MUTAGENICITY AND ITS TESTING

by
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Mutagenesis refers to those processes that cause changes in the genetic material (mutations) in individuals or cells, either spontaneously or by the actions of chemicals or radiation, whereby their successors differ in a consistent and heritable way from their predecessors. Although deleterious effects may not be manifested in certain circumstances for many generations, a proportion of mutagenetic changes will produce these effects in the offspring.

Just as the thalidomide disaster brought about an awareness of teratogenesis, a hitherto unrecognized toxic effect, so the dramatic developments in genetics, particularly at the molecular level, during the past two decades have evolved the new subject of mutagenesis or genetic toxicology. Here the hazard is not merely to the next generation, as in teratogenesis, but to all succeeding generations. Unlike teratogenesis, for which valid human evidence exists, chemical mutagens have yet to be clearly associated with specific human genetic disorders.

Although the burden of spontaneous genetic diseases involves about 2-4% of the population, the full extent of such diseases is unknown. At present, detection of any effects of a mutagenic chemical is unlikely because the background is noisy, an inherent time scale of generations operates and the probable exposure time to significant numbers of new chemicals is about one generation. Clearly, however, the kinds of genetic damage that can be brought about by chemicals in experimental systems are remarkably similar to those observed in spontaneous human disease. In one case, the biochemical defect in the rare sex-linked disease known as the Lesch-Nyhan syndrome has been exploited to form the basis of an *in vitro* mammalian cell test system for mutagens. Chromosomal anomalies are probably the most clearly definitive form of all human mutations, and some 60 different conditions have been described in foetuses surviving to birth. Similar chromosomal damage can be induced by chemicals in both *in vivo* and *in vitro* experimental systems.

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Several facts concerning human chromosomal anomalies are striking. For example, 5-10% of all human conceptions are estimated to be chromosomally abnormal. A high proportion of these defects are prenatally lethal and lead to spontaneous abortion, the exact frequency of which is difficult to estimate but probably lies between 15-20% of recognizable pregnancies. Finally, some 40% of spontaneous abortions are likely to have chromosomal abnormalities. However, this figure is influenced by the age of the foetus at abortion or miscarriage.

The chromosomal changes considered above involving segments, or even whole chromosomes, are now accepted as mutations in a practical genetic sense, even though the point of attack leading to the gross structural change may not be the DNA molecule and even when the genetic damage produced is so severe as to be lethal. In all cases, however, the operational genetic material received by the daughter cell is functionally inadequate. In some cases this situation is paradoxical because an excess of genetic material actually exists. What matters is the capacity of a chemical to bring about a heritable change in the genetic material by whatever means.

An understanding of recent developments in mutagenesis and their implications for carcinogenesis depends upon an appreciation of several basic facts concerning the molecular basis of heredity. The gene has long been postulated as the unit of genetic material, but a molecular basis for genetic processes was not available until the remarkable structure of deoxyribonucleic acid (DNA) and its significance was proposed by Watson & Crick in 1953. Their work stimulated an enormous amount of research into the molecular biology of cellular synthetic processes and genetic mechanisms. Now a gene can be defined in terms of the chemistry of DNA. DNA is a complex molecule in which sequences of four specific purine (adenine and guanine) and pyrimidine (thymine and cytosine) bases are linked across a double sugar-phosphate backbone to form a long, double helical structure. This structure has sequences of complementary base pairs (adenine-thymine or guanine-cytosine) running between the sugar-phosphate molecules which form the threads of the double helix. The crucial element of this ordered molecular arrangement is that the base sequence extending from one thread of the helix, taken in triplets, forms the genetic code. This coded information can be transcribed to a complex molecular messenger system consisting of

ribonucleic acid (mRNA) and transferred to other systems in the cell where synthesis of polypeptides can occur, each transcribed triplet coding for a specific amino acid.

