JOURNAL OF VIROLOGY, Oct. 1968, p. 1102-1106

Vol. 2, No. 10
Printed in U.S.A.

Target Volume Analysis of Vaccinia Virus: Influence of Virus Dispersion and Noninfectious Particles

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Received for publication 19 April 1968

These data indicate that 90% of monodisperse vaccinia virus (VV) has a D_{37} dose of $1.0 \times 10^4 \pm 0.2 \times 10^4$ rad when exposed to the direct effect of gamma radiation from ^{137}Cs and ^{60}Co sources. This D_{37} dose yielded a target size of approximately 10^8 daltons, close to the molecular weight of VV deoxyribonucleic acid. This target corresponds to the first component of the survival curve of VV. Noninfectious virus seemed to play a role on the slope and the proportion of the survival curve second component, suggesting that multiplicity reactivation takes place.

Target theory provides a satisfactory method of studying dose-effect relationship of ionizing radiation on viruses. In fact, it has been possible to establish a ratio close to unity between target volume and the amount of genetic material of viruses that contain single-stranded nucleic acids. However, for those viruses containing double-stranded nucleic acids, target size appears to be about one-tenth that of the genetic material (11, 21). Vaccinia virus (VV) belongs to this category because its deoxyribonucleic acid (DNA) molecule of 1.6×10^8 daltons is double-stranded (8, 17), and it has been the subject of many radiobiological studies that suggest a target volume definitely smaller than its DNA molecule (3, 13, 14, 24). Moreover, it has been shown that VV presents a compound survival curve upon gamma radiation (6, 15). The curve can be resolved into two components, one of relative radiosensitivity and one of relative radioresistance. It may be postulated that the D_{37} of the first component could provide an estimate of the virus target volume, whereas the D_{37} of the second component could be a composite of simultaneous diverging processes of inactivation and reactivation.

In this paper, we show that the D_{37} of the first component of purified and monodisperse suspensions of VV, exposed to the direct effect of gamma radiation, yields a target volume similar to the size of its DNA molecule. It is also shown that the inactivation rate and the proportion of the second component depend on the amount of noninfectious virus, suggesting that multiplicity reactivation takes place.

MATERIALS AND METHODS

Virus strains. The following strains of vaccinia virus (VV) were used. (i) A dermal virus maintained through numerous passages over a period of more than 60 years in calf skin (CD virus) was kindly made available to us by R. Palacios, Instituto Bacteriológico de Chile, Virus Department. The same virus was also maintained in chick embryo chorioallantoic membrane (CAM). (ii) The viruses 7N and RP were donated by Frank Fenner of Australia, and were maintained by us in CAM.

Virus multiplication. To determine virus multiplication on calf skin, approximately 10^7 plaque-forming units (PFU) in about 10 ml of glycerol, water, and serum (50:40:10, v/v) were smeared on the previously shaved, washed, and lightly scratched animal's flank. After 4 days, an extensive lesion was observed in the inoculated area. The animal was then bled to death, the dermal lesion was scraped and assayed virologically and bacteriologically, and the preparation was stored at −20 C. The batches that were used contained more than 10^8 PFU/ml and less than 10^4 microorganisms/ml.

To determine virus multiplication on chick embryo chorioallantoic membrane, approximately 10^4 PFU were inoculated in 0.1 ml of water on the CAM of 11-day chick embryos, following the technique of Westwood et al. (23). The CAM, with confluent pocks, were dissected 48 hr later and frozen at −20 C.

Virus purification. Virus grown in CAM was purified following Joklik's technique (9). The purified virus was suspended in water with 10% heated horse serum (60 C for 30 min) and frozen at −20 C.

The calf dermal virus was ground in a mortar and suspended in water with 10% horse serum, 200 μg of penicillin, and 200 μg of streptomycin per ml. It was left for 3 hr at room temperature, kept overnight at 4 C, and centrifuged at 1,000 × *g* for 20 min. The

supernatant fluid was purified as described for CAM virus and stored at −20 C.

Virus bioassay. Bioassays were performed in 48-hr cultured fibroblasts from 10-day-old chick embryos in 1-oz bottles and overlayed with agar. The virus was allowed to adsorb for 2 hr at 37 C prior to addition of the agar overlay supplemented with 3% horse serum. Neutral red (0.01%) was added 5 days later in a second agar overlay. The number of plaques were scored on two successive days. Bioassays were also performed in the CAM of chick embryos by using 10 embryos per dilution and by scoring the number of pocks 48 hr later.

Electron microscopy. The instrument, Siemens Elmiskop I, was kindly made available to us by the Professor of Histology at the School of Medicine, University of Chile. The specimens were prepared by means of a sample drawn from the viral suspensions just before irradiation (16). The count of viral particles (VP) was made by following Sharp's technique (18). In order to apply his equation, more than 1,500 particles were counted in each instance.

