Investigation of the Mechanism of Changes of RS Character of Neurovaccine in Passages in the Presence of Bromodeoxyuridine

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SUMMARY

Neurovaccinia virus propagated in the presence of bromodeoxyuridine loses the capacity to produce necrosis in the skin of rabbits. Mechanisms underlying this loss were studied. The population under study consisted predominantly of virus particles capable of producing necrosis (RS+) and a small portion of particles which had lost this capacity (RS-). An RS- clone isolated from the population induced an inhibitor of interferon type in rabbit skin, which markedly inhibited reproduction of RS+ virus strains and consequently the capacity of these strains to produce necrosis.

INTRODUCTION

The capacity of some viruses of the variola-vaccinia subgroup to produce necrosis upon intracutaneous inoculation of rabbits has been described by Fenner (1958) and Ghendon & Chernos (1964).

In the course of passages in L-cell cultures TK- strain (Kit et al. 1963) of neurovaccinia virus in the presence of 5-bromodeoxyuridine we obtained a variant (RS-) which had completely lost the capacity to produce dermal necrosis in rabbits (Ghendon, Chernos & Rozina, 1967). Similar results were obtained by Ferrari et al. (1965) with vaccinia virus passaged in the presence of iododeoxyuridine.

In the clonal analysis of our RS- variant of vaccinia virus, of 16 clones under study only one (M-156) lost the capacity to produce dermal necrosis in rabbits, while the other 15 clones were definitely RS+ (Chernos & Apridonidze, 1968). Nevertheless, the whole population from which these clones had been isolated produced no necrosis by the intracutaneous route in rabbits. Thus, this property was determined not by the virus particles prevailing in the population but by those infective particles which were much less numerous.

We report here an investigation of the mechanism of this phenomenon.

METHODS

Viruses. A neuropathogenic variant of vaccinia virus, MM strain, and rabbit pox virus, UTRECHT strain, were used which produced necrosis when inoculated intracutaneously in rabbits (RS+ marker), as well as a variant of the MM strain which had undergone 20 consecutive passages in L-cell cultures (TK-) in the presence of 25 µg./ml. 5-bromodeoxyuridine and had lost its capacity to produce necrosis (RS-). The virus pools were prepared from chorioallantoic membranes of chick embryos inoculated according to the method of Westwood, Phipps & Boulter (1957). Western equine encephalitis virus was passaged in chick embryo fibroblast tissue culture.
Cell cultures. Primary cultures of chick embryo fibroblasts prepared by trypsinization were used for virus assay. The virus concentration was determined by counting plaques under the agar overlay described by Porterfield & Allison (1960).

Animals. Albino chinchilla rabbits weighing 2 to 2.5 kg. were used.

RESULTS

For interpretation of the clonal analysis of the RS⁻ population of neurovaccinia virus we assumed that mutant particles composing it, in particular the M-156 RS⁻ variant, possessed a higher interfering activity and inhibited multiplication in the skin of rabbits of highly pathogenic RS⁺ virus which produced necrosis. To test this assumption experimentally, tests were made in which the M-156 mutant was mixed with neurovaccinia virus, rabbit pox virus or RS⁺ M-155 clone recovered from an RS⁻ population of neurovaccinia virus. The resulting mixture was inoculated intracutaneously in rabbits. Tenfold dilutions of neurovaccinia, rabbit pox or M-155 clone viruses were mixed 1:1 so that the mixture contained the number of p.f.u. indicated in Table 1 of RS⁺ virus strain and 10⁶ p.f.u. of M-156 mutant in 0.2 ml. In controls, RS⁺ strains were mixed instead of the mutant with an equal amount of medium 199. The mixture was inoculated intracutaneously in 0.2 ml. doses in two rabbits, and the results were read at 5 days. M-156 clone in control produced no necrosis by the intracutaneous route using 10⁶ p.f.u. Neurovaccinia and rabbit pox viruses produced necrosis after intracutaneous inoculation of 100 p.f.u. or more (Table 1). At the same time 100 or even 1000 p.f.u. of neurovaccinia or rabbit pox virus mixed with 10⁶ p.f.u. of M-156 mutant failed to produce necrosis. However, when the dose of RS⁺ viruses in mixture was increased from 10⁴ to 10⁵ p.f.u., addition of M-156 mutant did not prevent the development of necrosis. Thus the M-156 mutant was capable of preventing necrosis produced by RS⁺ strains at a ratio of p.f.u. of the mutant to p.f.u. of RS⁺ virus 1000:1, that is, under conditions in which the number of infective particles of M-156 mutant was 1000-fold greater than that of RS⁺ particles of 'wild' virus type.

