PRELIMINARY STUDIES ON THE DEVELOPMENT OF NEOPLASIA IN THE SKIN OF MICE PAINTED WITH METHYLCHOLANTHRENE AND INJECTED WITH CORTISONE AND VACCINE VIRUS*

By F. Duran-Reynals

Department of Microbiology, Yale University School of Medicine, New Haven, Conn.

The experiments to be described here are part of a general study carried out in our laboratory with the object of discovering whether ordinary viruses (that is, viruses that, under ordinary circumstances, induce immediate, more or less inflammatory lesions that end in necrosis) can start or cause neoplastic and malignant lesions under other circumstances. The concepts upon which this line of research is based can be discussed here only in a broad, general way, and may be summarized as follows:

(1) Concerning such factors as composition, morphology, antigenicity, and epidemiology, one finds the same properties, in the same degree of variability, in viruses that induce neoplasia and cancer (in such animals as birds and mice) as in ordinary viruses. Even at the risk of expressing a platitude, the basic difference between both groups of viruses is that the gross, clinical lesions, *as usually perceived by the observer*, result either in cell stimulation or in cell destruction. This difference reduces itself to various degrees of compatibility between the virus and the cell, ranging from (a) temporary cell stimulation, as in pox viruses, to (b) longer-lasting cell stimulation, as in rabbit fibroma and (c) indefinite cell stimulation, as in sheep pulmonary adenomatosis or avian tumors.

(2) The cell-stimulating or cell-destroying and inflammatory effects *of the same virus* are conditioned, in whole or in degree, by (a) the variation of the virus, as in different strains of pox and fibroma viruses; (b) the age of the host, as in sheep pox and avian tumors; and (c) the phase of the disease; that is, the stimulation of the cell preceding its necrosis, as in pox viruses, or following it, as in sheep pulmonary adenomatosis and in influenza.

The problem of the compatibility of the virus and the cell, however, reduces itself ultimately to the common and fundamental factor[1,2] of the degree of severity of the infection relative to the degree of virulence of the virus. Generally speaking, acute inflammation and cell necrosis occur in the highly susceptible host attacked by a highly virulent virus whereas, if a balance of power is established, either by a weakening of the infectious agent or by a critical degree of strengthening of the host defenses, cell proliferation will ensue.

* The part of this study dealing with the role of the ground substance of the mesenchyme in natural resistance to infection was supported by Research Grant USPHS A-561 (C8) from the National Institute of Arthritis and Metabolic Diseases, Public Health Service, Bethesda, Md. The remainder of the study was supported by grants from the Jane Coffin Childs Memorial Fund for Medical Research, New Haven, Conn.; the National Cancer Institute, Public Health Service, Bethesda, Md.; and The American Cancer Society, New York, N. Y.

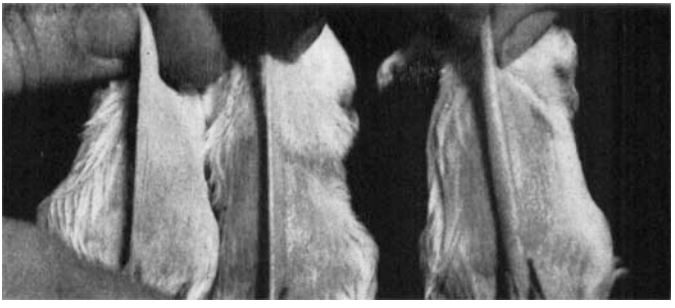

FIGURE 1. Effect of hormone treatment on the dermal ground substance of female Swiss mice. The mouse at the right, showing thickening of the skin, was treated with 100 γ of estradiol benzoate; that of the middle, showing thinning of the skin, was treated with 5 mg. of cortisone; and that of the left was an untreated control.

Pox viruses were chosen for our work because of the broad host range of the virus in the case of vaccinia, and because the duality of effects on cells, dependent on most or all of the factors listed above, is especially manifest with these viruses.

