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OBSERVATIONS ON THE REACTION OF THE SKIN TO OILS AND TARS.

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A MICROSCOPICAL study of the skins of mice painted with definite carcinogenic agents and with agents which appear to be incapable of exciting the production of tumours has, in the main, enabled us to confirm the observations of previous workers on the different histological changes which take place. A brief description of our findings will first be given.

An obvious thickening of the surface epithelium and a hyperplasia of the epithelium lining the hair follicles may, with a powerful agent, be seen within a week, after one or two applications only. A hyperplasia of the sebaceous cells is rarely so noticeable and, as a matter of fact, continued painting over a long period of time may have very little apparent effect on these cells. Retention of the follicular excretion is an early phenomenon, followed later by sub-epithelial inflammation. The latter is in the nature of congestion of the vessels, a certain amount of fibroblastic response and some lymphocytic infiltration. The most outstanding feature of the sub-epithelial inflammation is, in many cases, the accumulation of a large number of mast cells. Practically all workers with tar have remarked upon these cells, and the view is generally taken that the mast cells have probably nothing whatever to do with the genesis of the cancer production. We are of a similar opinion: in cases of spontaneous breast tumours of mice we have often observed large foci of mast cells in the subcutaneous tissues, situated between the new growth and the skin epithelium. We also agree with the view that these cells have, in all probability, an entirely different origin from the mast cells of the blood. We have found the mast cells sometimes to be so numerous that they have more than once been mistaken for an epithelial tumour. We have noted that the amount of inflammation in the deeper tissues is frequently proportional to the amount of epithelial hyperplasia. This is particularly evident when one is dealing with a weak agent where the epithelial hyperplasia is often focal instead of being diffuse. We are not quite clear as to the relation of the two processes to one another, but incline to the view that the activity of the surface epithelium is often a result of stimulation from within. We have noted that whether the inflammation is caused by a new growth such as a breast tumour or by metazoal, protozoal or bacterial parasites, the epithelium directly over the inflamed tissue is almost invariably greatly thickened. Occasionally one finds an accumulation of pigment and pigment-containing cells in the subcutaneous tissues, which may be dense enough to constitute a small melanoma. It is not uncommon to find that the usually very granular mast cells have

lost most of their basophile granules, pigment granules being substituted for them. When working on the action of carcinogenic agents applied to the skin of mice the presence of sarcosporidia in the muscle and bladder worms in the fatty and fibrous tissues frequently complicates the inflammatory picture.

We have been able to confirm the repeatedly established experimental fact of the multiplicity of site of origin of the malignant tumours of the skin. Not only experimental but also clinical evidence has been brought forward in recent years to show that carcinomata do not necessarily arise from a single cell only. We are in entire agreement with Deelman and the many other workers who have shown that applications of tar may lead to the development of multiple primary epitheliomata of the skin, and we have found a similar state of affairs to exist when carcinogenic oils, etc. are substituted for gas tar. In the skin of a single animal one can sometimes see many stages of the changes undergone by a tissue in its development from the normal to the malignant. Thus as one approaches the painted area from the normal epithelium varying degrees of hyperplasia are met with, the transition from normal to hyperplastic being often very gradual. This gradual thickening of the epithelium is probably, in most cases, a true indication of the amount of carcinogenic substance which has come in contact with the skin, notwithstanding the fact that tumours sometimes arise in situations far removed from the area of skin painted. An increase in the number of mitotic figures with oedema of the epithelial cells is met with as one approaches nearer the centre of the site painted, the epithelium being intensely hyperplastic. Warts and papillomata may be encountered, and finally one or more frankly malignant tumours. It is often difficult to decide as to when malignancy has actually supervened, both on benign tumours and on a hyperplastic skin. The epithelium lining the hair follicles being usually in a very active condition renders the diagnosis of doubtful cases difficult and at times impossible.

