Tumor Promoters: Carcinogenesis Gets More Complicated

The relation between chemicals and cancer appears to be one of those complex modern issues with the potential for getting even more complicated than it is already. Research on a group of chemicals called tumor promoters illustrates this point very well. It shows that cancer can be produced by combining a single exposure to a known carcinogen, but in a dose so low that it would not normally cause cancer, with prolonged exposure to very small quantities of an agent—the tumor promoter—that is not carcinogenic by itself.

The phenomenon of tumor promotion has been known for almost 40 years, but initially there were reservations about its significance because it could only be studied by inducing the development of skin cancers in mice. A phenomenon found only in mouse skin might not be representative of carcinogenesis in general. More recently, however, a number of experiments have indicated that promotion is important in the development of several cancers, including those of the lung, colon, bladder, and liver, in a variety of species.

The extent of the promotion phenomenon—whether or not it is implicated in the development of all chemically induced tumors, for example—is not known. Carcinogenic chemicals can and do cause cancer by themselves without assistance from a promoter. Nevertheless, many carcinogens may have the ability both to initiate and also to promote tumor formation. Thus, promotion may be a very widespread occurrence.

Another important aspect of the work on tumor promoters is that it lends support to the idea that carcinogenesis is a multistep process. The classic promotion experiment by which tumors are induced in mouse skin involves at least two steps. In the first, the skin is exposed to a low dose of the carcinogen; in the second, the skin thus exposed is treated with the promoting agent. Only after weeks of repeated treatment with the promoter are tumors produced.

The distinction between initiation and promotion has important implications for public health because the initiation step of the process eventually leading to tumor formation is irreversible. But promotion is reversible. If the exposure ceases before the cells gain the ability to multiply in the absence of the promoter, then tumor formation may be avoided. Moreover, investigators have shown that certain agents inhibit tumor promotion even in the presence of the promoting agent.

Croton oil, the promoting activities of which were discovered by Isaac Berenblum, who is now at the Weizmann Institute in Rehovot, Israel, was used in the early experiments on cancer induction in mouse skin. The oil is obtained from the plant *Croton tiglium* L. and is a complex mixture of chemicals. In the late 1960's, Eric Hecker of the Deutsches Krebsforschungszentrum in Heidelberg, Germany, and Benjamin Van Duuren of the New York University Medical Center independently isolated and identified the active ingredients of croton oil. They turned out to be esters of the plant alcohol phorbol.

The ester designated TPA, for 12-*O*-tetradecanoylphorbol-13-acetate (Fig. 1), is especially effective as a promoter of tumor formation. According to Roswell Boutwell of the University of Wisconsin, the combination of an initiating carcinogen plus TPA is at least ten times as effective in inducing tumors as the carcinogen alone. For example, in a promotion experiment carried out in Boutwell's laboratory, application of a carcinogen plus TPA in a total dose of 380 nanomoles produced as many mouse skin tumors in 18 weeks as did a dose of 7600 nanomoles of the carcinogen alone.

But the phorbol esters are not the only chemicals with promoting activity. Several unrelated compounds also appear to promote tumor formation although not as effectively as TPA. Moreover, the list of suspected promoters includes materials, such as the drug phenobarbital, the artificial sweeteners saccharin and sodium cyclamate, and even the bile acids, which are normally present in the intestine, to which humans are, or have been, widely exposed.

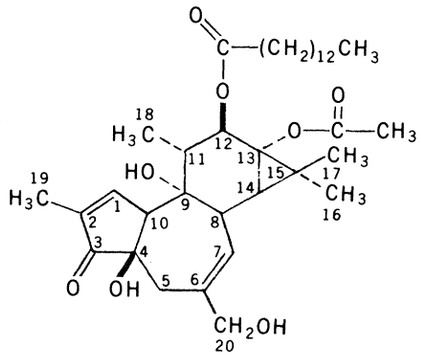

Fig. 1. Structure of 12-*O*-tetradecanoylphorbol-13-acetate (TPA).

In fact, Bandaru Reddy, John Weisburger, and Ernst Wynder of the American Health Foundation think that promotion by the bile acids may provide a mechanism to explain the postulated role of a high fat diet as a cause of human colon cancer. Epidemiological studies have suggested, but do not necessarily prove, a link between high-fat diets and a high incidence of this cancer.

In a more direct test of the hypothesis, the Health Foundation workers examined the effect of dietary fat content on the incidence of cancers induced in rats by exposure to a carcinogen. Rats on a high-fat diet developed more colon cancers than rats on a low-fat diet. In addition, the rats on the high-fat diet excreted more of the bile acids and their derivatives. The bile acids are required for the normal absorption of fats. Thus, an increased quantity of these acids in the intestines in response to a high-fat diet is not surprising.

