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The antigenicity of γ-irradiated vaccinia virus

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Inactivation of vaccinia virus by heat and many chemicals destroys its protective antigenicity. However, Collier, McClean & Vallet (1955) preserved measurable immunogenicity in suspensions of vaccinia virus inactivated by ultra-violet light in a Habel–Sockrider apparatus. Because of this we thought it worthwhile to investigate the effects of ionizing radiations on the protective antigenicity of the virus.

METHODS

Virus

The Lister Institute vaccine strain was used. Suspensions of virus from sheep vaccinial pulp were partially purified by one cycle of differential centrifugation and diluted in phosphate and citric acid buffer, pH 7·2, 0·004 M phosphate to a titre of about 10^7 pock-forming units per millilitre (p.u./ml.) before irradiation. Dried virus samples were prepared by freeze-drying in 5 % peptone (Collier, 1955). The vaccines were dried in a centrifugal freeze-drying machine, and the dry material was in the form of wedges in 0·5 ml. ampoules. The single batch of dried rabbit virus used had an initial titre of $4{\cdot}1 \times 10^8$ p.u./ml.

Irradiation

The virus preparations were irradiated by the Technological Irradiation Group of the Isotope Research Division of the Atomic Energy Authority. We are indebted to the Group for the irradiations and for the following information. Exposures were made to γ-rays emitted by cobalt-60 sources enclosed within concrete shields. During the course of the experiments different sources were used, and dose rates and conditions of exposure varied from 4×10^5 rad./hr. (± 10 %) at *c.* 45° C. to $1{\cdot}08 \times 10^5$ rad./hr. (± 5 %) at 15° C. Total doses were determined by a ferrous sulphate technique. As a result of handling, the dried wedges were generally broken into crumbs of peptone in the ampoules. We cannot, therefore, give any data of sample dimensions relative to beam dimensions.

Infectivity titrations

These were done by pock counts on chick embryo chorio-allantoic membranes (Westwood, Phipps & Boulter, 1957). Dilutions were made in McIlvaine's phosphate + citric acid buffer, pH 7·2, 0·004M phosphate. Five embryos were inoculated with each dilution tested. To establish complete inactivation of

infectivity, embryos were inoculated with undiluted material; more than 5 were used when possible. When the results of inspection after 2 days incubation at 36° C. were doubtful, the membranes—aseptically harvested—were extracted in buffer and subinoculated to fresh embryos. In one experiment irradiated virus was concentrated by centrifugation before inoculation.

Antigenicity tests

Each inactivated preparation was tested in five rabbits. The animals were bled for normal serum before inoculation and then given two subcutaneous injections, each of 1 ml., of the test suspensions at an interval of 2 weeks. Two weeks after the second injection the animals were bled again, and challenged by cutaneous scarification with a potent, glycerolated smallpox vaccine known to produce at least a semiconfluent lesion at a dilution of 10^{-4} in normal rabbits. The lesions were inspected daily from the third day and scored on the fifth day. In normal rabbits vaccinial lesions generally show no signs of scabbing until the ninth or tenth day. Immunized animals, however, often develop lesions which fade precociously or become black and necrotic at the centre—the so-called abortive lesions. These were recorded in our test rabbits.

Virus neutralizing antibody in the serum samples was titrated by mixing serum dilutions with a constant challenge dose of virus, ten times the minimal amount necessary to produce a confluent lesion on a normal rabbit. The virus and serum mixtures were incubated for 1 hr. at 22° C. and then inoculated by scarification of normal rabbits. The neutralizing titre of the serum was taken as the highest dilution which reduced the response to not more than ten vesicles. Antibody titrations by pock inhibition on the chick embryo chorio-allantois were done as described by Boulter (1957).

RESULTS

When virus, suspended in liquid, was irradiated in a Co-60 source the temperature reached 45° C. after 5 hr. The dose rate was such that, at the maximum dose used, the virus suspensions were heated at 45° C. for 8 hr. Because of this, and also because of some irregular results thought to be due to changes in the suspensions of virus while travelling in the post, dried virus was used for several experiments (Tables 1–3; fig. 1).

