VACCINIA AND ECTROMELIA IN THE MOUSE.

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THE virus of infectious ectromelia can be inactivated with ultra-violet radiation; such inactive virus can evoke immunity, and can suppress the lethal effects of living virus inoculated intraperitoneally into mice at the same time. Another example of the "interference phenomenon" is thus in evidence (Andrewes and Elford, 1947). Burnet and Boake's (1946) finding of a close relationship between the viruses of vaccinia and ectromelia prompted us to investigate the properties of irradiated vaccinia virus and the ability of vaccinia to interfere with the effects of ectromelia in mice.

MATERIALS AND METHODS.

The strain of ectromelia we used was the Moscow strain obtained from Professor V. D. Soloviev, and described in our earlier paper. The vaccinia virus was propagated in rabbit testes or by rubbing into the shaved skin of a rabbit. In the latter case the virus was concentrated and purified by differential centrifugation by our colleague Dr. A. S. McFarlane. Inactivation by ultra-violet radiation was carried out as described earlier (Andrewes and Elford, 1947).

Immunization with irradiated vaccinia.

Ectromelia inactivated by ultra-violet radiation proved capable of provoking definite immunity in mice. Vaccinia, now shown to be closely related to ectromelia, is, however, generally believed to engender only a low grade immunity when inactivated by any means.

A washed virus-suspension produced lesions on intradermal inoculation of 0·1 c.c. into a rabbit's skin when diluted 10^{-8}. This was first diluted 1 : 50 in buffered saline (M/60 phosphate pH 7·4). Irradiation for 4″ under the conditions used earlier for ectromelia sufficed to inactivate it, as shown by intradermal tests on rabbits; treatment for 1·7″ did not inactivate. The 4″ vaccine was then given intraperitoneally to mice in 0·5 ml. doses: the mice were challenged with living ectromelia (1 in 1000 dilution) intraperitoneally as shown in Table I.

TABLE I.—*Immunizing Value of Irradiated Vaccinia.*

Vaccination course.	Two doses a week apart. Challenge 1 week later.	One dose. Challenge 1 week later.	One dose. Challenge 2 weeks later.	Unvaccinated controls.
Results of challenge.	SSSSSS	+++++S	+++++++	+++++++

\+ = death from ectromelia.
S = survived 3 weeks.

This result suggested that irradiated vaccinia had good immunizing value provided that two doses were given. Unexpectedly we found no references in the literature to tests of irradiated vaccinia. We therefore carried out tests of the immunizing potency of this vaccine in rabbits and guinea-pigs. In a first experiment 2 rabbits received 5 c.c. doses of U.V.R. vaccine intraperitoneally 11 days apart, and were challenged intradermally with graded doses of live virus 8 days after the second dose ; in a second trial the two doses of 5 c.c. were given 6 days apart and the challenge was 6 days later. There was no conclusive evidence of immunization. Similar experiments were carried out on 5 guinea-pigs which received (first experiment) two doses of 3 c.c. intraperitoneally a week apart, or (second experiment) two doses of 5 c.c. subcutaneously a week apart. Challenge was made by injecting dilutions of vaccinia into the foot-pads and shaved skin of the belly. The results were irregular, but indicated some immunity against 1 to 10 minimal infective skin doses, i.e. about as much as was found by Bland (1932) in his trials of formolized vaccinia in guinea-pigs. The efficacy of the U.V.R. vaccinia vaccine against ectromelia in mice is, therefore, to be attributed to other causes than an intrinsic superiority of vaccine inactivated in that way.

Immunization with living vaccinia.

We readily confirmed the finding of Burnet and Boake (1946) that living vaccinia will protect mice against ectromelia. Complete protection against 100 lethal intraperitoneal doses of ectromelia was afforded by a single dose of concentrated vaccinia given intraperitoneally 14 days beforehand. When challenge with ectromelia was made into the foot-pad, deaths were prevented but local lesions were not. An immunizing effect of live vaccinia given intraperitoneally was apparent after 24 hours: on intraperitoneal challenge with ectromelia after that time, 5/6 mice survived ; the only death was not due to ectromelia. This result raised the question as to whether we were dealing with immunization or interference, and we felt, as a result of our earlier work with ectromelia vaccines, that the problem was more likely to be clarified by study of the results of inoculations into foot-pads.

Vaccinia in the mouse's foot-pad.