Irradiation. The ^{137}Cs (Faculty of Sciences, University of Chile) was used at a dose rate of 1.0×10^4 rad/hr, repeatedly checked by means of ferrous sulphate dosimetry (7). The ^{60}Co (USAEC Atoms for Peace Exhibition, Santiago, Chile) was used at dose rates of 5.5×10^4 rad/hr and 1.34×10^5 rad/hr. Virus was exposed to radiation in the liquid state in water with 10% horse serum (5 mg of protein per ml) and in the solid state (lyophilyzed from the same medium). After the virus was lyophilyzed, the tube containing the pellet was filled with nitrogen. The temperature during irradiation was 18 ± 3 C. In order to attain monodispersion of the virus particles, the virus suspension was sonically treated with a Biosonik Sonicator (120 w, 20 kc) at peak output three times for 30 sec prior to irradiation.

RESULTS

Analysis of the target volume of vaccinia virus. When the survival of vaccinia virus to gamma radiation is analyzed, the curve obtained can be resolved into the exponential components:

$$N \div No = a\, e^{-K_1 r} + b\, e^{-K_2 r}$$

The component characterized by "a" and "K_1" will be referred to as first component; the other one, "b" and "K_2," second component.

Because the rate of inactivation may be influenced by indirect effects of ionizing radiation, the virus was irradiated in the presence of 5 mg of protein per ml in the liquid as well as in the solid state, in the presence and in the absence of oxygen, and with different dose rates. The results presented in Table 1 are remarkably similar for the different experimental conditions, suggesting that the slopes of the inactivation curve result from the direct effect of gamma radiation on the virus. The bioassays used to determine K_1 have been done with virus particle-to-cell ratios in the order of 0.001; those used for K_2 have been done with particle-to-cell ratio approaching unity.

The state of dispersion of the virus particles, owing to the characteristics of this survival curve, may influence the proper evaluation of K_1. Dispersion and purity of the virus suspensions to be irradiated were routinely checked under the electron microscope. It is expected that monodisperse particles should inactivate as a single target model, although the shoulder suggesting multitarget inactivation may remain unnoticed unless small doses of radiation are used. The results presented in Table 2 indicate that polydisperse suspensions, containing small clumps of virus particles, are inactivated according to a multitarget model. The same virus preparations yield survival values that suggest the expected single target model, when properly monodispersed.

TABLE 1. *Survival curve of vaccinia virus irradiated in the liquid and solid state with different dose rates*

Dose rate (rad × 10^4/hour)[a]	State[b]	$N \div No = a\, e^{-K_1 r} + b\, e^{-K_2 r}$		
		"a"[c]	$K_1 \times 10^4$ rad	$K_2 \times 10^5$ rad
13.4	Liquid	0.99	1.11	1.4
13.4	Solid	0.99	1.15	1.5
5.5	Liquid	0.99	1.14	1.4
5.5	Solid	0.99	1.15	1.3
1.0	Liquid	0.80	1.00	1.4

[a] The 13.4 and 5.5 × 10^4 rad/hr dose rates were obtained from the ^{60}Co source; the 1.0×10^4 rad/hr was obtained from the ^{137}Cs source. Irradiations started with 0.3×10^4 rad.

[b] The monodisperse virus was irradiated in the liquid state in the presence of 5 mg of protein per ml; oxygen was present. The virus in the solid state was irradiated in the absence of oxygen as described in Materials and Methods.

[c] Since a + b = 1, "b" is omitted from the Table.

TABLE 2. *Survival of monodisperse and polidisperse suspensions of vaccinia virus after gamma radiation*

Dose (rad × 10^4)	Virus survival: N ÷ no. × 100	
	Monodisperse	Polidisperse
.3	69	100
.6	48	100
.9	34	100
1.2	24	60
2.4	6	37
4.8	1.5	13

The data presented in Fig. 1 would indicate that 90% of monodisperse vaccinia virus has a D_{37} dose of $1.0 \times 10^4 \pm 0.2 \times 10^4$ rad when exposed to the direct effect of ionizing radiation. In order to calculate the target size of vaccinia virus, we may use the equation of Hutchinson and Pollard (7), D_{37} (rads) $\times$ MW $= 0.96 \times 10^{12}$, which is applicable to targets where the primary ionization is the effective event, and which is expressed as the effective molecular weight. Using the D_{37} dose of 1×10^4 rads, an effective molecular weight of approximately 1×10^8 daltons is obtained. The DNA of vaccinia virus has a molecular weight of 1.5 to 1.7×10^8 daltons (8, 17).

