Reactivation of Heated Vaccinia Virus by UV-Irradiated Vaccinia Virus (35179)

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(Introduced by H. M. Meyer, Jr.)

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Reactivation of poxviruses which have been inactivated by certain physical or chemical means has been studied by a number of workers (1–7). Joklik developed the theory that the mechanism involved is nongenetic, depending on protein elements of the viral coat to derepress some portion of the host cell genome (8). The cell then produces an uncoating protein which enables the release of viral DNA for replication. Kates and McAuslan (9) have observed the synthesis of poxvirus messenger RNA before viral cores have become uncoated. They believe that this early messenger RNA codes for a variety of "early" enzymes including those essential for the final uncoating of the virion. Whether the host cell genome or the viral genome codes for this protein, denaturation of the virus by heat or urea treatment prevents the completion of the uncoating process. When cells are multiply infected with heat-inactivated virus and active virus, the active virus provides the necessary elements for uncoating itself, as well as the inactive virus and both can replicate. Virus whose genome has been damaged by nitrogen mustard can reactivate heated virus even though it cannot replicate itself (10). However, attempts to use UV-irradiated virus as the reactivating virus have generally failed. The purpose of our investigations was to try to provide the necessary conditions for reactivation of heated virus with UV-inactivated virus by (A) irradiation just to the point of extinction of biologic titer and (B) deliberate aggregation of the virus mixtures to provide a high multiplicity of infection for at least a portion of the cells.

Materials and Methods. Viruses. The WR (mouse neurotropic) strain of vaccinia virus was originally obtained from the American Type Culture Collection. This strain was passed 327 times in L cells in Dr. D. Gordon Sharp's laboratory. The virus used in these experiments was passed in our laboratory once or twice on the chorioallantoic membrane (CAM) and extracted with Genetron (11). The IHD strain of vaccinia was obtained from the American Type Culture Collection, passed once or twice on the CAM and extracted with Genetron as above. The IHD strain and the WR 327 strain of vaccinia were chosen for these experiments because of the easily discernible difference in the type of pock produced by each on the CAM. The IHD strain causes a pale gray diffuse pock with a hemorrhagic center while the WR 327 strain produces a small white opaque pock.

The pocking efficiency of the crude WR 327 virus preparations used was approximately 100 virus particles/pock-forming unit (vp/pkfu), and that of the purified WR 327 virus was 700 vp/pkfu. The IHD strain used had a pocking efficiency of approximately 25 vp/pkfu prior to heating.

Virus titrations. Infectivity measurements and recognition of reactivated virus were made on the CAM of 11–12-day-old embryonated eggs by the method of Westwood *et al.* (12). The inoculum was 0.1 ml of the virus appropriately diluted in Dulbecco's phosphate buffered saline (PBS) (pH 7.2). Ten to 18 eggs were used per dilution and eggs were incubated for 48 hr at 36°. When a second passage of virus from infected membranes was made, the virus was extracted with Genetron and inoculated on the CAM.

Heat inactivation. The IHD strain of vaccinia virus, diluted in PBS, was sealed in glass ampoules and heated, fully submerged,

TABLE I. Pock Production on the CAM by Mixtures of Heated Plus Untreated Virus.[a]

	Heated IHD			Untreated WR 327		
	Inoculum (vp/CAM)	(pkfu/CAM)		Inoculum (vp/CAM)	(pkfu/CAM)	
Expt.	(1st passage)	1st passage	2nd passage	(1st passage)	1st passage	2nd passage
1	1.25×10^8	1.6	—[c]	2.5×10^4	180	—
2	1.25×10^8	0.2	—	3×10^3	39	—
3	1.25×10^7	0.1	Confluent	3×10^3	NC[b]	TNTC
4	5×10^7	3.7	Confluent	5×10^3	76	TNTC

[a] Heat inactivated IHD and active WR 327 mixed in proportions indicated above and inoculated on the CAM.

[b] NC = not counted; TNTC = too numerous to count.

[c] Not done.

in a water bath at 60° for 15 min.

Virus purification. When purified virus was used, the purification process was that of Joklik (13).

