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STUDY OF GENOME HOMOLOGY OF SOME ORTHOPOXVIRUSES

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Summary. — Homology between DNAs of some orthopoxviruses: vaccinia, neurovaccinia, cowpox, ectromelia, variola and white-pox was studied. By the method of molecular hybridization of labelled virion DNAs of the viruses studied with an excess of unlabelled vaccinia DNA and further treatment of the hybridized products by the single-stranded-specific nuclease S_1, it was shown that the concentration of unlabelled vaccinia virus DNA, converting 50 % of homologous labelled DNA into a hybrid, converted under similar experimental conditions into a hybrid 50 % of labelled neurovaccinia virus DNA, 48 % of white-pox virus DNA, 43 % of variola virus DNA, about 30 % of cowpox virus DNA and 27 % of ectromelia virus DNA. Based on the quantitative data obtained the relationship between viruses of this subgroup and the possible origin of vaccinia virus is discussed.

Key words: *Orthopoxvirus*; *hybridization*; *genetic relationship*

Introduction

It is known that representatives of a certain virus group with common biological properties have a similar genome structure and a common sequence of nucleotides in their DNA. Orthopoxviruses have a rather similar structure (Westwood *et al*., 1964; Avakyan and Bykovsky, 1964), show a close relationship in antigenic structure (Woodroofe and Fenner, 1962) and are able to form recombinants on joint cultivation (Woodroofe and Fenner, 1962). At the same time these viruses are biologically distinct which suggests that they differentiated in the process of evolution long ago and have acquired specific properties. The development of molecular hybridization techniques, in particular hybridization of labelled virion DNA with an excess of unlabelled DNA and further treatment of the hybridized products with nuclease, makes possible quantitative assessment of the degree of genome homology of the viruses under investigation.

Materials and Methods

The following orthopoxviruses were used: 1) vaccinia virus L-IVP, dermal strain, derived in the Moscow Research Institute of Viral Preparations by adaptation of the Elstree strain from the Lister Institute (England) on calf skin; 2) neurovaccinia virus, strain (MM), a neurovariant of vaccinia virus which undergone about 300 continuous passages in the rabbit brains; 3) cowpox virus, strain Brighton (England); 4) ectromelia virus, strain Moscow; variola virus, strain India-385; and 6) variola-like white-pox virus, strain V-71, isolated from a rodent in Dahomeo. Viruses 1-3 were propagated on chorioallantoic membranes (CAM) of 12-day-old chick embryos (Westwood *et al.*, 1957). Ectromelia virus was propagated in foot pads of white mice; viruses 5 and 6 were kindly supplied by Dr. S. Marennikova. The viruses were purified and concentrated by differential and gradient centrifugation according to Joklik (1962).

The labelled viruses were isolated by the following procedure. Chick embryo cell (CEC) culture (48 hr) was inoculated with virus purified through a layer of 36 % sucrose. Before use, the virus was subjected to sonication. The multiplicity of infection was 30—60 pock-forming units (PkFU) per cell. After 60 min of adsorption at 4 °C, the inoculum was removed, the cells were washed and 0.5 % lactalbumin hydrolysate containing ^{3}H-thymidine in a final concentration of 100 μCi/ml was added. The mixture was incubated for 20—24 hr at 37 °C, the cells were mechanically scraped off and disintegrated in phosphate buffered saline (PBS), pH 7.2, in a Dounce homogenizer and the nuclei were sedimented by centrifugation at 750 × g for 10 min. The cytoplasmic extract was used for virus purification (Joklik, 1962). DNA from the labelled virus was extracted according to Randall and Gafford (1969) and Oda and Joklik (1967). The latter method proved to be more effective as it yielded up to 70 % of DNA.

Nuclease S_1 used in this study was obtained from α-amylase according to Vogt (1973). In our experimental conditions the enzyme digested 95—97 % of single-stranded ^{3}H-DNA from CEC and less than 5 % of double-stranded DNA.