In general terms, among our animals, there has been a great tendency for the malignant growths which have developed to remain local and ultimately to cure. A cure or tendency to cure has only become manifest after ulceration has taken place. We have never observed recession in a tumour covered with intact skin, but once ulceration supervenes the majority of the malignant cells are unable to withstand the condition of sepsis prevailing and finally succumb. The rarity of metastases among our animals is very curious in view of the frequency with which they have been reported by other workers. Many hundred animals bearing tumours have been included among our *post mortems*, but we have very rarely observed metastases. It is true that microscopical metastases may have been overlooked, organs of many of the animals not being subjected to microscopical examination. On the other hand, a large number of sections have been studied without success. Our diagnosis of malignancy has rested on the microscopical appearances utilised for the diagnosis of malignant growths in human pathology, but unless the tumour was very definitely malignant it has been classified as benign.

We have failed to observe, among our malignant tumours arising from the application of carcinogenic agents, a single instance of a sarcoma. We have on several occasions come across the spindle-celled variety of epithelial new growth, but never a true sarcoma. There has been a good deal of controversy in the past as to whether the sarcomata reported as arising from the application of tars, etc. are or are not really of connective tissue origin. At the present time it is generally accepted that these tumours are genuine sarcomata, this decision having been arrived at owing to the successful repeated transmission of the tumours, the ease with which this manipulation is performed and the fact that even after 40 passages the tumour remains spindle-celled and morphologically resembles exactly a connective tissue tumour: personally we see no reason why a cell which has mutated in a certain direction should revert to its former state after 40 passages. We agree that a tar may be capable of exciting connective tissue to develop malignancy, but feel certain that our spindle-celled tumours were of epithelial origin. In every instance we were able to trace their connection with the surface or follicular epithelium without difficulty. Even in the primary hyperplasia of the epithelium there is often a marked difference in the morphology of the cells. While usually they remain more or less normal in shape except for a certain amount of oedema, etc. it is not uncommon to find the whole epithelium, from the surface to the basement membrane, consisting of squamous or spindle-shaped cells: from the latter cells spindle-celled epitheliomata may eventually arise.

While all the malignant tumours which arose in our animals had their origin from the surface epithelium or hair follicles, we observed four sebaceous adenomata, which macroscopically we had classified as warts or papillomata. From the fact that one of these tumours, the largest, developed over the painted area of an animal which had received only four applications of a comparatively harmless oil, we are inclined to the opinion that the sebaceous adenomata do not bear the same relation to malignancy as do warts and papillomata. We had evidence that a few of our malignant tumours had a follicular origin; we feel certain, however, that none of them were sebaceous carcinomata.

Among the many aspects of the problem of the development of malignant tumours on mice subjected to the application of what are called carcinogenic agents, perhaps one of the most interesting is that of the relation of epithelial hyperplasia to malignancy. In the course of extensive experiments wherein we have utilised more than 20,000 animals we have had an opportunity of studying this question in some detail, and as it appears to us a matter of importance it may be worth while recording some of our observations bearing on this subject. We have made a microscopical study of some 3000 skins of mice rendered hyperplastic by a variety of agents, many of the specimens being taken from animals which died within a few weeks after the commencement of the experiment. Although we have failed to gain any outstanding information as to the mechanism of cancer production we have nevertheless

obtained a fairly clear mental picture of the passage of events as far as the hyperplasia is concerned.

There are some workers who are of opinion that there is little or no relation between epithelial hyperplasia and malignancy, and who believe that the degree of irritation and the so-called precancerous state resulting from the application of an irritant are factors quite apart from cancer development. It is, of course, recognised by all that there are many mechanical, physical and chemical agencies which excite the epithelium to a hyperplastic response and under the influence of which the epithelium never takes on malignancy. We are not disputing this apparently fundamental truth, but we believe that epithelial hyperplasia is a very important part of the cancerous process in the vast majority of cases when carcinogenic agents are used. In human cancer of organs other than the skin it seems that a benign hyperplasia often precedes the ultimate malignant tumour. For example, the hyperplasia of the epithelium of the intestine in the neighbourhood of definite polyposis adenomatosa intestinalis, which disease is known to be so frequently the site of subsequent malignant disease; the preliminary benign hyperplasia associated with the later development of benign and malignant thyroid tumours, etc.

