THE ANTIGENICITY OF ULTRA-VIOLET IRRADIATED VACCINIA VIRUS

By L. H. COLLIER, D. McCLEAN and L. VALLET

The Lister Institute of Preventive Medicine, Elstree, Herts

(With Plate 15 and 2 Figures in the Text)

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INTRODUCTION

No antigen with satisfactory immunizing properties has hitherto been obtained from inactivated vaccinia virus. Bland (1932) studied the relative immunizing power of virus preparations inactivated by heat, phenol and formalin; his paper contained an account of earlier attempts to obtain a satisfactory antigen. Bland's results in the immunization of rabbits and monkeys were poor, but more encouraging with guinea-pigs, though there was no definite evidence that the phenol- or formalin-inactivated virus was more effective than the heat-treated antigen. There was some doubt, however, in the guinea-pig experiments, whether the first weak dose of living virus used as a test for immunity, although it produced no obvious lesions, contributed to the apparent partial immunity of the animals at a later test with a stronger dose of virus. Parker & Rivers (1936) studied the antigenic activity of purified elementary body suspensions of vaccinia inactivated by 0·3 % formaldehyde and of virus-free extracts of vaccinia. Humoral antibodies and a certain degree of resistance to infection, probably not enduring, were

produced by repeated injections. The antigenicity of virus inactivated with alcohol was investigated by McClean (1945), who reported that two spaced injections of very large doses of vaccinia virus inactivated with 50% ethanol induced a slight immunity and detectable neutralizing antibody in the serum; the very large amount of purified virus suspension required and the poor immunity response precluded the use of this material in the prophylaxis of smallpox. The dose of inactivated virus was large in terms of minimal infective units per ml. The actual weight of inactivated virus injected into each rabbit was, however, only about 50 μg., of which only an unknown proportion consisted of immunizing antigen. In the same year Levinson, Milzer, Shaughnessy, Neal & Oppenheimer (1945) and Milzer, Oppenheimer & Levinson (1945) reported a new method for the production of potent inactivated vaccines with ultra-violet irradiation. Technical details were not available at that time, but bacteria and viruses were described as inactivated 'in a fraction of a second by exposing continuously flowing thin films with a depth of less than 1 mm. to a newly developed lamp which is a powerful source of both total and extreme (below 2000 A.) ultra-violet'. The authors emphasized that bacteria and viruses could be rapidly inactivated with minimal loss of antigenicity, and they reported successful immunization with inactivated rabies, St Louis encephalitis and poliomyelitis viruses. Preliminary experiments with vaccinia virus were also successful (private communication). In 1951 a Habel–Sockrider (1947) ultra-violet apparatus became available in this country, and it was possible to extend the work of Levinson and his colleagues.

Andrewes, Elford & Niven (1948) reported that irradiated vaccinia virus immunizes mice against ectromelia, but affords little protection to rabbits and guinea-pigs against vaccinia. The immunity to vaccinia may have been poor because the immunizing doses were usually given intraperitoneally, not subcutaneously, and the intervals between the two immunizing doses and between the last dose and the challenge with living virus were too short for good immunization. In their apparatus, the time of exposure to ultra-violet light was determined by measuring the rate of flow of the suspension. With the simple arrangement they used, however, the flow rate would decrease as the level in the reservoir fell, and it is unlikely that precise determinations of the minimum degree of irradiation for inactivation could be made. Excessive irradiation may have destroyed the antigenicity of their virus preparations.

Irradiated virus might induce a firm immunity to smallpox, but even if it failed to do this, it might stimulate the production of circulating antibodies and so modify the response to subsequent vaccination with living virus. Primary Jennerian vaccination of infants is associated with a low but definite risk of complications, and some of these complications are serious. The risks are greater in the primary vaccination of older children and adults and, moreover, any measure which would reduce the malaise following vaccination would be welcome. As a preliminary to tests in man it was important to ensure that virus preparations could be completely inactivated by irradiation, and that any immunity which developed in experimental animals could not be attributed either to traces of living virus or to accidental exposure of the animals to infection.

MATERIALS AND METHODS

Source of virus

In two experiments ordinary vaccinial pulp, as used in the preparation of vaccine lymph but without the addition of phenol or glycerol, was employed. The ground pulp was suspended in distilled water (1/15, w/v) to reduce the absorption of ultra-violet rays. In all other experiments the virus was irradiated in the form of purified elementary body suspensions (E.B.S.) prepared by differential centrifugation from crude vaccinial pulp. The purified virus used was the Lister Institute strain, which had been adapted to the rabbit by repeated skin passage. The method of purification employed was a modification of that described by Craigie (1932), with Salaman's (1937) modification of fractional resuspension of the virus deposit obtained by angle centrifugation. The suspensions were largely freed from contaminating bacteria by incubation for 48 hr. at 22° C. with 0·25 % phenol. The virus was then deposited by angle centrifugation, the supernatant containing the phenol discarded, and the deposit taken up in 10 ml. of 0·004 M phosphate-citric acid buffer, pH 7·2. After further low-speed centrifugation to remove clumps of elementary bodies, the supernatant was carefully removed, and titrated in embryonated eggs. The E.B.S. was then diluted with 0·004 M McIlvaine buffer so as to contain approximately 10^7 infective particles per ml. and it was irradiated in this form.

Infectivity titrations

E.B.S. were titrated by the pock-counts in embryonated eggs (Collier, 1955); titres are expressed as the logarithm of the number of pock-producing units per ml. of the original suspension.

Irradiation

The apparatus described by Habel & Sockrider (1947) was modified for the ultra-violet irradiation of virus suspensions. It (Pl. 15), consisted of an aluminium cylinder 5·1 cm. in diameter, 71 cm. long, mounted on bearings so that it would be rotated about its axis on an inclined frame at 1000 r.p.m. by a constant-speed electric motor. A low-pressure mercury lamp was mounted axially in the cylinder. The suspension to be irradiated was fed on to the inner surface of the rotating cylinder by compressed air using McCartney siphons, where it formed a thin 'flowing' film suitable for irradiation. When it reached the lower end of the cylinder, it was flung off by centrifugal force and collected in a special cup that led into a receiving vessel. The rate of rotation of the cylinder was considerably greater than that used by Habel & Sockrider. A more stable film could be obtained at the higher rate. The lamp was a 24 in. Hanovia low-pressure mercury lamp, from which over 90 % of the ultra-violet emission was at 2537 A., at about 3 W.

The rate of flow of the E.B.S. was kept constant using a 'Rotameter' flowmeter, calibrated for water at 15° C., through which the suspension passed. The flow-meter was unsuitable for vaccine lymph, because the larger particles would have been trapped in the meter tube; the flow rate of lymph was measured as the time to

fill a 50 ml. bulb on the output side of the irradiator. By injecting dye into water, as it was fed into the rotating cylinder, and measuring the time that elapsed before it appeared in a glass collecting cup at the output end, the minimum value of the exposure time for each rate of flow was estimated. Flow rates from 1·8 to 8 l./hr. gave exposure times between 15 and 5·5 sec., respectively. Increase of flow rate beyond 8 l./hr. to reduce the exposure time further was not practicable because the reduction in exposure time with increase of flow is less at higher rates. To increase the range of exposure for these experiments, the effective output of the lamp was reduced by screening sections at each end, enclosing them in tubes of stiff paper. This reduced the time during which suspensions flowing through the cylinder were exposed to irradiation on the cylinder surface. Very long exposures were obtained by putting the material through the irradiator several times. The term 'Relative Exposure' is used to indicate the effective time of exposure of the suspension to radiation, taking into account these various factors.

