Role of Subliminal Pharmacology Programs in Support of Carcinogenesis Research

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THE TECHNOLOGICAL and industrial achievements of this mechanized and nuclear era have evolved a chemical environment for man. In this environment may be evidenced atmospheric, soil, or stream pollution, chemicals in industrial operations and household products, and even chemical food additives developed in modern food processing. Some of these environmental chemicals are, and others may be, potential hazards to public health. Attention has been focused on some of these compounds which, although not labeled as poisons, may contribute toxicologically to induction of cancer. The examples of environmental carcinogens, such as aromatic amines, end products of petroleum cracking process, exhaust of internal combustion engines, and X-rays applied to man and animals, are well documented. A good illustration of a certain chemical that was never marketed on the basis of its established potent carcinogenicity is the experimental insecticide acetylaminofluorene.

Synthetic products continuously introduced in cleansing agents, cosmetics, food containers, food coloring, and food additives form a wide spectrum of materials which must be tested thoroughly for carcinogenicity prior to general use. On September 6, 1958, the U.S. Congress passed an amendment to the Federal Food, Drug and Cosmetics Act (Public Law 85–929) stipulating that all food additives must be tested for possible toxicity. In addition, certain requirements relative to the carcinogenic potential of food additives and degradation products from processing methods, including the newer concept of preservation by means of ionizing radiation, are specified (*1*). Similarly, the Miller Pesticide Amendment of 1954 established a procedure for setting safe amounts for residues of pesticides on fruits and vegetables. The color additives amendment of 1960 deals with potential carcinogenicity of coal tar colors and establishment of their harmlessness by the Food and Drug Administration.

Since many toxic or carcinogenic agents at low dosage levels may express, because of their latent effect, only a weak subthreshold or subliminal pharmacological action which is difficult to detect by current methodology, further development and refinement in methodology is imperative. This paper advances some newer concepts concerning methodology and training in subliminal pharmacological chronic toxicity and carcinogenicity research.

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Toxicity and Carcinogenicity Studies

In revealing a source of environmental carcinogens, clinical suspicions and subsequent epidemiologic surveys supported by statistical studies may yield important evidence. Studies of occupational groups may be more easily interpreted than studies of large segments of the general population for whom causal relationship is difficult to establish. Anomalous situations and indeed poor correlation may prevail between data in experimental and human studies in general population investigations. For example, evidence of association of lung cancer in man to chromate exposure is somewhat equivocal; however, the findings in animals (rats, rabbits, guinea pigs, and mice) indicate the

association is negative (*2*). Hence, there is always the problem of selecting the correct species for a specific carcinogenicity test, since it is inevitable that tests on laboratory animals may not parallel the human experience. This criticism, however, may hold for any evaluation of biological activity.

In studies of toxicity, subacute or chronic, this situation of animal-to-man correlation prevails just as much as in studies of carcinogenicity. Despite this situation, a body of documented evidence supports parallelism between man and animal in carcinogenesis data. By the same token, evidence on carcinogenesis from tests restricted to a single animal species cannot establish negative results unless fortified by similar findings from several species. In this respect an analogous situation prevails in the assessment of chronic toxicity.

Chronic toxicity investigations have become well-established procedures for evaluation of drugs, cosmetics, food additives, and products of food processing. In most cases only minor modifications are required in the design of the long-term chronic toxicity experiments to provide for evaluation of carcinogenicity. This dual consideration of chronic toxicity and carcinogenicity in the investigation of new products may help prevent the introduction of new hazards or conversely lend support to the establishment of the safety of the product in question.

Among the requisites for chronic toxicity evaluation are the specifications for more than one test species, lifespan studies, an adequate number of test animals in the control group, and final histopathological examination of control and treated animals. These requirements are also relevant to quantitation of results in carcinogenicity studies. In essence, the possible production of cancer is but one of the more important manifestations of intoxication during a long-term investigation. The contractual research program on wholesomeness of irradiated food, conducted recently by the Surgeon General of the Department of Army, is perhaps one of the most comprehensive large-scale programs comprising in an experimental design simultaneous evaluation of nutritional, toxicological, and carcinogenic factors.