The role of noninfectious virus on the "K_2" and "b" values of the survival curve. An average VV suspension has a ratio of virus particles to PFU (VP/PFU) of about 50, and is normally assayed on a large excess of susceptible cells, VP/cell ratios of 0.001 or smaller. However, when VP/PFU increases as a result of viral inactivation, the bioassay may be done in conditions where the number of virus particles approaches or exceeds the number of cells, unless the bioassay is performed from a large volume of viral suspension placed on similar large numbers of susceptible cells. It may be expected that VP/cell ratios close to or greater than unity would favor multiplicity of infection. It can be seen in Table 3 that VP/cell ratios smaller than 0.002 have no effect on virus titer; however, when VP/cell ratios increase, there is a significant increase in virus titer. This was illustrated by experiments 1 and 4. The increase in virus titer, suggesting that multiplicity reactivation is taking place, modifies the "K_2" value of the survival curve, particularly with large doses of radiation, unless the bioassay is performed on a large number of susceptible cells.

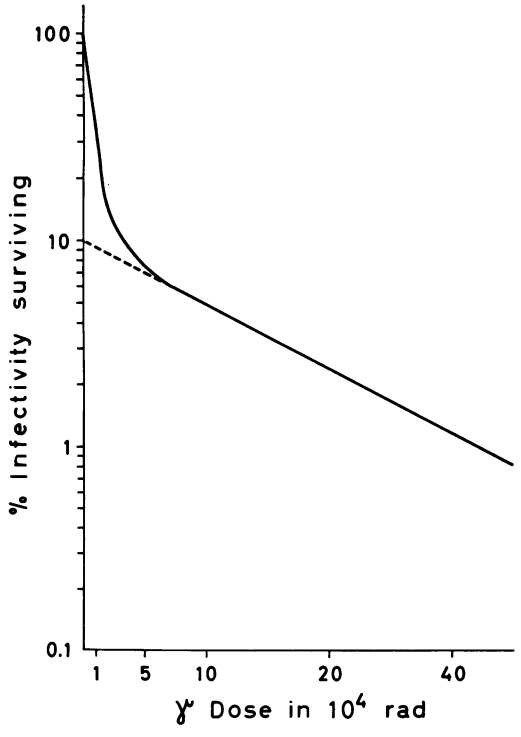

FIG. 1. *Vaccinia virus mean survival curve after gamma irradiation derived from 9 experiments. The curve can be represented by the following equation:*

$$N \div No = 0.90\, e^{-1.0\times10^4 \text{ rad}} + 0.10\, e^{-1.4\times10^5 \text{ rad}}$$

Another way to study the role of noninfectious virus on the shape of the survival curve is to irradiate VV that has different proportions of noninfectious virus. If we irradiate VV that has lost PFU activity as a result of storage at -20 C, we obtain the results presented in Table 4. When a virus batch has lost PFU activity, it also shows a greater "b" value. This is true for the three strains studied, and is marked for the CD strain. This same strain was used to study the changes in the ratio between viral particles and PFU. When freshly assayed, this preparation had a VP/PFU ratio of 1.3, which increased to 57 upon storage at $-20°$ C; at the same time "b" increased from 1 to 20%.

TABLE 3. *Relation between the virus titer and the VP/cell ratio used in the bioassay*

VP/cell ratio[a]	Expt no.	Total volume tested (ml)	no. of plaques	Virus titer (PFU/ml)
0.00002	3	8.8	83	9.5×10^6
0.0002		1.8	190	10.0×10^6
0.002		0.6	560	9.2×10^6
0.0002	5	16.0	24	1.5×10^5
0.002		3.2	57	1.8×10^5
0.02		1.2	300	2.5×10^5
0.002	4	9.2	22	2.4×10^3
0.02		2.0	91	4.5×10^3
0.2		0.6	348	5.8×10^3
0.02	1	16.4	41	2.5×10^3
0.2		1.6	83	5.2×10^3
2.0		0.6	569	9.5×10^3

[a] The number of cells per unit of bioassay is constant, approximately 1×10^6. The inoculum per unit is 0.2 ml. The same virus preparation was used for these experiments, and it was inactivated by irradiation to varying extents.

TABLE 4. *Influence of noninfectious virus on the proportion of the second component of vaccinia virus*

Expt no.	Strain	Interval in days between purification and irradiation	PFU/ml	Second component ("b" value × 100)	VP/PFU
1	7N	8	4.5×10^7	25	Nd[a]
		338	1.2×10^7	53	
2	RP	8	6.0×10^7	10	Nd
		340	7.0×10^6	30	
3	CD	9	3.0×10^8	1	1.3
		136	7.2×10^6	20	57.0

[a] Nd = not done.

DISCUSSION

In the preliminary stage of our studies (15), we were able to observe a compound survival curve for VV after gamma irradiation. This finding has been confirmed by other workers (6). Further improvement in the measurements of the parameters of the survival curve allow a closer appreciation of the virus target size and give a better understanding of some of the factors involved in yielding the shape of this curve.