The results of five experiments with dried virus were consistent, but were irregular at the higher radiation doses, from the point where the rate curve flattens (Table 3; fig. 1). Other ampoules of virus treated at these doses were tested several months later. The virus titres were usually about equal to those in ampoules tested immediately after irradiation, but in occasional ampoules no virus was detected. Because of the apparent stability of a minute fraction of the dried virus, even when exposed to doses of γ-rays as high as 11×10^6 rad., tests of these preparations for residual immunogenicity of inactivated virus were clearly precluded. A few suspensions of virus were irradiated at several doses in a Co-60 source in which temperatures could be held at less than 20° C. The preparations were all small samples of elementary body suspensions (EBS) partially purified for smallpox

Table 1. *Inactivation of dried vaccine by γ-rays*

Dose (Mrad.)	Surviving virus (p.u./ml.)*
0	3×10^8
3·5	$\sim 2 \times 10^3$
4·0	$3{\cdot}5 \times 10^2$
4·5	$2{\cdot}6 \times 10^2$
5·0	2·0
5·5	6·0

* Titrated on chick chorio-allantoic membranes.

Table 2. *Inactivation of dried vaccinia virus by γ-rays*

	1		2	
Dose (Mrad.)	Surviving virus (p.u./ml.)	Log V_0/V	Surviving virus (p.u./ml.)	Log V_0/V
0	$1{\cdot}3 \times 10^7$	0	$1{\cdot}4 \times 10^7$	0
1	$2{\cdot}3 \times 10^4$	2·76	$1{\cdot}3 \times 10^4$	3·05
2	$4{\cdot}4 \times 10^3$	3·47	$8{\cdot}0 \times 10^2$	4·25
3	$3{\cdot}9 \times 10^2$	4·53	47	5·47
4	60	5·34	15	5·97
5	2	6·82	2	6·85
6	1	7·12	*	—

V_0 = initial virus titre; V = titre at any specified irradiation dose.
* No pocks on titration membranes. Passage of membrane extracts +ve.

Table 3. *Inactivation of dried vaccinia virus by γ-rays*

	1		2		3	
Dose (Mrad.)	Surviving virus (p.u./ml.)	Log V_0/V	Surviving virus (p.u./ml.)	Log V_0/V	Surviving virus (p.u./ml.)	Log V_0/V
0	3×10^9	0	$1{\cdot}2 \times 10^7$	0	$4{\cdot}1 \times 10^8$	0
0·58	6×10^5	1·7	$1{\cdot}8 \times 10^5$	1·83	$8{\cdot}4 \times 10^6$	1·7
1·74	$1{\cdot}9 \times 10^4$	3·19	$2{\cdot}8 \times 10^3$	3·63	$1{\cdot}5 \times 10^5$	2·44
2·32	$2{\cdot}7 \times 10^3$	4·05	$1{\cdot}9 \times 10^3$	3·80	$7{\cdot}6 \times 10^4$	3·74
3·48	81	5·57	8·8	6·13	*	—
5·22	5	6·78	0	> 7·08	*	—
5·8	0†	> 7·48	2·0	6·78	*	—
6·96	4	6·88	3·8	6·50	*	—
8·12	> 15	< 6·30	0	> 7·08	30	7·14
9·28	8·7	6·54	2·0	6·78	36	7·07
11·6	15‡	6·30	2·5	6·68	28	7·17

1 and 2, preparations of vaccine virus; 3, highly purified rabbit virus.
† Fresh ampoule retested: 4 p.u./ml.; log V_0/V = 6·88.
‡ 2 ampoules retested: 2 p.u./ml; 3 p.u./ml.
* Not enough embryos for these samples to be titrated. Whole sample reconstituted to 0·4 or 0·6 ml. and inoculated to two or three embryos. Virus was present in all.

vaccine production, diluted to a titre of about 10^7 p.u./ml. Two suspensions were completely inactivated by 10^6 rad. and were not immunogenic. Two further samples, EBS 50/58, inactivated by 6×10^5 rad., and EBS 57/58, inactivated by