The thickness of the film formed in the cylinder and the exposure time at constant rate of rotation depended on the rate of flow, the angle of elevation and the physical characteristics of the suspension. The behaviour of E.B.S. resembled that of water. Both increase of flow rate and decrease of angle of elevation of the cylinder caused an increase in film thickness. The film thickness used was about 0·1 mm. In view of the very low absorption of the suspension at 2537 A., any screening of particles either by absorption of the medium or by the virus particles themselves would be quite negligible.

Tests for inactivation of virus

Cultivation on the chorio-allantois of the chick embryo proved to be a much more sensitive indicator of living virus than scarification of rabbit skin, and was used exclusively throughout the tests. The material to be tested was concentrated tenfold by centrifugation and 0·2 ml. of the resulting suspension inoculated on to the membranes of four fertile eggs. Thus the equivalent of 8 ml. of the suspension to be used for immunization was tested for inactivation of virus. After 72 hr. incubation the eggs were examined for the presence of any characteristic infective foci. In tests on several of the later batches of irradiated material, the apparently sterile egg-membranes were minced and passed in a further set of eggs to exclude the presence of living virus which had failed to produce pocks. However, positive subcultures were never obtained.

Bacterial content of irradiated material

Although irradiation substantially reduced the bacterial contamination of lymph suspensions they were not sterilized. It was therefore necessary to incubate irradiated material for 48 hr. with 0·5 % phenol before injecting it into experimental animals. E.B.S. were, however, largely freed from contaminating organisms during purification; after irradiation they were found to be sterile in both aerobic and anaerobic culture.

Immunization experiments.

During immunization the groups of rabbits were kept in a house a quarter of a mile from the vaccine lymph laboratories and animal house, and they were cared for by attendants who had no contact with animals infected with vaccinia. In most of the experiments, the rabbits were given two injections of irradiated material at 14 days' interval. Blood samples were taken before each injection. A fortnight after the second injection the animals were again bled and then challenged by scarification on the flank with increasing dilutions of the laboratory 'control' lymph. Their reactions to this vaccination were compared with those of a group of normal rabbits to the same lymph. In addition, virus neutralizing antibody in the serum samples taken before, during and after immunization was titrated by mixing increasing dilutions of the sera with a fixed dose of the control lymph, which was ten times that required to give a confluent or semi-confluent reaction on a normal rabbit. After 1 hr. incubation at 22° C. the virus and serum mixtures were inoculated by scarification on normal rabbits, the inoculations being so arranged that the two or three samples from any one rabbit were always titrated on one animal. The results were read on the 4th, 5th and 6th days, and the neutralizing titre of the serum sample was taken as the highest dilution that would reduce the resulting reaction to not more than ten discrete vesicles. A neutralizing titre of less than 1/4 was not regarded as significant. These tests provide a record of both the resistance to vaccination developed by the treated animals and the increase in circulating antibody.

Table 1. *Titration of control vaccine lymph by scarification in normal rabbits*

Dilution of lymph	Rabbit number						
	1	2	3	4	5	6	7
1/1,000	c	c	c	c	c	c	c
1/2,000	c	c	c	c	c	c	c
1/4,000	c	c	c	c	c	c	c
1/8,000	c	c	sc	c	c	sc+	sc+
1/16,000	sc	c	3	sc+	sc	sc+	sc
1/32,000	5	sc+	2	0	5	sc−	8
1/64,000	3	sc	1	3	1	2	6
1/128,000	2	4	0	3	1	0	2

c = confluent reaction over the whole area; sc+ = lesions covering 70–80 % of the area; sc = lesions covering 50–70 % of the area; sc− = lesions covering less than 50 % of the area. Discrete vesicles are indicated by the appropriate figure.

Titre of control vaccine lymph in normal rabbits

Table 1 shows the response of seven normal rabbits to the control lymph used in direct tests for immunity to vaccination and in the titrations of circulating antibody. Confluent reactions developed with dilutions up to 1/4000 and confluent or semi-confluent reactions at 1/8000 and 1/16,000. At higher dilutions up to 1/128,000 semi-confluent or discrete vesicles were produced. Vesicles reached their maximum development on the 5th or 6th day after vaccination, and did not show

any sign of scabbing or healing before the 9th or 10th days. These results will be compared with those following the challenge of the groups of experimental animals.

EXPERIMENTAL RESULTS

Inactivating dose of irradiation

The ultra-violet absorption coefficients of both diluted lymph and purified E.B.S. were determined, and the doses of irradiation necessary to inactivate both materials was ascertained by preliminary experiments in which the exposure was varied.

The extinction coefficient at 2537 A. of lymph suspensions varied from 160 to 189. This high level of absorption, and the comparatively large particles they contained, made these suspensions difficult to irradiate uniformly. The extinction coefficient of E.B.S. containing 6·08 units of virus was only 0·60 or less at this wavelength and uniform exposure and rate of flow could be obtained with these preparations.

Whereas the lymph required exposure to 3 W. at 2537 A. with a rate of flow of 1·3 l./hr. at 5° elevation for complete inactivation, E.B.S. were inactivated using a flow rate of 6·2 l./hr. at 15° elevation with 0·75 W., only one-quarter of the arc length of the lamp being exposed. With E.B.S. of the higher titre used in this work, the flow rate was reduced as a routine to 3·0 l./hr. at 15° elevation with one-quarter of the arc length exposed. Any experimental variations in the dose of irradiation are recorded below as fractions or multiples of this exposure, which is taken as unity (relative exposure 1).

Immunization of rabbits

Irradiated lymph. Since, as has already been explained, vaccine lymph is unsuitable material for irradiation and, with one exception, all subsequent experiments were done with elementary body suspensions, the preliminary vaccine lymph experiment will only be summarized. The two large subcutaneous doses of irradiated material, followed a fortnight later by two intravenous doses, produced satisfactory resistance to subsequent challenge with living virus; only a few scattered abortive vaccinial vesicles were produced at the lower dilutions of the test lymph as compared with the confluent reactions regularly obtained in normal animals. Fourteen days after the second subcutaneous dose of irradiated material neutralization of virus was shown up to serum dilutions of from 1/16 to 1/64. There was no apparent rise in titre after the subsequent intravenous doses.

Irradiated E.B.S. A similar preliminary experiment in five rabbits given two subcutaneous doses of 5 ml., followed by two intravenous doses of the same volume of E.B.S. 60 with titre of 6·04 units/ml. before irradiation produced similar results. Subsequent challenge with living virus produced abortive vaccinial lesions only in the areas inoculated with the lower dilutions of lymph. These abortive lesions show a small necrotic centre with surrounding induration; they heal early and do not develop into a typical vesicle and pustule. Neutralization of virus was shown up to serum dilutions of from 1/16 to 1/32 a fortnight after the second subcutaneous dose and up to 1/64, the highest dilution tested, after the second intravenous dose.

The influence of smaller doses of antigen

Irradiated lymph. Five rabbits were given two subcutaneous doses of only 1 ml. of irradiated lymph at a fortnight's interval, and their reaction to challenge with the control active lymph tested 14 days after the second dose. Abortive discrete lesions at the lower dilutions only were obtained in three of the five rabbits, one rabbit was completely refractory to vaccination and the fifth produced semi-confluent reactions at the two lowest dilutions only (Table 2).