Elsewhere[3] my associates and I have reported results with fowl pox virus comparable in several ways to those to be described here. While studies on this virus have been continued, and will be reported in the future, the model system of vaccinia in mice has proved far more convenient for experimentation.

The present experiments indicate that infection by vaccinia [or, more cautiously, that changes occurring in the lesions induced by this virus in mice, prepared with a special combination of methylcholanthrene (MC) and cortisone] result in the *start* of lasting neoplasia and malignancy. We are not concerned at present with the *perpetuation* of the neoplasia as a direct result of the virus infection, although such a possibility will be discussed briefly.

The experiments evolved from our laboratory studies dealing with the general problem of the role played by the mesenchyme and its ground substance in natural resistance against infection and cancer.[4-6] Since it is known that some hormonal effects result in pronounced changes of this ground substance, we looked for a model system in which both an increase and a decrease of the ground substance could be induced easily and regularly in the same animal. Such a system was found in young noninbred female Swiss mice.

When these animals were injected subcutaneously with 100 γ of estradiol benzoate (EB), there was a rapid and marked production of dermal ground substance, selectively occurring in the flanks and the back of the mouse. The phenomenon was grossly manifested by a thickening of the skin, sometimes detectable 1 day after inoculation, that attained its maximum at the fourth or fifth day, and persisted for about 2 weeks (FIGURE 1). When stained with toluidine blue, the thickened skin gave a strong metachromatic reaction, and it yielded extremely viscid extracts; both the metachromasia and the viscidity disappeared promptly upon the addition of hyaluronidase.

On the other hand, opposite phenomena were induced by cortisone. When this hormone was injected subcutaneously into normal mice in 5 successive daily doses of 1 mg. each, a thinning of the skin of the flanks and the back was rapidly induced (FIGURE 1), and the metachromatic material, which normally exists in appreciable amounts in these regions, disappeared entirely. The same phenomena were observed when cortisone was injected into mice in which large amounts of ground substance had been induced by previous EB treatment.

As summarized elsewhere,[6] several studies have been carried out in Swiss mice thus treated in order to learn about the physiology of the ground substance (as a problem of intrinsic interest) and about the behavior of several infectious agents (and also cancer cells) when injected intradermally in different regions of the skin, that is, in the ground substance variously altered by previous treatment with hormones.

One of the infectious agents was vaccinia, a very virulent strain of Levaditi neurovirus kept by passages through rabbit testes. Normal female Swiss mice, the noninbred strain from Carworth Farms, New City, N. Y., proved to be quite resistant to this virus. An intradermal inoculation of 0.1 cc. of a 1:10 extract of the infected rabbit-testicle tissue generally would induce, 5 or 6 days after inoculation, lesions measuring only a few mm. in diameter. This natural resistance against the virus was further increased by treatment with a single dose of 100 γ of EB; when the virus was injected intradermally 5 days after the hormone it induced either no gross lesions or lesions measuring 1 mm. or less in diameter. Cortisone, however, had an entirely opposite effect; when the virus was injected intradermally between the third and fourth daily injection, at a total dose of 5 mg. of the hormone, it regularly induced, by the fifth to the sixth day, severe lesions that often measured 1 cm. or more in diameter. These lesions healed in 2 to 3 weeks, although leaving large and conspicuous scars that persisted for a considerable time.

These pronounced and opposite effects which estrone and cortisone, respectively, produced on the *immediate*, acute vaccinial infection in a host so susceptible to naturally occurring and experimentally induced cancer, prompted us to study again the possibility that, as a *late* effect of the virus infection, neoplasia may develop in specially prepared hosts.* For this purpose vaccine virus was injected into mice having received previously a number of paintings of MC, alone and in combination with either EB or cortisone, to test whether neoplasia would evolve in a way different from that induced by the MC and/or the hormones, in the absence of virus infection.