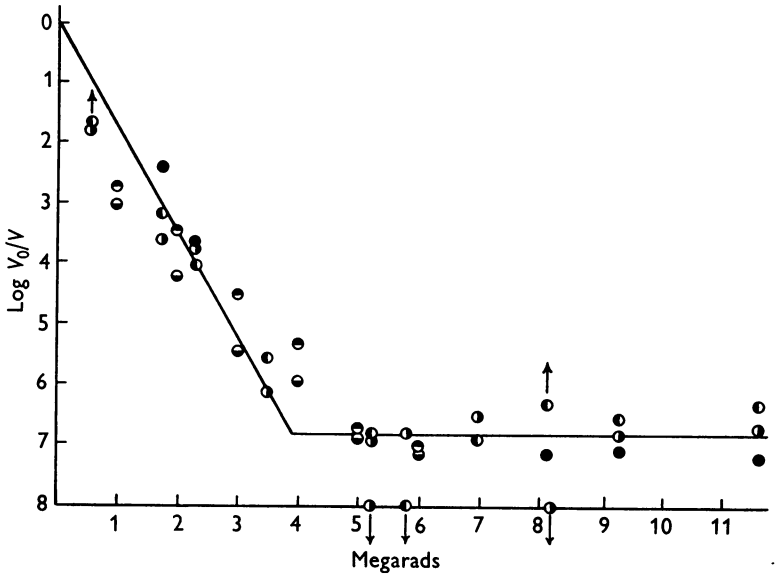

Fig. 1. Inactivation of dried vaccinia virus by γ-rays. V_0 = initial titre of virus. V = titre at any specified radiation dose. ◐, Exp. no. 1; $\log_{10} V_0 = 7{\cdot}15$. ◑, Expt. no. 2; $\log_{10} V_0 = 7{\cdot}11$. ◒, Expt. no. 3; $\log_{10} V_0 = 7{\cdot}48$. ◓, Expt. no. 4; $\log_{10} V_0 = 7{\cdot}08$. ●, Expt. no. 5; $\log_{10} V_0 = 8{\cdot}62$.

Table 4. *Inactivation of vaccinia virus suspensions by γ-rays*

	Surviving virus (p.u./ml.)	
Mrad.	EBS 50/58	EBS 57/58
0	$1{\cdot}1 \times 10^7$	$1{\cdot}2 \times 10^7$
0·2	$1{\cdot}6 \times 10^4$	$7{\cdot}0 \times 10^3$
0·4*	60 (5)	25 (0)
0·6*	0 (0)†	0 (0)†
0·8*	‡	0 (0)†
1·0	0	0
1·5	‡	0

Each suspension divided into 7–12 ml. samples. One sample exposed at each irradiation dose.

* Inoculation repeated: second titre in brackets.

† Remainder of sample used for immunization of rabbits.

‡ Not tested: bottles broke in transit.

6×10^5 and 8×10^5 rad. (Table 4), were each used to immunize five rabbits. EBS 50/58 (6×10^5 rad.) induced both circulating antibody and resistance to challenge in the rabbits, whereas EBS 57/58 (6×10^5 and 8×10^5 rad.) induced only resistance to challenge 2 weeks after the second injection (Table 5), unequivocally

raised titres of circulating antibody being absent. The sera from the animals used to test EBS 57/58 (8×10^5 rad.) contained no pock-inhibiting antibody.

As a confirmatory experiment, EBS 106/58 diluted to about 5×10^7 p.u./ml.,

Table 5. *Immunogenicity test of γ-irradiated vaccinia virus: resistance to challenge and circulating antibody*