A sequence of bases carrying the information necessary for the synthesis of a specific product. For example, an enzyme or a protein such as globin, is one unit of genetic material - a gene (in this case a structural gene). Other genes exert controlling functions. One DNA molecule is made up of sequences of many genes, all precisely ordered. Each DNA molecule is associated with complex enzyme systems of maintenance and repair, the whole linear arrangement forming the structure known as a chromosome. A further remarkable feature of the DNA complex is that it is not only concerned with the transcription of information via messenger RNA to cell synthesizing systems, but it can precisely replicate itself at cell division. In this way, an identical copy of the genetic information can be transferred to either daughter cells at somatic cell division (mitosis) or to future gametes at the more complex process of meiotic cell division which occurs when germ cells divide to produce ova and spermatozoa. The common ground between mutagenicity and carcinogenicity is that the interaction of chemicals with DNA produces the serious consequences. If a chemical has the capacity to covalently bind to DNA, it can result in a disturbance of the base sequence. If this upset is perpetuated in the post-replication DNA, the genetic information of a daughter cell will differ, sometimes dramatically so, from that of the parent cell. In other words the chemical interaction with the parent DNA has produced a mutation in one or both daughter cells.

One of the most significant advances in the understanding of how mutational events were related to carcinogenicity was the realization that it was not usually the administered chemical (the pre-carcinogen) that interacted with DNA but one of its metabolic products (the ultimate carcinogen). The study of the metabolic fate of chemicals in living systems now forms a major growth area in both carcinogenic and mutagenic research. The discovery that many of the key enzymes responsible for the biotransformation of pre-carcinogens to carcinogens could be isolated from cell extracts gave great impetus to these studies and also greatly improved testing systems. The particular relevance of these metabolic studies to the human situation lies in the fact that many of the chemicals that have been synthesized in recent times are

quite different from those already present in nature. Consequently, it is difficult to know how the metabolic systems that have been evolved to deal with substances likely to be encountered in natural circumstances will react to a totally new chemical situation: what may be crucial is that different animals may deal with a particular chemical by quite different metabolic pathways. When a particular chemical contains doubtful elements in its toxicity profile, the determination of exactly how this chemical is metabolized in the human is essential before decisions are finalized concerning its use.

Reference has been made to the DNA molecule and its sequences of two pairs of complementary bases linking the two deoxyribose sugar-phosphate longitudinal components of the double helix. These four different bases, taken three at a time, have 64 possibilities of forming triplets, and as proteins contain about 22 different amino acids, coding units are ample (codons) for polypeptide formation. The remarkable feature of this code is that it is exactly the same for all organisms - i.e. man and bacteria share the same code. The coding is described as degenerate because more than one codon exists for some amino acids. Some codons have an instructional function for the starting or stopping of the transcription process to messenger RNA. These facts are important for the understanding of one class of mutations: point mutations. The chemistry of DNA and its repair processes are such that one of the consequences of the binding of a chemical to the DNA is to effect an eventual exchange of bases at the region of the interaction, thus changing the coded information. Certain base changes in the codons of a number of amino acids can produce a termination codon. If this codon is within a polypeptide sequence, transcription will cease at that point. This effectively deletes an entire gene, whereas a change or deletion of one amino acid in a gene product may not be so serious. Mutational effects that are essentially confined to one gene are known as point mutations. To understand how mutations involving whole sequences of genes (i.e. chromosomal mutations) can occur, more information is necessary about the disposition of the chromosome in the cell during cell division.

A chromosome is the remarkable complex of the long, filamentous DNA molecule together with special protein and enzyme systems. As the evolutionary scale is ascended, the quantity of DNA per cell increases, as does the

relative mass of the associated proteins and enzyme systems. This increase in DNA is not only due to the presence of greater numbers of genes but also to the appearance, in a species-specific way, of long repetitive runs of bases between sequences of genes. In higher animals these redundant, nontranscribing sequences are relatively enormous. In humans the redundant DNA greatly exceeds the total of the transcribing DNA, i.e. the gene DNA. In simple organisms such as bacteria, the DNA consists essentially of a single gene string together with associated protein and enzyme systems, often in the form of a circular molecule free in the cell cytoplasm. Such a relatively simple arrangement is characteristic of all prokaryotic organisms. In all other higher organisms including humans (collectively, the eukaryotes), the DNA is arranged into a series of chromosomes. For example, the human has 46 chromosomes, each a complex arrangement of a coiled and supercoiled DNA molecule together with elaborate arrangements of special proteins, RNA and enzyme systems. All are segregated within the cell by a nuclear membrane except during phases of cell division. Each higher eukaryotic somatic cell carries a double set of genetic information, one from each parent. Thus, each chromosome is paired, with one exception: the sex chromosomes, the female X and the male Y chromosome. The combination XX produces the female and XY the male.

