enabled this work to be done. We are indebted for material to members of the staffs of St. Bartholomew's Hospital, St. Peter's Hospital for Stone, the Metropolitan Hospital, All Saints' Hospital and the Royal Masonic Hospital.

REFERENCES.

GIROUD, A.—(1938) *Ergeb. Vitamin Hormonforschung*, **1**, 68.
JAWORSKY, M., ALMADEN, P., AND KING, C. G.—(1934) *J. biol. Chem.*, **106**, 525.
KENNAWAY, E. L., KENNAWAY, N. M., AND WARREN, F. L.—(1944) *Cancer Res.*, **4**, 245.
LOEPER, M., COTTET, J., AND LESURE, A.—(1936) *C. R. Soc. Biol., Paris*, **122**, 388.
MANCEAU, P., POLICARD, A. A., AND FERRAND, M.—(1936) *Bull Soc. Chim. Biol.*, **18**, 1369.
POLICARD, A. A., AND FERRAND, M.—(1936*a*) *C. R. Soc. Biol., Paris*, **122**, 200.—(1936*b*) *Ibid.*, **123**, 1081.
ROE, J. H., AND KUETHER, C. A.—(1943) *J. biol. Chem.*, **147**, 399.

INFECTIOUS ECTROMELIA: EXPERIMENTS ON INTERFERENCE AND IMMUNIZATION.

C. H. ANDREWES AND W. J. ELFORD.

From the National Institute for Medical Research, London, N.W. 3.

Received for publication June 19, 1947.

IN 1936, Greenwood, Hill, Topley and Wilson reported that they could immunize mice against the virus of infectious ectromelia by the following procedure: A dose was first given of infective liver filtrate fully inactivated by treatment with 0·2 per cent formalin for 10 days in the cold. A second dose was given 7 days later of virus similarly treated with formalin, but only for 3 days; this still contained living virus as judged by tests in normal mice, but produced no infection in mice previously injected with "wholly dead" vaccine. After the two doses, first of inactive, second of "attenuated" virus, mice withstood a big dose of living virus. Greenwood *et al.* made no detailed study of the mechanism of this immunizing procedure, which was used as a tool in their studies of experimental epidemiology. Similar findings are recorded for the virus of African horse sickness (Du Toit and Alexander, 1930); here also immunization against living virus can be achieved by injecting horses first with tissue suspensions wholly inactivated with 1 : 1000 formalin, later with virus incompletely killed by treatment with weaker formalin; 1 : 2000, 1 : 3000 and 1 : 4000 formalin were used for treating the virus used for the successive doses. It seemed to us unlikely that incomplete formalinization would affect virus particles uniformly, attenuating them all without killing them. Recent work on the interference phenomenon in the virus field suggested that the results might be better explained on the basis that a partly inactivated vaccine contained a mixture of live and dead virus, the dead component of the mixture interfering with the full activity of the live virus. We accordingly carried out experiments similar to those of Greenwood *et al.*, but in order to avoid the complications introduced by the presence of formaldehyde or urotropin we used

virus inactivated with ultra-violet light. We were in any case on surer ground in so doing, for virus inactivated by ultra-violet light has been shown to interfere with live virus in the case of bacterial viruses (Delbrück and Luria, 1942) and influenza (Henle and Henle, 1944).

Methods: (a) *Inoculation of mice.*

Earlier work was carried out with the Hampstead strain of ectromelia described by Marchal (1930). Later we used also a Moscow strain kindly given us by Prof. V. Soloviev. The two were antigenically alike. Mice injected intraperitoneally with either strain developed diverse pathological lesions, varying with dosage and other factors. After a heavy dose most mice died within a few days with extensive liver necrosis. Others, more resistant, or after a smaller dose, died later with general peritonitis associated with fat necrosis due to pancreatic damage. Yet others developed generalized skin-lesions involving feet, tail, mouth and nose, as described by Marchal; some of these recovered. Though we took full precautions against spread of infection from one cage to another, the Hampstead strain of virus proved to be much less infectious than was described by Marchal; even within a cage cross-infection occurred irregularly. The Moscow strain was, however, highly infectious. On inoculation into the pad the Hampstead virus produced good local lesions: fatal general infection might or might not follow. The Moscow virus, on the other hand, usually killed mice after pad-infection; with higher dilutions mice commonly died with hepatic necrosis without showing any local foot-lesion.