Intraplantar injection into mice of vaccinia virus, either as washed concentrated dermal virus or suspension of infective testis, produced the following changes: Swelling of the affected foot appeared in 48 to 72 hours, and reached a maximum about the fifth day ; it never became nearly as pronounced as with ectromelia. Superficial necrosis and desquamation of epithelium were apparent by the sixth day ; during the second week, as the activity of the inflammation rapidly subsided, deformation of the foot was often apparent. Adjacent toes frequently became fused together, or toes stuck out at odd angles owing presumably to fibrotic contraction in deeper structures. Occasionally necrosis of a whole toe occurred, but the characteristic gangrene of ectromelia was absent. The whole process had passed its peak of activity at a time when lesions in mice inoculated at the same time with ectromelia were only beginning to appear.

A few attempts were made to transmit vaccinia serially in mice, removing pads, grinding them up, inoculating to pads of fresh mice ; 2-day and 4-day

lesions were used, but in each case the lesions became rapidly less on passage. Attempts at adaptation were not, however, carried out intensively.

Interference tests in the foot-pad.

When a mixture was made of equal parts of undiluted living vaccinia and 1 in 1000 ectromelia, and this was injected into the foot-pads of 6 mice, typical ectromelia lesions developed, but only one mouse died, and that not from ectromelia. Of 6 controls receiving the same amount of ectromelia only, 5 died of ectromelia, and the sixth showed extensive lesions of ectromelia (ascites and fat necrosis) when killed 3 weeks later. When the vaccinia was injected into a foot-pad 24 hours ahead of 1:1000 ectromelia, again typical ectromelia lesions appeared, but none of 6 mice died. These results closely resembled those described earlier (Andrewes and Elford, 1947), when U.V.R.-inactivated ectromelia interfered with the action of living virus mixed with it and injected into the foot-pad. In both instances local lesions were relatively unaffected, but deaths were prevented. The next experiments suggested caution in concluding that a local interfering action could explain the two types of experiment equally.

Results of injecting vaccinia and ectromelia into opposite feet.

When the two living viruses were injected at the same time into opposite foot-pads, the results were the same as when they were mixed and injected together (see Table II).

TABLE II.—*Injection of Vaccinia and Ectromelia into Opposite Feet.*

Control (ectromelia alone).	Undiluted vaccinia into opposite foot.	1 in 10 vaccinia into opposite foot.
+++++++	FFFFFF	+++FFF
+---+F*	FFFFFF	FFFFFF

\+ = specific ectromelia death.
* = non-specific death.
F = ectromelia foot lesion and survived for 3 weeks.

Two other experiments gave similar results. In these tests a local "interfering" effect in the foot could not explain the life-sparing effect. It could alternatively be suggested that vaccinia, of which the lesions came up some days before those of ectromelia, might be very rapidly inducing an immunity which was effective in time to halt the march of the ectromelia. That this is the explanation is suggested by two facts. In mice receiving vaccinia in one foot and ectromelia in the other, the development of the ectromelia lesions was precocious as compared with those in mice receiving ectromelia only; they were evident after 5 days as against 6 days in the controls, and their evolution seemed a little more rapid. Such a result suggests that the vaccinia infection had altered the mouse's reactivity to the antigenically related ectromelia. Inoculation of herpes virus into the opposite pad failed to influence the outcome of ectromelia. In the next experiment, twelve mice were inoculated into the foot-pad with vaccinia and twelve with 1 in 1000 ectromelia. Pairs of mice were killed daily, and heart's blood and liver suspensions were tested for ectromelia by plantar inoculation into mice and for vaccinia by intradermal tests in

rabbits. Ectromelia was recovered from the heart-blood and liver after 5 days but not earlier, whereas vaccinia had reached the heart-blood after 3 days; it was not recovered from liver. Smith (1929) has shown that in rabbits anti-vaccinial antibodies can be demonstrated as early as the 2nd day after injection, and some experiments by one of us (Andrewes, 1931) suggested that active immunity to vaccinia might be present within 24 hours of injection of inoculation of virus and antiserum into opposite sides of a rabbit. In contrast to the results with living vaccinia, U.V.-inactivated ectromelia vaccine inoculated into one pad had no influence on deaths resulting from injection of living ectromelia in the other pad. In most ectromelia mice surviving as a result of concomitant vaccinia infection, macroscopic lesions of liver, spleen and other viscera were absent.

Effect of delaying vaccinia injection.

Five groups of 6 mice were injected into the left foot with ectromelia diluted 10^{-4}. One group received vaccinia into the right foot at the same time; to the others the vaccinia was given similarly after 1, 2, 3 and 4 days respectively. Table III shows the result.