The normal process of growth and maintenance of bodily tissues is brought about by the multiplication, as needed, of the appropriate cell types. In this process of somatic cell division, each daughter cell receives an identical set of parental genes. Thus, the daughter cells receive the necessary information for them to function exactly as the parent cell. The process of DNA replication and subsequent separation into daughter cells is known as mitosis. During the normal functioning life of a cell, the chromosomes are present in an extended, filamentous form invisible in the optical microscope and even in the electron microscope unless special techniques are used. Messenger RNA is formed at appropriate loci where the DNA duplex splits to allow the building up of an mRNA molecule along a length of one strand of the DNA. Prior to mitosis, the production of mRNA ceases, and the duplex molecule of the DNA is systematically divided at each base pair and a complementary DNA half molecule is built up for each side of the duplex. Thus, two molecules of DNA are formed, each containing one half molecule of the parent DNA and a newly synthesized complementary half molecule.

Thus, two exact replicates of the original codon sequence are prepared for the daughter cells. After replication, the chromosomes undergo a remarkable process of looping and folding to condense down to highly compact linear paired bodies that are now easily visible with the optical microscope. These paired bodies are the daughter chromosomes, or chromatids, joined together by a special regional body known as a centromere. Once condensed, the pairs of chromatids are drawn to a central plane of the cell by a special paired body known as the spindle apparatus; one thread of the spindle is attached from opposite poles of the cell to each centromere. The function of the spindle apparatus is to pull one chromatid of each pair, the centromere splitting, to opposite poles of the cell. Thus, each half of the cell now contains a full complement of daughter chromosomes. These chromosomes are then enclosed in a nuclear membrane, and cell membranes are formed across the equator of the parent cell to divide it into two daughter cells, each now with a new nucleus.

Genetic mutations are produced by damage to, or interference with, the DNA of germ cells. These are groups of cells set aside early in the development of an embryo to be the precursors of the future gametes (ova or spermatozoa). Germ cell development or gametogenesis is quite distinct from somatic cell development into tissues. Each gamete possesses half the number of chromosomes (a haploid set) present in a somatic cell which, as has been mentioned, possesses a double set (a diploid set) of chromosomes, one from each parent. The division of germ cells to form gametes is a special process known as meiosis, which is more complex than mitosis. At germ cell division the chromosomes do not replicate but briefly assemble together in homologous pairs. After a complex interchange of genetic material, these homologous pairs are separated by the spindle apparatus so that each forming daughter cell receives one full set of chromosomes, the DNA of each chromosome now being derived in a complicated fashion from each parent of the parent cell. After separation, the daughter cells divide again by mitosis. Thus, in the full process of meiosis, each diploid germ cell produces four haploid daughter cells. These cells undergo quite different processes of development, depending upon whether they are destined to be ova or spermatozoa.

One of the commonest forms of mutation occurring in humans comes about because one thread of the spindle apparatus may fail to function correctly during the process of separation of the chromosomes in meiosis and draw its attached chromosome into the daughter cell. Thus one daughter cell will receive an extra chromosome by default and correspondingly, the other a deficit. This malfunctioning of the spindle apparatus, known as non-disjunction, is usually lethal unless one of the small chromosomes is involved. A non-disjunction involving chromosome 21 results in Down's syndrome or trisomy 21, one of the commonest of all genetic defects seen in humans.

Some indication has been given of the way in which point mutations and non-disjunctions can arise. However, the important matter of damage to individual chromosomes remains. This damage has been demonstrated in humans after exposure to toxic chemicals, as well as in humans with certain categories of genetic disease. The production of a discontinuity in both strands of the DNA of a chromatid can result in certain visible abnormalities in the condensed chromosomes at mitosis. These abnormalities can be observed by special techniques at the stage just before the spindle fibres separate the sets of chromosomes - the metaphase of mitosis (or meiosis). These abnormalities are best interpreted as resulting from processes that produce one or more complete breaks in a chromosome with, in many cases, a rejoining of the separate fragment, either to the same chromosome or to another chromosome. If the fragment, or fragments, remain free, the effect is usually lethal. If a rejoining occurs, the possibilities are numerous. The most important of these possibilities are translocations, inversions or deletions. In translocations, a segment is transferred from one chromosome to another. This transfer can have important genetic consequences as it can result in a deficiency or excess of chromosome material in a resultant zygote. Although usually lethal, survival may occur if the fragment is small but usually with deleterious consequences. Inversions result when a fragment of a chromosome is separated and rejoined to the same chromosome but from the other end, thus reversing the order of the genes. Deletion or deficiencies arise when two breaks occur in the same chromosome, the fragment between the breaks separates and the two free ends of the chromosome join. Some deletions are believed to be an important source of genetic damage; if small enough, they could resemble, in effect, point mutations.