Obviously, as in past experiments with fowl pox in chickens and with vaccinia in rabbits, such planning of the experiments was based on another tentative assumption, or rather on a further elaboration of our main hypothesis; that is, that carcinogens would be instrumental in the hypothetical induc-

* In the past, the same problem, was extensively studied in rabbits prepared by skin paintings with MC and later injected intravenously and intradermally with vaccinia. As a general conclusion, it can be stated that the virus infection did not appreciably modify the usual events ending in dermal carcinogenesis.

tion of neoplasia by "ordinary" viruses, as they are known to be in some cases of induction of cancer by "neoplastic" viruses—as is the case of MC in the papilloma-carcinoma sequence in the rabbit, and in the case of estrone in the breast cancer of mice.

Accordingly, the Swiss mice were painted, on both flanks, 10 times in 12 days, with a 1 per cent solution of MC in benzol; 3 days after the last painting they were injected, some with 100 γ of EB in a single dose, and some with 5 mg. of cortisone in 5 daily doses. Vaccine virus—0.1 cc. of a 1:10 suspension of infected rabbit testicle—was injected intradermally on the fourth day after the administration of EB and between the third and fourth injection of cortisone. The virus was also injected into mice prepared only with EB, cortisone, and MC, respectively; into normal mice; and into other groups of mice painted with benzol, with and without hormone treatment. Other control groups included mice having received the same treatment, as described above, but not injected with the virus.

In this experiment, treatment with MC did not change (at least in any pronounced degree) the course of the dermal virus infection, either in the mice not treated with hormones that showed the same natural resistance to the infection (FIGURE 2a); in the mice treated with estrone, that showed the same increase of the natural resistance (FIGURE 2b); or in those treated with cortisone, that showed the same enhancement of the infection (FIGURE 2c). Neither did the treatment with MC modify essentially the histological character of these lesions, namely, temporary epithelial stimulation followed by inflammation and necrosis. Benzol treatment likewise had no effect on the virus infection.

The presence of the virus in the lesions from all the groups of mice was checked, in one experiment, by titrating extracts of the lesions in the rabbit skin at the fourth, seventh, eleventh, and eighteenth day after infection. The persistence and the titer of the virus appeared to agree with the persistence and severity of the lesions observed in the differently prepared hosts. Thus, the results were as follows: in the mice treated with MC and EB the virus persisted until the fourth day with a titer of 10^{-2}; in the mice prepared with MC alone the virus persisted until the seventh day with a titer of 10^{-3}; and in the mice treated with MC and cortisone the virus persisted until at least the eleventh day with a titer* of 10^{-6}. These preliminary results, therefore, indicate that, in the skin of mice treated with MC and cortisone, the virus finds a better ground for its multiplication than it does in the mice treated otherwise.

In view of these findings, and also for reasons to be given later, we attempted to maintain the virus by skin passages not only through normal mice, but also through differently treated mice. At the present stage of the experiments, it can be said only that, in the mice treated with both MC and cortisone, characteristic skin lesions have been induced regularly in eight successive passages. These lesions are less inflammatory and more prolifera-

* The data on the virus titer may be subject to variation depending on whether only the strict necrotic lesion or larger pieces of skin are extracted. This point is now being studied.