Table 2. *Titration of control vaccine lymph on rabbits immunized with two 1 ml. doses of irradiated lymph*

Dilution of lymph	Rabbit number				
	9	10	11	12	13
1/1,000	sc	4	0	5	3
1/2,000	sc−	0	0	1	2
1/4,000	4	0	0	2	0
1/8,000	2	?2	0	0	0
1/16,000	?1	0	0	0	0
1/32,000	0	0	0	0	0
1/64,000	0	0	0	0	0
1/128,000	0	0	0	0	0

sc and figures = same significance as Table 1.

All the rabbits developed an antibody titre ranging from 1/8 to more than 1/64. With two rabbits there was detectable antibody response to only one dose of antigen (Table 3). In this table the results of each test are given in full so that the

Table 3. *Neutralizing antibody titre of sera from rabbits before, during and after immunization with two doses of irradiated lymph*

Rabbit no.	Bleeding	Serum dilution						Antibody titre
		1/2	1/4	1/8	1/16	1/32	1/64	
9	Before immunization	c	c	c	c	c	c	< 2
	Before 2nd dose	9	c	c	c	c	c	2
	Before challenge	0	1	1	9	sc−	c	16
10	As above	sc+	sc+	sc+	c	c	c	< 2
		1	0	5	2	sc+	c	16
		4	2	2	0	3	sc+	32
11	As above	c	c	c	c	c	c	< 2
		10	sc	c	c	c	c	2
		0	5	2	sc−	sc+	c	8
12	As above	6	sc	sc	sc−	sc	sc	2
		0	1	4	4	3	12	32
		0	0	0	1	3	8	>64
13	As above	sc+	c	c	c	c	c	< 2
		2	sc+	c	c	c	c	2
		1	2	4	sc+	sc+	c	8

c, sc+, sc, sc− and figures = same significance as in Table 1. Antibody titre is expressed as the reciprocal of the highest dilution showing neutralization of virus.

method used in this work will be clear; subsequently only the neutralizing end-point of each serum as defined under Methods will be shown.

Irradiated E.B.S. At a fortnight's interval five rabbits were given two subcutaneous injections of 2 ml. of irradiated E.B.S. 64 with a titre of 7·89 units/ml. before irradiation. Only three rabbits survived for challenge by vaccination; no. 30 died from trauma after the final intracardial bleeding and another (no. 32), which proved to be pregnant, died the second day after challenge. Blood samples were, however, taken from all five rabbits; in this experiment no blood was collected after the first injection. Two of the three surviving rabbits (Table 4) had abortive lesions only, but the third (no. 35) had unmodified semi-confluent lesions at the lower dilutions of virus. All three developed circulating antibody, but the sick pregnant rabbit (no. 32) which died after challenge had developed no circulating antibody (Table 4).

Table 4. *Titration of control vaccine lymph and neutralizing antibody titre in rabbits immunized with two doses only of irradiated* E.B.S. 64

Dilution of lymph	Rabbit number				
	30	31	32	34	35
1/1,000	No test	9 ab	Rabbit	6 ab	sc+
1/2,000		6 ab	died 2nd	?	sc+
1/4,000		6 ab	day after	2 ab	sc−
1/8,000		2 ab	challenge	0	0
1/16,000		0		0	2
1/32,000		0		0	1
1/64,000		0		0	0
1/128,000		0		0	0
Serum samples					
Before immunization	<2	<2	<2	<2	<2
Before challenge	16	32	<2	8	2

c, sc and figures = same significance as Table 1. ab = abortive lesions. Antibody titre is expressed as the reciprocal of highest dilution showing neutralization of virus.

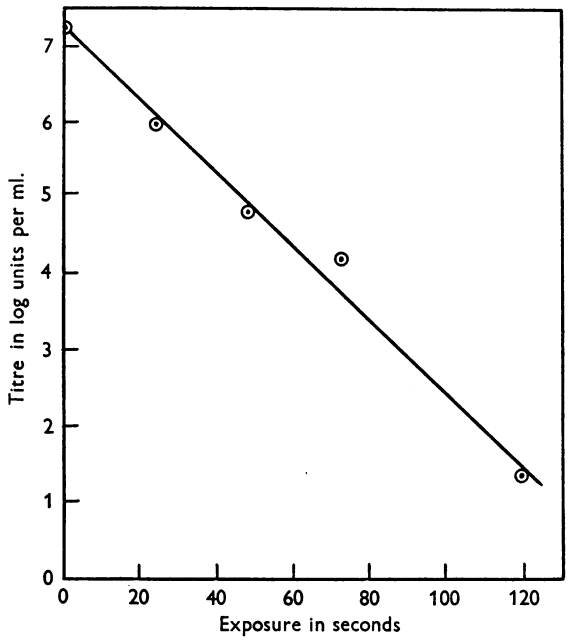
Fourteen days after the challenge, the pooled sera of the three surviving rabbits had a neutralizing titre of 1/150, and a similar pool from the rabbits 9–13 (Table 3) used in the previous experiment with irradiated lymph had an antibody titre of 1/300.

Relation of irradiation to antigenicity

Irradiation required to inactivate vaccinia E.B.S. It was important to determine the smallest dose of irradiation that could be relied on completely to inactivate the virus in the suspensions used and the influence of larger doses of irradiation on their antigenicity.

Taylor, Sharp, Beard, Finkelstein & Beard (1941) demonstrated an exponential relationship between the infective titre of surviving Eastern equine encephalomyelitis virus and its exposure to ultra-violet radiation. This relationship is dis-

cussed by Lea (1946), who cites examples of its application to both viruses and bacteria. In view of its bearing on the complete inactivation of vaccinia, the survival-exposure relation for vaccinia E.B.S. was investigated. For the irradiation a quartz cell of 20 ml. capacity was mounted in front of a low-pressure mercury lamp which had similar emission characteristics to that used in the Habel–Sockrider apparatus. The contents of the cell were stirred by an oscillating glass rod. The layer of suspension presented to the lamp was 1·0 cm. in thickness. As over 80% of the incident radiation was transmitted by this layer, it could be



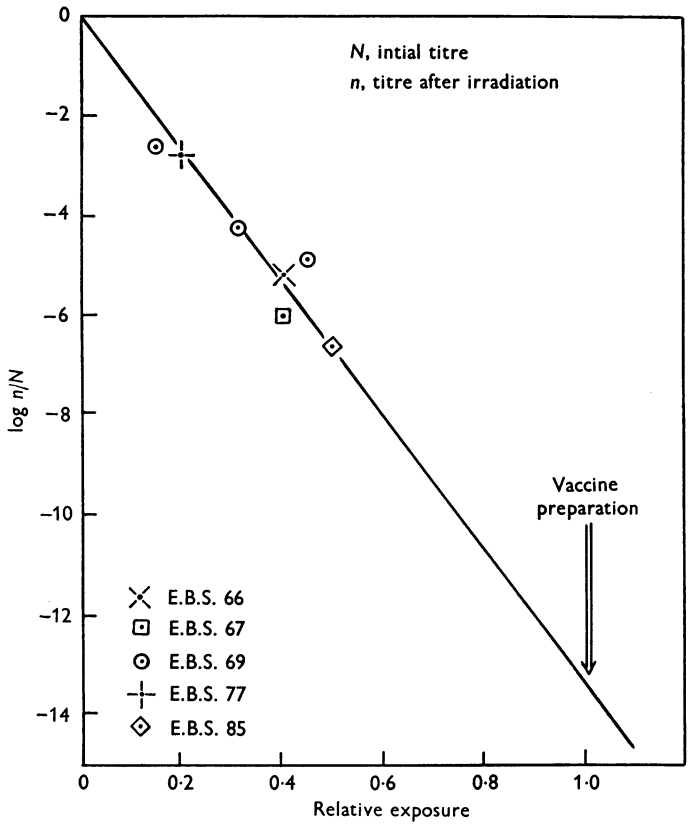
Text-fig. 1. The effect of exposure to ultra-violet radiation on the titre of an elementary body suspension, irradiated in a quartz cell.

