Establishment of timetables for the phasing out of animal experiments for cosmetics

Carcinogenicity

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General considerations

Substances are defined as carcinogenic if they induce tumours (benign or malignant), increase their incidence or malignancy, or shorten the time of tumour occurrence when they are inhaled, ingested, dermally applied, or injected. The process of carcinogenesis is now recognised as resulting from the transition of normal cells into cancer cells via a sequence of stages and complex biological interactions. The modeling of such complex adverse effects cannot be accomplished at present by the use of non-animal tests. It should be noted that a well performed carcinogenicity bioassay in animals will in many situations also be a chronic toxicity assay, since the two endpoints can be combined in the guidelines for animal testing.

Since the induction of cancer involves genetic alterations which can be induced directly or indirectly, carcinogens have conventionally been divided into two categories according to their presumed mode of action: genotoxic carcinogens (= initiators) and non-genotoxic carcinogens (epigenetic carcinogens = promoters). Most potent mutagens are also carcinogens in animal experiments. Information of genotoxic carcinogenic potential of a substance may be obtained from the mutagenicity/genotoxicity studies. Two in vitro tests, cell transformation and gap junction intercellular communication (GJIC) have been proposed as tests that may provide information on possible non-genotoxic, as well as, genotoxic carcinogens. In
cases where the *in vitro* short-term tests suggest possible carcinogenic potential, carcinogenicity bioassay in animals will be useful to determine potency and target organs.

It is generally accepted that carcinogenesis is a multi-step process strongly influenced by factors such as age, diet, environment, hormonal balance, etc. The complexity of this process makes it not only difficult to extrapolate findings in animals to humans, but also difficult to develop *in vitro* alternative test models. Phases which play a role in the development of tumours are initiation, promotion and progression. It seems to be very difficult to build in all these phases a single *in vitro* alternative model.

A large number of systems and models dedicated to the prediction of carcinogenicity have been developed (QSARs and SARs). It has been demonstrated that carcinogenicity is generally only poorly predicted, and the best models tend to be those that can integrate mechanism-based reasoning with biological data (M. Cronin et al, Environ Health Perspective, 111, 1392-1402, 2003)
1. Inventory of the alternative methods currently available

Cell transformation assay

B21/not formally validated yet

Short description, scientific relevance and purpose
Mammalian cell culture systems may be used to detect phenotypic changes in vitro induced by chemical substances associated with malignant transformation in vivo. Widely used cells include SHE, C3H10T1/2 and Balb/3T3, and the tests rely on changes in cell colony morphology and monolayer focus formation. Less widely used systems exist which detect other physiological or morphological changes in cells following exposure to carcinogenic chemicals. Cytotoxicity may be determined by measuring the effect of the test material on colony-forming abilities (cloning efficiency) or growth rates of the cultures.

Developer of the method
Low pH SHE: LeBoeuf RA (1987)
Balb/3T3: Aaronson SA and Todaro GJ. (1968)
C3H10T1/2: Mondal S. and Heidelberger C. (1970)

Known users
Academics. Pharmaceutical and cosmetic industry in screening purposes only.

Status of the validation and standardisation
It is considered by the European Community, although not formally validated. The OECD is working on the rewriting of a detailed review paper (DRP31) aiming at reviewing all the available data on the 3 main protocols for cell transformation assays. This should be circulating by the first quarter of 2004. Data are available today after different “validation studies” done in the past. After comments, a decision will be made concerning the writing of a specific OECD Test Guideline. This ongoing work should progress in the coming 3 years. Depending on the results and conclusions of the DRP31 process, the need for specific “validation” study could be discussed.

Field and limitations of application
The in vitro test endpoints have been established based on induction of tumorigenicity. Some of the test systems are capable of detecting tumour promoters. Some cell types and substances may require an appropriate external metabolic activation system. When primary cell types are used that possess intrinsic metabolic activity, additional metabolic activation is not used. The scoring of transformed colonies and foci may require some training and experience.