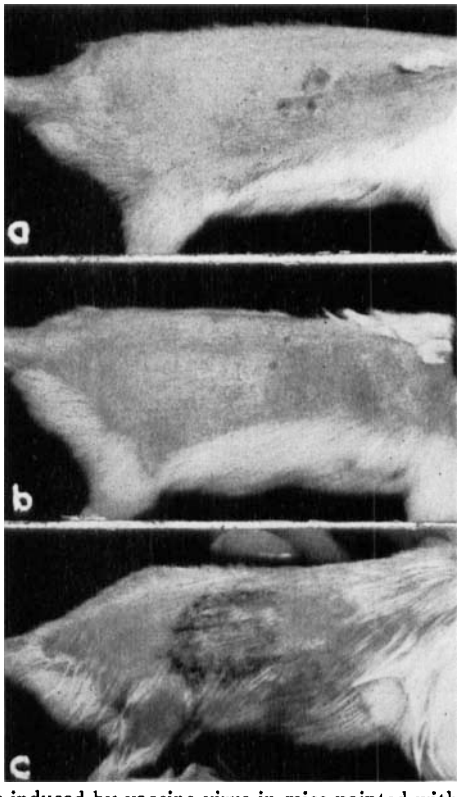

FIGURE 2. Lesions induced by vaccine virus in mice painted with methylcholanthrene and then injected with estradiol benzoate (2b), cortisone (2c), or not injected (2a). The results are 6 days after intradermal injection of the virus.

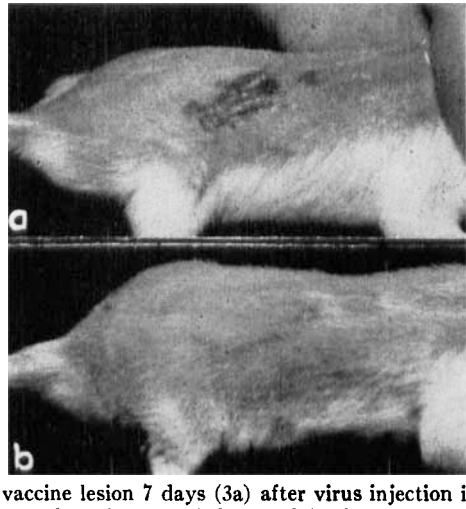

FIGURE 3. Acute vaccine lesion 7 days (3a) after virus injection in a mouse prepared by methylcholanthrene and cortisone, and the scar left after 26 days (3b).

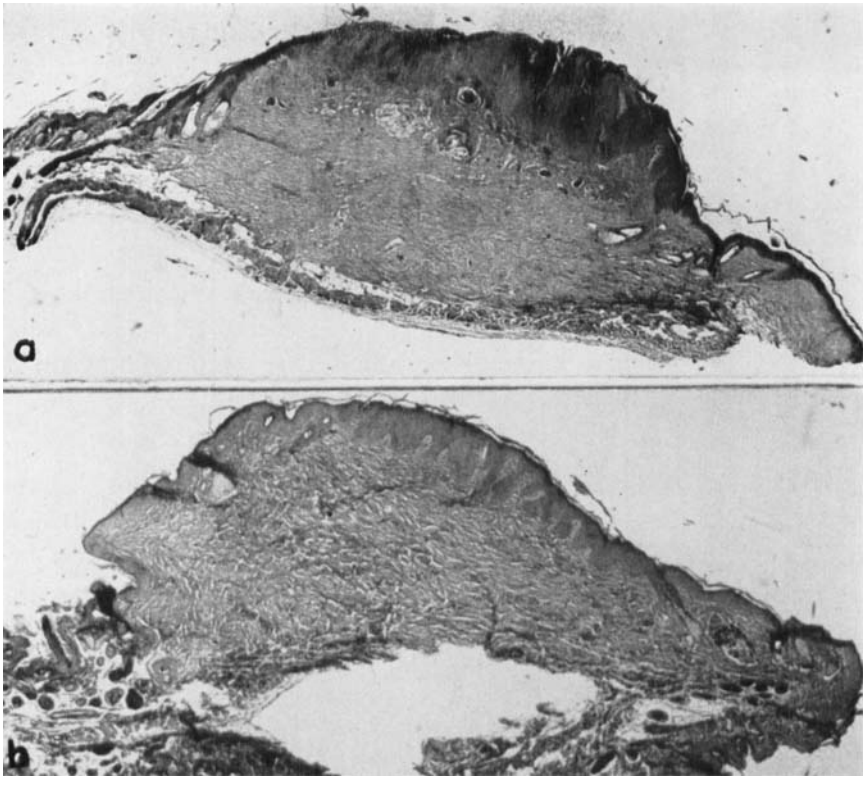

FIGURE 4. Benign neoplastic lesions in the site injected with vaccine virus in mice prepared with methylcholanthrene and cortisone. The lesion in FIGURE 4a (×19) is 52 days old from the day of the virus injection; that of FIGURE 4b (×80) is 94 days old.