assumed that, with stirring, uniform irradiation was obtained throughout the E.B.S. in the cell. By this method, exposure times were directly controlled and more accurately measured than in the Habel–Sockrider apparatus; the radiation intensity, however, was considerably less, and the exposure times longer. The inactivation curve (Text-fig. 1) shows an exponential relation between infectivity and exposure time.

The results of graded exposures in the Habel–Sockrider apparatus below that used for the preparation of vaccine are shown in Text-fig. 2. For direct comparison of several E.B.S. of different initial titres, survival is expressed as the logarithm of the ratio of surviving infectivity to initial titre. Here again inactivation is exponential.

In our experiments, suspensions with a titre of about 7·5 units/ml. were reduced by a relative exposure of 0·5 to about 0·9 unit/ml. The conditions necessary to produce a fall in titre from 7·5 to 0·9 units in other irradiators can be found by

experiment, using graded exposures. Having done this, a vaccine may be prepared by irradiating an E.B.S. of the same initial titre using double this exposure. It must be emphasized that relative exposure 1·0 is a purely arbitrary level of radiation which was found by experiment to inactivate our preparations completely without destroying their antigenicity.



Text-fig. 2. The effect of graded exposures to ultra-violet radiation on elementary body suspensions irradiated in the Habel–Sockrider apparatus.

With three different E.B.S. increase in the dose of irradiation rapidly destroyed the antigenic activity of the virus. All three E.B.S. produced a satisfactory response after relative exposure 1; when the dose of irradiation was doubled (relative exposure 2) there was only a suggestion of immunity in a minority of the rabbits measured either as response to vaccination or by circulating antibody. Relative exposure 5 completely destroys the antigenicity.

In the Habel–Sockrider apparatus the suspension is spread in a very thin film, and is exposed to ozone generated by the lamp. To eliminate these conditions as a partial cause of the inactivation, ozone generated externally was fed into the Habel–Sockrider apparatus at the output end through the electrode orifice, the lamp being omitted, while E.B.S. was flowing through under the conditions used for relative exposure 1.

The ozone was generated by mounting an ultra-violet lamp, of the type used in the irradiator, in an aluminium tube. Air blown through the tube was ozonized, forming a mixture resembling that occurring inside the irradiator.

Samples of E.B.S. flowing through the tube in the absence of both ozone and radiation, and in the presence of ozone, but without radiation, were titrated in eggs; their titres were sufficiently close to that of an untreated control to indicate that neither the spreading of the E.B.S. as a film nor its exposure to ozone caused the inactivation of the virus.

Immunization of monkeys

It was desirable to prove that irradiated virus is antigenic in some animal other than the rabbit and, in view of possible use in human volunteers, that no ill effects are likely to follow the use of the inactivated virus. Dr F. O. MacCallum of the Virus Reference Laboratory, Colindale, generously collaborated in tests on monkeys which were housed at Colindale and therefore had no other known contact with vaccinia virus.

Normal monkeys. Two normal monkeys (M. 46 and M. 47) were vaccinated with the same control lymph undiluted and in tenfold dilutions up to 10^{-3} with the following result:

	Dilution of lymph			
	10^{0}	10^{-1}	10^{-2}	10^{-3}
M46	Confluent	Semi-confluent	1 vesicle	0
M47	Confluent	4 large vesicles	2 vesicles	0

The lesions were normal, without evidence of acceleration, reaching their maximum on the 7th and becoming scabbed on the 8th day. The result indicates that monkeys, like men, do not react to such high dilutions of lymph as rabbits, which normally have a confluent or semi-confluent reaction at a dilution of 1/8000 to 1/16,000 of this lymph. This may be because the virus is passed alternately through rabbits and sheep, and is therefore partially adapted to the rabbit. The titre of circulating antibody in these monkeys showed no significant rise in 8 days but rose to 1/16 and 1/60 respectively by the 23rd day.

Immunized monkeys. Two monkeys (M 37 and M 38) were given two subcutaneous doses, spaced as for rabbits, each of 2 ml. of irradiated E.B.S. 67 originally containing 7·98 units/ml. This suspension unfortunately proved to be a poor antigen in an experiment in rabbits which will be described later. The usual blood samples were taken from the monkeys before the second irradiated doses and again immediately before challenge a fortnight after the second dose. These animals were also bled again 13 and 19 days after vaccination. Haemagglutination inhibition tests were done at Colindale on the sera for these samples, in addition to the usual virus neutralization tests at Elstree.

The challenge with the control lymph diluted 10^{-1}, 10^{-2} and 10^{-3} produced four or five discrete lesions at the lowest dilution only in both monkeys, and these were already scabbing at the 6th day. Virus neutralization tests on the sera from these monkeys show considerable difference in the response of the two animals: M 37

showed detectable antibody a fortnight after the first dose and this rose to a titre of 1/32 after the second dose. M 38, however, showed no response to the first dose and only partial neutralization at 1/4 after the second. Tests on blood samples 13 and 19 days after challenge again showed a better response in the first monkey with a neutralizing titre of 1/320. That of the second monkey had only risen to 1/40. Parallel tests at Colindale on the same samples of serum showed no increase in anti-haemagglutinin following the two doses of irradiated vaccine. The challenge with vaccine lymph, however, caused a sharp rise in anti-haemagglutinin titre in both monkeys. Chu (1948) reports that the haemagglutinin normally present in vaccine pulp is discarded during purification by differential centrifugation. The E.B.S. used in these experiments were tested for specific haemagglutinin activity, by Chu's method, with negative results. The failure to produce anti-haemagglutinin after irradiated vaccine was therefore due to the absence of haemagglutinin in the E.B.S. and not to its destruction by ultra-violet light.

Two more monkeys (M 70 and M 106) were each given 2 ml. doses of irradiated E.B.S. 74 which had been dried from the frozen state and successfully used in a group of rabbits in an experiment on the preservation of the virus by drying, to be described later. The usual blood samples were taken before, during and after immunization. Challenge a fortnight after the second dose gave the following result:

	Dilution of lymph			
	10^{0}	10^{-1}	10^{-2}	10^{-3}
M 70	7 vesicles	2 vesicles	0	—
M 106	Confluent	6 vesicles	2 vesicles	0

Modification of the vaccinial lesions was poor compared with similar tests in rabbits, but they reached their maximum on the 5th and healing had already commenced by the 7th day. Good antibody production (Table 5) occurred before

Table 5. *Neutralizing antibody titre of sera from two monkeys before, during and after immunization with two doses of dried irradiated* E.B.S. 74 *and after challenge with test vaccine lymph*

Serum samples	Monkey number	
	70	106
Before immunization	> 2	> 2
Before 2nd dose	4	> 2
Before challenge	80	160
12 days after challenge	800	1,600
18 days after challenge	400	1,600

The figures have the same significance as in previous tables.

challenge, which makes it surprising that there was not more modification of the response to living virus. After challenge, virus neutralization had risen tenfold by the 12th day with no further increase by the 18th—a rapid rise suggesting a secondary type of response.