In long-term evaluation of toxicity (chronic toxicity) using rats as test animals, the challenge of the chemical, drug, or food is usually extended through at least four successive generations, or for a total evaluation period of 2 years. The parent generation (F_0) is maintained through this 2-year span to provide data on such criteria as lifespan, growth, food consumption, and, at termination, histopathological findings for data on tumor incidence. The succeeding generations F_1, F_2, and F_3 of the parent generation are maintained no longer than 4 months before sacrifice in order to acquire information on reproduction and lactation performance. When the dog is the test animal, the testing period may be as long as 6 to 8 years, but in some studies, for expediency, this may be reduced to 2 years.

The significance of following a biological response from one generation to another is equally demonstrated in carcinogenesis studies as well as toxicological evaluations. For example, methylcholanthrene fed to rats invokes an increased incidence of leukemia in the progeny (*3*), and, similarly, urethan given to a pregnant mouse reveals that carcinogenic action may be demonstrated in the fetus through lung tumorigenesis (*4*). Here, generation studies are important in studying tumor-inducing products which cross the placental barrier. In these situations as in others the carcinogenesis evaluation becomes an integral part of the broad program in assessment of chronic toxicity; conversely, subliminal pharmacology studies may provide a supporting role to development of evidence for carcinogenesis (*5*).

For short-term toxicity studies (subacute toxicity), the test material is applied in repeated doses (with continuous feeding of processed foods) during 10 percent of the animal's lifespan. Assuming that little is known concerning the toxicity or response of a chemical, food additive, or processing degradation product, a range of doses are tried before certain doses are selected. If no effects are observed at the 10 percent dose level in the diet, no increase in level is warranted to check for safety in pure compounds. With complex systems, such as foods with a limiting threshold or potentially weak toxic or carcinogenic effect, the challenging dose is set sufficiently higher than the normal intake to elicit a response. For evaluation

of foods, the level is frequently established at 20–35 percent of total calories or that proportion of total solids.

For a standard test diet, a diet of known composition is essential in order to support normal growth and reproduction and not confound the findings from the feeding trials because of a dietary variant. For example, in carcinogenesis studies in which p-dimethylaminoazobenzene was administered to rats, Japanese workers produced liver tumors when they used rice as a source of dietary protein for these animals. Kinosita (*6*) was unable to reproduce these hepatomas as successfully when he used wheat as a source of protein. Similarly, in toxicological studies on feeding irradiated beef to rats on a special diet where menadione was intentionally omitted, Johnson and associates (*7*) were able to produce hemorrhagic diathesis in 40 days. This result was not duplicated by other workers who selected synthetic rations of different carbohydrate and protein sources. Addition of methionine to the diet caused a regression in incidence of hemorrhagic diathesis (*8*).

The conventional chronic toxicity procedures provide certain criteria or measurements for assessment of the magnitude of biological response and ultimate establishment of safety of various products. Some of these, such as growth, food consumption, longevity, reproductive performance, lactational performance, blood and tissue enzyme levels, hematology, histopathology on sacrificed animals, and tumor incidence or carcinogenicity, have been referred to previously, but only for comparative performance of control and experimental groups of animals. Several of these biological effects may reflect only grossly the influence of an agent on a physiological response. In this category are such criteria as growth, food intake, lifespan, and gross pathology. Well in advance of the more obvious responses are the more subtle physiological effects, reflected at the cellular level, from the influence of a toxic agent or absence of an essential nutrient. The biochemical measurement of levels of tissue and blood enzymes and the histochemical characterization of certain biochemical entities may be considered as more refined techniques for this purpose.

Further refinement and improvement in methodology is imperative to keep pace with rapid advances in biochemistry and pharmacology. Further motivation for sophistication of procedures to adequately assess the hazards associated with subliminal pharmacology or long-term subtle toxic and latent carcinogenic effects has been the result of recent Federal legislation, such as Public Law 85–929, relating to food additives.

