Infectivity and Capacity for DNA Replication of Vaccinia Virus Irradiated by $\gamma$-Rays

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SUMMARY

Purified vaccinia virus was irradiated by $\gamma$-rays under direct-effect conditions. The ability of the irradiated samples to form plaques (infectivity) and to induce viral DNA synthesis was determined. The radiosensitive volume of the viral unit causing infection ($1.9 \times 10^{-17}$ cm$^3$) is very small compared with the volume of the whole viral DNA ($\sim 10\%$).

The inactivation of the DNA replication function follows a simple exponential law. The radiosensitive volume necessary for the replication of DNA ($1.6 \times 10^{-18}$ cm$^3$) represents only $8.5\%$ of the DNA necessary for infectivity and $0.85\%$ of the total viral DNA. This indicates existence of a dissociation between two functions of vaccinia virus, the synthesis of viral DNA and infectivity.

INTRODUCTION

The use of $\gamma$-rays has often given much information on the size and structure of viral particles as well as on the relationship between this structure and infectivity. The earliest recorded information on the action of ionizing radiations on a virus seems to be that of Green (1904), who subjected various bacteria and also vaccinia virus to the action of $\beta$-rays. Baker (1935), Gowen & Lucas (1939) and Lea & Salaman (1942) proved that inactivation followed the well-known exponential relation between survival and dose. Since then, numerous authors have studied the influence of $\gamma$-rays on vaccinia virus infecting capacity (Jordan & Kempe, 1956; McCrea, 1960; Wilson, 1961; Palacios et al. 1963; Jimenez & Ohlbau, 1966).

The present study was undertaken to determine the action of $\gamma$-rays, first on vaccinia virus infectivity, secondly on the DNA replication capacity of the virus. Our first observations concerning these two points are reported here.

METHODS

Virus. The vaccine MERIEUX 37 strain (LISTER strain which has undergone ten passages on KB cells at 37$^\circ$) was used.

Tissue cultures and media. These methods were described by Burkard et al. (1968).

Virus purification. The virus was purified by the method used by Joklik (1962). Roux bottles containing 24 hr cultures of KB cells were infected with an input multiplicity of 4 p.f.u./cell. After adsorption for 1 hr each bottle received 50 ml. of medium and was incubated at 37$^\circ$. Twenty-four hr later the cells and media were collected, frozen and thawed three times and subjected to ultrasonic treatment for 5 min. to set free the remaining intracellular virions (MSE Disintegrator, 100 w, 20 kc). The virus
suspension thus obtained was centrifuged in a Sorvall RC2 centrifuge at low speed (750g) for 10 min. The sedimented cellular debris contained no more than 5% of the total amount of virus and was discarded. The supernatant fluid was then centrifuged at 15,000g for 40 min., in order to sediment virus particles. The pellet, resuspended in 50 ml. of tris buffer (0.001 M, pH 9), was layered on 3 ml. portions of 36% sucrose and centrifuged for 2 hr at 23,000g in the SW 25 rotor of a Spino L2 ultracentrifuge. The virus pellet was resuspended in 4 ml. of tris buffer and exposed to ultrasonic vibration for 1 min. in order to break aggregates of virus and then immediately layered on a 20% to 40% sucrose gradient and centrifuged for 45 min. at 23,000g.

The band containing the virus could be seen distinctly 2 cm. from the bottom of the tube and a second wider band was also visible near the top of the gradient. The bands were collected through pinholes from the bottom by fractions of 15 drops each. The infectivity of each fraction, was determined. The optical density was measured at 260 nm. in a PMQ II Zeiss spectrophotometer (Fig. 1). The first peak contained the virus; the second, which was devoid of infectivity, was composed of viral DNA, haemagglutinin and also cellular DNA. The fractions containing the virions were mixed and dialysed for 24 hr against two changes of 200 vol. of tris buffer. The virus thus purified had an infectivity of 10^8 p.f.u./ml. and contained 4 x 10^{10} virus particles according to Joklik’s data showing that one optical density unit corresponds to 1.2 x 10^{10} elementary particles.

**Measurement of viral DNA synthesis in the infected cells.** This technique has been previously described (Burkard et al. 1968). Monolayers of KB cells were infected at a multiplicity of 10 p.f.u./cell. After an adsorption period of 1 hr the labelled medium (0.06 µg/ml.) was added (Lactalbumin—yeast extract 10% calf serum,[3H]thymidine) and the cells were incubated at 36°C. At the 5th hr., when DNA synthesis occurred, the cells were treated by trypsin+EDTA and centrifuged at 1000g for 5 min. After two
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washings with phosphate-buffered saline, the pellet was suspended in hypotonic buffer (RSB) for 20 min. at 0°C; then the cells were broken in a Dounce tissue grinder and centrifuged at 600g for 5 min. The cytoplasmic fraction containing the viral DNA was treated by trichloracetic acid (10%) for 30 min. at 0°C and the precipitate obtained washed three times with 5% trichloracetic acid. The pellet was finally dissolved in a liquid scintillator and the radioactivity determined in a Beckman liquid scintillation system.