For virus filtrates to be used in the studies to be described, we usually infected 9 mice intranasally with potent virus, killed them after 3 or 4 days and removed the lungs. Lungs of any dead mice in the group were pooled with these, and a $2\frac{1}{2}$ per cent suspension made in buffered saline (0·85 per cent NaCl + M/60 Sörensen phosphate buffer pH 7·4). After centrifugation the suspensions were clarified either through paper-pulp and sand filters or through coarse (A.P.D. 2 μ and 1 μ) gradocol membranes and then filtered through 0·7 μ membranes. Suspensions made thus from lung material proved much clearer and easier to filter than those made from liver, and gave filtrates of higher titre. Broth, which helps virus-filtration so much, was not used in these experiments, in order to avoid adding substances which might interfere with ultra-violet inactivation.

In all experiments, unless otherwise stated, surviving mice were killed and autopsied after 21 days.

(b) *Ultra-violet light inactivation.*

The apparatus employed in these experiments for the controlled exposure of infectious fluids to ultra-violet light is shown diagrammatically in Fig. 1 (*a*) and (*b*).

The liquid flows at a known rate through a capillary tube of transparent quartz (1·5 mm. bore; 1 mm. wall), a known length of which is exposed to the light issuing from the slot in the case surrounding a ∩ type low-pressure mercury discharge lamp. The length of capillary exposed can be varied by altering the effective length of the slot by means of the sliding cover—the maximum length is 10 cm. The light emitted by this type of burner (supplied by the Thermal Syndicate, Ltd.) consists to the extent of 95 per cent of $\lambda = 2536$ Å, the intensity at 100 cm. horizontally from the lamp being $1{\cdot}2 \times 10^3$ ergs/cm.^{2}/sec. The

quartz capillary is in the same horizontal plane as the ∩ tube and situated 2·5 cm. from one arm. Rates of liquid flow have varied from 0·25 to 2·5 c.c./min., with actual exposure varying from 1·25 sec. up to 125 sec. The exposures greater than 50 sec. were attained by using a transparent quartz coil made of tubing of the same bore and wall thickness as the straight capillary.

Two variables enabled the rate of flow to be adjusted, (1) the glass capillary tube *R* of 0·5 mm. bore and length which varied from 5 to 25 cm., and (2) the value of *h*, the mean height of liquid producing flow. The glass and quartz tubes, after thorough cleaning in hot chromate-sulphuric acid mixture, were sterilized in the hot air oven and then assembled as in Fig. 1 (*b*) with sterilized

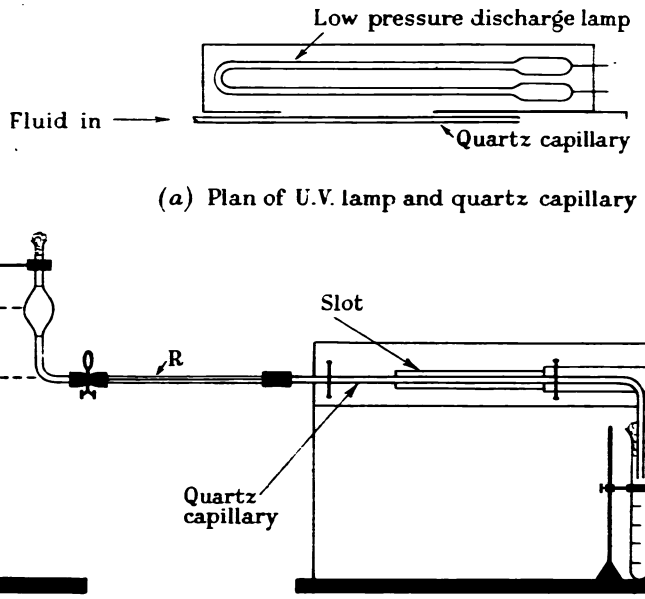

FIG. 1.—Apparatus for controlled exposure of infectious liquids to ultra-violet light.

rubber unions. In operation, it is important to stop the flow of liquid when the meniscus reaches the lower graduation of the bulb, for should it be inadvertently allowed to pass the resistance *R* the rate of flow of the final portion of liquid will be much increased and the whole experiment will be vitiated.

The discharge tube generates some heat, and the fluid passing at the lowest rates of flow became slightly warmed; starting at 20° C. the liquid in the receiver would be 25° C. Strictly, therefore, we were investigating the combined effect of ultra-violet and heat radiation.

Results: Amount of irradiation necessary to inactivate.