Inoculum	Rabbit no.	5th day response to challenge virus diluted 1/1000	1/2000	1/4000	1/8000	1/16,000	Neutralizing antibody titre
EBS 50/58	986	1 ab	1 ab	0	0	0	> 1/64
6×10^5 rad.	987	3	0	0	1	0	> 1/64
	988	1 ab	0	1 ab	0	0	1/32
	989	1	0	0	0	0	1/32
	990	2	0	0	0	0	1/8
EBS 57/58	981	sc+, ab	6 ab	3 ab	4 ab	0	< 1/2
6×10^5 rad.	982	sc+, ab	9 ab	2 ab	4 ab	0	< 1/2
	983	c, ab	sc, ab	3 ab	0	1 ab	1/4
	984	c, ab	4 ab	1 ab	0	0	1/2
	985	8 ab	0	0	0	0	< 1/2
EBS 57/58	976*	—	—	—	—	—	< 1/2
8×10^5 rad.	977	4 ab	0	2 ab	1 ab	1 ab	1/4
	978	sc+, ab	5 ab	6 ab	1 ab	1 ab	1/2
	979	sc−, ab	3 ab	1 ab	0	1 ab	< 1/2
	980	2 ab	0	0	0	0	< 1/2

* Not challenged: killed after second bleed. ab = abortive lesion, c = confluent lesion, sc+ = semi-confluent lesion covering 70–80 % of area, sc = semi-confluent lesion covering 50–70 % of area, sc− = semi-confluent lesion covering < 50 % of area.

Table 6. *Immunogenicity test of γ-irradiated vaccinia virus: resistance to challenge and circulating antibody*

Inoculum	Rabbit no.	5th day response to challenge virus diluted 1/1000	1/2000	1/4000	1/8000	1/16,000	Neutralizing antibody titre
EBS 106/58	26/58	2	0	0	1	0	1/4
6×10^5 rad.	27/58	0	1	1	0	0	1/8
	28/58	4 ab	1	0	0	0	1/8
	29/58	1 ab	0	0	0	0	1/8
	30/58	2 ab	1 ab	1 ab	0	0	1/8
EBS 106/58	21/58	1 ab	0	0	0	0	> 1/16
8×10^5 rad.	22/58	3 ab	5 ab	1	1 ab	1 ab	< 1/2
	23/58	5	4	1	0	2	< 1/2
	24/58	1 ab	0	0	0	0	1/8
	25/58	7	3	1	6	2	< 1/2

ab = abortive lesion.

was sent for irradiation at two dose levels. Four bottles of virus suspension (*c.* 100 ml.) were to be exposed to γ-rays—two of them to 6×10^5 rad. and two to 8×10^5 rad. Unfortunately, one bottle received $9{\cdot}6 \times 10^5$ rad. instead of 8×10^5. After irradiation, the contents of similarly irradiated bottles were pooled and

rabbits immunized with 11 ml. of each pool. The remainder of each pool was then concentrated by centrifugation to 1 ml., and the whole used to inoculate ten 12-day chorio-allantoic membranes; 2 days later, no pocks were found on any membrane. Five rabbits were inoculated with each of the preparations inactivated by 6 and 8×10^5 rad. (Table 6). Inactivation by 6×10^5 rad. left a virus with more immunogenic activity, measured both by resistance to challenge and circulating antibody response, than inactivation by 8×10^5 rad.

DISCUSSION

Pollard (1953, 1956) discusses the theoretical reasons for expecting inactivation by ionizing radiations to leave a high proportion of the surface antigens of a virus undamaged. On the assumption that protective antigenicity resides in the surface antigens, virus suspensions inactivated by ionizing radiations should make effective and easily prepared vaccines. Dick, Schwerdt, Huber, Sharpless & Howe (1951) exposed suspensions of Type 2 poliomyelitis virus to a high-intensity electron beam. Doses of about 25×10^5 roentgen equivalents physical (r.e.p.) consistently inactivated infectivity and left reasonable antigenicity, whether the irradiation was done at atmospheric pressure or *in vacuo* at $-196°$ or $-76°$. Jordan & Kempe (1956) inactivated mouse neurotropic vaccinia virus at $-76°$ by γ-rays from a Co-60 source. Virus partially purified from infected mouse brain by differential centrifugation was more readily inactivated than unpurified suspensions, suggesting that in the crude preparations the impurities were protective, and that even at very low temperatures indirect radiation effects occurred. Exposure to $1{\cdot}5 \times 10^6$ r.e.p. made the virus non-infectious by the tests used. Some antigenicity was retained. Traub, Friedemann, Brasch & Huber (1951) bombarded suspensions of rabies virus with high-intensity electrons; they also inactivated infectivity while retaining antigenicity. They reported that the absorbed energy varied from 1·5 to $4{\cdot}7 \times 10^6$ r.e.p.; it is, nevertheless, impossible to construct an inactivation curve from their results. The antigenicity of their vaccines varied within a 100-fold range, but there was no correlation between the inactivating dose of electrons and the antigenicity of the vaccines.