TABLE III.—*Effect of Delaying Vaccinia Injection.*

	Vaccinia.			
Same day.	1 day later.	2 days later.	3 days later.	4 days later.
FFF	FFF	+++	+++	−+−
FFF	FFF	GGG	+++	−+−

\+ = specific ectromelia death.
G = generalized ectromelia skin lesions, but survived 2 weeks.
F = ectromelia foot lesion but survived 2 weeks.

Clearly, delay of 24 hours in injecting vaccinia still permits it to save the lives of the mice, but at 48 hours the vaccinia is almost, and at 72 hours quite ineffective. A probable analogy may be drawn between this experiment and the results of vaccinating human beings shortly after exposure to smallpox.

Histological studies.

The lesions in ectromelia have been described in detail by Marchal (1930), by Greenwood, Hill, Topley and Wilson (1936), and recently, in a study of the natural transmission of the virus, Fenner (1947) has confirmed the widespread distribution of inclusion bodies. Since our primary object has been to determine whether protection by vaccinia is accompanied by any alteration in the character of the histological lesions, studies have, in the main, been confined to examination of the foot-pads and those organs which regularly present pathological changes—the spleen and the liver. The kidney in ectromelia occasionally shows naked-eye and microscopic abnormalities (Marchal, 1930; Fenner, 1947), so this organ was also included in the tissues regularly examined. We also studied the changes in the foot-pads, spleen, liver and kidney after vaccinial inoculation.

Ectromelia lesions.

The development and morphology of the inclusion bodies were fully described by Marchal, and it is only necessary to describe briefly the tissue changes in the

foot-pads. These do not become evident until the 6th day, the earliest sign being the development of localized foci of thickened squamous epithelium, brought about by enlargement and vacuolation of the cells of the *rete Malpighii*. Following the development of inclusion bodies, the nuclei become pyknotic, the cytoplasm disappears, and fluid-containing spaces are formed which coalesce with one another and rupture either into the dermis or externally. Oedema develops in the subcutaneous tissue around the 6th–8th day, and spreads outwards into the dermis and inwards, involving muscle and periosteum. Ulceration of the surface is rapidly followed by extensive infiltration of all the foot-pad structures by polymorphs; muscles, walls of arteries, tendons, joints and bone marrow are all involved. The arteritis is frequently followed by actual thrombosis, and gangrene of the extremities results, the extent depending upon the degree of vascular obstruction. Massive invasion by micro-organisms also takes place, and inclusion bodies are as a rule no longer demonstrable after this is established. The occurrence of liver and spleen necrosis is well known, and only a brief description is necessary. In the liver the process first affects small groups of cells within the lobules, the patches increasing in size and finally coalescing. In the spleen, necrosis is preceded by a tremendous hyperplasia of the primitive cells of the secondary nodules of the Malpighian bodies, formation of mature small lymphocytes being in abeyance. As in the liver, cell death occurs at first in small foci and quickly involves larger areas. As Fenner (1947) has pointed out, gross changes in the kidney occur irregularly, and in our series only one example has been obtained in which the renal architecture was markedly altered, and this was complicated by extensive bacterial invasion of the organ. A generalized capillary dilatation in the glomeruli was noted throughout, however, in the series, accompanied by increased prominence of the endothelial cells and occasional small foci of endothelial proliferation with mitoses. Small foci of round-cell infiltration in the cortex were sometimes found.

Vaccinial lesions.

In contrast to ectromelia, reaction to injections of vaccinia in the foot-pads occurs very quickly. Within 24 hours polymorphs have accumulated in considerable numbers in the subcutaneous tissue and between the muscle fibres, and numerous mitoses are seen in the germinal layers of the squamous epithelium, so that in 48 hours it has increased in thickness by several cell layers. Between the 3rd and 4th day, inclusion bodies of the nature of Guarnieri bodies appear in the *rete Malpighii*, the cells of which become increasingly vacuolated. Simultaneously a remarkable development of granules in the stratum granulosum takes place and hyperkeratinization is also apparent. Soon the nuclei of the affected patch become pyknotic, the cytoplasm disintegrates and the patches of degeneration are invaded by polymorphs. Ulceration quickly follows. Inclusion bodies are much less numerous than in ectromelia, and are confined to covering epithelium and hair follicles and are not found in the accessory skin glands as in ectromelia. The cellular reaction in the tissues of the foot-pad increases in intensity and spreads widely, but the walls of arteries never become affected. From the third day onwards fibrocytes enlarge, and soon a generalized but not uniform increase of connective-tissue cells is evident, and the subsequent formation of collagen is responsible for the fibrotic contraction and deformity of the foot structures seen at a later stage. Around the 12th day healing begins to take

place, and epithelium grows over the ulcerated areas. Secondary bacterial invasion is never so extensive as in ectromelia, healing is accompanied by fibrosis, and is not preceded by gangrene of the extremities as in ectromelia. No lesions have been found in the internal organs in the series examined.