tive than those in any other circumstances, but in their evolution and in their gross and microscopic features they seem to answer the essential requirements demanded by vaccinia infection; extracts of these mouse lesions injected in the skin of rabbits reproduce an infection seemingly identical to that currently induced by the rabbit-passage virus. The virus can be maintained also, but apparently less successfully, in passages through mice treated with MC alone or with cortisone alone. On the other hand, the virus cannot be maintained (or can be maintained only for a short number of passages) through untreated mice.

No further conclusions can be drawn from this work until more experiments —now under way—provide an answer to questions regarding such points as the nature of the agent maintained in the passages; the changes in infectivity of the agent for rabbits and the changes in the inflammatory and cell-stimulating power for mice with advancing mouse passages; and the dependence of the agent on treatment of the mice with both MC and cortisone. These points may be of great importance in trying to understand the phenomena I shall now describe.

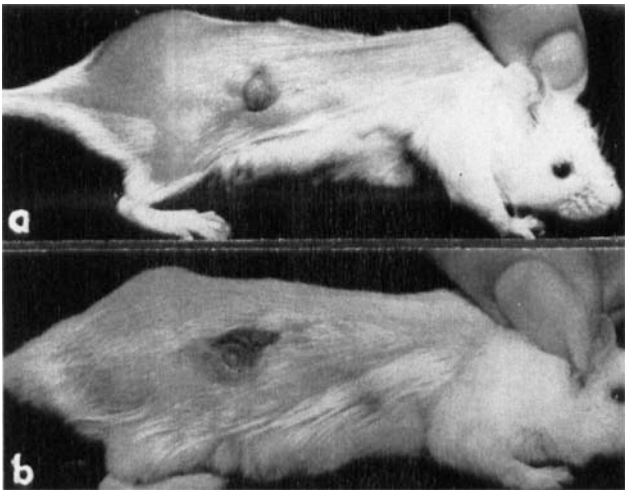

FIGURE 5. Two stages—35 (5a) and 63 (5b) days—in the evolution of an epidermoid carcinoma in a mouse prepared with methylcholanthrene and cortisone and injected with vaccine virus. The derivation of the neoplasm from the scar is clearly manifest in FIGURE 5a.

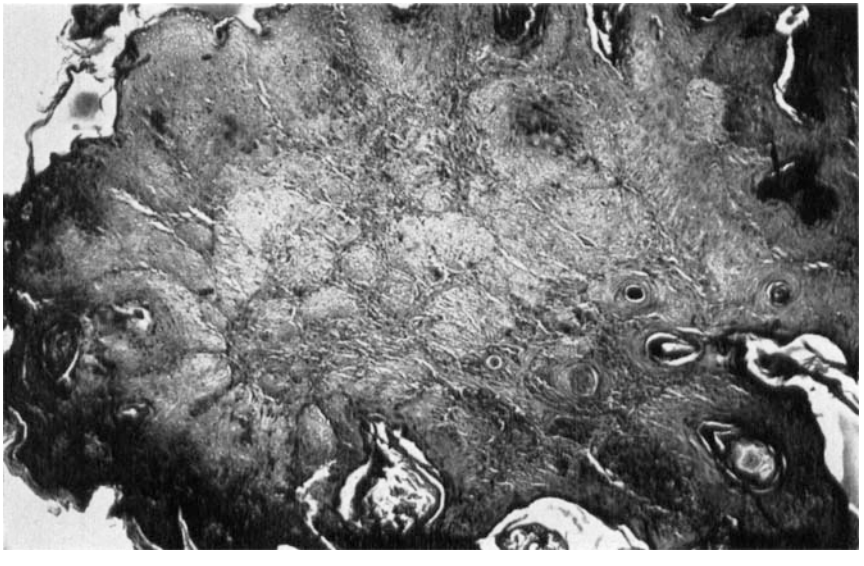

FIGURE 6. Epidermoid carcinoma in the mouse of FIGURE 5. ×60.