In another experiment, Reddy, Weisburger, and Wynder found more colon tumors in rats treated first with a carcinogen and then with bile acids than in animals exposed only to the carcinogen. No tumors were found in animals treated only with bile acids. Thus, the investigators propose that increased production of the bile acids, caused by a high-fat diet, promotes the development of colon cancer.

Although cigarette smoke contains a number of known carcinogens, investigators think that their concentrations are not adequate to account for the full incidence of cancers associated with smoking. The smoke also contains several promoters, however. According to epidemiological data gathered by Wynder and also by E. Cuyler Hammond and his colleagues at the American Cancer Society, heavy smokers who kick their habit experience a progressively decreasing risk of developing cancer; after 15 years of not smoking their risk will be only slightly higher than that of individuals who have never smoked. The occurrence of this reduction in risk is more consistent with promoters being the major cause of the cancer-inducing properties of cigarette smoke than it is with carcinogens playing that role because the continued presence of promoting chemi-

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cals is required if cancer is to develop.

Evidence implicating saccharin and sodium cyclamate as promoters rather than as true carcinogens comes from the laboratory of R. Marian Hicks at the Middlesex Hospital Medical School in London. In the absence of a carcinogen, Hicks and her colleagues found a very low incidence of bladder tumors in animals fed the sweeteners. In Hicks' cautious words, the incidence was "so low that it cannot be stated unequivocally that either sweetener is a solitary bladder carcinogen." The incidence of bladder tumors was very high, however, in ani-

New Moons: Encounters of the Serendipitous Kind

Earthbound planetary astronomers, who are forced to peer through a turbulent atmosphere and then across millions and billions of miles of space, obviously must take second place in some cases to the wave of unmanned probes that are sweeping the solar system. But it appears that less glamorous telescopic observations can still lead to unexpected significant discoveries. Recent confirmed telescopic discoveries include the rings of Uranus, which continue to pose difficult problems of celestial mechanics, and an asteroid-like body, Chiron or Object Kowal, circling the sun in an unlikely orbit beyond Saturn. Two more telescopic discoveries were announced on 7 July, a proposed moon of Pluto and a possible moon of the asteroid Herculina, the first ever suggested for such a small body.

Like the earlier finds, these were stumbled upon while the observers were intently studying something entirely different. James Christy of the U.S. Naval Observatory (USNO) was attempting to measure more accurately the orbital characteristics of Pluto when he noticed a small bulge in its image on a series of photographic plates taken through the USNO's 155-centimeter telescope at Flagstaff last April and May. After satisfying himself that poor atmospheric conditions or faulty tracking by the telescope were not to blame, he checked plates of Pluto taken in 1965 and 1970 and found seven that showed the same phenomenon. Christy then decided that the bulge was a moon so close to Pluto that it could be noticed only when they were at their greatest apparent separation. Using the 1978 observations and reported variations in the brightness of Pluto to estimate the period of revolution, Robert Harrington, also of USNO, was able to predict future appearances of the bulge as well as explain its appearance in the past.

Initial reaction to the announcement of a Plutonian moon seemed to depend on the availability of the USNO plates. Those who have seen them are convinced of the reality of the proposed new satellite, while those who have not tend to remain "interested but not convinced," as Daryl Mulholland of the University of Texas at Austin describes himself. Explaining his own hesitation, Mulholland points out that the apparent separation of Pluto and its proposed satellite, now estimated to be 0.8 second of arc, would be difficult to distinguish even under the best of viewing conditions. Some of those hesitating to accept the claim would prefer that the two bodies be completely resolved photographically or subjected to more sophisticated instrumental analysis. Unfortunately, the opportunity to gather new data, as opposed to searching photographic archives, passed for this year shortly after the announcement, when Pluto moved too close to the sun in the sky.

Apart from confirming unequivocally the existence of a satellite, better data would help further refine estimates of Pluto's mass. The interrelated properties of mass, diameter, and density have never been known accurately for Pluto, it turns out, although estimates predate the discovery of the planet. Its mass was first estimated as ten times that of the earth, but that value plummeted by the early 1970's to about 17 percent of the earth's mass at most. Using a distance of 17,000 kilometers estimated from the USNO photographs as the separation of Pluto and its moon, the observed period of revolution, and Kepler's Third Law, Harrington has calculated that the mass of the planet-moon system is more like 0.17 percent of the earth's mass. The actual separation is still rather uncertain, falling somewhere between 15,000 and 20,000 kilometers, according to Harrington. The effect of this uncertainty on the estimated mass is considerable, since it is the cube of the separation that enters the calculations. Improvements will probably be made when Pluto again moves into a better sky position, but the ultimate accuracy of the determination remains to be seen.