Some workers do not believe that the hyperplasia is related to malignancy because if one ceases to apply a carcinogenic agent it will not prevent the subsequent development of an epithelioma, and because tumours may arise on skins which otherwise are normal in appearance. We have frequently verified both these facts, but we have not interpretated our observations in quite the same manner as others seem to have done. In our opinion, the question is as usual a relative one, for although we have seen tumours arise long after we have ceased to apply the carcinogenic agent, the tumour graphs fall very far short of those obtained with the control animals where the painting has continued without interruption: in other words, we have found that hyperplasia or tumour development have to be far advanced to be reasonably sure that recession will not take place. These observations are in agreement with those of most other workers who have studied this question. And, although it is true that a tumour may arise on a skin which shows very little or no hyperplasia of the epithelium elsewhere, this does not necessarily mean that there was not a preliminary hyperplasia at the site of the tumour development. In the early stages of an experiment consisting of the application of a very weakly carcinogenic substance one so commonly meets with skins which show a normal appearance of the epithelium except at one or more tiny sites where the epithelium may be heaped up in a remarkable manner (excluding artefacts resulting from a fold of the skin or the angle of sectioning). We have always recognised these isolated hyperplastic foci as potential sites of future tumour formation. When a little more advanced these isolated thickened areas of the epithelium can be seen with the naked eye as minute, slightly raised spots on the otherwise smooth skin of the animal.

The lowly carcinogenic American petroleum oils with which we have been

conducting experiments on a fairly extensive scale are good examples of agents which after prolonged application will give this patchy epithelial hyperplasia. Any tumours which develop are usually solitary, because the potency of the oil is low. A more active petroleum oil will probably lead to a regular, although perhaps not very marked, epithelial hyperplasia and tumours may be multiple. When one is dealing with a carcinogenic shale oil or a gas tar there results almost invariably an intense epithelial hyperplasia, with marked sub-epithelial inflammation, tumours often being multiple. The thickening of the skin is as a rule relatively regular. On the other hand, it is well known that there are substances which will excite a marked hyperplastic response of the epithelium while tumour development may be quite infrequent. We found a good example of such a substance in a sample of a refined Pennsylvanian petroleum oil which yielded but two benign tumours among 100 mice painted for 60 weeks, while the skin of most of the animals gave a good hyperplastic response. There are also substances, such as oleic acid, which produce a certain degree of regular hyperplasia but with which other workers and ourselves have consistently failed to excite tumour development.

Now although oleic acid has been registered non-carcinogenic owing to our inability to induce tumours as a result of its application to mice, there is a possibility that our insuccess is due to the fact that the life of the mouse is not long enough. As a matter of fact quite recently we observed a tiny papilloma in an animal treated with oleic acid for 15 weeks only. All along we felt convinced that the simple hyperplasia we were examining was really similar pathologically to that arising in the early stages of an experiment in which a definite carcinogenic agent was being used. To subject our hypothesis to experimental proof we proceeded to render the skins of a batch of mice hyperplastic by prolonged application of oleic acid, and then subsequently to paint the animals with tar. The animals treated with the oleic acid developed tumours on an average 4 weeks earlier than the controls. We draw the conclusions from these experiments not only that the thickened skins failed to protect the animal from the effect of the tar but that the skins were really in a hypersensitive state and ready to respond actively to a carcinogenic agent and take on the uncontrolled growth of malignancy. We feel bound to assume also that this oleic acid hyperplasia is really a very early stage of a malignant development of the epithelium, which is arrested at this primary stage although prepared for advancing into the wart and epitheliomatous stages given the stimulation of a suitable agent. Waterman's polarisation experiments support this assumption. It might be suggested that the age, diet and other conditions of maintenance rendered the animals more susceptible to the tar applications than were the younger, freshly purchased control animals. We have excluded these possibilities by painting with tar 45 animals, which had been treated for 24 weeks with pure lactic acid and whose skins had given no evidence of becoming hyperplastic. They responded in a manner practically identical to the control animals.