As stated before, after the healing of the lesions induced by the rabbit virus, a large scar remained in those mice that had been prepared with MC and cortisone (FIGURE 3)—and also with cortisone alone—while in the other groups the scars were either much smaller or unnoticeable. It was in the mice treated with MC and cortisone that the central observation of this study was made, for it was exclusively in these mice, anywhere from three

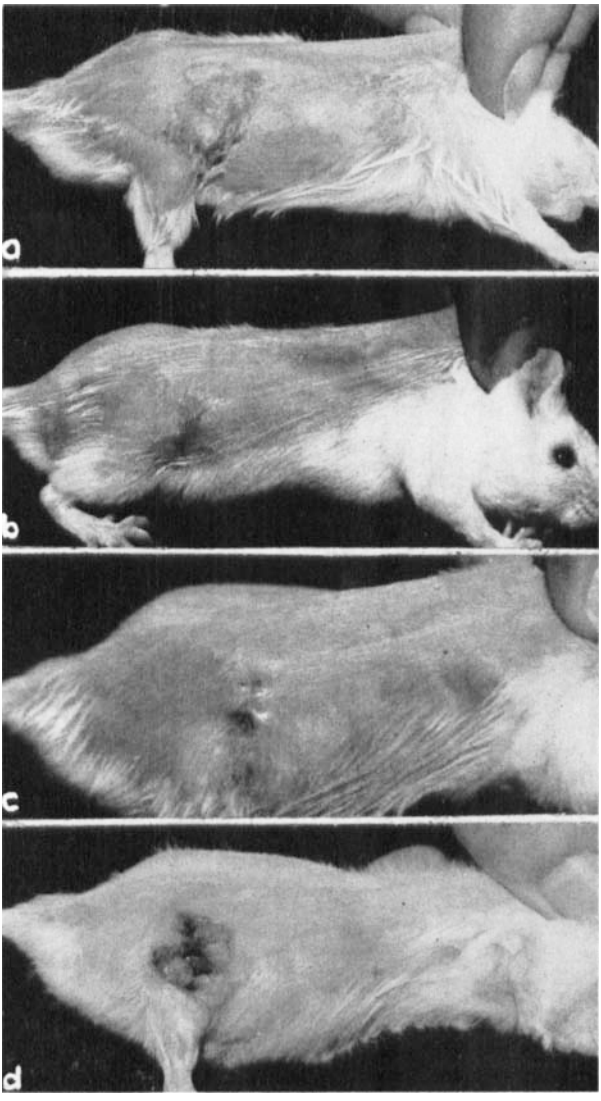

FIGURE 7. Evolution of a lesion—7 days (7a), 27 days (7b), 5 months (7c), and 6 months (7d)—from acute vaccinia infection to a sarcoma in a mouse treated with methylcholanthrene and cortisone.

weeks to a few months after the virus infection and strictly limited to the virus-infected site clearly revealed by the scar, that signs of growth became manifest.

In some cases these growths were benign fibrous or papillomatous structures that could regress after a few weeks of growth (FIGURE 4). In other cases, an initially mild lesion grew steadily and became, in a matter of weeks, an epidermoid carcinoma (FIGURES 5 and 6). In still other cases the lesion apparently remained benign for several months; in such cases the lesion