The hybridization test of ^{3}H-thymidine-labelled virion DNA of the virus studied with an excess of vaccinia virus DNA was performed in 0.05 M Tris.HCl, pH 8.5 + 0.07 M NaCl buffer (Frankel *et al.*, 1973) at 67 °C for 63 hr in a volume of 0.2 ml. DNA preparations previously fragmented (see below) by sonication were denatured by incubation at 100 °C for 10—20 min depending on the ionic strength of the solution and were rapidly cooled on ice. A standard amount of ^{3}H-thymidine-labelled test DNA (about 1000 count/min per sample) was mixed with increasing amounts of vaccinia virus DNA (from 0.1 to 25.65 μg per sample) in a standard volume of 0.2 ml. Each sample was tested in duplicate. By the end of the test the hybridized mixture was diluted to 1 ml with buffer containing 0.03 M sodium acetate, pH 4.6 and 0.01 M $ZnSO_4$. Denatured calf thymus DNA (25 μg/ml) and nuclease S_1 (100 μl per sample) was added to the samples. Control samples were incubated without enzyme. The incubation lasted for 30 min at 45 °C. The reaction was stopped by cooling on ice. Thereafter 2 drops of 0.1 % casein and trichloroacetic acid to a concentration of 5 % were added to the samples which were then incubated on ice for 15 min for the formation of precipitate. The samples were washed on "Synpor-6" (Chemapol, Prague) filters and counted in toluene scintillator for 10 min.

Each test included a control series. The count in samples with undenatured ^{3}H-DNA treated with S_1 nuclease was taken for 100 %. The control of the effectiveness of ^{3}H-DNA denaturation and enzyme activity showed which part of single-stranded ^{3}H-DNA remained nuclease-resistant. The control samples contained nonhybridized and enzyme-treated denatured ^{3}H-DNA. The value obtained (in %) made it possible to define the nuclease-resistant "nucleus" of single-stranded DNA and was taken into account in introducing a correction. The control for reassociation of ^{3}H-DNA was represented by samples in which the denatured ^{3}H-DNA was incubated without unlabelled DNA and subsequently treated with nuclease S_1. The reassociation was taken into account in introducing a correction. Annealing with cell DNA (from CEC) served as control for the possible presence of cell DNA impurity. Hybridization with cell DNA was generally negative. The nuclease-resistant fraction was determined as the proportion (in %) of the count in samples with enzyme out of the count in control samples which was taken for 100 %. In estimating the percentage of hybridization a correction was made the S_1 nuclease-resistant fraction of single-stranded DNA according to the formula

$$x = a - \frac{b \times (100 - a)}{100 - b} \tag{1}$$

where x = the percentage of hybridization, a = the percentage of nuclease-resistant material in the sample, b = the percentage of nuclease-resistant material in the control sample containing no virus-specific DNA (Kaverin *et al.*, 1975).

The kinetics of DNA reassociation was described in the following system of coordinates: the product of the initial molar concentration of nucleotides C_0 (in mmol/ml) by time (the given time of reassociation t in sec) on the abscissa in log scale was plotted against the magnitude of DNA reassociation (in %) on the ordinate. In estimating C_0t, a correction was made for the ionic strength of the solution. For quantitative evaluation of hybridization, the median hybridization value C_0t_{50} was used.

Reagents. Sodium dodecyl sulfate (SDS) and NaCl were from Serva (FRG), pronase from Calbiochem, 2-mercaptoethanol and Tris. HCl from Merck (FRG).

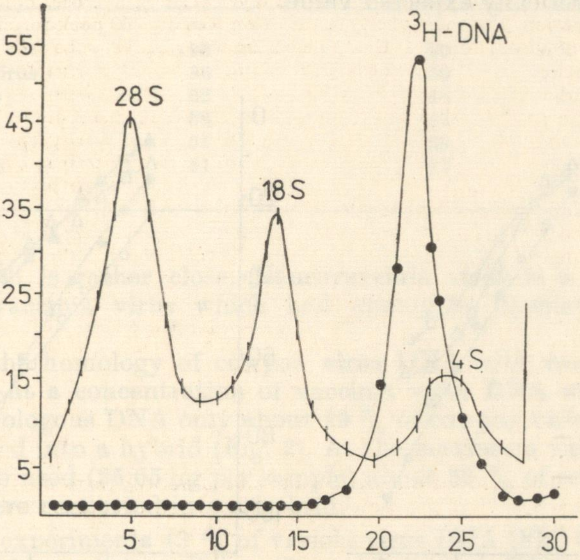

Fig. 1.

Sedimentation profile of dermal vaccinia virus ^{3}H-DNA in a 15—30 % sucrose gradient Centrifugation at 27000 rev/min; 25 °C; SW 3×25 rotor of an MSE Superspeed 65 centrifuge. Abscissa: fraction number; ordinate: count/min × 10^{-2}

Results

The optimal conditions for performing hybridization tests: incubation temperature, duration of incubation, size of DNA fragments and others were determined in preliminary experiments. The duration of the reaction was 63 hr, as within this interval the self-hybridization of labelled DNA was not yet registered. DNA was fragmented by sonication. The size of DNA fragments was determined by centrifugation in a 15—30 % sucrose density gradient in buffer (0.1 M NaCl; 0.01 M Tris. HCl; 0.001 M EDTA, pH 7.4) with 0.2 % SDS. The DNA fragmentation procedure by sonication used in the present study yielded fragments with sedimentation coefficients in the order of 7 S (Fig. 1).

