccessful isolations was a spontaneous tissue degeneration. In our second case we attempted to use for inoculation a vesicle fluid which had previously been infected with bacteria.

The first cytopathogenic changes appeared in the fibroblast cultures 5-8 days after inoculation. Fig. 1 gives a good illustration of the cell group consisting of swollen refractory cells, which sharply contrasts with the surrounding intact cells. The change spreads centrifugally, per continuitatem, primarily in the direction corresponding to the longitudinal axis of the fibroblast cells. The spread is faster the denser the fibroblast culture. In the beginning only a few foci may be observed, but their number gradually increases up to the 10th day, and afterwards new foci do not appear any more. On isolation an average of 8-10 foci developed on a single culture.

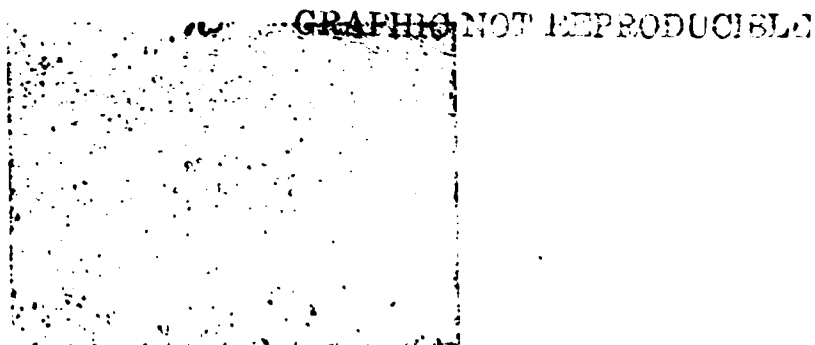


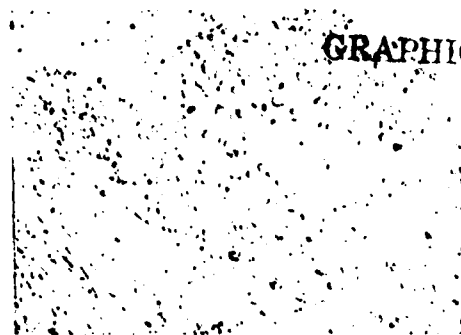
Fig. 1. Two-Day Old Cytopathogenic Alteration, on the Seventh Day Following Infection with Herpes Zoster Vesicle Fluid, in Fibroblast Cell Layer (Magnification approx. 300 x).

As the size of the foci increases, the central cells undergo granular degeneration and necrotization, as a result of which the continuity of the cell layer is interrupted in the center of the foci.

On examining the stained preparation it may be seen that the cells have become rounded on the area of the foci, and are of different sizes. A granular aggregate has formed in the nuclei which has condensed into an eosinophilic mass. The eosinophilic inclusion occupies a position in the center of the nucleus, pressing the chromatin granules and nucleoli to the nuclear membrane.

The cells in a more advanced stage are characterized by pycnotic nuclei, with a heap of small, irregular, rounded basophilic granula (chromatin residues); and among them only the remnants of the inclusion may be noted. The final result of this process is the total disintegration of the cell.

Examination of Patients' Sera. In the next phase of the experiment we wanted to verify by means of serological tests that the cultured agent indeed originates from the patients' vesicle fluid and to prove, together with other data, that the isolated agent can be made responsible for the syndrome. The antibody reaction was determined from the increase, in the sera taken in the acute and the convalescent stage, of the neutralizing antibody titer vis-a-vis the isolated pathogen.



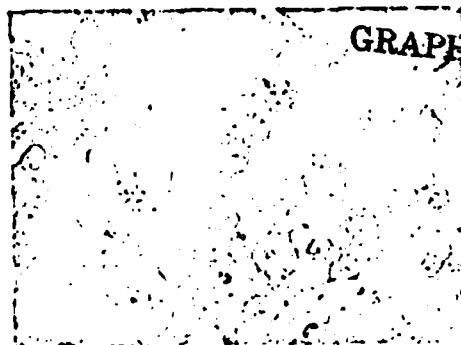
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Fig. 2. Eight-Day Old Cytopathogenic Alteration in Fibroblast Cell Layer (Magnification approx. 300 x).