Whatever the end result is, Pluto is likely to be the smallest of the major planets. Dale Cruikshank and his colleagues at the University of Hawaii have made an estimate of 3000 kilometers for its diameter on the basis of its reflectivity (*Science*, 23 April 1976, p. 362). This estimate was based on the assumption that Pluto was a single body. Harrington suggests that the moon is only two to three times smaller than Pluto, whose diameter would thus be even smaller than Cruikshank's estimate. Apparently, Pluto forms a "double planet" with its satellite. By comparison, Mercury has a diameter of 4680 kilometers, whereas Pluto's nearest neighbor, Neptune, has a diameter of 44,800 kilometers. Mulholland quips that, if the satellite does exist, the pair might better be considered a "double asteroid" system.

Just such a system has been suggested by Edward Bowell of Lowell Observatory as the most reasonable explanation of observations made by him and Michael A'Hearn at Lowell, and by Keith Horne of the California Institute of Technology and James McMahon, an amateur, in two locations in California. They were hoping to measure the diameter of the asteroid Herculina during its occultation of a star. In addition to the predicted single blinking out of the star as Herculina passed in front of it, a secondary extinction was observed 2 minutes before the predicted occultation at two of the three locations. The observations, including the failure to see the secondary extinction at one location, are consistent with Herculina having a satellite with a diameter of 46 kilometers, about one-quarter that of Herculina itself, at a distance of 977 kilometers. Bowell favors this explanation but acknowledges that other objects in the vicinity of Herculina might have been responsible. Visual confirmation is probably impossible, but more data on similar phenomena may be available from other anomalous occultation observations that have been reported recently. In any case, both Pluto and the asteroids will be receiving particular attention in the near future from earthbound planetary astronomers.—RICHARD A. KERR

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mals receiving a single dose of a carcinogen and eating diets containing either saccharin or sodium cyclamate.

Finally, several investigators, including Carl Peraino of Argonne National Laboratory, have shown that phenobarbital, a commonly used—and abused—sedative drug for humans, appears to act as a promoter of liver tumors.

Although the public health implications of tumor promotion are reason enough for studying the phenomenon, cancer researchers are also enthusiastic about the research because they hope it will enable them to identify the biochemical change or changes underlying the transformation of normal cells to malignant ones. Numerous alterations have been observed in transformed cells but investigators have not yet been able to separate those that actually cause transformation from those that result from transformation. The division of at least some kinds of chemical carcinogenesis into the two stages of initiation and promotion may help to advance the dissection of the process as a whole.

Nevertheless, the mechanism of neither of these stages is understood at present. Emmanuel Farber of the University of Toronto says ignorance of the nature of initiation is a weakness of promotion research because nobody knows just exactly what is being promoted. Most, although certainly not all, investigators think initiation involves an alteration in the cell's DNA by the initiating agent. The fact that a single low dose of the agent is sufficient to produce a permanent change supports this suggestion. Tumors develop in mouse skin, for example, even if a year is allowed to elapse between application of the initiator and treatment with the promoter. Thus, the cells appear to have a "memory" for the initiator, a result implying that initiation involves a genetic change that can be passed from one generation of cells to the next. The chemical nature of initiating agents is consistent with this hypothesis; they are, or can be converted to, compounds that attack DNA.

The nature of the gene change produced by the initiating agent is unclear, although it does not appear to affect the cells until they are also exposed to the promoter. Farber, basing his conclusions on a model he has developed for studying early cancerous changes in the liver, has suggested that the initiating carcinogen induces a change in some cells that makes them more able to multiply in the presence of toxic agents, including promoters, than are cells that have not undergone the initiating event. Selective division of the initiated cells might then lead to the development of tumors if the animal is exposed to another toxic agent. Additional work will be needed to confirm Farber's hypothesis.

Many other investigators have concentrated on studying the effects of promoters with the hope of identifying the particular effect actually causing malignant transformation. If this causative event can be spotted, it may then point the way to the initiating gene change.

The problem is the large number of effects produced by promoters, a situation which has resulted in an abundance of theories on the mechanism of promoter action because each researcher tends to focus on the effect he or she is studying. Nevertheless, although there is now no generally accepted unifying theory to explain all of the diverse actions of promoters, there are some tantalizing clues about how they work.