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An extensive body of knowledge has been acquired concerning the experimental interaction of chemicals with DNA to produce mutations in many forms of life ranging from micro-organisms to plants to mammals. If the exposure situation were adequate, mutations induced in this way could, without doubt, also be produced in humans. Such mutations could indeed be occurring, but given the paucity of knowledge of the true extent of genetic diseases in the human population, at least a generation will likely pass before the damage caused by a chemical could be detected. In the meantime many governments have accepted that chemical mutagenesis could be a significant potential danger to human health, and that steps should be taken to prevent exposure to such chemicals. Although present concern about mutagenicity is undoubtedly influenced by the belief that mutagenicity and carcinogenicity are linked, concern should not diminish the need to consider mutagenicity as a danger in its own right.

Preventive measures depend upon agreed methods of identifying the mutagenic properties of a chemical. To this end a large number of test systems have been proposed. The basic problem is that mutagenic events are rare, (one in millions). Observations on tens of millions of bacteria are technically easy, but such numbers are out of the question when whole animals such as mice are considered. The most extensive data on the interactions of chemicals with DNA have been derived from bacteria, in particular Salmonella typhimurium and Escherichia coli. Various strains of these bacteria have been greatly modified to facilitate the chemical interaction with the bacterial DNA. A number of ingenious procedures have been devised so that of the tens of millions of bacteria exposed to a chemical, only the mutants are subsequently able to multiply and hence become visible on a test layer of growth medium. Simpler procedures have been developed for mammalian cells grown in tissue culture, but the handling of millions of cells is not so easy. Hence, to increase the probability of an interaction with DNA, large doses are used and observations are made in systems using hundreds of thousands of cells. In experiments involving the treatment of whole mammals (in vivo test systems), such large numbers of target cells are difficult to observe. The direct testing of point mutations in mice, for example, involves the observation of at least 30-40 thousand progeny before a statistically significant result can be expected.

The fact that no single test system so far developed can be relied upon to detect all possible chemical mutagens has to be accepted. At present the data base assembled for each test system shows that each of them has "blind spots" for certain chemicals or classes of chemicals. Thus, the argument has been raised that a series of tests must be used, and much international effort has gone into considerations of what should constitute the most appropriate "battery" of tests and what general strategy should be adopted. Support is strong for a combination of selected test procedures designed to probe the hereditary machinery at increasing levels of complexity and also to take into account some of the barriers that a chemical must pass to reach the DNA in mammalian cells. One such battery of tests has been published by the Department of Health and Social Security of the United Kingdom (see bibliography) in their guidelines for the testing of chemicals for mutagenicity. The recommended tests in the "basic package" of four test screening procedures for mutagenic properties of chemicals are as follows:

Test 1: A test designed to demonstrate the induction of point mutations (base pair change and frameshift mutations) in established bacterial test systems such as Salmonella typhimurium, Escherichia coli or Bacillus subtilis. The tests should be conducted with and without the use of appropriate systems.

Test 2: A test designed to demonstrate the production of chromosome damage in appropriate mammalian cells grown in vitro with and without the use of appropriate metabolic activation systems.

Test 3: The induction of mutations in mammalian cells grown in vitro or a test designed to induce recessive lethals in Drosophilia melanogaster.

Test 4: A test designed to demonstrate the induction of chromosomal damage in the intact animal using either the micronucleus test or, preferably, the metaphase analysis of bone marrow or other proliferative cells; or the induction of germ cell damage as demonstrated by the dominant-lethal test in the rat or mouse.

The committee that drew up these guidelines suggested "that this recommended basic package screening procedure should detect, if fully and properly exploited, the great majority of the potential mutagens among chemicals