UV inactivation. The WR strain of vaccinia virus, washed twice in PBS, was diluted to contain 3×10^9 virus particles (vp) (approximately 3×10^7 pkfu) in a 3-ml volume and was UV irradiated in a 60-mm petri dish or 50-ml beaker under a G-E germicidal lamp at a distance of 50 cm for 90 sec. The dish was rotated during irradiation.

Virus particle counts. The number of virus particles in each preparation was determined with electron microscopy by the method of Sharp (14).

Aggregation of virus. Concentrated suspensions of virus were centrifuged in the Sorvall swinging bucket rotor 30 min at 16,300*g* to encourage aggregation of particles. The pellets were resuspended by pipetting.

Results. Effect of heat on IHD strain. In order to demonstrate complete loss of infectivity by the heated virus, we inoculated CAM's with the heated IHD at concentrations ranging from 2.5×10^7 to 2.4×10^8 vp/egg. After 48-hr incubation, the membranes were excised and treated with Genetron. The resulting supernatant fluid was inoculated undiluted again on the CAM. A similar third passage of this material was also made. There was no development of pocks on these membranes at any time.

Reactivation of heated virus by untreated virus. IHD virus, completely inactivated by heat, was mixed with varying dilutions of untreated WR 327, aggregated by centrifugation and inoculated on the CAM (Table I). WR pocks appeared on the membranes in numbers consonant with the concentrations of the inocula. IHD pocks were rare, averaging between 0 and 3.7/membrane, despite the fact that the inocula contained 4×10^5 pkfu prior to heating. On second passage of the infected membranes, both confluent IHD pocks and discrete WR pocks were too numerous to count (TNTC). This second passage acts as an amplification step to reveal the presence of active virus.

In one experiment, the same first passage material was passed a second time at lower dilutions (Fig. 1). At a 10^{-2} dilution, IHD pocks were confluent and WR pocks were in countable numbers. Further dilution permitted the production of more WR pocks than IHD pocks. When both virus strains are present in large numbers, the cellular damage produced by the IHD virus may prevent the formation of the proliferative type of pock produced by the WR virus.

UV inactivation of WR 327. In order to determine the amount of UV irradiation necessary to destroy the infectivity of a given amount of WR 327, inactivation experiments were performed, as described in Materials and Methods. Samples were withdrawn from the irradiation dish at intervals, suitably diluted and titrated on the CAM. After 60-sec irradiation, the original pock titer of $10^{6.6}$ pkfu/ml had decreased 5.4 logs. If the slope of the line remains the same through 90-second irradiation, there should be a 7.5 log

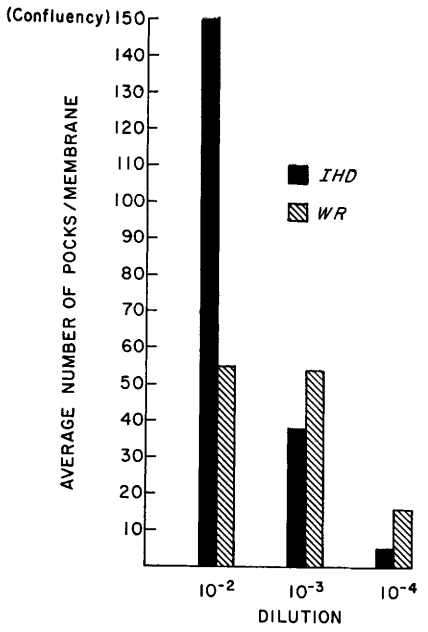

FIG. 1. Effect of dilution on predominant pock type: Second CAM passage of mixtures of heated IHD vaccinia and untreated WR 327 vaccinia. The IHD pock count declines in a linear fashion with dilution. The WR pock count declines much more slowly. When there is space on the CAM for full expression of both pock types, the WR pocks are more numerous than the IHD pocks.

decrease in infectivity titer—more than enough treatment to completely destroy the infectivity of the virus inocula used, providing there is no multiplicity reactivation.