We were able to obtain pairs of sera from four patients. From two patients we obtained blood only in the acute stage of the disease. The serum pair of one patient proved to be cytotoxic during the virus-neutralization tests.

We noted that the sera of patients S. S., K. I. and Sz. I. obtained during the acute phase had only a minimal neutralizing ability vis-a-vis the agents isolated from their vesicle fluids; the virus-neutralization capacity of the acute sera of

I. O. and N. Gy. was more significant, but these were obtained as early as five days after the onset of the disease. The convalescent sera of S. S., I. O. and Sz. I. possess definite neutralizing ability, and two of these, those of S. S. and Sz. I. exhibited a strong rise in neutralizing titer compared with the acute serum pairs (50- and 12-fold increase, respectively).



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Fig. 3. Fibroblast Cells Infected with Herpes Zoster Virus, with Type A Intranuclear Inclusions (Magnification approx. 1400 x).

Serological Comparison of Virus Strains. Our next task was to decide whether we had isolated the same virus strain from all clinical material. To determine this, we wanted to find the answer to two questions:

1. Do the patients' acute and convalescent sera neutralize their own virus strain and that isolated from another zoster patient to the same extent?

Of our six patients, there is a 4% difference between the neutralizing ability of I. O.'s acute and convalescent sera with respect to his own, Z-2, virus strain, and to another, Z-1, virus strain. The serum pair of Sz. I. exhibited a 5% difference in their neutralizing ability vis-a-vis the two virus strains. The acute sera of K. I. and N. Gy. neutralized their own virus strains and the Z-1 virus strain to the same extent. These deviations observed in the extent of neutralization are within the limits of error of the method employed.

2. Are the antibodies proliferating during the disease in the convalescent serum of a given herpes zoster patient to the other isolated virus strains in the same way as they are to their own virus strains?

To decide this, we made an attempt to demonstrate, by using patient S. S.'s convalescent serum, virus particles in cells infected with his own virus strains as well as with the other five virus strains, employing an indirect immunofluorescence method for this purpose. In all cases the infected cells exhibited an intense fluorescence, indicating that antibodies specific to each virus strain had proliferated on S. S.'s convalescent serum.

No fluorescence was obtained with acute serum in the case of any virus strain, which corresponds to the minimal antibody content found in the neutralization test.

Table 2.

Virus-Neutralizing Ability of Acute and Convalescent Sera in %

(1) A beteg jelölése	(2) Az izolált vírus jelölése	(3) A vírus eloszlásának időpontja a betegben (kezdőbeteg)	(4) A vírus neutralizáló képessége a homolog vírus-származékkal
S. S.	Z-1.	2. 21.	100% 98%
I. O.	Z-2.	7. 21.	92% 92%
K. I.	Z-3.	3. —	100% —
N. Gy.	Z-4.	7. —	40% —
G. A.	Z-5.	citotoxikus (5) szövődmény	
Sz. I.	Z-6.	2. 21.	90% 6%

*The decrease, in %, of the number of foci relative to the number of control foci (developing in the presence of calf serum).

1 -- Patient's initials; 2 -- Code of strain isolated; 3 -- Number of days after onset of illness when blood sample was taken; 4 -- Neutralizing ability of serum vis-a-vis the homologous virus; 5 -- Pair of cytotoxic sera.

Utility of Immunofluorescence Method for Confirmation of Etiological Diagnosis. During the further course of this study we used the immunofluorescence method for the confirmation of those herpes zoster diagnoses which were made only on the basis of clinical symptoms.

Of the serum pairs of the four patients examined, two acute sera did not contain antibodies which could be detected with our method, while the other two acute sera already had a significant antibody content. All four of the convalescent sera had a high antibody content.