The experiments on monkeys indicate that quite a high titre of antibody can be produced by irradiated virus, though the immunity to skin challenge is not as definite as would have been expected from comparable rabbit experiments. It is possible that if this material were used for basal immunization in man the modification of the reaction to subsequent orthodox vaccination might also not be as great as in rabbits.

The duration of immunity

Six rabbits were given the usual two doses of irradiated E.B.S. 67 which had an original titre of 7·98 unit/ml. This was the preparation also given to the first two monkeys (M 37 and M 38). Blood samples were taken before the first and after the second dose, and it will be seen (Table 6) that the antibody response was poor.

Table 6. *Neutralizing antibody titre of serum samples from rabbits immunized three times during a period of* 10 *months*

Serum samples	Rabbit number					
	41	42	43	44	45	46
Before immunization	<1/2	<1/2	<1/2	<1/2	1/2	<1/2
2 weeks after 2nd dose	1/4	<1/2	1/4	1/2	1/8	1/4
Before 2nd immunization	<2	—	<2	<2	2	<2
2 weeks after 1st dose	160	—	16	160	160	16
2 weeks after 2nd dose	16	—	8	4	160	16
6 weeks after 2nd dose	8	—	8	—	32	4
10 weeks after 2nd dose	4	—	16	—	16	<2
18 weeks after 2nd dose	2	—	8	—	8	<2
22 weeks after 2nd dose	4	—	2	—	8	2
3 days after 3rd dose	8	—	4	—	16	2
7 days after 3rd dose	32	—	80	—	160	640
14 days after 3rd dose	32	—	160	—	80	—

The figures have the same significance as in previous tables.

Fifteen weeks after the first dose the animals were bled again and then given the first of another pair of injections of irradiated E.B.S. 74 with an original titre of 7·86 units/ml. which was known from other experiments to be a good antigen. The antibody response of the five surviving rabbits to this second pair of injections was good (Table 6). Instead of challenging these rabbits, they were kept and blood samples were taken at approximately monthly intervals to observe the persistence of circulating antibody which is shown in the same table. There was a gradual fall in antibody titre until, at 22 weeks, this was only detectable in the four surviving rabbits at serum dilutions 1/2 to 1/8 (Table 6). The rabbits were then given a single further dose of 2 ml. of the same irradiated vaccine (dried E.B.S. 74) and blood samples taken at the 3rd, 7th and 14th days to detect any accelerated secondary immune response (Table 6). There was a detectable increase in circulating antibody after 3 days in three out of four rabbits, a rapid rise to a maximum titre in 7 days in three out of four animals, and the neutralizing titre was higher than that following either of the preceding immunizations in two out of four animals. Subsequent challenge of the three surviving rabbits with living virus showed that

they were highly resistant, and the few vesicles produced at the lower dilutions were all of the abortive type. It appears that, in rabbits, circulating antibody can be detected for at least 22 weeks after immunization with irradiated vaccinia, but that the antibody level falls steadily during this period. The accelerated response to a single re-inforcing dose of the same material is, however, additional evidence of its antigenicity.

Preservation of the antigen

The antigenicity of some of our ealier irradiated E.B.S. deteriorated on storage in the liquid state at ± 4° C. and the preservation of this material by drying from the frozen state was therefore examined. 700 ml. of E.B.S. 74, with an initial titre of 7·86 units/ml., was irradiated in the usual way. Sterile 20 % peptone solution, pH 7·4, was added to give a final concentration of 2 % peptone in 225 ml. of this suspension, which was dried from the frozen state in 5 ml. amounts, and stored at 4° C. under nitrogen in sealed ampoules. The remainder of the irradiated virus (in buffer at pH 7·4) was held at the same temperature. Experiments reported elsewhere by one of us (L.H.C., 1955) had shown that the addition of peptone increased the stability of dried smallpox vaccine stored at temperatures from 0 to 37° C. Preliminary tests with the fluid moiety in five rabbits indicated that this irradiated E.B.S. possessed satisfactory antigenicity.

Table 7. *Titration of control vaccine lymph and antibody titre in two groups of rabbits immunized with irradiated* E.B.S. *stored respectively in the fluid and dry state*

Dilution of lymph	Rabbits receiving fluid suspension					Rabbits receiving dried suspension				
	702	703	704	705	706	707	708	709	710	711
1/1,000	c	c	2 ab	sc ab	sc	1 ab	sc + ab	1 ab	1 ab	0
1/2,000	sc	c	1 ab	sc ab	sc	0	sc ab	0	0	2 ab
1/4,000	sc	sc +	1 ab	sc ab	6	0	3	1 ab	1 ab	0
1/8,000	6	5	1 ab	5	5	0	0	1 ab	0	0
1/16,000	2	3	0	2	0	0	0	0	0	0
1/32,000	1	1	0	0	2	0	0	0	0	0
1/64,000	1	0	0	1	1	0	0	0	0	0
1/128,000	1	0	0	1	1	0	0	0	0	0
Antibody titre	< 2	2	8	< 2	2	> 640	2	20	160	80

c, sc and figures = same significance as in previous tables; ab = abortive lesions.

After 11 weeks' storage the antigenicity of the fluid and dried portions were retested in two groups of five rabbits with the usual pair of spaced subcutaneous injections of 2 ml. of suspension. Tests for susceptibility to subsequent challenge with living virus and for circulating antibody revealed no deterioration in the dried material and very little in the fluid portion. Storage was therefore continued to a total period of 27 weeks when the antigenicity tests were repeated with the results shown in Table 7.

Of the rabbits receiving the fluid suspension only one (no. 704) showed marked suppression of the lesions following challenge with living virus, though the lesions

on one other rabbit (no. 705) were also of the abortive type and all showed some diminution in susceptibility. The rabbits that had received the dried suspension, however, all showed marked reduction of reaction, and those vesicles that developed were all of the abortive type. Only one of the rabbits that had received the fluid suspension developed a significant titre of circulating antibody, and this was the same animal that had shown the most marked suppression of vaccinial lesions. All the rabbits receiving the dried material developed circulating antibody, three of them to a titre 1/80 or more. Thus a liquid-irradiated E.B.S. stored at 4° C., that slowly loses its antigenicity over a period of 6 months, can be preserved if it is freeze-dried in the presence of peptone. Storage experiments with the dried material stored at 37° C. for 2 months have revealed no detectable loss of antigenicity. It is hoped that gelatine will prove as effective a preservative agent as peptone, for its use would avoid the risk of sensitivity reactions to peptone in man.