Promoters and Cell Differentiation

An effects of tumor promoters that has been receiving much attention recently is interference with the normal development of cells. Several investigators, including Leila Diamond and Thomas O'Brien of the Wistar Institute of Anatomy and Biology, Howard Holtzer of the University of Pennsylvania, and I. Bernard Weinstein and Hiroshi Yamasaki of the Columbia University College of Physicians and Surgeons, have observed that phorbol ester promoters inhibit the differentiation of a variety of cell types in culture. As a consequence of developing to maturity, cells lose their capacity to divide. But if their differentiation is inhibited and they remain in an immature state, they may continue to divide, perhaps in the uncontrolled manner characteristic of malignant cells.

Weinstein and Yamasaki have suggested how inhibition of differentiation might account for the increased proliferation observed in mouse skin cells treated with phorbol esters, if the results obtained in cultured cells can be applied to the skin system. Normally when an immature skin cell divides, one of the daughter cells retains the capacity to continue dividing while the other one differentiates, thus losing its growth potential. If differentiation of this cell is inhibited by a tumor promoter, however, the result could be an increase in the proportion of cells capable of dividing.

Moreover, most promoters are known to stimulate cell division, although this effect cannot account by itself for tumor formation. Many nonpromoting chemicals also stimulate cell proliferation.

In general, phorbol ester promoters induce in cultured cells changes characteristic of malignant transformation whether it is brought about by chemicals, viruses, or irradiation. Among these changes are the effects on cell differentiation and proliferation that have already been mentioned and also alterations in the activities of several enzymes and in a variety of cell surface properties. Most of these studies have been performed with cultured cells, however, and there is some question as to whether the observed alterations reflect what happens in the living animal.

Nevertheless, detailed biochemical studies of transformation are difficult, if not impossible, in living animals. Consequently, investigators are trying to develop cultured cell systems that are good models of promotion. Charles Heidelberger and S. Mondal of the University of Wisconsin and also Nancy Colburn and Stuart Yuspa of the National Cancer Institute have already shown that it is possible to devise culture systems in which cells are transformed by a two-step process analogous to that for the development of mouse skin tumors. Such systems may help to dissect the chemical changes caused by promoters. Meanwhile, of course, researchers have been pursuing their studies of the changes already identified, both in mouse skin and cultured cells, in the hopes of pinpointing the specific biochemical events leading to the malignant state.

Two such changes undergoing intensive study are the increases in the enzymes ornithine decarboxylase (ODC) and plasminogen activator. The activity of ODC is especially well correlated with promoting activity, according to Boutwell and to Yuspa, Colburn, and their colleagues. These investigators observed a large increase in ODC activity within 2 hours of exposure of mouse skin or cultured cells to TPA. Moreover, when they tested a series of compounds with varying degrees of promoting activity, they found a close correlation between the degree of promotion and increased ODC activity. Thus far all promoters tested have been found to increase ODC activity, but no nonpromoters do so.

The enzyme ODC is needed for the synthesis of a group of cellular chemicals called polyamines, which are known to stimulate cell division. Thus, enhanced production of ODC could lead to greater cell proliferation. Because promoters only transitorily induce production of the enzyme in normal cells, whereas in initiated cells high production of the enzyme eventually becomes permanent, Boutwell and his colleagues have proposed that initiation involves the loss of a gene that would normally control the in-

activation of ODC. According to this theory, the gene still functions in normal cells, where it shuts off synthesis of the enzyme, but its loss in initiated cells leads to uncontrolled ODC action.

The fact that a number of agents, including putrescine, which is the product of the reaction catalyzed by ODC, several vitamin A derivatives (retinoids), and inhibitors of prostaglandin synthesis, inhibit both the increase in ODC activity produced by promoters and promotion itself lends support to the hypothesis that the enzyme activity is a prerequisite for promotion.

But ODC is not the only enzyme implicated in this phenomenon. Thus, other investigators have developed alternative theories to explain tumor promotion.

According to Walter Troll and his colleagues at the New York University Medical Center, for example, proteases are also produced in mouse skin in response to TPA. And agents that inhibit protease activity block development of tumors in this living system.

Weinstein and Michael Wigler, also at Columbia, have identified in cultured cells a specific protease, called plasminogen activator, whose activity is increased by treatment of the cells with phorbol ester promoters. The amount of the enzyme produced correlates with the promoting activity of the phorbol ester. Many promoters chemically unrelated to the phorbol compounds have no effect on the production of plasminogen activator, however.