Before applying the target theory, a possible indirect effect of radiation should be considered. In order to avoid such damage, the virus was irradiated in the liquid state in the presence of 5 mg of protein per ml, because this is known to afford protection against indirect effects (22). Irradiation of virus in the solid state and in the absence of oxygen has given results similar to that of irradiation performed in the liquid state. No differences were observed when dose rates were used that differed by a factor of 10. in a previous study (15), when the suspension medium was replaced with fresh medium after a total dose of 9×10^5 rad, the slopes of inactivation remained unchanged. Based on these experimental data, it seems reasonable to interpret our results as a direct effect of gamma radiation on the virus particle itself. However, before we can use them to calculate target volume, it is necessary to consider virus dispersion because of the characteristics of the survival curve of this virus The use of doses beginning with 3,000 rads, together with observations under the electron microscope, clearly showed that only monodispersed virus preparations yielded survival curves, suggesting a single target model.

In these experimental conditions, we have found that the D_{37} dose of VV is 1×10^4 rad. Using this D_{37} dose and the equation of Hutchinson and Pollard (7), the target size for VV is approximately 1×10^8 daltons. The only structure of a size in this order within the VV particle is the DNA molecule (8, 17). The larger D_{37} dose (3.5×10^4 rad), reported previously by us (15), was a result of aggregation of the virus particles in the specimens exposed to radiation. Even if this D_{37} dose, or the one reported by Friesen et al. (6), is used to calculate the target volume of VV, it is larger than the one expected for a typical virus-containing double-stranded DNA. In fact, a relation between radiosensitivity and genetic complexity has been observed (11, 21). For viruses that contain single-stranded nucleic acids, the relation between target volume and genetic material molecular weight is close to unity; however, for viruses that contain double-stranded DNA, the target volume is about one-tenth of the DNA molecular weight. Efforts have been made with bacteriophage systems to elucidate the relative biological significance of the different radiation-induced damages suffered by DNA molecules. As a result, it has been found that the relatively greater radiosensitivity of viruses containing single-stranded DNA is due to the fact that single-strand breaks, which normally are not lethal in double-stranded DNA, result in molecular cleavage and therefore inactivation (4). Viruses containing double-stranded DNA are inactivated both by double-strand breakage and by base damage; single-strand breaks, on the contrary, were found to be ineffective (5). However, other phages that also contain double-stranded DNA are exceptionally sensitive to ionizing radiation. One such case is phage α, which apparently owes its unusual radiosensitivity to the fact that single-strand breaks are lethal (5). Another exception is the greater sensitivity of the replicative form of bacteriophage ϕX174, which apparently is also due to the fact that single-strand lesions can inactivate the DNA molecule (20). A third exception, presented here, is the unusual sensitivity of VV to gamma radiation. Since similar refined analyses are not available in this case, and predictably they will be difficult to perform, it may be suggested by simple analogy that the radiosensitivity of this virus is also probably due to the same mechanism; i.e., single-strand lesions are lethal. Why single-strand breaks are lethal in some viruses containing double-stranded DNA and are not lethal in other viruses, remains an open question.

The presence of a relatively more radioresistant component in the survival curve in every strain studied poses a different problem. The possibility of more than one virus population is discarded because the viral progeny after a dose of 10^6 rad, when only the radioresistant component is pres-

ent, shows after further irradiation the presence of the two original components (15). The results presented here, which show that noninfectious virus contributes to the existence of multiplicity reactivation, afford a possible explanation for the change in slope of the survival curve. At this time, it is hardly surprising to find multiplicity reactivation of this virus after gamma radiation, since it has been shown to occur after ultraviolet light irradiation (1, 19), after exposure to nitrogen mustard (12) and after irradiation with soft X-rays (K. S. Kim and D. G. Sharp, Bacteriol. Proc., p. 170, 1967). It is possible that other factors, such as virus aggregation (20), the mechanism of VV multiplication (10), and the peculiar distribution (2) and topography of the multiplication sites (1) also play an important role.

ACKNOWLEDGMENTS

We are grateful to George Hodgson for encouragement and enlightening discussions on this subject and for valuable editorial help. We thank José Tohá for his permission to use some unpublished data. We also thank Rosa Jiménez, Juan Vergara, Carlos Oberti, and the staff of the Virology Department for collaboration and advice. We express our gratitude to Raul Palacios and Frank Fenner for their generous gifts of VV strains.

We acknowledge the technical assistance of Yolanda Miranda and Carlos Reyes.

This investigation was supported by research contract 558/RB from IAEA and by grant 63/28 from Comisión de Ayuda a la Investigación Científica, Facultad de Medicina, Universidad de Chile.

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