Comprehensive studies dealing with the route and rate of absorption, levels of storage in the tissues, and ultimate metabolic fate are now required in order to elucidate the mechanism of biological action of a material under investigation. Classic and conventional toxicological methods involving, for example, long-term animal feeding trials, which are time consuming, have and are providing important toxicity data but do not adequately meet the requirements just described. Equally important as and associated with such requirements in subliminal pharmacology is the need for more rapid methods and extension of certain bioassay procedures, including identification of potential carcinogens not now characterized, for better elucidation of the mechanism of carcinogenesis. To accomplish such objectives, an accelerated and coordinated program of research and training is essential. Various approaches in this direction are therefore suggested in the following discussion of methodologies.

Development of Methodology

The preceding discussion indicates that for some time end points for evaluating stress agents, either toxic or carcinogenic, have been gross observations on the intact animal and gross or microscopic study of tissues and organ systems. Following this, biochemical techniques have advanced to the point where the influence of physical or chemical agents on the intact animal could be reflected in enzyme levels at the cellular level, and progress in this direction is continuing at a rapid pace.

With the adaptation of isotopically labeled compounds, direct metabolism studies of various stress situations would contribute markedly to evaluation of the effect of such agents on the animal or organism. In some cases synthesis of a labeled compound is not practical, and if

it is, the direct metabolic study may not reveal the full impact of the toxic or carcinogenic agent on the living animal. The effect of ionizing radiation on animals, for example, would not be investigated by use of labeled compounds with direct metabolism studies, but rather the approach would be in terms of indirect effects on certain metabolic pathways. All of the procedures used in pursuit of evaluation of environmental stresses on animals have in essence been based on normal metabolism and any aberrations related thereto.

More recently investigators have turned their attention to the influence of a stresser on certain metabolic systems. Domingues and associates (*9*) have described an instrument and technique for the continuous measurement of respiratory carbon dioxide patterns in metabolic tracer studies. The development in 1955 by Tolbert (*10*) of a continuous $C^{14}O_2$ analyzer facilitated metabolic studies on nutrients. In preliminary studies, Conrad and associates (*11*) determined the digestibility in the rat of uniformly labeled carbon 14 soy bean cellulose, which was later followed by use of the continuous $C^{14}O_2$ analyzer. In essence, such studies are founded on the principle that the main source of energy for biological processes is derived from oxidation of nutrients, and any agent, toxic or carcinogenic, which interferes with biological oxidations will produce aberrations in normal metabolic rates and reactions. In maintaining a homeostatic state, the body has certain adaptive capabilities in modifying the forces which tend to disrupt metabolic pathways. The reflection of the modification on enzyme systems (dehydrogenases, peroxidases, or oxygen carriers), such as in the altered rate of glucose oxidation, has an important correlation to toxicity or tumorigenesis.

Indirect Metabolic Procedures

Previous discussions have stressed the necessity for evaluation of thousands of compounds (food additives, pesticides, and industrial chemicals) for potential toxic and tumorigenic properties. Tolbert (*10*), reporting on the metabolism of glucose by continuous $C^{14}O_2$ measurements, indicated that tumorous rats had depressed glucose use, and later as the tumor became enlarged metabolism showed a moderate increase. While many studies have used radioglucose as an indicator of metabolic function under influence of stressers (toxic or tumorigenic), other metabolites, such as amino acids and certain triglycerides, have been proposed and can be used. However, glucose is commonly selected because of its involvement in many enzymatic syntheses in the body. It is a metabolite that has an end product in urine or expired CO_2 from lungs with a rapid turnover rate. Either carbon 1 or carbon 6-C^{14} glucose compounds can be readily synthesized and easily identified.

Essentially in investigation of sublethal effects of toxicants or stressers, 24-hour excretion measurements on $C^{14}O_2$ are made with the radioglucose alone (glucose-1-C^{14} or glucose -6-C^{14}) and then with the radioglucose plus toxicant. Both rate and concentration of expired $C^{14}O_2$, $C^{12}O_2$ and ratio of $C^{14}O_2$ to $C^{12}O_2$ are obtained. Alteration in excretion patterns is used to determine the effect of an agent after various periods of intubation to rats (for example, 9th, 27th, 90th, and 180th day) or by subacute oral feeding. Tumorigenic and toxic agents, such as safrol and 2-acetylaminofluorene, as well as other materials, such as carbon tetrachloride and parathion, have been studied by Zeitlin (*12*). Stresser effects may be expressed either in terms of depressed or stimulated rate of oxidation of radioglucose.