To define the homology of vaccinia virus genome and with that of the other viruses studied it was necessary to determine the character of reassociation of homologous molecules of vaccinia virus DNA. Subsequently, such test was included as control in hybridization tests with heterologous DNAs. Under the experimental conditions used the reassociation of homologous molecules of dermal vaccinia DNA was 95 + 5 % (Fig. 2). C_0t_{50} for reassociation of homologous DNAs was achieved at a concentration of excess DNA of about 8×10^{-3} mg/ml for 63 hr and equals 0.54 mmol × sec/ml, which is close to the theoretically expected value.

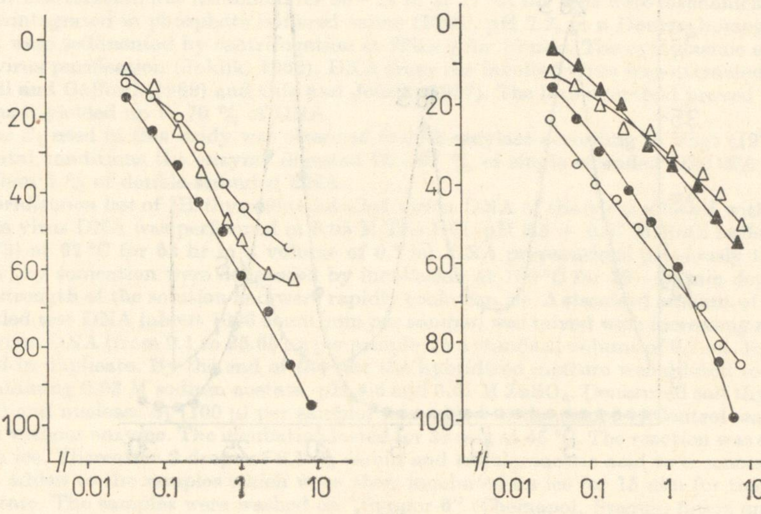

Fig. 2. **Fig. 3.**

Dermal vaccinia virus DNA hybridization with labelled ^{3}H-DNA of orthopoxviruses
Abscissae: C_0t (mmol × sec/ml); ordinates: % of DNA hybridized
DNA from dermal vaccinia virus was hybridized with ^{3}H-DNA from:

Fig. 2:
● dermal vaccinia virus (selfhybridization)
▲ variola virus
○ white-pox virus

Fig. 3:
● dermal vaccinia virus (selfhybridization)
○ neurovaccinia virus
▲ cowpox virus
△ ectromelia virus

The study of the homology of neurovaccinia virus DNA with vaccinia virus DNA showed that, in the same experimental conditions as used with the homologous vaccinia virus DNA, about 86 % of ^{3}H-DNA of neurovaccinia virus was converted into a hybrid (Fig. 2). At a concentration of vaccinia virus DNA, which caused reassociation of 50 % of homologous sequences, also 50 % of neurovaccinia virus DNA sequences were converted into a hybrid. These data are in good agreement with the biological relationship of these

Table 1. Genetic relationship of some orthopoxviruses to vaccinia virus

^{3}H-DNA compared	Percentage of hybridization at maximum standard concentration of DNA	Percentage of hybridization in the conditions of C_0t_{50}	Percentage of homology with vaccinia virus DNA
Vaccinia virus	95	50	100
Neurovaccinia virus	86	50	90
White-pox-virus	62	48	65
Variola virus	56	43	59
Cowpox virus	55	29	57
Ectromelia virus	51	27	54

viruses, which is rather close. Neurovaccinia virus is a neuropathogenic variant of vaccinia virus which had undergone numerous rabbit brain passages.

Tests on the homology of cowpox virus DNA with vaccinia virus DNA showed that at a concentration of vaccinia virus DNA which reassociated 50 % of homologous DNA only about 29 % of cowpox virus DNA sequences were concerted into a hybrid (Fig. 2). At the maximum vaccinia virus DNA concentration used (25.65 μg per sample) about 55 % of cowpox virus DNA sequences were converted into a hybrid.