The top line of Table I shows that a filtrate irradiated for 2 seconds was not completely inactivated, while one irradiated for 4 seconds apparently was; also that both vaccines had good immunizing power. Two doses of 0·5 ml. were

given intraperitoneally one week apart. After another week a challenge dose of about 200 MLD was given, also intraperitoneally. The results are shown in the lower part of Table I. The filtrate from which the vaccine was made had a moderately high titre, killing 2 of 3 mice specifically when diluted 10^{-5}.

TABLE I.—*Effect of Time of Irradiation on Activity and Antigenicity.*

	Controls (unvaccinated).	UVR 1 sec.	2 sec.	4 sec.
Challenge	††††††	†††ESS	†SSSSS	SSSSSS
	††††††	..	SSSSS	†SSSSS

† = specific ectromelia death.
E = generalized ectromelia.
S = survived 3 weeks.

In a similar experiment with virus of possibly higher titre (10^{-5} killed 4/5 mice, 10^{-6} 1/5) irradiation for 4 sec. did not wholly inactivate, since 1 of 6 injected mice died. Treatment for 16 sec. was, however, effective; no mice died and virus could not be recovered on passage. In a number of later experiments 16 sec. irradiation never failed to render all filtrates non-infectious.

Immunizing power of ultra-violet-ray-treated filtrates.

As already seen in Table I, two intraperitoneal doses of vaccine protected against a heavy dose of virus given subsequently by the same route. This result was confirmed in all subsequent experiments. Even when the titre of the filtrate was as low as 10^{-4}, vaccine made by irradiation for 16 sec. was effective. The second dose of this vaccine was not necessary, a single intraperitoneal dose giving good immunity against a challenge 14 days later. Irradiation for 25, 50, or even 65 sec. still yielded an active vaccine; but after 105 and 125 sec. antigenic power was apparently lost.

Activity of these vaccines, made from the Hampstead strain of virus, was only certainly demonstrable when both vaccine and challenge-dose were given by the intraperitoneal route. Intraperitoneal vaccination failed to protect against the development of foot lesions, nor did subcutaneous vaccination prevent deaths after intraperitoneal challenge. Limitation to the intraperitoneal route for both injections was not, however, so necessary in work with the Russian strain to be described in a later paragraph.

Interference phenomenon.

In a mixture of live and inactive virus given intraperitoneally, the activity of the live virus was wholly or largely suppressed.

Table II shows the results of titrating a virus intraperitoneally using as diluent (*a*) buffered saline, (*b*) undiluted 4 sec. vaccine, (*c*) undiluted 16 sec. vaccine.

All mice of the lots surviving an injection of virus-vaccine mixture were challenged at the end of 3 weeks with a dose of virus which killed 6/6 controls: all 34 of them survived.

TABLE II.—*Interference Phenomenon (Intraperitoneal Injections).*

Virus diluted in	10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.
(*a*) Control (buffer)	†††††*	††††SS	†**SS	†††††*	††††SS
(*b*) In 4 sec. vaccine	†SSSS	†SSSSS	†ESSSS	SSSSSS	SSSSSS
(*c*) In 16 sec. vaccine	..	..	*SSSSS	†ESSSS	..

† = specific death.
E = generalized ectromelia.
S = survived 21 days.
* = non-specific death.

It will be seen that in the presence of 4 sec. or 16 sec. vaccine, a very definite sparing effect is evident against the effects of live virus.

Similar results were obtained in subsequent experiments.

Nature of the interfering agent.

All evidence suggested that the interfering agent consisted of inactivated particles of virus. A potent 16 sec. vaccine lost all interfering activity after filtration through a gradocol membrane (A.P.D. 0·2μ) which was known to retain all virus. Further, after centrifugation at 8000 r.p.m. for 1 hour in an Ecco centrifuge, the supernatant fluid was found to be devoid of interfering activity, while the deposit, resuspended in the original volume of fluid, was still active.

Treatment with ultra-violet rays for longer periods removed the interfering property roughly *pari passu* with antigenicity. After 25 and 50 sec. of irradiation activity was still present, while after 65 sec., and in one test after 50 sec., activity was lost. These results do not differ greatly from those reported earlier for the effect of ultra-violet rays on antigenic activity. Henle and Henle (1947) found that in the case of influenza viruses, antigenicity persisted longer after irradiation than did interfering activity.