The use of indirect metabolic procedures as just described should assist investigators in toxicological and carcinogenesis studies to detect at a cellular basis the influence of the following: (*a*) dose-effect relationship, (*b*) time-concentration-effect relationship, and (*c*) absorption, distribution, and metabolic fate of the agent administered.

The alteration of carbohydrate metabolism, especially glucose metabolism by carcinogenic chemicals, has been reported (*9*, *10*). The degree of alteration or use, or both, depends on the structure or property of the toxicant or tumorigenic agent. In addition to measuring glucose metabolism as influenced by stress, one can also characterize metabolites in the metabolic pools of the body and ascertain the difference in the labeled pool formation with or without a stresser agent administered to the test animal.

Radioisotopes in Direct Measurement

The preceding discussion emphasizes the importance of indirect indicators, such as alteration of metabolism of specific nutrients, in the evaluation of stress of a particular compound on the animal. Just as radioactively tagged materials, such as radioglucose, can be used in the indirect determination on metabolism, so the toxicant to be studied can be "tagged" and its metabolic fate established. For example, it might be useful to know whether a "tagged" food additive is altered in the gastrointestinal tract. If such is the case, the metabolic degradation products or metabolites should be identified and their physiological action determined. Since isotopic tracers facilitate the location, concentration, and chemical form of the metabolite deposited, one can possibly arrive at conclusions useful in predicting ultimate toxic or carcinogenic action of the metabolite or its precursor.

It is possible to use radiometabolites in tissue culture and to study the growth morphology and metabolism of tissue culture of certain animals under the influence of toxicants or carcinogens and, hence, to evaluate the effect of these stressers. In another procedure substrates, such as carbohydrate, fat, and protein, are labeled and placed with mitochondria, which are essentially reproducible enzyme systems, and the effect of an added toxicant on this system is determined.

Labeled compounds can be used effectively in tracing the translocation of compounds from plants to animals to man. Hence, radioactive labeling of compounds not only permits the establishment of the localization and level of concentration at a particular anatomical site, but in turn provides a predictive basis for its physiological and toxicological effects. While this can be done experimentally, one might recall that the occurrence of fallout radionuclides (in plants, animals, and man) is a special case illustrative of this mechanism. The biological effects of these radionuclides, particularly strontium 90, are continually being studied.

The evaluation of the toxicological hazards and carcinogenic properties of spray residues, such as pesticides, fungicides, soil fumigants, and rodenticides, on foods is a constant problem. The use of radioactive tracers can serve a most useful purpose in assessing the fate and ultimate effects of such materials on man. Certain procedures such as washing remove such residues, and here the efficacy of removing the toxic compound and of quantitating the volumes of water required for removal can be suitably determined by use of radioactive tracers.

Recent Federal legislation (Public Law 85–929, Food Additives Amendment) is not only stringent regarding constituents in foods, but also pertains to migration of polymers and plasticizers from food containers and packaging materials into the food. The potential toxic and carcinogenic properties of these coating compounds must be established, and radioactive tracers are uniquely suited for this purpose.

Specific Procedures

Isotope dilution. Chemicals added to foods or contaminants in foods (pesticides) which are available in a radiochemical form can be assayed for concentrations in the food, excreta from animal or man, or in the body tissues.

The alleged toxicity or carcinogenicity of these compounds can be more accurately evaluated from known concentration levels. In these situations the labeled compound is added to a food or other material (excreta or tissue) in which an unknown amount of the identical nonlabeled compound is present. The dilution of the labeled compound by the identical nonradioactive compound existing in the food or other material is determined after it is chemically separated from the medium in which it was suspended. By measurement of reduction in radioactivity caused by dilution of the labeled compound with the stable compound, the concentration of the toxicant or carcinogen present can be determined.

The reverse situation of the above can be employed where a known amount of unlabeled compound is added to a substance containing the same substance radioactively labeled; from this reduction in radioactivity, the original level of the compound in question is ascertained. This method is particularly useful where a mixture of labeled substances is present.

Neutron activation analysis. Some toxic compounds or carcinogens can be labeled as "unusual isotope or element" (for example, calcium 48) which in turn, by neutron activation,

is converted to the radioactive form (calcium 48 activated to calcium 49, the radioactive form). By means of a pulse height analyzer the radiation from this substance is compared with that from a known amount of a standard of the same "unusual element" used for the quantitation.