In similar experiments 43 % of variola virus DNA (Fig. 3) were converted into a hybrid in conditions of C_0t_{50} for vaccinia virus DNA. At the maximum vaccinia virus DNA concentration used, a bout 56 % of variola virus DNA sequences were converted into a hybrid. Under conditions of C_0t_{50} for vaccinia virus DNA, 48 % of white-pox virus DNA were converted into a hybrid and at the maximum concentration of vaccinia virus DNA used nearly 62 % of white-pox virus DNA were hybridized (Fig. 3). At C_0t_{50} for vaccinia virus DNA, about 27 % of ectromelia virus DNA sequences were converted into a hybrid. The maximum concentration of vaccinia virus DNA which converted the homologous DNA into a hybrid almost completely, converted only 50 % of ectromelia virus DNA into the hybridized form (Fig. 2).

Orthopoxvirus homology might be expressed in per cent of homologous reassociation, the latter being taken for 100 %. Heterologous reassociation was estimated by the following equation (Akhundov and Mednikov, 1976)

$$x_{ji} = a_{ji}/a_{ii} \times 100 \qquad (2)$$

where a_{ii} = the number of homologous DNA regions and a_{ji} = the number of heterologous DNA regions denatured during incubation. If the number of heterologous DNA areas reassociated at maximum concentration of DNA during hybridization is taken for "a" (in %), the degree of relationship between the virus species studied might be expressed in per cent with respect to vaccinia virus used for comparison. Thus the value of homology for neurovaccinia virus

$$x = \frac{86}{95} \times 100 = 90\ \%.$$

If we insert into the equation (2) the values of the maximum percentage of hybridization for the DNAs of the viruses tested, we can define the degree of genome homology of these viruses with the vaccinia virus genome. Based on data presented in Table 1, the viruses might be arranged in a certain sequence, the degree of the genetic relationship being best demonstrated by comparing the C_0t_{50} values. As expected, neurovaccinia virus proved to be the most closely related and ectromelia virus the least related to vaccinia virus. White-pox virus and variola virus appeared to be more closely related to vaccinia virus as compared with cowpox virus.

The significance of the differences in curves of virus DNA hybridization was confirmed by mathematical processing of the results.

Discussion

Six orthopoxviruses with different degree of biological relationship were selected for a comparative study. This selection was based on some important factors. At the present time of complete eradication of smallpox the problem of poxvirus ecology is of utmost importance. Irrespective of the great practical virological achievements in smallpox eradication in the world we do not yet know wheter there exist natural reservoirs of variola virus, what is the nature of the white-pox viruses and which is the association between them, monkeypox viruses and variola virus. Great contribution into the elucidation of this problem was made by S. S. Marennikova and co-workers who stressed the potential danger of white-pox and monkeypox viruses in the new epidemiological conditions and disclosed the possible source of white-pox viruses (Marennikova and Shelukhina, 1978*a*, *b*; Marennikova *et al.*, 1978).

The present data clearly showed that neurovaccinia virus is the most closely related to vaccinia virus, which is in good agreement with the biological characteristics of these two viruses. This correlation itself indicates that the methodical approach used was adequate. It is quite logical that ectromelia virus was the most distant from vaccinia virus, which is also in good correlation with their biological properties. Of particular interest is the question of the degree of relationship between vaccinia virus on the one hand and variola virus and cowpox virus on the other. C_0t_{50} values revealed a high degree of genetic relationship between vaccinia virus and variola virus (48 % hybridization under C_0t_{50} conditions for vaccinia virus DNA) as compared with cowpox virus (29 % hybridization under C_0t_{50} conditions for vaccinia virus DNA). These data make it possible to assume that the Elstree strain originated from variola virus and is not a "white mutant" of cowpox virus. The high degree of genetic relationship between variola and variola-like viruses also deserves attention. Our data showed that variola virus DNA in hybridization tests behaved in a similar but not identical way as white-pox virus DNA. It is possible to assume, therefore, that this strain of white-pox virus is related to, but not identical with, variola virus.

Recently some scientists have applied novel molecular and biological approaches to the study of the genetic relationship between orthopoxviruses (Müller *et al.*, 1977; Arehard *et al.*, 1978). The "restrictase" analysis used by them differs significantly from our method: a comparison of restrict distribution clearly reveals qualitative differences between the viruses but it does not permit quantitative evaluation of the degree of relationship between the viruses compared.

The results obtained by Müller *et al.* (1977) as concerns vaccinia, rabbit pox and ectromelia viruses are in good agreement with our results. The combination of our method with restrictase analysis would not only answer the question of similarity or difference between close poxviruses but also make it possible to study the evolution of this large virus group.

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