Lesions of ectromelia modified by vaccinia.

Inoculation of vaccinia in the opposite foot-pad in no way alters the pathological changes due to ectromelia at the site of injection, although, as already stated, the process begins slightly earlier. One observes, however, a striking effect on the incidence of splenic and hepatic lesions, splenic necrosis being consistently absent, and liver necrosis absent or minimal. In the spleen, the demarcation between red and white pulp is almost obliterated by the hyperplasia of the lymphocyte precursors in the Malpighian bodies, but in contrast with the process when ectromelia alone is given, no necrosis takes place. In the liver, necrosis is generally absent; only occasionally the remains of a few necrotic cells are seen, although small accumulations of mononuclear cells in portal tracts and parenchyma are quite frequent, but as such foci are common in uninoculated animals, their significance is doubtful. In the kidney, dilatation of glomerular capillaries is quite striking, but is not accompanied by any increased cellularity.

DISCUSSION.

The experiments first described show that vaccinia inactivated with U.V. radiation can act as an immunizing agent, but suggest that it is not likely to be much more effective than other killed vaccinia vaccines.

Other experiments show that vaccinia can interfere with the lethal action of ectromelia injected at the same time. One cannot conclude, however, that this is a phenomenon altogether comparable with other instances of the "interference phenomenon" now so familiar amongst viruses. It is in fact very difficult to decide whether to interpret the results as due to interference or immunity. On the one hand one may visualize the pre-emption of cells by vaccinia, which thus inhibits the access or multiplication of the ectromelia; on the other hand, the more rapidly multiplying vaccinia may quickly engender an immunity against the slower ectromelia. Since vaccinia virus and its antibodies are likely to be well distributed in the body within a few days of injection, we have failed to devise a crucial experiment to decide between these alternatives. Nevertheless the facts presented incline us to believe that orthodox immunity rather than interference explains the results.

SUMMARY.

Vaccinia virus inactivated by ultra-violet irradiation will immunize mice against ectromelia. Such vaccines protect poorly against vaccinia in rabbits and guinea-pigs.

Vaccinia virus inoculated in a mixture with ectromelia into a mouse's foot-pad will interfere with the lethal outcome of the ectromelia; so, too, will vaccinia injected into the pad opposite to that receiving ectromelia. This sparing effect is possibly analogous to other examples of the "interference phenomenon," but can with equal or greater probability be explained as a result of immunity

rapidly induced by the vaccinia virus. The effect is still apparent when the vaccinia injection is delayed until 24 hours after the ectromelia.

Comparative study of the histological changes induced in the mouse's foot by ectromelia and vaccinia respectively supports other evidence that the vaccinial lesion evolves much the more rapidly.

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THE PREPARATION OF PURIFIED AND CONCENTRATED DIPHTHERIA TOXOID FROM A SEMI-SYNTHETIC MEDIUM.

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PART A.—TOXIN PRODUCTION.

MUELLER, in 1939, published a formula for the preparation of a semi-synthetic medium for the production of diphtheria toxin. In this medium he obtained a toxin titre of Lf 62 equal to 23 Lf per mg. of medium nitrogen. He stated that the optimal concentration of NaCl was about 0·5 per cent, and later Mueller and Johnson (1941) described methods for reducing the salt content of the casein hydrolysate. Using these methods Mueller and Miller (1941) obtained the very high toxin titre of Lf 100, equal to 37 Lf per mg. medium nitrogen.

For some time Mueller's first medium was used in these laboratories with consistent results. The only variation employed was to use a slightly lower concentration of casein nitrogen, namely 0·2–0·22 per cent. A toxin titre of Lf 62 was regularly obtained, but by further modification much higher yields of Lf per mg. of medium nitrogen have been achieved.

Experimental.

Unless otherwise stated *C. diphtheriae* P.W.8, strain "Toronto" was used, and cultures incubated at 34° C. for 7 days.

One of the earliest experiments carried out with Mueller's medium (1939) was to determine the limiting factors for growth. One factor proved to be the