Immunization with small amounts of living virus

In view of the small margin between the dose of irradiation required to inactivate the virus and that which destroyed its antigenicity, it might be contended that the immunity developed by most of the rabbits was due to the injection of small doses of living virus which our tests had failed to detect. This criticism might be met by observing the immunity developed by groups of rabbits deliberately given small doses of living virus. Accordingly, we consulted Dr Richard Doll, of the Medical Research Council's Statistical Research Unit, as to the chance that any or all of our rabbits had received living virus; he also advised us on the plan of experiments with living virus to ensure that these animals received larger doses of living material than could have been given by chance in the experiments already described. From an examination of our infectivity tests on the batches of irradiated material it was concluded that, if it could be assumed that every living virus particle would produce a pock on the egg membrane, it was unlikely that there were many more than 300 living particles in a litre of irradiated vaccine. If, however, there were 300, it would not be at all unlikely that a sample of 10 ml. might contain at least four living particles. Since each of our rabbits had received only two injections of either 1 or 2 ml., the maximum number of living particles that any of our animals might have received was reduced to a very small number.

It cannot, of course, be assumed that every living virus particle will produce a pock on an egg membrane, but Beveridge & Burnet (1946) say: 'The approximate linear relationship between virus concentration and pock count indicates that each focus is initiated by one infective particle, but it by no means follows that the pock count corresponds closely to the absolute number of virus particles deposited on the membrane.' Apparently they think that although one particle can initiate a pock every particle may not be adsorbed to a susceptible cell. Since no more delicate indicator of the presence of living virus is available we assumed that a mean of not more than two infective particles is required to produce one pock and therefore any preparation passing our inactivation test would be unlikely to contain more than 800 living particles per litre. With this number of living particles

our chance of getting five sterile samples of 2 ml. would be about 0·04. Therefore, a sample of 10 ml. might contain up to thirteen living particles, and the maximum number any of the animals might have received in one injection is still small—certainly not more than five.

In the experiments with living virus we proposed that, having determined the titre of an E.B.S. by pock counting, we should make a dilution so that 0·5 ml. would contain a calculated number of particles; of a total volume of 5·0 ml., 0·5 ml. should be injected into each of five rabbits, and a similar volume should be inoculated into five eggs, to control the number of pock-producing units actually given to the rabbits. The entire volume would therefore be distributed equally between rabbits and eggs, and no sampling error should arise due to virus being left in the tube. It was calculated that we should need a minimum of five pocks on the eggs before we could reasonably conclude that there was some living virus in the other half of the material. The probability that half the material would be free of virus when the whole material contained x infective units is $1\ (\frac{1}{2})^x$, and with 5 units this would be 0·3. In order to be reasonably certain that each sample contained an adequate number of units, it was suggested that we should work with between 20 and 30 infective units. A mean dose of 25 would ensure that each rabbit received between 16 and 36 infective units; if the pock count was 60 the dose for each animal would be between 45 and 75 units.

In the first experiment with living virus, an E.B.S. was diluted in 5% buffered sheep serum so that 0·2 ml. amounts gave a mean pock count of 25 particles in five eggs. A further 3 ml. of buffered serum was added to 2 ml. of this diluted E.B.S. to render the 0·5 ml. doses given to rabbits equivalent to the 0·2 ml. amounts inoculated into eggs. Precautions were taken to obviate proliferation of the virus in the epidermis at the point of needle entry during subcutaneous injection of this material; the needle used for drawing the fluid into the syringe was not used for the injections, a fresh needle was used for each rabbit and the site was swabbed with iodine before and after injection. The animals were inspected daily to exclude the development of vaccinial lesions.

Five rabbits received two doses of this material at an interval of 14 days. The second dose gave a mean pock count of 24. Blood samples were taken before the first dose and a fortnight after the second, immediately before the challenge. The vaccination results shown in Table 8 were equivocal. Rabbit 36 developed lesions to a dilution of the test lymph similar to those obtained in unimmunized controls. Rabbits 37 and 39 both showed definite suppression of lesions, whereas in nos. 38 and 40, although the apparent titre of the lymph was reduced, semi-confluent lesions developed up to a dilution of 1/4000. It was noted, however, that in none of these rabbits were the vaccinial lesions of the abortive type previously described. Only rabbit 37 showed significant antibody formation. Thus only one of the five showed both resistance to challenge and titre of circulating antibody comparable with those animals given irradiated virus although four out of five showed some resistance to challenge.

In a second experiment with living virus the dilutions were made by the same technique so that one group of six rabbits received two doses each of approximately

20 virus particles and another similar group two doses of a mean of 60. Precautions against epidermal lesions due to living virus and the intervals between doses and between blood samples were the same as in the previous experiment. The results of challenge and titration of circulating antibody are shown in Table 9.

Table 8. *Titration of control vaccine lymph and antibody titre on rabbits immunized with small doses of living virus*

Dilution of lymph	Rabbit number				
	36	37	38	39	40
1/1,000	c	2	sc+	3	sc+
1/2,000	c	0	sc−	1	sc−
1/4,000	sc+	0	sc−	0	sc−
1/8,000	sc+	1	3	0	2
1/16,000	sc+	0	8	0	0
1/32,000	sc−	0	2	0	0
1/64,000	3	0	0	0	1
1/128,000	4	0	0	0	0
Antibody titre	<2	8	2	2	<2

c, sc and figures have same significance as in previous tables.

Table 9. *Titration of control vaccine lymph and antibody titre on two groups of rabbits immunized respectively with doses of* 20 *and* 60 *infective particles of virus*

Dilution of lymph	Rabbits receiving doses of 20 particles						Rabbits receiving doses of 60 particles					
	59	60	61	62	63	64	65	66	67	68	69	70
1/1,000	c	c	c	c	c	c	c	c	c	c	c	c
1/2,000	c	sc+	sc+	sc	sc+	c	c	sc+	c	c	c	sc
1/4,000	sc+	sc+	sc+	4	12	c	sc−	sc+	c	sc	3	4
1/8,000	0	3	sc	2	3	sc−	3	8	c	sc−	sc−	2
1/16,000	sc−	7	sc	?	0	sc−	5	4	sc+	2	2	3
1/32,000	1	5	7	0	1	4	1	2	7	4	3	0
1/64,000	0	0	1	0	1	1	0	0	0	0	0	1
1/128,000	0	0	0	0	0	0	0	0	0	0	1	0
Type of lesion	ab	ab	n	n	ab	n	n	n	n	n	n	ab
Antibody titre	2	<2	<2	4	<2	<2	<2	<2	<2	<2	<2	16

Symbols and figures have same significance as in previous tables; n = normal.

Although there was a considerable variation in the reaction of individual rabbits to challenge, most of them showed some reduction in the reaction to the control lymph; this is particularly marked in rabbits 62, 63, 65, 66, 69 and 70. On the other hand, only three rabbits in the group receiving 20 particle doses and one in the group receiving the larger dose developed lesions of the abortive type. Antibody titrations showed that only two rabbits in the first group produced detectable antibody to a titre of 1/2 and 1/4 respectively. The only animal in the second group with detectable antibody did, however, show the relatively high titre of 1/16, and

this was the rabbit in that group which had shown the most marked suppression of vaccinial lesions. Apart from this animal, the lack of correspondence between the partial suppression of vaccinial lesions and the traces of detectable antibody in the individual rabbits suggested that any immunity produced was very slight. There was no significant difference between the responses of the groups that had received doses of 20 and 60 particles respectively.