Recently we have performed a more conclusive experiment. A batch of animals painted for 41 weeks with an almost inert oil responded to the subsequent application of fraction 1 of our turpentine synthetic tar more readily than the control animals. The oil itself produced a little epithelial hyperplasia, and when the tarring was commenced only 40 animals were living. In these animals the first wart appeared on the 7th week, when 20 animals remained alive, while at that time no animal among the 89 controls showed a tumour, the first wart appearing here on the 8th week. In the control animals the first epithelioma made its appearance on the 19th week, while among the animals previously painted with the oil the first epithelioma appeared as early as the 11th week. What is more, on the 11th week there remained only 10 live animals in the latter group against 84 controls. An additional batch of 100 animals painted with the oil only failed to develop a single tumour, the painting being continued for 60 weeks.

Another experiment was carried out with a methyl sulphite extract of a refined Pennsylvanian petroleum oil. This extract renders the skin of mice more hyperplastic in 4 weeks than does oleic acid in 40 weeks. With the acute hyperplasia induced by the oil extract there is usually concurrent sub-epithelial inflammation, while the more chronic hyperplasia produced by oleic acid is not often accompanied by much change in the subcutaneous tissues. There appears, however, to be an essential difference in the sensitiveness of the epithelium to the subsequent application of tar. Mice subjected to 8 bi-weekly skin applications of the methyl sulphite extract of this oil and then painted with tar developed tumours perceptibly later than the control animals. Our conclusions from this experiment are that the hyperplasia had so little advanced towards the cancerous state that we were unable to demonstrate any difference between it and the normal: we rather imagined that the thickened skin offered some protection from the tar.

Although a methyl sulphite extract of this particular oil is a powerful hyperplastic agent we have found that it is not a very powerful carcinogenic agent. Thus our experiments go to show that although hyperplasia of the epithelium is almost invariably a precursor to cancerous growth, the nature of a hyperplasia is not always the same. We are aware of this fact from clinical experience alone. If we compare the epithelium of an animal painted for 40 weeks with oleic acid and that of an animal painted for 4 weeks with a methyl sulphite extract of a certain oil, we may find in both animals an epithelium 6 to 10 layers of cells thick. If we assume the number of divisions which the basal cells have undergone since the commencement of the experiment to be the same, which is obviously not the case, the essential difference between the two is that of contact time with the agent applied. One group of cells has had 40 weeks wherein to adapt itself or react to the influence of the oleic acid. Between each division of each individual cell there has been a considerable interval of time so that ample opportunity has been available for each generation to acquire to the fullest extent the changes excited by contact with the oleic acid.

The progeny of such cells would presumably differ from those of cells under the influence of a hyperplastic agent for a short time. Selection and resistance also come into the question, and there are obviously many other possible aspects to be considered.

In studying hyperplasia and the precancerous state we have also varied the total number of applications. Two hundred mice were divided into 10 groups of 20 animals, each group receiving a varying number of applications of a synthetic tar: the first group had two and the last group 20 applications. They were painted twice a week so that the last applications were made on the 10th week. All surviving animals were killed 30 weeks after the commencement of the experiment, and the skins, together with those of the animals which died during the course of the experiment, were studied microscopically in respect of the hyperplastic response of the epithelium and the degree of recession after the discontinuance of the applications. Three tumours developed in the group of animals which received 20 applications, the earliest appearing on the 10th week. The only other tumours observed were two tumours in the animals which had received 14 applications and one in those receiving 16 applications, the earliest appearing on the 11th and the 22nd week respectively, *i.e.* 4 and 14 weeks after the last application. Microscopically, it was found that the degree of response of the epithelium to the tar, in general terms, corresponded to the number of applications, it being most marked in the animals which had received 20 applications of the tar. On the other hand, in each group of animals the epithelium appeared to recede towards the normal more or less to an equal extent.