On the other hand, in experiments with RS⁺ M-155 clone isolated from the same virus population from which RS⁻ M-156 clone had been obtained, the latter prevented necrosis produced by RS⁺ M-155 clone when the number of virus particles of RS⁺ clones was 1000-fold greater than that of RS⁻ particles. We suggest, therefore, that the inability of our RS⁻ population of neurovaccinia virus to produce dermal necrosis in rabbits was indeed due to the small number of RS⁻ virus particles present in the population.

Table 1. The capacity of RS⁻ variant (M-156) to inhibit necrosis in experiments with RS⁺ strains of viruses of the variola vaccinia subgroup

<table>
<thead>
<tr>
<th>No. of p.f.u. of RS⁺ strains</th>
<th>Neurovaccinia (RS⁺)</th>
<th>Rabbit pox (RS⁺)</th>
<th>Clone M-155 (RS⁻)</th>
<th>Neurovaccinia Clone M-156</th>
<th>Rabbit pox Clone M-156</th>
<th>Clone M-155</th>
</tr>
</thead>
<tbody>
<tr>
<td>10²</td>
<td>10</td>
<td>12</td>
<td>—</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10³</td>
<td>16</td>
<td>17</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10⁴</td>
<td>17</td>
<td>19</td>
<td>8</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>10⁵</td>
<td>18</td>
<td>24</td>
<td>12</td>
<td>8</td>
<td>12</td>
<td>0</td>
</tr>
</tbody>
</table>

* 10⁶ p.f.u.
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We were interested to find out whether reproduction of M-156 mutant was necessary for manifestation of its above-mentioned inhibiting activity and whether this mutant could change the capacity of RS+ viruses to produce necrosis or inhibit multiplication of wild virus in the skin of rabbits. Rabbits were inoculated intracutaneously with 10⁶ p.f.u. of neurovaccinia virus, 10⁶ p.f.u. of M-156 mutant, and a mixture containing 10⁵ p.f.u. of neurovaccinia virus and 10⁶ of M-156 mutant in active or inactivated form. Inactivation was achieved by ultraviolet irradiation (10,000 erg/mm² with the thickness of the virus-containing fluid layer 1 mm). At 72 hr in one group of rabbits areas of the skin into which the virus had been inoculated were cut out and 1 g. of skin was minced in 2 ml. of medium 199 in a homogenizer of ultraturrax type. Since neurovaccinia virus produced plaques in chick embryo fibroblast tissue culture at 40° and M-156 clone had lost this capacity, titration was done at 40° in determinations of the titre of RS+ virus in areas of the skin infected with a mixture of viruses or only RS+ virus. In the other group of rabbits development of necrosis was observed. Neurovaccinia virus multiplied well in the skin of rabbits while M-156 mutant did so very poorly. When rabbits were inoculated with a mixture of neurovaccinia virus and either infective or inactivated M-156 mutant, reproduction of the RS+ strain was almost completely inhibited (Table 2). At the same time development of necrosis in rabbit skin was also inhibited. This evidence thus demonstrated that the capacity of M-156 mutant to prevent necrosis by RS+ virus strains was due to marked inhibition of RS+ strain multiplication, multiplication of RS- mutant being not necessary for manifestation of this inhibiting activity.

### Table 2. The effect of RS- variant (M-156) on multiplication of RS+ strains and their capacity to produce necrosis

<table>
<thead>
<tr>
<th>Viruses</th>
<th>Infective virus isolated from rabbit skin (p.f.u./0.1 ml.)</th>
<th>Diameter of necrosis (mm.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurovaccinia, MM strain</td>
<td>6 × 10⁵</td>
<td>15</td>
</tr>
<tr>
<td>M-156 clone</td>
<td>5 × 10⁶</td>
<td>0</td>
</tr>
<tr>
<td>Neurovaccinia + clone M-156</td>
<td>4 × 10⁶</td>
<td>0</td>
</tr>
<tr>
<td>Clone M-156 inactivated by u.v.-irradiation</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Neurovaccinia + clone M-156 inactivated by u.v.-irradiation</td>
<td>6 × 10⁵</td>
<td>0</td>
</tr>
</tbody>
</table>