Recommendations of use in the view of animal replacement
None of the in vitro test endpoints has an established mechanistic link with cancer.

Ongoing development
Protocol standardisation
References

Gap junction intercellular communication (GJIC)

Short description of the method
Gap junction intercellular communication (GJIC) is the intercellular exchange of low molecular weight molecules (less than 1000-1500 D) through gap junction channels between adjacent cells, and has been found to be of importance in regulation of cell growth and differentiation. Dysfunction in this type of communication has been observed to result in abnormal cell growth and behaviour, and associated with several human pathological conditions. Structure activity studies have shown relationship between the ability of substances to inhibit GJIC, and to induce tumours in rodents, but not between inhibition of GJIC and genotoxic activity. This suggests that inhibition of GJIC is involved in non-genotoxic cancer induction, and a candidate endpoint in screening assays for identification of non-genotoxic carcinogens and tumour promoters not detected by conventional genetic toxicology tests.

Developer of the method
Yotti LP. et al (1979), Murray AW. and Fitzgerald DJ (1979)

Known users
Academics

Status of the validation and standardisation
No formal validation of the method has been performed. Several methods exist for the determination of GJIC in different cell types.

Field and limitation of application
Method for identification and characterization of cancer causing substances without genotoxic activity.

Recommendations of use in the view of animal replacement

Ongoing development
Work is ongoing to determine:
- Molecular mechanisms involved in regulation of GJIC
- Role of altered GJIC in human diseases
- Importance of substances with adverse effect on GJIC in relation to human diseases including cancer
- Supplemental role of GJIC in addition to other assay end points
References


3. Reduction / refinement

Since no replacement methods are to date available in the area of carcinogenicity, we considered also reduction and refinement methods. In all transgenic models described below a limited number of animals 20 – 25 animals/sex/treatment group can be used. Another advantage is that duration of the experiment in transgenic models is 6 – 9 months instead of a two years bioassay. However, in addition wild type animals should be included in test battery to demonstrate that no genetic drift may affect interpretation of the results.

Transgenic mouse models

The transgenic mouse lines were chosen with the objective of developing short-term assay in vivo that could be used to differentiate carcinogenic from non-carcinogenic chemicals and mutagenic from non-mutagenic carcinogens. Mouse with well-defined genetic alteration resulting in over-expression or inactivation of a gene intrinsic to carcinogenesis, but insufficient alone for neoplastic conversion, appear to offer susceptible targets for carcinogenic chemicals.

Mainly three mouse models are considered of a greatest potential usefulness: Tg.AC, heterozygous (+/-) p53-deficient, and TgHras 2 lines.

Tumour genes altered in these mice have been often reported to be mutated and/or amplified (Ha-ras) or mutated and/or lost (p53) in both human and rodent tumours.

As general consideration, it has to be kept in mind that the genetic backgrounds of these 3 models are different, which undoubtedly influences responses to carcinogens.

Tg.AC mice

These animals carry a v-Ha-ras oncogene fused to the promoter of the zeta globin gene (Leder et al 1990). The v-Ha-ras transgene has point mutations in codon 12 and 59 and the site of integration of the transgene confers on these mice the characteristic of genetically initiated skin as a target for tumorigenesis.

The Tg.AC model is thus analogous to the classical, widely studied model for two stage carcinogenicity in mouse skin (Boutwell, 1964).

The presence of the v-Ha-ras transgene in Tg.AC mice obviates the obligatory exposure to initiating agents that was necessary in the earlier model of carcinogenesis.

The transgene is not expressed constitutively in skin of the Tg.AC mice, so untreated skin is indistinguishable from the skin of the wild-type parent strain.

Repetitive dermal applications of recognised mouse skin tumour promoters to untreated skin of Tg.AC mice result in a dose-related development of benign squamous-cell papilloma which can progress to malignancy.

The Tg.AC mice line is useful to differentiate carcinogens from non-carcinogens but it cannot be used to distinguish genotoxic complete carcinogens from agents that have only tumour promoting capability.