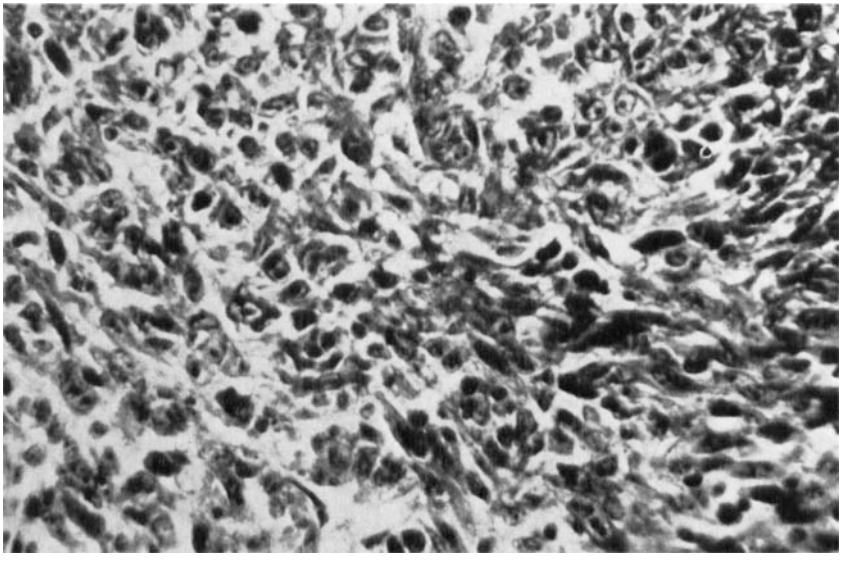

FIGURE 8. Sarcoma in the mouse of FIGURE 7. ×450.

became a sarcoma (FIGURES 7 and 8). Two of these sarcomas became readily transplantable.

I shall now summarize the results obtained in a few representative experiments. To simplify, I shall call the growths that develop at the site injected with vaccine virus "site tumors," and those that develop at random away from the site of the virus injection in the dermal areas painted with MC I shall call "random tumors."

In one experiment, of 18 mice, 13 (72 per cent) developed site tumors, benign in 7 cases and malignant in 6. Regressions have occurred in 5 of the benign tumors, with possible regrowth in 2 of them. Random malignant tumors have occurred in 3 cases (17 per cent), coinciding in 1 mouse with a benign site tumor. Several mice of this experiment are still alive, and the above data may be altered.

In a second experiment only benign tumors have developed thus far in 10 mice; 8 are site tumors and 2 are random tumors, also benign and in both cases coinciding with a site tumor. Most of the mice of this experiment are still alive.

In a third experiment 1 benign and 5 malignant site tumors developed in 8 mice without any random tumors. This experiment was terminated 52 days after virus injection, so that random tumors may have developed at a later date.

The over-all results thus far available from these and from still another experiment are as follows: of a total of 42 mice, 28 (66 per cent) developed site tumors, 16 benign and 12 malignant; 8 of these mice (19 per cent), have developed random tumors, 4 benign, and 4 malignant.

These results are being fully confirmed in experiments under way with

neurovirus in Swiss mice and probably in mice from some inbred strains. Also—and this may be more significant—infection with dermovirus, a variant far less inflammatory and virulent than the neuro strain, seems to result in the development of site tumors more frequently and rapidly than with the latter strain.

On the other hand, tumors at the site of the vaccinia lesion have not yet developed in a total of 295 mice variously treated with the following: MC alone; MC plus EB; EB alone; EB plus benzol paintings; cortisone alone; cortisone plus benzol paintings; benzol paintings alone; nor, of course, have they developed in normal mice. Nor did site tumors develop in groups of mice treated as above, but injected with heat-killed virus and with benzol, which induces ulcers grossly similar to those induced by the virus in mice treated with MC plus cortisone.

A number of random tumors have appeared in all of the mice treated with MC either alone or in any combination, with or without virus. At present, however, we cannot give an estimate of the incidence of these tumors because the number varies from group to group, suggesting that each of the hormones and also the virus, or both in combination, may influence the number and type of these tumors; such an estimate requires careful analysis. This may likewise be true of a number of internal tumors, for example, in the lungs and lymph nodes, that have developed in mice painted with MC. No tumors of any kind have developed in mice treated with either of the hormones alone or with benzol, or with benzol alone.