Irradiation. In order to protect the virus against indirect effects we have combined the chemical protection provided by a high concentration of organic inert substance (10% calf serum) with the physical protection of the solid state which greatly reduces the migration of free radicals (−70°C). In this case the indirect effects of radiations were shown to be negligible. The virus suspensions were exposed to radiation doses starting at $3 \times 10^4$ r. to $1.5 \times 10^6$ r. with a rate of 600 r./min. from a $^{60}$Co source. Doses were measured with a Fricke dosimeter.

RESULTS

Inactivation by direct effect

To be sure that the indirect effects of the radicals produced in the irradiated water were eliminated, virus samples containing different amounts of calf serum were irradiated at −70°C. Above a concentration of 5% serum, the rate of inactivation remained constant and it was concluded that all indirect effects had been eliminated. In addition we verified that inactivation is independent of the dose rate, thus confirming the absence of effect due to long lived toxic products which take time to act.

Infectivity

Four inactivation experiments were made with different initial viral concentrations, in the range of $10^6$ to $3 \times 10^8$ p.f.u./ml. (Fig. 2). The biphasic curve obtained can be resolved into two exponentials of different slope. On the assumption that this reflects a mixed population containing two components of different target sizes, we have determined the two values of the 37% inactivation dose (D37). The D37 for the more radiosensitive component is $70,000 \pm 5000$ r., giving a target volume of $1.9 \times 10^{-17}$ cm$^3$ and a molecular weight of $1.94 \times 10^7$ daltons for the viral unit causing infection. If this value were a sphere, its diameter would be equal to 33 nm. The D37 for the less radiosensitive component is $230,000 \pm 20,000$ r., thus corresponding with a target volume of $6 \times 10^{-18}$ cm$^3$. The two volumes have been calculated using the conversion factor $7.54 \times 10^{11}$ primary ionizations per cm.$^3$ of nucleic acid per roentgen (Pollard, 1954).

Viral DNA synthesis

The amount of viral DNA whose synthesis was induced by irradiated virus at various doses was determined by tritiated thymidine incorporations in the acid-insoluble cytoplasmic fraction of KB cells removed 5 hr after infection (Fig. 2). The amount of DNA synthesized following infection by irradiated virus is expressed as a percentage of that induced by non-irradiated virus. The inactivation of the DNA replication function followed a simple exponential law. In addition the function of the viral DNA synthesis was less sensitive to $\gamma$-rays than was infectivity as indicated by
Fig. 2. Effect of γ-ray dose on infectivity and DNA replication capacity of vaccinia virus. Infectivity at various initial concentrations of virus: △, 3.0 × 10⁶ p.f.u./ml.; ○, 1.1 × 10⁸ p.f.u./ml.; ●, 1.0 × 10⁷ p.f.u./ml.; ▲, 1.0 × 10⁶ p.f.u./ml.; □, thymidine incorporation in acid insoluble cytoplasmic fraction of infected cells; the samples were removed 5 hr after infection.

Fig. 3. Time course of vaccinia virus DNA synthesis determined by incorporation of tritiated thymidine in acid-insoluble cytoplasmic fraction of infected KB cells. ●, Non-irradiated virus; ○, irradiated virus (D = 4.2 × 10⁵ r); △, uninfected cells.
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The relatively high value of the \( D_{37} \): \( 830,000 \pm 50,000 \) r. When the infectivity was reduced to 1% of its original value the viral DNA synthesis was only reduced to about 50%. This was confirmed by an autoradiographic study.

The value of the ‘\( D_{37} \)’ allowed us to determine the radiosensitive volume necessary for the replication of viral DNA \( (V = 1.6 \times 10^{-18} \text{ cm}^3) \), which represents a sphere of 14.6 nm. in diameter. The molecular weight of this target is \( 1.63 \times 10^6 \) daltons. DNA synthesis by the irradiated virus was slightly slower than that of the un-irradiated virus (Fig. 3).

**DISCUSSION**

The data on inactivation by \( \gamma \)-rays of vaccinia virus infectivity are summarized in Table 1. Our \( D_{37} \) value of \( 7 \times 10^4 \) r. is in fair agreement with the results of other workers.

In several plant viruses and T7 bacteriophage Epstein (1953) showed excellent agreement between radiosensitive volume and nucleic acid volume. This does not seem to be valid for vaccinia virus. The volume of the vaccinia virus DNA determined by Hoagland *et al.* (1940) was \( 1.9 \times 10^{-18} \text{ cm}^3 \), whereas the radiosensitive volume we have determined by the target theory is \( 1.9 \times 10^{-17} \text{ cm}^3 \). If viral DNA represents the target involved in viral replication, only 10% of this DNA is radiosensitive.