It has been suggested in the “Workshop on the utility of transgenic assay for risk assessment” (Washington, DC, February 2003) organised by ILSI-HESI Alternative to Carcinogenicity Testing Committee, that Tg.AC model is the second most
commonly requested assay and is considered to be appropriate for evaluation of products intended for dermal application.

**Heterozygous p53+/- deficient mice**
Mice that are heterozygous for the suppressor gene p53 are at increased risk for tumour development. The heterozygous p53-deficient mice used in rapid (26-week exposure) studies for the identification of mutagenic carcinogens have one functional wild-type allele and one inactivated null allele and the mice remain generally free of sporadic neoplastic disease during short-term studies. Inactivation of the remaining wild-type p53 allele by mutation or loss due to exposure to a mutagenic carcinogen would be expected to give a selective growth advantage to clonally derived neoplasia and result in shortened tumour latency. Positive controls need to be included in the test battery.

It has been reported in the “Workshop on the utility of transgenic assay for risk assessment” (Washington, DC, February 2003) organised by ILSI-HESI Alternative to Carcinogenicity Testing Committee, that the p53 +/- assay is the most commonly requested assay and it is also considered appropriate in providing data for regulatory purposes.

**CBF1-Tg-Hras2 mice**
CBF1-Tg-Hras2 mice carry five or six copies of a human c-Ha-ras proto-oncogene which is expressed both in spontaneous tumours and normal tissue

It has been reported in the “Workshop on the utility of transgenic assay for risk assessment” (Washington, DC, February 2003) organised By ILSI HESI Alternative to Carcinogenicity Testing Committee, that Tg-H-ras2 model is considered appropriate for evaluation of both genotoxic and non-genotoxic compounds even if further evaluation using additional non-direct acting agents is suggested. Positive controls need to be included in the test battery.

**Recommendations of use in the view of animal replacement**
p53-deficient, and TgHras have been adopted for the evaluation of carcinogenic potential of pharmaceuticals to replace a mouse life time assay. Evaluation of skin mutation and/or neoplasia in dermally exposed transgenic mice (e.g. Tg.AC, p53) may be relevant after further validation (3-4 years) and can be used in the mechanistic evaluation.
References

3. Final Comments

The process of carcinogenesis is recognised as resulting from a sequence of stages and complex biological interactions. The modeling of such complex adverse effects cannot be accomplished at present by the use of non-animal tests.

The experts suggested that the carcinogenic potential of a substance could be detected by a combination of the existing *in vitro* genotoxicity tests, cell transformation assays and gap junction intracellular communication assay (GJIC). Although several *in vitro* tests exist to detect the genotoxic potential of a compound, they present crucial limitations as absence of metabolic capacity, as well as the use of cell lines not relevant to predict the endpoint at target organ (see also chapter on “Genotoxicity and Mutagenicity”). Moreover, non-genotoxic carcinogens act through a variety of mechanisms that are problematic to predict and to detect. As a general limitation, cancer is a long-term process that is difficult to mimic with relatively short-term *in vitro* tests.

The carcinogenicity bioassays are rarely used for cosmetics but the bioassay is still needed to determine potency and target organ of a carcinogenic compound.

The opinion of the experts is that a battery of *in vitro* short-term tests will not be sufficient to fully replace the carcinogenicity bioassay in rodent in the next ten years.

The experts also suggested that *in vivo* assay with transgenic animals may provide important reduction and refinement in animal use.

In conclusion, taking into consideration the present state of the art of the carcinogenicity tests, the experts were unable to suggest a deadline for full replacement.
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* This table estimates the time needed to achieve ESAC endorsement for individual alternative tests assuming optimal conditions. It does not indicate the time needed to achieve full replacement of the animal test, nor does it include the time needed to achieve regulatory acceptance. “Optimal conditions” means that all necessary resources, for example technical, human, financial and coordination, are met at all times in the process and that the studies undertaken have successful outcomes.