We assumed, on the basis of the results obtained, that the inhibiting capacity of the M-156 variant was due to induction by this virus of an inhibitor of interferon type. To test this assumption experiments were made in which extracts were prepared of rabbit skin infected with M-156 variant or wild virus. Rabbit skin was inoculated with 10⁶ p.f.u. of RS+ strain of neurovaccinia virus or the same dose of M-156 variant in the active or u.v.-inactivated form. At 24 hr the area of the skin was cut out, minced in 10 ml. of medium 199 in a homogenizer of ultraturrax type, clarified at 3000 rev./min. and freed from virus particles in MSE Superspeed ultracentrifuge at 60,000 g for 1 hr (rotor 2410). The supernatant fluid was heated at 55° for 1 hr and used in the experiment. In some experiments the extract instead of being heated was incubated at pH 2.5 for the inactivation of the virus. The absence of the infective virus in extracts was checked by inoculation of chick embryo tissue culture or chorioallantoic membrane of 12-day-old chick embryo. Neurovaccinia virus, MM strain, and rabbit pox virus were mixed with the heated extract at a ratio of 1:1 so that 0.2 ml. contained 10⁴ p.f.u. of rabbit pox virus or 10⁴ p.f.u. of neurovaccinia virus. In controls, the active virus was mixed with medium 199. The mixture was inoculated intracutaneously in rabbits.
M-156 mutant, both in active form and after inactivation by u.v. induced in rabbitskin formation of a substrate capable of preventing necrosis produced by neurovaccinia or rabbit pox virus (Table 3). At the same time an extract of skin infected with neurovaccinia virus was unable to prevent necrosis when mixed with active neurovaccinia virus.

The inhibiting effect of rabbit skin extract against neurovaccinia and Western equine encephalitis viruses was manifested only in a homologous system and not in a heterologous system (Table 4). The extract potency was not diminished after treatment at pH 2.5 and 4° overnight. Infected rabbit skin extract was diluted with medium 199 1:4 and added to the cell culture. After 24 hr contact the cells were washed with Hanks’s balanced salt solution and infected with neurovaccinia virus at multiplicity of 10 p.f.u./cell or Western equine encephalitis virus at multiplicity 3 to 5 p.f.u./cell. After 1 hr adsorption period at 24° the cells were washed to remove unadsorbed virus and incubated at 36° for 24 hr. Virus yield was measured by titration in chick embryo fibroblast cell culture. The results indicate that there is a strong inhibition in homologous system only.

### Table 3. The capacity of the extract of infected rabbit skin to prevent necrosis produced by RS+ viruses

<table>
<thead>
<tr>
<th>Extract of rabbit skin infected with viruses</th>
<th>Neurovaccinia</th>
<th>Rabbit pox</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-156</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M-156 inactivated with u.v.-rays</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Neurovaccinia</td>
<td>7</td>
<td>—</td>
</tr>
<tr>
<td>Uninfected rabbit skin</td>
<td>12</td>
<td>17</td>
</tr>
</tbody>
</table>

### DISCUSSION

The results obtained are of interest first of all from the point of view of investigation of genetic characters of virus populations. Properties of virus populations are usually determined by the properties of the dominant type of virus particles. The appearance in our virus populations of even a few mutants with increased interfering activity radically changed the capacity of the population to produce necrosis by intracutaneous inoculation of rabbits despite the fact that the number of RS+ particles in the population was many times greater than that of RS- particles. Hence it follows that the properties of virus population are not always determined by the properties of dominating virus particles.

Vaccinia virus is able to induce interferon production in the skin of rabbits (Nagano & Kojima, 1958; Nagano, 1967). On the basis of our studies (Table 3) we suggest that the RS- clone M-156 induced in rabbit skin production of a sufficient amount of an inhibitor...
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of interferon type in contrast to RS+ strains of vaccinia virus. When rabbits are inoculated intracutaneously with a mixture of M-156 mutant and RS+ virus multiplication of the latter and consequently production of necrosis is inhibited by interferon-like inhibitor induced by M-156 clone.

The capacity of RS- M-156 clone to prevent necrosis by RS+ virus strains varied considerably with different viruses. Thus multiplication of neurovaccinia or rabbit pox virus was inhibited at an RS- clone to RS+ strain ratio of 1000:1. At the same time manifestation of skin pathogenicity of RS+ clone M-155 isolated from the RS- population of neurovaccinia virus was inhibited by clone M-156 even when the number of p.f.u. of the RS+ clone was 100-fold greater. It should be mentioned that the necrosis-producing capacity of all the strains studied was closely similar; necroses were produced by 100 to 1000 p.f.u. of virus. Thus, varying inhibiting activity of M-156 variant with regard to different RS- viruses appears to be explained by diverse sensitivity of these viruses to an interferon-like inhibitor. It may also be suggested that RS+ viruses under study had varying capacities of active inhibition of the effect of interferon, as has been demonstrated for some viruses of variolavaccinia subgroup (Ghendon, 1965; Bektemirov & Gumennik, 1967).

The results show that the inhibitor has similar properties to interferon.

Investigation of mutants of vaccinia virus possessing increased interferon-stimulating activity may be of interest from the point of view of search for a highly active interferon stimulator capable of inhibiting multiplication of a virulent virus in the organism. The use for this purpose of non-pathogenic virus mutants has a certain advantage since such viruses will induce not only interferon production but subsequently also the specific antibody response.

REFERENCES


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