**Table 1.**

<table>
<thead>
<tr>
<th>( D_{37} )</th>
<th>Authors</th>
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<tbody>
<tr>
<td>( 8 \times 10^4 )</td>
<td>Lea &amp; Salaman (1942)</td>
</tr>
<tr>
<td>( 2 \times 10^5 )</td>
<td>Jordan &amp; Kempe (1956)</td>
</tr>
<tr>
<td>( 4 \times 10^5 )</td>
<td>McCrea (1960)</td>
</tr>
<tr>
<td>( 1 \times 10^6 )</td>
<td>Wilson (1961)</td>
</tr>
<tr>
<td>( 3.5 \times 10^6 )</td>
<td>Palacios <em>et al.</em> (1963)</td>
</tr>
<tr>
<td>( 4 \times 5.6 \times 10^6 )</td>
<td>Friesen, Sankoff &amp; Siminovitch (1963)</td>
</tr>
<tr>
<td>( 2 \times 5 \times 10^6 )</td>
<td>Jimenez &amp; Ohlbaum (1966)</td>
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Not all hits occurring in the DNA appear to be lethal. Ginoza (1967) offered three explanations for the radioresistance of DNA viruses:

(a) Only double-strand scissions or simultaneous base pair damage can kill, and the efficiency for either of these possibilities is one tenth for vaccinia virus.

(b) The host cell can repair radiation lesions occurring in double stranded DNA but not those occurring in single stranded DNA.

(c) A critical target within the genome with a molecular weight of \( 1.94 \times 10^7 \) must be hit to inactivate, and hits elsewhere in the genome are innocuous.

Under direct effect conditions, ionizing radiations inactivate chiefly by producing breaks in the DNA chain (Eckert & Tisne, 1966). In a double-stranded DNA, single breaks are rarely lethal whereas double breaks are highly lethal (Freifelder, 1965–6; Kaplan, 1966). According to our data, the frequency of double breaks would therefore be 10%, nearly the same value as that obtained by Stent (1963) for the phages \( \lambda \) and the T series. However, in a recent study on the size of DNA molecules in phage T7 after \( ^{32} \text{P} \) decay, Reslova & Drobnik (1968) have shown that the efficiency of generation of double-strand breaks by \( ^{32} \text{P} \) transmutation was more than an order lower than the killing efficiency (0.076). Thus our results do not permit us to prefer any one of these three hypothesis in particular.

An inactivation curve with two components has been observed by different authors who reviewed the data on inactivation by \( \gamma \)-rays of vaccinia virus infectivity.
for vaccinia virus inactivated by $\gamma$-rays (McCrea, 1960; Palacios et al. 1963; Friesen, Sankoff & Siminovitch, 1963; Jimenez & Ohlbaum, 1966). It might be due to the existence of two virus populations of different radiosensitivity. Palacios suggested that the difference between the two populations is on the level of the DNA which could be single or double stranded (Pfau & McCrea, 1962). But for Jimenez the presence of cellular debris and serum may be responsible for the second component of the survival curve.

In this work we have assumed that $\gamma$-rays have no influence on viral adsorption. If the viral attachment zone of vaccinia virus is altered by ionizing radiations, as may be supposed by the work of Wilson (1961), the radiosensitive targets relative to infection and DNA synthesis would be even smaller.

To our knowledge, the influence of irradiations on the function of DNA synthesis of vaccinia virus has not been previously studied. We have determined the size of the radiosensitive volume necessary for the replication of DNA on the basis of the target theory: $V = 1.6 \times 10^{-18} \text{ cm}^3$. According to these results, the genome which directs DNA synthesis represents only 8.5% of the DNA necessary for infectivity and 0.85% of the total DNA. The fact that the DNA replication is so little affected by $\gamma$-rays indicates that the action of these radiations takes place in the later stages of the virus development. It is, however, to be noted that we have no information on the physical and biological integrity of the newly synthesized DNA.

We have found it interesting to compare our results with those of other authors who have studied the dissociation of the functions of irradiated oncogenic viruses. Latarjet, Cramer & Montagnier (1967) showed that infectivity of polyoma virus and its transformation capacity are equally resistant to the direct effect of ionizing radiations. The different results obtained by Benjamin (1965) and by Basilico & Di Mayorca (1965) would be due to the indirect effect not being entirely suppressed. Latarjet interpreted his results by conceiving that any double break on the double-stranded DNA molecules inactivates both infectivity and transformation functions. In a recent study, however, of the SV 40 virus irradiated by $\gamma$-rays at $-70^\circ$, Defendi, Jensen & Sauer (1967) found that some functions of the viral genome, the synthesis of the complement fixing antigen and the induction of DNA synthesis of the host cell were more radiation-resistant than infectivity. Our results are in agreement with those of Defendi, since we have shown a dissociation between two functions of the vaccinia virus, the synthesis of viral DNA and infectivity.

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