product), because such a product usually has to be incorporated into the diet at levels as high as 20 per cent to 60 per cent at the expense of a corresponding nutrient (e.g. modified versus unmodified starch; single cell protein versus soybean meal).

Variations in the use patterns of industrial and agricultural chemicals throughout the world preclude harmonization by OECD on one list of dietary contaminants. Notwithstanding this fact, common dietary constituents which are known to influence carcinogenesis (e.g. antioxidants, unsaturated fatty acids, selenium) should not be present in interfering concentrations. The potential impact of several common dietary contaminants upon carcinogenicity assessment necessitates that special attention be given to their presence. In this respect, substances of concern include pesticide residues, chlorinated and polycyclic aromatic hydrocarbons, oestrogens, heavy metals, nitrosamines and mycotoxins.

In addition, periodic analysis of the basal diet may be carried out by the testing laboratory for both nutrients and unintentional contaminants, including carcinogens. The results from such analyses should be retained and included in the final report on each test substance.

When the test substance is administered in the water or food, stability tests are essential. Properly conducted stability and homogeneity tests, prior to the chronic study, should be used to establish the frequency of diet preparation and monitoring required.

When diets are sterilised, the effects of such procedures on the test substance and dietary constituents should be known. Appropriate adjustments to nutrient levels should be carried out. The effect of chemical sterilants (e.g. ethylene oxide) on the bioassay should be ascertained.

During carcinogenicity tests, investigators should be aware of potential contaminants in the water used. Although water approved for human consumption is generally satisfactory, the investigator should ascertain the data available on the components in the water supply.

• <u>Test conditions</u>

Dose levels and frequency of exposure

For risk assessment purposes, at least three dose levels should be used, in addition to the concurrent control group. The highest dose level should be sufficiently high to elicit signs of minimal toxicity without substantially altering the normal life span due to effects other than tumours. Signs of toxicity are those that may be indicated by alterations in certain serum enzyme levels or slight depression of body weight gain (less than 10 per cent). For a diet-mixture, the highest concentration should not exceed 5 per cent with the exception of nutrients (see section on diet).

The lowest dose should not interfere with normal growth, development, and longevity of the animal; and it must not otherwise cause any indication of toxicity. In general, this should not be lower than 10 per cent of the high dose.

The intermediate dose(s) should be established in a mid-range between the high and low doses, depending upon the toxicokinetic properties of the chemical, if known.

The selection of these dose levels should be based on existing data, preferably on the results of subchronic studies.

Frequency of exposure normally is daily but may vary according to the route chosen. If the chemical is administered in the drinking water or mixed in the diet, it should be continously available. The frequency of administration may be adjusted according to the toxicokinetic profile of the test substance.

Controls

A concurrent control group which is identical in every respect to the exposed groups, except for exposure to the test substance, should be used.

In special circumstances, such as in inhalation studies involving aerosols or the use of an emulsifier of uncharacterised biological activity in oral studies, a concurrent negative control group should be utilised.

The negative control group is treated in the same manner as all other test animals except that this control group should not be exposed to the test substance or any vehicle.

• Route of administration

The three main routes of administration are oral, dermal and inhalation. The choice of the route of administration depends upon the physical and chemical characteristics of the test substance and the form typifying exposure in humans.

In general, the frequency of exposure may vary according to the route and type of administration chosen, and should be adjusted according to the toxicokinetic profile of the test substance, if available.

Oral studies

Provided it can be shown that the test substance is absorbed from the gastro-intestinal tract, the oral route of administration is preferred. The animals should receive the test substance in their diet, dissolved in drinking water, or given by gavage for the length of time specified in the section on duration of study, below. If the test substance is administered in the drinking water or mixed in the diet, exposure is continuous. If the test substance is mixed in the diet, the highest concentration to be tested should not exceed 5 per cent, with the exception of nutrients (see section on diet). Ideally, daily dosing on a 7-day per week basis should be used, because dosing on a 5-day per week basis may permit recovery or withdrawal toxicity in the non-dosing period and thus affect the result and subsequent evaluation. However, based primarily on practical considerations, dosing on a 5-day per week basis is considered to be acceptable.

Dermal studies

Cutaneous exposure may be selected to simulate a main route of human exposure and as a model system for induction of skin lesions. Special studies designed for induction of skin tumours are not presented in this Guideline.