Judged by antibody response and tissue response to challenge in immunized animals, the immunogenicity of our inactivated preparations was not closely related to the inactivating dose of γ-rays (Table 5, EBS 50/58 and EBS 57/58). This may, perhaps, be associated with the fact that inactivation of suspensions must have been by a mixture of direct and indirect effects. A further point, shown very clearly in Table 6, is that the response of immunized rabbits to skin challenge with a potent vaccinia virus preparation does not bear close relationship to the amount of circulating antibody. Little indeed is known of the quantitative relationships between circulating antibody and reaction to cutaneous challenge with vaccinia virus.

In our dried vaccine preparations indirect radiation effects were assumed to be negligible. Their absence may be expected to increase the dose of γ-rays necessary to inactivate completely, but cannot account for the survival of virus exposed to

more than 10^7 rad. The results of Jordan & Kempe (1956), however, suggest that the peptone in the dried vaccine may have protected it. It may also be argued that this apparent survival was due to multiplicity reactivation. The phenomenon can be excluded on the following grounds: Cairns & Fazekas de St Groth (1957) estimated the number of allantoic cells in the 12th-day chick embryo to be $4{\cdot}25 \times 10^5$/cm.^{2}; Overman & Tamm (1957) assumed that chorionic cells are at least three times as numerous as allantoic cells. The average area of chorio-allantoic membrane available for inoculation on 25 consecutively treated embryos was 16 cm.^{2}, i.e. about 2×10^7 cells/dropped area. In an inoculum volume of 0·1 ml. the number of infectious units in un-irradiated vaccine would be about 10^6/membrane. The mean ratio of total particle count to infectious units for the virus strain used in these experiments is *c.* 12 (Kaplan & Valentine, 1959). There are, therefore, about $(12 \times 10^6)/(2 \times 10^7) = 0{\cdot}6$ particles/cell; so that multiplicity reactivation may be disregarded as a cause of pock formation by heavily irradiated vaccinia virus.

Although the physical arrangement of the dried virus in its ampoules precluded proper mathematical treatment of the results, it nevertheless seems that the inactivation of infectivity followed first-order (or pseudo first-order) kinetics until the proportion of survivors was very small. We do not think that the survival of this minute fraction of the virus has any bearing on the results of Lea & Salaman (1942), nor on the general theory of sensitive volumes (Lea, 1955). Workers in the physical rather than the biological side of this field have, quite legitimately, seldom aimed at complete inactivation of their virus preparations. This is, of course, a necessary stage in the production of a safe vaccine.

In our opinion, the likeliest explanation of survival is that the virus is heterogeneous in its response to ionizing radiations as it is to heat (Kaplan, 1958), and to ultra-violet irradiation and β-propiolactone (unpublished observations). It was not possible, unfortunately, to determine experimentally whether the resistance to ionizing radiations was genetical. However, we have so far been unable to demonstrate a genetical basis for the heat-resistant fraction of vaccinia virus (Kaplan, 1958; and subsequent unpublished observations). It may be, therefore, that the resistant fraction of vaccinia virus is an expression of the heterogeneity of biological material in general.

SUMMARY

Suspensions and dried preparations of vaccinia virus were exposed to γ-rays in cobalt-60 sources. Suspensions completely inactivated by $6–8 \times 10^5$ rad. retained measurable immunogenicity. Dried preparations could not be completely inactivated even by doses of 11×10^6 rad., although the inactivation apparently followed first-order kinetics until about one particle in 10^7 survived, suggesting that the populations of vaccinia virus in the preparations were heterogeneous.

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