It appears, then, that, subsequent to an acute virus infection, tumors arise in the precise site of the virus lesion, although in our experiments this has happened only in mice prepared with MC and cortisone.

Now the following questions should be asked: is the virus *itself* instrumental in the development of these tumors? If so, in what capacity? As a specific tumor agent undergoing (on a very specially prepared soil) a change comparable, as some but not all workers believe, to the change that takes place in the Shope virus in the papilloma-cancer sequence in the rabbit? Or as a nonspecific agent that, through the injury followed by repair, *promotes* the development of potential malignancies already *initiated* by MC? Is there present in the rabbit testes another agent instrumental in the effects described?

What, then, is the part played by cortisone? Is it purely to induce larger lesions, thus magnifying the injury-repair effect? To complement in other ways the effect of MC?[7] To interfere with the full development of immune reactions of the host against the virus?

Obviously, no answers to these questions can be given at the present time. In the search for such answers we must (1) complete and extend our initial observations with other variants of vaccinia and other viruses (for example, herpes) infecting the skin and other tissues (especially lung) of differenr strains of mice and other animal species, and also investigate the possible effect of agents, viral or otherwise, that may accompany vaccinia in the rabbit testes or mouse skin; (2) ascertain whether a vaccinia antigen is present in transplants of the tumors that develop at the site injected with

vaccine virus, and investigate the effects of previous immunization of mice against vaccinia on the development of these tumors; and (3) attempt, in the course of the mouse passages or by other means, to isolate from vaccine virus variants that, in normal mice and/or other species, induce lesions progressively more proliferative than inflammatory that may culminate in lasting neoplasia.

Summary

The intradermal injection of a suspension of vaccine virus from infected rabbit testes into Swiss mice prepared by skin paintings with MC combined with injections of cortisone, results in the development of a much enhanced dermal infection. Frequently this is followed by the development of a variety of both benign and malignant neoplastic lesions arising strictly from the precise site (still evident from the scar) injected with the virus material.

These neoplastic lesions have never developed following injections of the virus material into mice prepared (1) only with MC; (2) only with cortisone—in which hosts the infection is also enhanced; and (3) with EB with and without MC—in which hosts the infection is suppressed; not to mention normal mice.

The significance of these findings is briefly discussed.

Acknowledgment

This study has been carried out with the able assistance of Phyllis Johnson and Barbara Stanley.

References

1. DURAN-REYNALS, F. 1950. Neoplastic infection and cancer. Am. J. Med. **8**: 490.
2. DURAN-REYNALS, F. 1957. Virus-induced tumors and the virus theory of cancer in the physiopathology of cancer. F. Homburger and W. H. Fishman, Eds. 2nd ed. Hoeber. New York, N. Y. In press.
3. DURAN-REYNALS, F. & E. BRYAN. 1952. Studies on the combined effects of fowl pox virus and methylcholanthrene in chickens. Ann. N. Y. Acad. Sci. **54**(6): 977.
4. DURAN-REYNALS, F. 1942. Tissue permeability and the spreading factors in infection. A contribution to the host-parasite problem. Bacteriol. Revs. **6**: 197.
5. DURAN-REYNALS, F. 1952. La substance fondamentale du mésenchyme et les facteurs de diffusion dans l'infection. Semaine hôp. **25**: 1047.
6. DURAN-REYNALS, F. 1957. The dermal ground substance of the mesenchyme as an element of natural resistance against infection and cancer. Henry Bunting Mem. Lectures. In press.
7. GILLMAN, T., J. PENN, D. BRONKS & M. ROUX. 1955. Influence of cortisone on connective tissue—epithelial relations in wound healing, hair regeneration and the pathogenesis of experimental skin cancers. Nature. **176**: 932.