Another experiment consisted in the application of the tar to mice at twice the usual interval, viz. once per week. We were surprised to observe the first tumour in this group of animals 35 days after the commencement of the experiment, almost in half the time taken for the development of the first tumour in the controls, but on the whole the more frequently painted animals responded earlier. A graph compiled from our results with animals painted every fortnight again indicates the subtleness of the epithelial activity, viz. an early first tumour but a late general response. We are disinclined to believe that our results are compatible with a difference in the degree of sensitiveness of the batches of animals to our carcinogenic agent. In our opinion, these experiments indicate the importance of the concentration, etc. of the agent utilised for the experimental production of malignant new growths. It seems that the potency of the agent must be of an exact degree in order to obtain a maximum response of the epithelium. Presumably the basal cells should be stimulated to the greatest amount possible, consistent with the very best conditions necessary for the general health. It will be remembered that in order to excite X-ray epitheliomata Bloch found that it was necessary to give careful attention to the question of dosage. Too small a dose induced only benign tumours, while if the dose was too large ulceration and necrosis of the skin occurred. Time and again we have noted how certain carcinogenic agents

which will excite the development of tumours quickly and in good number are not really very powerful cancer-producing materials. The warts which develop remain small and the majority may fall off, leaving behind an intact epithelium upon which malignant disease either does not supervene at all or is late in arriving. Some of our ethyl alcohol and methyl sulphite extracts of oils have acted in this way. With pitch we have found, in agreement with other workers, that while warts excited by the application of this substance are apt to fall, malignancy is not abnormally late in making its appearance.

The immune reactions of the host have a bearing upon these experimental results. Although we have found that it may be difficult to demonstrate microscopically tissue reactions to an injected oil, and that an oil may remain for many months apparently untouched at the site of injection, this does not mean necessarily that the host has not reacted to a certain extent to the injected material. When we apply an agent to the skin we know that there is a local reaction on the part of the epithelial cells, a reaction which manifests itself presumably as part of the defence of the body as a whole. But, at the same time, it is highly probable that the epithelial cells make an effort to protect themselves individually from the toxic action of the oil. As animal cells, contact with the oil should result in their acquiring a certain degree of tolerance for the oil or tar, in the same way as Protozoa become tolerant to organic and inorganic toxins. This tolerance may be a case of the survival of the fittest, as it is supposed to be with trypanosomes and spirochaetes, but there seems to be no reason why, at the same time, there should not be developed a true antibody immunity, both local and to a less extent general. It is not to be assumed that the immunity is necessarily a protective immunity, although the essence of the immune reaction is to protect the individual cell, and ultimately the body as a whole. On the contrary, we see no reason why at some particular moment the cells may not be in a state of hypersensitiveness. If it is agreed that cells which have been in contact with a carcinogenic agent for some time are not absolutely similar biologically to normal cells, then it will be conceded that the potency of an agent for the cells to which it is applied may be different at the beginning and the end of an experiment. A substance which may have 10 per cent. of the maximum cancer-producing power on epithelial cells at the beginning, may have 5 or 15 per cent. at the end of an experiment. Hence the difference in the results we obtained with animals painted once a week and those painted twice a week. At first the tar we were utilising induced a maximum response on the part of the epithelium when applied only every fortnight, but when the cells had become more tolerant it was necessary to paint more frequently.

Conclusions.

1. A number of substances applied to the skin of mice may induce a similar degree of hyperplasia, judged microscopically, but this hyperplasia may be quite different biologically.

2. A hyperplastic epithelium may, when the irritant is removed, recede or it may eventually become the site of a malignant tumour.

3. An acute hyperplasia, we imagine, is more likely to recede than a chronic hyperplasia. How far the sub-epithelial inflammation found in the former case is responsible for the recession we are unable to say.

4. Two agents which may have an equal capacity to induce an acute hyperplasia may be quite different as regards their power to excite the production of tumours.

5. When the weak tumour-producer is concentrated by means of alcohol or methyl sulphite tumours may be numerous, but such tumours rarely become malignant and as a rule fall off at an early date. Thus we arrive at a similar state of affairs when tumours are concerned as was found when dealing with hyperplasia.

6. Two agents may excite the production of tumours with equal facility, although the tendency to become malignant may be different.

7. We have so far not been able to observe any difference in the degree of malignancy of epitheliomata induced by the many agents we have tested, although the percentage of animals bearing malignant tumours in each experiment may vary greatly.

8. There appears to be evidence that the epithelial cells acquire a certain degree of tolerance to the agent applied. On the contrary they can be rendered hypersensitive to tar applications by treating them initially with certain reagents.

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