These procedures are described in detail by Rust in his excellent report relating to use of nuclear techniques in food toxicology and processing (*13*).

Radio release analysis of stable ions. A procedure has been developed by Richter and Gillespie (*14*) by which trace amounts of nonradioactive ions in biological materials can be measured. The general procedure is to pass the sample slowly through an ion exchange resin on which a previously chosen radioactive substance has been deposited. The ion for which concentration is being determined displaces the radioactive species stoichiometrically. The quantity displaced can be measured by counting radioactively the effluent as it comes off the bottom of the column. Concentrations of trace materials which are toxic or carcinogenic can be measured at levels as low as 0.2 ppm.

Other Indirect Procedures

The presence of metabolites in urine and feces extracts can be followed by chromatograms, starting with a labeled compound that is easily ingested and readily metabolized. The toxicity and carcinogenicity of metabolites can be followed by the nuclear techniques previously described. In such labeling procedures, micro methods are feasible and they have the advantage over conventional methods where excessive doses must be administered to measure the resulting metabolites. These procedures provide the means for acquiring data on absorption, localization, metabolism, and excretion, not heretofore attainable in gross measurements for toxicity and carcinogenicity.

Certain physical methods have been proposed as alternate rapid procedures for standard long-term biological evaluation of toxicants and carcinogens. For example, Lovelock and coworkers (*15*) have proposed that the toxicity or biological activity was attributable to the ability of toxicants and carcinogens to function as irreversible electron traps so that the normal transfer of electrons during oxidative phosphorylation was impaired. Hence, if such a concept is valid, any substance with a high electron absorption coefficient is potentially toxic, although to retain toxicity the substance must attain and remain active at an appropriate site within a living cell and irreversibly trap electrons there.

Carcinogenic activity may be a special illustration of this type of toxic action, since the recent observation by Allison and Lightbown (*16*) that the principal mechanism of carcinogenesis by different agents is the disturbance of mitochondrial electron transport. Lovelock and co-workers (*15*) have measured the electron absorption coefficients of cyclic (tri and tetra) aromatic hydrocarbons. It is assumed that those compounds with high electron absorption coefficients are more likely to be carcinogenic. Although the procedure just proposed is speculative and presents a most attractive explanation of biological activity, experimental verification as to its validity remains to be accomplished.

Need for Training and Research

There is a pressing need for more sophisticated methodology or refinement of current techniques to assess the potential carcinogenic or toxic effects of compounds which man encounters in his daily environment. To implement such training and research, programs for this development should be supported through funds provided certain universities and their departments. Many compounds, such as fungicides, foliar fertilizers, pesticides, food additives, and industrial chemicals, are potentially toxic or carcinogenic. Current Federal legislation has imposed strict requirements for decisions by food and drug regulatory groups as to the resolution of this problem.

A multidiscipline and a multidepartmental approach at various universities should advance knowledge and competence in the evaluations required for subliminal pharmacology and the associated assessment of carcinogenicity in major and minor environmental contaminants. Some initial steps have been taken toward establishing such basic centers for research and training in this field.

Summary

The increasing introduction of environmental chemicals which may be potentially toxic or carcinogenic necessitates the design of experiments to include simultaneous research on toxicity and carcinogenicity in animal studies. Because of their latent effect, many toxic or carcinogenic stress agents at low dosage levels may express only a weak subthreshold or subliminal pharmacological action which is difficult to detect by current methodology. Therefore, further development and refinement in methodology is imperative.

Comprehensive studies dealing with the route and rate of absorption, levels of storage in the tissues, and ultimate metabolic fate are required in order to elucidate the mechanism of carcinogenesis. Suggested procedures for these studies include evaluation of the effect of toxic or tumorigenic stressers on metabolic pathways by use of metabolic profiles, expansion of the use of the newer direct and indirect radioisotope and nuclear techniques, and other special physical methods.

A multidiscipline and multidepartmental approach at various universities toward training and research programs in subliminal pharmacology and the associated assessment of carcinogenicity in major and minor environmental contaminants is proposed.

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