Since it was possible that traces of living virus might be more antigenic in the presence of large amounts of inactivated material or of inert particulate matter than they were in serum, a third experiment was undertaken. Equal volumes of the following particulate suspensions were prepared: (1) E.B.S. inactivated by a five-fold dose of irradiation (Rel. Exp. 5); (ii) fine glass powder; (iii) a heat-killed 24 hr. culture of *Serratia marcescens*. Equal volumes of diluted living E.B.S., estimated from a preliminary titration to give a pock count on the final mixture of about 40 particles per 0·5 ml., were added to each of these suspensions. Groups of four rabbits were inoculated with each mixture, each rabbit receiving two doses of 0·5 ml. The spacing of the doses and the challenges were the same as in previous similar experiments. The actual number of living particles injected was controlled by pock counts; the score for each mixture was the mean of the counts on five membranes. The mean of the scores for all the mixtures prepared was 35, S.D. 14·51. The results of challenge after immunization and antibody titrations are shown in Table 10.

Table 10. *Titration of vaccine lymph on groups of rabbits immunized with a small dose of infective virus suspended in particulate matter. Virus neutralization by sera from these rabbits*

Dilution of lymph	Virus in broth				Virus with inactivated E.B.S.				Virus with glass powder				Virus with *Serratia marcescens*			
	R786	R787	R788	R789	R790	R791	R792	R793	R794	R795	R796	R799	R805	R806	R807	R808
1/1,000	c	2	1	0	c	0	0	c	0	c	6	4	3	sc+	c	6
1/2,000	c	2	6	0	sc+	2	1	c	0	c	4	3	2	8	sc	3
1/4,000	sc+	1	1	0	3	0	1	c	2	sc	6	0	3	8	0	1
1/8,000	10	0	0	0	2	0	0	sc+	0	sc−	2	0	0	3	0	0
1/16,000	12	0	0	0	1	0	0	sc+	0	6	0	0	0	5	0	0
1/32,000	6	0	0	0	0	0	0	sc−	0	2	0	0	0	1	0	0
1/64,000	5	0	0	0	0	0	0	sc−	0	2	0	0	0	0	0	0
1/128,000	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0
Type of lesion	n	ab	ab	—	n	ab	ab	n	ab	n	ab	ab	ab	ab	ab	ab
Antibody titre	<2	8	2	>64	<2	2	<2	<2	16	2	4	16	32	2	32	32

Symbols and figures have same significance as in previous tables.

It appears that the suspension of the virus in particulate matter certainly increases the antigenicity of very small doses of living virus. It is perhaps unfortunate that in this experiment the group immunized with virus suspended in broth reacted slightly more strongly than in the previous two experiments; nevertheless, those groups inoculated with the virus in particulate suspensions, particularly the glass powder and *Serratia marcescens*, reacted more strongly to the virus. It is, however, still true to say that the reactions of individual rabbits were much less uniform than in the experiments with full doses of irradiated material; all these rabbits also received a far greater dose of infective virus than could possibly have been given by accident in the experiments with irradiated material.

Confirmatory experiment

After an interval of 2 years the essential immunization experiment was repeated, starting with fresh material. In view of the ambiguous results obtained in the experiments already described with very small doses of living virus, the tests for inactivation were made even more stringent than hitherto; a fortyfold concentration of irradiated E.B.S. was inoculated in 0·2 ml. amounts into ten embryonated eggs instead of the usual tenfold inoculation in five eggs. The ten membranes were pooled, minced and passed on four more eggs. Thus the equivalent of 80 ml. of the litre of suspension used for immunization in 2 ml. doses was tested for living virus. No living virus was detected.

E.B.S. 85, with a titre of 7·07 units/ml., was exposed to four levels of irradiation: relative exposures 0·5, 1·0, 2·0 and 5·0. Samples from the three higher exposures were used for immunization in three groups of five rabbits each; relative exposure 0·5 was used for a further check point on the inactivation curve. The immunizing doses, the intervals between doses and before challenge, were the same as in the earlier experiments.

Table 11. *Titration of control vaccine lymph on rabbits immunized with* E.B.S. 85 *after three levels of irradiation*

Dilution of lymph	Relative exposure 1				Relative exposure 2					Relative exposure 5				
	R569	R571	R573	R576	R582	R584	R586	RN6	R593	R592	R574	R585	R567	R568
1/1,000	5	4	1	4	sc+	sc+	c	c	0	c	c	c	sc	c
1/2,000	1	1	1	3	sc−	sc+	sc	sc+	0	c	sc	sc	sc−	sc+
1/4,000	1	1	1	2	sc	sc	4	sc	3	sc	7	sc−	sc+	sc+
1/8,000	2	1	0	1	12	sc−	sc−	sc−	2	5	sc−	2	1	sc−
1/16,000	0	1	0	1	3	5	1	2	0	sc	4	3	sc−	4
1/32,000	0	0	0	0	0	5	0	2	0	0	1	2	5	0
1/64,000	0	0	0	0	1	1	0	3	0	1	1	1	4	0
Type of lesions	ab	ab	ab	ab	ab	ab	n	n	ab	n	n	n	n	n
Antibody titre	16	8	16	8	2	<2	<2	4	8	<2	<2	2	4	<2

Table 11 shows the results of this experiment, which provided satisfactory confirmation of previous work. Unfortunately, one rabbit of the group inoculated with virus given relative exposure 1 died between the two doses; the other four rabbits, however, showed definite resistance to subsequent vaccination, and a satisfactory titre of circulating antibody. Relative exposure 2 did not completely destroy the antigenicity of the virus since three of the five rabbits developed abortive lesions after vaccination, but there was only significant production of circulating antibody in two of these animals. Relative exposure 5 had apparently completely destroyed the antigenic activity.

In view of the negative test for living virus on the equivalent of 80 ml. of the suspension used for immunization, it can be assumed that a litre of this suspension did not contain more than about 150 infective units; the chance of getting ten sterile subsamples of 2 ml. would be 0·05.

DISCUSSION

These experiments indicate that after inactivation by exposure in a thin film to intense ultra-violet radiation for a very short time vaccinia virus is antigenic. The margin between inactivation of the virus and loss of antigenicity is, however, narrow, and careful control of the conditions of exposure and rigid tests for inactivation are necessary. Our exposure time in the Habel–Sockrider type of apparatus at relative exposure 1 was about eighteen times longer than that used by Levinson & Oppenheimer, but there are insufficient data available to determine whether the intensity of radiation was proportionately less than that used by them. From our experience, however, it appears that the duration of exposure, up to about 6 sec., is not a critical factor in vaccine preparation provided that complete inactivation of virus is achieved.

Accidential infection of the rabbits during immunization might explain the results obtained, but this is excluded by the precautions that were taken to isolate these animals during this period and by the consistency of their response in many experiments; it is extremely unlikely that accidental infection could have achieved this uniformity. The results of those experiments in which animals were deliberately given amounts of living virus far greater than could have escaped detection in our irradiated preparations exclude the possibility that the antibody response was due to this cause. Taking the experiments as a whole, there is a satisfactory correlation between the relative resistance to challenge and the rise in titre of circulating antibody in the individual animals. This agreement in the two tests for relative immunity strengthens the evidence that our preparations were antigenic.

The immunity following two spaced doses of irradiated virus, as measured by detectable circulating antibody, persists for at least 9 months in rabbits. A typical secondary immune response is obtained following a further dose of the same material after this period; this is additional evidence that the irradiated virus is antigenic. It is also satisfactory that a confirmatory experiment after a lapse of 2 years provided the expected protection to the rabbits. In this experiment tests for inactivation were more than usually stringent.