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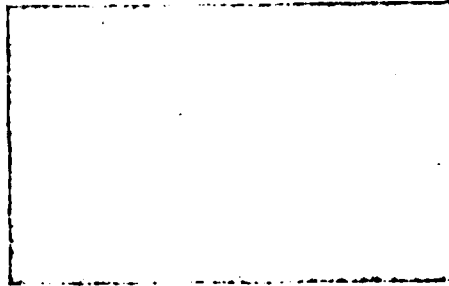


Fig. 4. Fibroblasts Infected with Herpes Zoster Virus Showing Specific Immunofluorescence. (Virus strain Z-2, convalescent serum of patient S. S. Magnification approx. 1000 x)

Discussion

The focal nature and development of the cytopathogenic changes brought about by agents isolated from herpes zoster vesicle fluid on human embryonic fibroblast cultures, and the cell-bound nature of the infection and its percontinuitatem spreading are in full agreement with Weller's data [1]. Our method of obtaining the vesicle fluid differed from that of Weller, inasmuch as in our case the fluids aspirated by means of a capillary were used for the inoculation of the tissue culture in the undiluted state before the onset of coagulation. Thus it was unnecessary to dilute with buffer solution or milk, while our isolation experiments were mostly positive. In contrast with Weller's subsequent observations [2] we did not succeed in isolating or maintaining a zoster virus strain on HeLa tissue culture either from vesicle fluid or by cell passage.

In 1948 Goodpasture and Anderson [3], and Blank, Coriell and Scott [4] inoculated human skin fragments grafted on the chorioallantoic membrane of the chick embryo with herpes zoster vesicle fluid, and in this way succeeded in demonstrating the presence, in the tissue fragments, of type-specific eosinophilic intranuclear inclusions. Weller and Stoddard [5] infected Maitland-type cultures of tissue fragments with varicella vesicle fluid, and likewise observed the formation of a large quantity of eosinophilic inclusions. Eosinophilic intranuclear inclusions could be detected also in fibroblast cells infected with zoster vesicle fluid cultured on a cover slip [2]. The intranuclear inclusions observed by us are in all respects identical to those of the other authors, and may be demonstrated in the infected cells already when the cytopathogenic change in the tissue culture is not yet observable by native examination.

For the demonstration of the patients' immune reaction several methods may be used. Until the problem of the cultivation of the virus was not yet solved, Netter and Urbain [6] and

Brain [7] agglutinated elemental bodies obtained from vesicle fluids with convalescent human sera; later several authors carried out virus neutralization tests (Weller, Witton and Bell [2]; Taylor-Robinson [8]) and complement-fixation tests were carried out using for antigen, concentrates of the nutrient fluids of infected tissue cultures (Weller, Witton [9]; Taylor-Robinson, Downie [10]).

Table 3.

	N. H.		D. L.		S. P.		G. B.	
	A	R	A	R	A	R	A	R
	 (1)							
Z-1. virus (2),	-	++++	-	++++	+++	++++	++	++++

A = acute serum; R = convalescent serum

Anti-Z-1 virus strain antibody content of the serum pairs of four herpes zoster patients, determined by the immunofluorescence method.

1 -- Sera; 2 -- Virus strain.

Our neutralization method differs somewhat from that of Weller [9] and Taylor-Robinson [8], inasmuch as the latter used diluted sera for the tests. However, even they did not obtain a total virus neutralization, which may be explained by the fact that the infective virus remained inside the cell.

By means of the neutralization tests used by us it was possible to prove, on the basis of the increase in titer occurring during the course of the disease, that the isolated agents originate from the patients. By means of cross-neutralization tests using a single, selected virus strain and the patients' sera we verified that we have isolated the same agent from all patients.

Weller and Coons [11] and then Koller et al. [12] were the first to show that the identification of pathogens may be done most reliably by means of the indirect immunofluorescence method, and we concur with their opinion. Our immunofluorescence results, too, confirm that we have isolated the same agent from all patients, and they prove their pathogenicity to the greatest possible extent. In our experience this method may be used (if herpes zoster virus strains are available) also for the confirmation of etiological diagnosis.

Summary

From the vesicle fluid of eight herpes zoster patients we isolated six virus strains. On the basis of the cytopathogenic changes observable natively on fibroblast cultures, of the eosinophilic intranuclear inclusions, of the cell-bound nature of the infection and of the neutralization tests carried out with the patients' acute and convalescent sera and the demonstration of virus within the cell by means of the immunofluorescence method, the virus strains were proved to be herpes zoster virus strains.

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