Both Weinstein and Troll speculate that the proteases produced in response to the promoting agents act to turn on genes by destroying the proteins that normally block the genes and prevent their expression. Expression of these genes would result in the appearance of the characteristic features of transformation, but these features would presumably become permanent only in cells that had previously undergone initiation.

Troll has specifically implicated defective DNA repair in his view of how promotion works. He thinks that promotion, through its stimulation of cell division, which requires DNA synthesis, might cause the cell to "notice" invisible damage to the DNA caused by the initiation. Recognition of that damage, in combination with the turning on of the appropriate genes by the protease might then lead to the activation of an error-prone pathway of DNA repair, which has been postulated to exist in mammalian cells. The result would be magnification of the DNA damage and consequent transformation of the cells.

However tumor promoters work, they must attach to, and possibly penetrate, their target cells before they can exert their effects. Several investigators are currently speculating that tumor promoters are like many hormones in that they have to bind to specific receptors on the cell surface in order to act. The characteristics of tumor promotion are known to resemble in some ways those of hormones. Both kinds of agents are effective in very small doses, for example. And tumor promoters, like hormones, need specific structures for their activity.

One possibility is that the promoters bind to a receptor for a naturally occurring material. Weinstein and Lih-Syng Lee, also at Columbia, have suggested a candidate for that material, namely epidermal growth factor (EGF), a substance that stimulates the division of epidermal cells. He points out the similarity between many—although not all—of the effects of TPA and those of EGF.

Do Promoters Compete for EGF Receptors?

Weinstein and Lee have recently shown that cells treated with low doses of phorbol esters bind less EGF than they normally would. The degree with which the growth factor binding is inhibited correlates with the tumor-promoting activity of the esters. This might mean that the promoters and EGF compete directly for the same receptors and that the effects of the promoters are mediated by their activation of a system normally responding to EGF. There is, however, an alternative explanation. Several investigators have observed alterations in cell surfaces as a result of exposure to tumor promoters. Thus, the inhibition of EGF binding might be the indirect result of these changes in the membrane rather than of a direct competition of the phorbol esters for the EGF receptors.

Although discussion of initiation and promotion might imply that chemical carcinogenesis is only a two-step process, such simplicity is unlikely. Most investigators now think that it involves as many as five or six discrete events. There is some evidence that promotion involves a series of steps. For example, Fredric Burns of the New York University Medical Center has observed a spectrum of properties in the tumors induced in mouse skin. The first tumors formed in this system are benign papillomas, which are similar to warts; some of these then undergo transformation to the malignant tumors called carcinomas.

According to Burns, most of the papillomas regress if exposure to the promoting agent ceases, but some of them continue to grow autonomously. Not surprisingly, the papillomas with the greatest autonomy develop more frequently into carcinomas. Longer exposure to the promoter produces more papillomas of the type that are likely to develop into carcinomas. In addition, if Burns exposes the apparently normal skin sites where regressed papillomas had once been located, to a second round of promotion, tumors form much more rapidly than they usually do in skin. Presumably the cells in those sites retain some of the changes induced by the first exposure to the promoting agent. These results indicate that there may be several stages in the progression of initiated cells to benign and then to malignant tumors.

A development that may help researchers to unravel the stages in tumor promotion is the discovery of several different inhibitors of the process. Among the substances having this effect are the protease inhibitors mentioned earlier, some of the anti-inflammatory steroids, the retinoids, and inhibitors of prostaglandin synthesis. The antipromotion activity of the last group of chemicals suggests that prostaglandins may also be required for promotion.

Moreover, the agents preventing promotion apparently act by different mechanisms. The retinoids, for example, inhibit ODC activity. The steroids have no effect on ODC but inhibit the production of plasminogen activator, according to the Columbia investigators. Use of inhibitors acting at different steps in the tumor promotion process may help investigators of carcinogenesis trace those steps in much the same way that biochemists have used specific inhibitors to trace complex biochemical pathways.

But there is an even greater significance to the existence of promotion inhibitors. Several investigators have pointed out the improbability of ever removing all chemicals capable of initiation—which only requires one exposure and is irreversible—from the environment. But the effects of promotion are reversible if the promoter can be removed in time, and even if it cannot be removed, it may be possible to use inhibitors to prevent cancers from developing. Thomas Slaga of Oak Ridge National Laboratory, for example, has observed a synergistic inhibition of promotion by a combination of a steroid with a vitamin A derivative at doses at which neither of these agents is toxic. Thus, although many questions about the mechanism of tumor initiation and promotion remain unanswered, the investigators hope that the research will lead both to a better understanding of chemical carcinogenesis and ultimately to strategies for preventing it.—JEAN L. MARX