Executive summary

Request for advice regarding new assay method

Chemical substances capable of modifying the body's genetic material (DNA) possess genotoxic properties. Mutations are irreversible changes to DNA. Such changes can affect any of the various types of cells in the body, and can lead to cancer. In addition, mutations in germ cells can also be transmitted to future generations. Assays for genotoxic properties in chemical substances form the basis for measures aimed at reducing these risks.

Genotoxicity can be established using a variety of assays. Mutagenicity assays are particularly important, as they can identify changes in DNA. Using in vivo assays, experimental animals are exposed to the substance under investigation. In vitro assays involve the use of cells or microorganisms. If in vitro and in vivo assays produce contradictory results, then more weight is usually given to the experimental animal results, since these organisms more closely resemble human beings. Indirect evidence is also taken into account when reaching a verdict with regard to genotoxicity. This can relate to (in vitro or in vivo) assays of the formation of DNA adducts (covalent bonding to DNA). In this advisory report, the in vivo data is similarly conclusive, therefore 'genotoxic' (or 'non-genotoxic') really means 'genotoxic in vivo' (or 'non-genotoxic in vivo').

Mutagenicity can manifest itself in the form of chromosomal abnormalities and gene mutations. These occur if DNA repair has not taken place prior to cell division. There are standardised, practical in vitro genotoxicity assays for chromosomal abnor-

malities, gene mutations, and DNA repair. The first two are mutagenicity assays, while the latter is used to determine indirectly whether or not a substance is genotoxic. Furthermore, these assays have in vivo counterparts, which are used to investigate chromosomal abnormalities and DNA repair. However, there is as yet no standardised, practical in vivo assay for gene mutations.

Nevertheless, new in vivo assays have been developed to identify substances capable of causing gene mutations. The new assays detect gene mutations using reporter genes. This means that the assay focuses on gene mutations in a particular gene (where a genetic characteristic is encoded), rather than on the entire DNA content of the cell. The reporter genes are either endogenous in origin (i.e. present in the natural situation) or transgenic (i.e. obtained from another species and introduced into the endogenous DNA via genetic modification).

The Minister of Health, Welfare and Sport has asked the Health Council of the Netherlands whether these new gene mutation assays with animals are suitable for use in the routine safety testing of chemical substances. A Health Council committee has assessed the question of whether such assays can be used to fill the above-mentioned gap in the safety testing of substances. The Committee consulted several Dutch and foreign experts from outside the Health Council while preparing its advisory report.

The Committee's procedures

In order to assess whether mutagenicity assays using reporter genes in experimental animals deliver reliable results, the Committee has compared results from the new assays with the conclusion derived from the previously described classical genotoxicity studies. Where the results are in agreement, this indicates the new assay's ability to determine whether or not a substance is genotoxic.

The Committee has restricted itself to verdicts on assays in experimental animals, using two different transgenic reporter genes. This is because these are the only assays in which the published test results are derived from a sufficiently large number of substances to enable sound conclusions to be drawn concerning assay characteristics such as sensitivity (chance of a genotoxic substance achieving a positive score) and specificity (chance that a non-genotoxic substance will achieve a negative score).

Published assay results derived from a sufficiently large number of substances are only available via Medline for the following experimental animals with transgenic reporter genes: Big Blue® rat and mouse strains, and the Muta™Mouse mouse strain. Assay results are available for a total of 64 genotoxic and non-genotoxic substances. However, the assay procedures showed considerable variation. They differ, for instance, in terms of the level and duration of exposure, and in terms of the organs analysed.

Verdict concerning the reliability of the new assays

On the basis of the assay results, the Committee concludes that assays using reporter genes in the selected rat and mouse strains are, in theory, suitable for the investigation of substances' ability to cause gene mutations. However, the assays are not yet sufficiently reliable for routine use. Firstly, the data cannot be used to derive a standard protocol, setting out broad outlines of the way in which the assay must be performed. Furthermore, while the assay characteristics sensitivity and specificity are high, support for the figures in question is inadequate. Therefore, the figures are provisional.

This is because the substances on which the figures are based are not representative. Relatively few non-genotoxic substances have been tested. In addition, within the group of substances designated as genotoxic, there is a preponderance of highly genotoxic substances. Accordingly, the Committee is only able to assign an indicative value to calculated assay characteristics such as sensitivity and specificity. Furthermore, the reproducibility of positive and negative results has been insufficiently investigated.