Interference was only demonstrable if the filtrate before irradiation had a titre of about 10^{-4} or better. When this was so, a vaccine had a definite delaying effect on deaths of mice even when mixed with an equal volume of living undiluted virus filtrate. A reduction of mortality was seen when the proportions of live : inactive virus were 1 : 9 or more (Table II). If less vaccine was used, the effect was not demonstrable. When, for instance, we took a mixture of 16 sec. vaccine and 1 in 100 live virus, this only killed 1 of 6 mice ; but a 1 : 10 and a 1 : 100 dilution of this mixture killed, in each instance, 5 of 6 mice ; the intraperitoneal inoculation in all cases was 0·5 ml. Such a result recalls the familiar reactivation by dilution of an apparently neutral virus-antiserum mixture—the " dilution phenomenon." The mechanism may, however, be quite different in the present instance.

Some suppression of viral activity was seen when vaccine was injected intraperitoneally 18, 24, 48 or 72 hours before active virus, or along with it. When virus preceded vaccine by 18, 24, 48 or 72 hours no such suppression occurred (Table III).

TABLE III.—*Intraperitoneal Injection of Vaccine Before Virus and vice versa.*

	18 hours.	24 hours.	48 hours.	72 hours.	Mixture.
Vaccine before virus 1 : 100	††SSS	††ESSS	ESSSSS	†SSSSS	*†SSSS
Virus 1 : 100 before vaccine	††††††	†††††E	††††††	††††††	..

Virus 1 : 100 killed 6/6 mice at the beginning of the experiment and 5/6 after keeping 5 days in the cold.

Other routes of injection.

The Hampstead strain of ectromelia was irregularly lethal in our hands when given intracerebrally, subcutaneously or intravenously; we were, however, able to study the effect of virus-vaccine mixtures given intranasally or into the foot-pad. We were unable to demonstrate any consistent interfering effect when we used the intranasal route. When we injected into the foot-pad mixtures which were harmless when given intraperitoneally, local foot-lesions were produced as readily as if no inactive virus had been present. Strangely enough, however, there were no deaths in the groups receiving the mixtures (Table IV). An experiment with the Moscow strain gave a similar result.

TABLE IV.—*Interference Phenomenon (Intraplantar Injections).*

	Dilutions in buffered saline.		Dilutions in vaccine.	
	V/10^3.	V/10^4.	V/10^3.	V/10^4.
1st exp. . .	††††FF	†††FFF	FFFFFF	FFFFF0
2nd exp. . .	††††††	FFFFFF	FFFFFF	FFFFFF
	FFFFFF	000000	FFFFFF	FF0000

† = specific death.
F = local foot lesion.
0 = no lesion.

Mixture with irradiated (16 sec.) virus produced no evident effect on foot-lesions on intraplantar injection, but none of 12 mice so treated died, while 7 of 12 controls did so. When vaccine was injected into pads 24 hours before 1 : 10,000 virus, only 1 of 12 mice died, and in addition foot-lesions were less severe.

Experiments with the Moscow strain.

The necessity, with the Hampstead strain, of giving both vaccine and challenge dose by the same route, the intraperitoneal, raised the question of whether the phenomenon was one of immunity in the ordinary sense at all. The resistance induced within 24 hours was presumably a manifestation of "interference"; could not that found after a fortnight be equally due to a persistence of the same effect, rather than to true immunity? The question was not easily settled in experiments with the Hampstead strain of ectromelia, but the more invasive, more infectious Moscow virus enabled us to show that irradiated vaccine could immunize against virus introduced at another site, and hence that local interference could not by itself explain our findings.

The Moscow virus proved to be inactivated by the same dosage of ultra-violet-radiation as was effective for the other strain. Two intraperitoneal doses of 0·5 ml. 16 sec. vaccine a week apart induced some immunity against intra-plantar injection of living virus and against susceptibility to contact infection. For the last test, mice were exposed in the same cage with newly infected mice (intraperitoneal or pad) 7 days after their second vaccine dose, and left for 3 weeks.

The vaccination failed to protect completely against local foot lesions, though their severity was modified. In this and in two other experiments there was definite reduction of mortality (Table V). In another experiment all 6 vaccinated

TABLE V.—*Immunization with Moscow Strain.*

	Mice receiving 2 doses 16 sec. vaccine i.p.	Control mice.
Intraperitoneal challenge V 1 : 100	SSSSSS	††††††
Intraplantar challenge V 1 : 100	FFFFFF	††††††
	FFFFF0	††††FF
Intraplantar challenge V 1 : 1000	FFFFFF	††††††
	FFFFFF	†††FFF

† = specific death.
F = foot lesion.
0 = no lesion.

mice survived contact exposure to infected mice during 23 days; within this period 5 6 similarly exposed controls died of ectromelia. It seems then that the resistance found 14 days after vaccination probably represents true immunity.