Inhalation studies

This Guideline provides some detail on inhalation studies since the technical problems involved are of greater complexity than for the other types of assay. It is recognised, however, that intratracheal instillation may constitute a valid alternative in specific situations.

Long-term exposures are usually patterned on projected industrial experience, giving the animals a daily exposure of 6 hours after equilibration of chamber concentrations, for 5 days a week (intermittent exposure), or on a possible environmental exposure, with 22-24 hours of exposure per day, 7 days a week (continuous exposure), with about an hour for feeding the animals and maintaining the chambers. In both cases, the animals are usually exposed to a fixed concentration of test materials. A major difference to consider between intermittent and continuous exposure is that with the former there is a 17-18 hour period in which animals may recover from the effects of each daily exposure, and an even longer recovery period during weekends.

The choice of intermittent or continuous exposure depends on the objectives of the study and on the human experience that is to be simulated. However, certain technical difficulties must be considered. For example, the advantages of continuous exposure for simulating environmental conditions may be offset by the necessity of watering and feeding during exposure, and by the need for more complicated (and reliable) aerosol and vapour generation and monitoring techniques.

Exposure chambers

The animals should be tested in inhalation chambers designed to sustain a dynamic flow of 12 to 15 air changes per hour to assure an oxygen content of about 19 per cent and an evenly distributed atmosphere. Control and exposure chambers should be identical in construction and design to ensure exposure conditions comparable in all respects except for the exposures to the test substances. Slight negative pressure inside the chamber is generally maintained to prevent leakage of the test substance into the surrounding area. The chambers should minimise the crowding of test animals. As a general rule to ensure the stability of the chamber atmosphere, the total volume of the test animals should not exceed 5 per cent of the volume of the chamber.

Physical measurements

The following measurements should be taken with care to avoid major fluctuations in the air concentrations or major discrepancies in the operations of the chambers:

(a) Air flow: the rate of air flow through the chamber should preferably be monitored continuously.

(b) Chamber concentrations: during the exposure period the actual concentrations of the test substance should be held as constant as practicable.

(c) Temperature and humidity: for rodents, the temperature should be maintained at 22°C (± 2°) and the humidity within the chamber at 30-70 per cent, except when water is used to suspend the test substance in the chamber's atmosphere. Preferably both should be monitored continuously.

(d) Particle size measurements: particle size distribution should be made on chamber atmospheres involving liquid or solid aerosols. The aerosol particles should be of respirable size for the test animal used. Samples of the chamber atmospheres should be taken at the breathing level of the animals. The air sample should be representative of the distribution of the particles to which the animals are exposed and should account for, on a gravimetric basis, all of the suspended aerosol even when much of the aerosol is not respirable. The size analyses should be carried out frequently during the development of the generating system to ensure the stability of the aerosol and only as often thereafter during the exposures as necessary to determine adequately the consistency of the particle distributions to which the animals had been exposed.

• Duration of study

It is necessary that the duration of a carcinogenicity test comprise the majority of the normal life span of the animals to be used. It has been suggested that the duration of the study should be for the entire lifetime of all animals. However, a few animals may greatly exceed the average lifetime, and the duration of the study may be unnecessarily extended and complicate the conduct and evaluation of the study. Rather, a finite period covering the majority of the expected life span of the strain is preferred since the probability is high that, for the great majority of chemicals, induced tumours will occur within such an observation period.

The following guidelines are recommended:

(a) Generally, the termination of the study should be at 18 months for mice and hamsters and 24 months for rats; however, for certain strains of animals with greater longevity and/or low spontaneous tumour rate, termination should be at 24 months for mice and hamsters and at 30 months for rats.

(b) However, termination of the study is acceptable when the number of survivors of the lower doses or control group reaches 25 per cent. For the purpose of terminating the study in which there is an apparent sex difference in response, each sex should be considered a separate study. In the case where only the high dose group dies prematurely for obvious reasons of toxicity, this should not trigger termination.

In order for a negative test to be acceptable, it should meet the following criteria:

(1) No more than 10 per cent of any group is lost due to autolysis, cannibalism, or management problems.

(2) Survival of all groups is no less than 50 per cent at 18 months for mice and hamsters and at 24 months for rats.

3. DATA AND REPORTING

- Observations

A careful clinical examination should be made at least once each day. Additional observations should be made daily with appropriate actions taken to minimise loss of animals to the study, e.g. necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals. Careful observations should be performed to detect onset and progression of all toxic effects as well as to minimise loss due to diseases, autolysis, or cannibalism.