The assay characteristics give the Committee no reason to suppose that the above-mentioned rat and mouse strains differ from one another in terms of their suitability for distinguishing substances which are genotoxic from those that are not.

Both false negative and false positive results have been obtained. The false negative results may have been generated by the way in which the assay is performed, for instance, either the exposure time or expression time may be too short. Alternatively, if the method's degree of sensitivity is relatively low, this might account for these findings. Both explanations are indicative of failure on the part of the new assay.

In the case of false positive scores, the Committee feels that these can be explained in terms of deficiencies in the assay methods which were used as a standard. The new assay systems make it possible to determine whether substances are capable of inducing gene mutations in vivo. This is not possible using the methods with which they are being compared. The Committee therefore assumes that the substances in question are indeed genotoxic, but they are not identified as such by the usual in vivo assays. It recommends that, while a positive score using the new assay systems should provisionally be considered an indication of genotoxic properties, a negative score cannot be taken as proof that the substance in question has no such properties.

The false negative scores obtained for the reproductive organs form a separate case. The assay systems are unable to test substances for mutagenicity in germ cells at certain stages of maturation. There is a technical reason for this. When using reporter genes, it is not possible to demonstrate mutagenic action in the stages following meiosis (reduction division). Substances which are only mutagenic in these stages therefore score negative in germ cells.

Verdict regarding viability in routine safety testing

In vivo assay systems using transgenic reporter genes are highly flexible. They differ from the existing, standardised in vivo assay systems in that, theoretically, cells in any type of organ can be investigated. However, germ cells at an advanced stage of development in the reproductive system are an exception. This permits a tailor-made approach, in which the assay's design can be attuned to previous toxicological findings with regard to the substance in question. This reduces the risk of false negative results. However, the term 'routine use' presupposes the existence of a standard protocol. The Committee feels that the available data does not support the drafting of a protocol of this kind at the present time.

The Committee concludes that it is not (yet) appropriate to standardise the use of assay systems based on the transgenic animals referred to above. However, the Committee does feel that the situation is sufficiently well understood for the use of these assay systems to be permitted on an ad hoc basis, provided that the test design is well substantiated. The procedure must therefore be geared to existing knowledge concerning the toxicity of the substance to be investigated. Aside from germ cells at an advanced stage of development, cells from any type of organ or tissue can be investigated.

Recommendations for further research

We must wait and see whether or not it will ultimately be possible to perform the assays with greater reliability and in accordance with a standard protocol or, in any event, with smaller procedural variations. The Committee makes some suggestions regarding an assay design which can bring the realisation of that objective closer. Initially, given components of the protocol should at least be established, e.g. which organs should be analysed in association with which exposure route. Not only does this diminish the differences in assay design to some degree, it also makes the protocol applicable to a wide range of substances whose safety has not been fully investigated, if at all. In the case of substances for which data is available that might open up additional avenues in terms of assay design, it should remain possible for the time being to depart from this in order to better attune assay design with the data in question. For the time being, therefore, the procedure used should always be properly explained by the researcher. The Committee believes that the protocol can best be developed within the framework of the OECD. This is because the results of assays carried out in accordance with OECD protocols are recognised by all members of the OECD.

It is also vital that more substances be assayed, in order to increase the reliability of the assay systems. In this connection, the Committee is considering substances which

have been shown to be mildly genotoxic. They are also considering the use of substances which have been identified as being non-genotoxic. This is because these two categories lag far behind in terms of numbers.

Recommendations for inclusion in safety testing

In vivo assays using transgenic reporter genes can demonstrate their viability as part of the genotoxicity assaying currently required by the EU. The EU requires a dossier containing standard toxicological data supplied by the manufacturer or the party which uses the substance for commercial purposes or which is placing it on the market. In the genotoxicity section, the results are requested of in vitro and in vivo assays which have been carried out step by step. In addition to production volume, it is the use to which the substance in question is to be put (e.g. crop protection) which determines exactly which genotoxicity assays are permitted or required in each of the various steps.

Despite these differences in terms of testing strategy, the Committee believes that the possibility of using the new assay systems across all policy areas involving substance regulation to be worthy of consideration. The assay is already permitted in a few of these areas, provided that there is a well-supported assay design. The Committee recommends that the assay be approved for use in the remaining areas, subject to the same conditions.

While the assay is a valuable addition to the current testing repertoire, it is laborious and time-consuming in comparison to the established assays. Furthermore, there is no standardisation, which is a major precondition for validation. The Committee therefore feels that, for the time being, it is suitable as an ad hoc supplement to the current assays.