Irradiated suspensions of virus can be dried from the frozen state in 2 % peptone, and their antigenicity is unimpaired after 6 months' storage at 4° C. and after 2 months at 37° C. For experiments in human volunteers, however, it is proposed to dry the virus in gelatine to obviate the risk of sensitivity reactions to parenteral injections of peptone.

Similar immunization experiments on a few monkeys indicate that these animals, too, respond by the production of circulating antibody but the response may be more variable than that obtained in rabbits and the modification of the lesions resulting from subsequent vaccination with living virus may not be as marked as it is in rabbits. No local or general reactions, however, follow the subcutaneous injection of irradiated material into monkeys and there were no complications following subsequent vaccination. There is, therefore, no evidence of any undesirable sensitization due to the preliminary immunization. In view of the experience of Henderson & McClean (1939) that the immune response of rabbits to

intracutaneous and subcutaneous injections of living vaccinia virus is much greater than that of children and young adults, it will be interesting to observe the antibody response of man to irradiated material and to see how far the reaction to subsequent vaccination with living virus is modified. Meanwhile, the experiments on animals recorded here justify the hope that the preliminary injection of irradiated virus may provide a basal immunity which will reduce the *malaise* and the incidence of complications following Jennerian vaccination. Post-vaccinial encephalitis is exceedingly rare after re-vaccinations when there is some basal immunity to the virus. Generalized vaccinia also only develops in the absence of circulating antibody. It is therefore reasonable to hope that the provision of a basal immunity by means of inactivated virus may reduce the incidence of these complications. The danger of death following the vaccination of patients with allergic eczema is so great that vaccination of these individuals is contra-indicated even in the presence of smallpox; immunization with irradiated virus might provide some protection for these people.

SUMMARY

Vaccinia virus which has been inactivated by exposure to ultra-violet irradiation under strictly controlled conditions produces an immunity response in rabbits and monkeys. This can be measured by relative insusceptibility to challenge with living virus and by the titration of circulating antibody.

Experiments with small doses of living virus in excess of those that might have been given accidentally indicate that the immune response to the irradiated material was not due to traces of living virus that had escaped detection.

There is a logarithmic relationship between exposure to irradiation and destruction of virus. The exposure necessary to produce complete inactivation can be deduced from a knowledge of this relationship. Excessive exposure destroys the antigenicity of the preparations.

The antigenicity of irradiated virus can be preserved for at least 6 months at 4° C. and at least 2 months at 37° C. by drying from the frozen state.

Circulating antibody is detectable in rabbits for at least 22 weeks following immunization, and a single subsequent reinforcing dose is followed by a secondary immune response.

The possibility that preliminary immunization with irradiated virus may diminish the risk of complications and reduce the severity of the reaction to subsequent Jennerian vaccination with living virus is discussed. This material may provide a basal immunity in those individuals in which vaccination is contra-indicated.

It is a pleasure to acknowledge the help of Dr Richard Doll of the Statistical Research Unit of the Medical Research Council and of Dr F. O. MacCallum at the Central Public Health Laboratory, Colindale.

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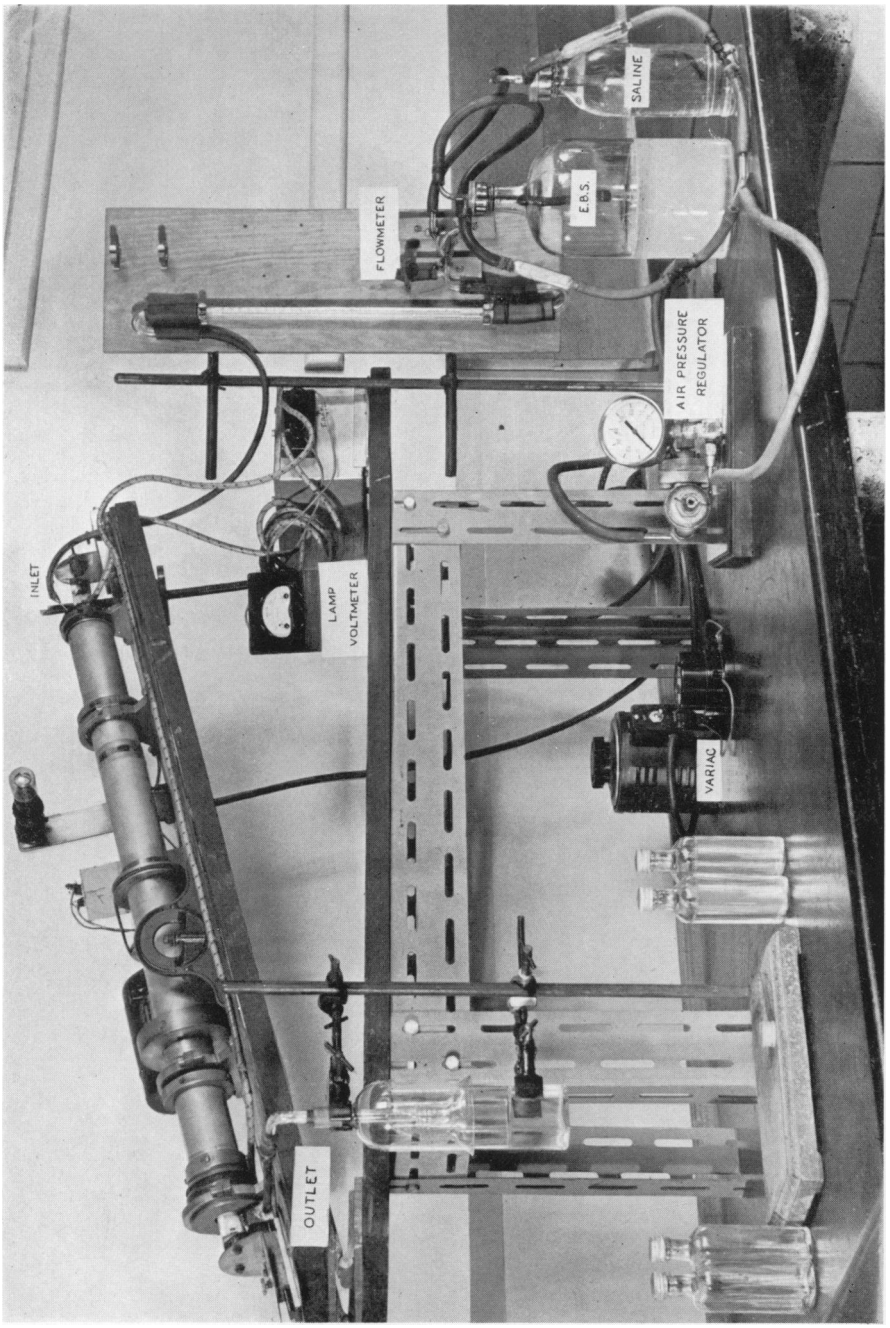
EXPLANATION OF PLATE

Plate 15. The modified Habel–Sockrider apparatus set up for the irradiation of an elementary body suspension.

(*MS. received for publication* 11. VIII. 55)


INLET
OUTLET
LAMP VOLTMETER
FLOWMETER
E.B.S.
SALINE
AIR PRESSURE REGULATOR
VARIAC

(*Facing p.* 534)