Must effective vaccines contain living virus?

We have shown that a little live virus may be masked by an excess of inactive virus; this raises the question of whether some so-called killed vaccines may not contain masked living virus, and even of whether a little living virus may not be essential for their activity. In hopes of settling this question we inoculated mice intraperitoneally with mixtures of vaccine and live virus in the proportion of 100 : 1, 1000 : 1, and 10,000 : 1. The virus used turned out to be active only to a dilution of 10^{-3}, so the mice receiving the 10,000 : 1 mixture were not further considered. Three mice of each of the other two groups were killed after 3, 7 and 14 days, and suspensions of their livers passed intraperitoneally to more mice to test for the presence of virus. From the 100 : 1 mixture virus was recovered after 3 and 7 but not 14 days; lung suspensions, tested after 14 days, also proved negative. No virus was obtained from livers of the 1000 : 1 group after 3, 7 or 14 days. It is possible, therefore, for the live virus to be masked so thoroughly that serial passage fails to reveal it; but in the experiment recorded this was so only when minimal quantities of live virus were used.

Another line of approach indicated that effective vaccines need not contain living virus. As already described, we could not demonstrate any local interfering effect when we gave mixtures of vaccines and minimal amounts of virus

into the foot-pad. We did obtain vaccines which effectively immunized but yet produced no lesions on pad-inoculation. We have therefore no reasons for doubting that ectromelia virus completely inactivated by ultra-violet rays may still be able to immunize.

DISCUSSION.

The experiments described indicate that ectromelia virus inactivated by ultra-violet radiation will immunize mice against living virus, and that this immunizing action need not depend on the presence in the vaccine of any living virus. The latter conclusion was not easily reached, for inactivated virus was found capable of interfering with or masking the activity of considerable quantities of live virus mixed with it. Though the conclusion as regards ectromelia is that a little living virus in a vaccine is not necessary for its activity, our results call in question the validity of some published work on durable immunity after killed vaccine injections. Casals and Olitsky (1945), for instance, found lifelong immunity in mice after injection of irradiated virus of Russian spring-summer encephalitis. Before such a result is accepted, one would like to know whether the possibility has been excluded that a little living virus has been masked by an excess of inactivated virus. Again, when inactivated vaccines are found to be unexpectedly labile, the possibility should be considered that the lability may be due to the dying off of a small but necessary fraction of masked living virus. Though we have not carried out parallel experiments with formolized virus, our results could provide a rational basis for the vaccination methods used by Greenwood *et al.* (1936) in ectromelia and by Du Toit and Alexander (1930) in horse sickness. Experiments now in progress show that living vaccinia virus can "interfere with" the lethal results of ectromelia infection in mice. It remains to be shown whether the same fundamental mechanism is necessarily concerned in all examples of virus "interference."

SUMMARY.

Ectromelia virus inactivated by ultra-violet irradiation can interfere with or mask the activity of living virus mixed with it. It is not necessary to conclude that irradiated ectromelia vaccines only immunize because of a masked living virus component. The findings do, however, call for examination of the possibility that masked living virus may be present in other inactivated virus vaccines and may even play a part in their efficacy.

REFERENCES.

CASALS, J., AND OLITSKY, P. K.—(1945) *J. exp. Med.*, **82**, 431.

DELBRÜCK, M., AND LURIA, S. E.—(1942) *Arch. Biochem.*, **1**, 111.

DU TOIT, P. J., AND ALEXANDER, R. A.—(1930) Report of the Director, Onderstepoort, p. 85.

GREENWOOD, M., HILL, A. B., TOPLEY, W. W. C., AND WILSON, J.—(1936) 'Experimental Epidemiology.' *Spec. Rep. Ser. med. Res. Coun., Lond.*, No. 209, p. 90.

HENLE, W., AND HENLE, G.—(1944) *Amer. J. med. Sci.*, **207**, 705, 717.—(1947) *J. exp. Med.*, **85**, 347.

MARCHAL, J.—(1930) *J. Path. Bact.*, **33**, 713.