Reactivation of heated virus by UV-treated virus. Three tubes of virus were prepared: (A) a mixture of heated IHD and WR 327 (irradiated for 90 sec) in amounts listed in Table II; (B) UV-treated WR 327 alone; and (C) heated IHD alone. The 3 virus suspensions were aggregated by centrifugation, resuspended by gentle pipetting and inoculated undiluted on the CAM. The irradiated control samples (B) produced a few white pocks in numbers roughly equivalent to the number of WR pocks produced in the heated plus irradiated samples. The heated control samples (C) produced no pocks.

Table II records the results of the virus mixture experiments. A few IHD pocks were observed in most experiments on first passage. In three out of four experiments, a few WR 327 pocks were evident. Second CAM passage of virus extracted from the first membranes produced confluent IHD pocks in all but one experiment, and a moderate number in the fourth experiment, where confluent WR 327 pocks made recognition of the IHD pock difficult. Where IHD pocks reached confluency, WR 327 pocks were present in small numbers, except in experiment 3 where none were visible.

In Expt. 4, the first passage membranes were pooled in two groups: (A) membranes showing only IHD pocks; and (B) membranes bearing no pocks of either type. Second passage of this material resulted in confluent IHD pocks from both groups and a few WR 327 pocks in both.

Extinction of infectivity and reactivation. An experiment was performed to determine the time of irradiation required to completely inhibit the replication of the WR 327 virus

TABLE II. Pock Production on the CAM by Mixtures of Heated Plus UV Irradiated Virus.[a]

	Heated IHD			UV Irradiated WR 327		
Expt.	Inoculum (vp/CAM) (1st passage)	(pkfu/CAM) 1st passage	(pkfu/CAM) 2nd passage	Inoculum (vp/CAM) (1st passage)	(pkfu/CAM) 1st passage	(pkfu/CAM) 2nd passage
1	2.5×10^7	0	++	4.0×10^7	4	Confluent
2	1.25×10^8	0.5	Confluent	2.9×10^7	11	++
3	1.25×10^8	0.15	Confluent	3.1×10^7	0.65	0
4	2.4×10^8	3(3 CAM's)	Confluent	2.1×10^7	0	1
		0(7 CAM's)	Confluent		0	33

[a] Heat inactivated IHD and UV irradiated WR 327 mixed in proportions indicated in the table and inoculated on the CAM. ++ = estimated count, ~50.

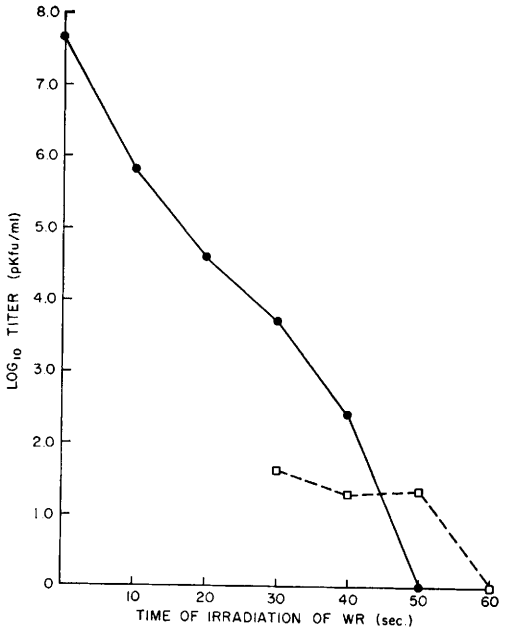

FIG. 2. Rate of UV inactivation of WR 327 and reactivation of heated IHD (1st passage): (●) WR 327; (□) IHD. Done with purified virus; pocking efficiency approximately 7-fold less than the virus used in Table I.

without destroying its ability to reactivate heated virus. For this experiment, purified preparations of both WR 327 and IHD were used. The WR 327 virus was irradiated for 70 sec, with samples being removed every 10 sec for mixing with the heated IHD, and titration on the CAM. The decrease in infective titer of the WR 327 with irradiation is illustrated in Fig. 2. Pocks were produced by WR 327 in the 0 through 40-sec samples but not in the 50 through 70-sec samples. Pocks characteristic of IHD were recognized only in the 30- through 50-sec samples. It would seem reasonable to expect IHD pocks in the 0 through 20-sec samples, but none were visible. Whether this was due to the difficulty of recognition in an area crowded with WR 327 pocks, or whether reactivated IHD virus was not expressed in pocks is not known.