Clinical signs and mortality should be recorded for all animals. Special attention must be paid to tumour development; the time of onset, location, dimensions, appearance and progression of each grossly visible or palpable tumour should be recorded.

Body weights should be recorded individually for all animals once a week during the first 13 weeks of the test period and at least once every 4 weeks thereafter.

Food intake should be determined weekly during the first 13 weeks of the study and then at approximately three-month intervals unless health status or body weight changes dictate otherwise.

If clinical observations suggest a deterioration in health of the animals during the study, a differential blood count of the affected animals should be performed.

At 12 months, 18 months and prior to sacrifice, a blood smear is obtained for all animals. A differential blood count is performed on samples of those animals in the highest dosage group and the controls. If these data, particularly those obtained prior to sacrifice, or data from the pathological examination indicate a need, then differential blood counts are performed for the next lower group(s) as well.

- Pathology

The pathological examination, macroscopy as well as microscopy, is often the cornerstone of the carcinogenicity study. These aspects should therefore receive all necessary attention and should be described and reported in detail, including diagnosis.

Necropsy procedures

A well-performed gross necropsy may provide optimal information for microscopic examination and may in certain cases facilitate a more restrictive microscopic examination. An inadequate gross necropsy cannot be replaced by microscopic examination no matter how well-performed. Gross necropsy should be carried out under the guidance of a trained laboratory animal pathologist.

Complete gross examination should be done in all animals including those which died during the experiment or were killed in moribund conditions. Prior to sacrifice of all animals, samples of blood should be collected from all animals for differential blood counts. All grossly visible tumours or lesions suspected of being tumours should be preserved. An attempt should be made to correlate gross observations with the microscopic findings.

All organs and tissues of all animals should be preserved for microscopic examination. This usually involves samples of the following organs and tissues: brain, pituitary, thyroid (including parathyroid), thymus, lungs (including trachea), heart, salivary glands, liver, spleen, kidneys, adrenals, oesophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, uterus, urinary bladder, lymph nodes, pancreas, gonads, accessary genital organs, female mammary gland, skin, musculature, peripheral nerve, spinal cord, sternum with bone marrow

and femur (including joint) and eyes. Although inflation of lungs and urinary bladder with a fixative is the optimal way to preserve these tissues, the inflation of the lungs in inhalation studies is a necessary requirement for appropriate histopathological examination. In special studies such as inhalation studies, the entire respiratory tract should be preserved, including nasal cavity, pharynx, and larynx.

If other clinical examinations are carried out, the information obtained from these procedures should be available before microscopic examination, since it may give significant guidance to the pathologist.

Histopathology

Microscopic examination is as essential as a gross necropsy to the proper conduct of a carcinogenicity study. While an all-inclusive examination of all tissues is perhaps theoretically desirable, the resource limitations dictate a more selective approach. As a minimum, the following is recommended for microscopic examinations:

(a) All grossly visible tumours or lesions suspected of being tumours in all groups;

(b) All preserved organs and tissues of: (a) all animals that die or are killed during the study, and (b) animals of the highest dose group and controls. While notation should be made of all histopathological lesions, those which were hyperplastic, pre-neoplastic and/or neoplastic should be fully described;

(c) If a significant difference is observed in hyperplastic, pre-neoplastic or neoplastic lesions between the highest dose and control groups, microscopic examination should be made on that particular organ or tissue of all animals in the study;

Note: in the event that the survival of the high dose group is substantially less than the control, then the next lower dose group should be examined as described previously (see above).

(d) In case the results of the experiment give evidence for substantial alteration of the animals' normal longevity or the induction of effects that might affect a neoplastic response, the next lower dose level should be examined as described above; and

(e) The incidence of tumours and other suspect lesions normally occurring in the strain of animals used (under the same laboratory conditions – i.e. historical control) is desirable for assessing the significance of changes observed in exposed animals.

- Test report

Each test report must identify:

- the laboratory where the test was performed by name and address;
- the inclusive dates of the test; and
- the individual responsible for the conduct and report of the study.

The test report must include all information necessary to provide a complete and accurate description of the test procedures and an evaluation of the results. It should contain a summary of the data, an analysis of the data, and a statement of the conclusions drawn from the analysis. The summary must highlight data or observations and any deviations from control data which may be indicative of toxic effects including hyperplasia, pre-neoplasia, or neoplasia.

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