These membranes were harvested and the virus extracted and passed again on the CAM (Fig. 3). Each of the 0 through 40-sec samples produced both IHD and WR 327 pocks. The 50-sec sample contained only IHD pocks. No WR 327 pocks appeared after more than 40 sec of irradiation and no infective virus of either type was demonstrated beyond 50 sec of irradiation.

Discussion. A comparison of the reactivation initiated by untreated virus and by irradiated virus indicated that irradiation decreased both the infectivity and the reactivating ability but the ability to replicate disappeared before reactivating ability was completely lost. Irradiation for 90 sec reduced the viability of the unpurified WR strain more than 7 logs, to the point where infectivity was almost extinguished. The few WR pocks appearing during first passage of the heated plus irradiated virus mixtures indicate the presence of some viable WR 327 virus, probably due to multiplicity reactivation facilitated by aggregation of the virus mixture (15, 16). However, this quantity of reactivated WR 327 is not sufficient to account for the reactivation of the heated IHD. In the experiments with heated plus untreated virus (Table I), there were an average of 70 pkfu of active WR to 1 pkfu of reactivated IHD. When the same ratio was calculated from Table II for heated plus irradiated virus, it became approximately 10 WR pocks to 1 IHD pock which indicated that WR 327 virus unable to form pocks was participating in reactivating the heated virus.

When the reactivating ability of purified virus irradiated for increasing periods of

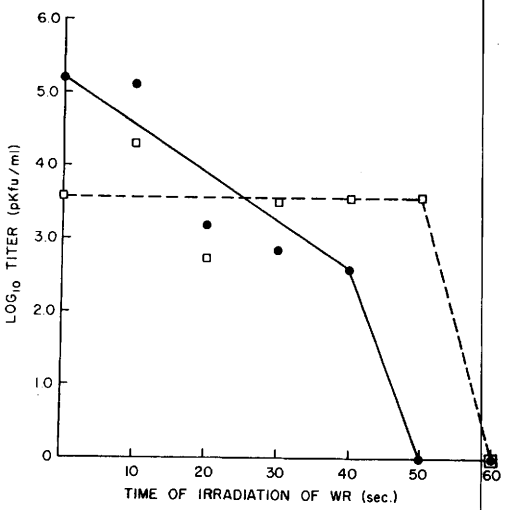

FIG. 3. Second CAM passage of heated plus irradiated virus: (●) WR 327; (□) IHD.

time was examined, it was found that after 50 sec of exposure to UV rays, no infectivity remained in the irradiated virus but some reactivating ability persisted. Sixty sec of irradiation destroyed both the virus ability to replicate and to reactivate heated virus. The amplification provided by second passage of these virus mixtures revealed that heated virus was reactivated in all samples up to and including 50-sec irradiation, even though it was not expressed in recognizable pocks on first passage.

UV irradiation produces random damage to the viral genome. Perhaps the particles which preserve their reactivating ability but lose their own replicating ability are those which suffer irradiation damage only in portions of their genome not connected with the production of "early" enzymes. Evidence has been given (9, 17, 18) that some "early" enzymes are coded for before the virion is fully uncoated. One of these "early" enzymes is thought to be the inducer protein which Joklik (8) postulated as necessary for final uncoating of the viral core. If the portion of the parental genome carrying the code for these enzymes were unaffected by irradiation, "early" enzymes could be induced by the irradiated virus. These enzymes would then be available to assist in the uncoating of heated virions present in the same cell. The heated particle could then replicate even though the irradiated particle could not.

Summary. Reactivation of heated IHD vaccinia virus was accomplished by the addition of either untreated or irradiated WR vaccinia. The irradiated WR virus which was unable to produce pocks on the CAM was nonetheless capable of providing the uncoating function for heat-inactivated IHD virus. Irradiation decreased both the reactivating ability and the infectivity of the WR virus but the reactivating ability was more resistant to irradiation than was infectivity.

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