Identification of Neurotoxic Chemicals in Cell Cultures

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Summary
In order to identify the neurotoxic potential of drugs or chemicals in vitro, a combination of different in vitro cell culture tests is required. Depending on the type and use of a given test compound, a sequential exposure of freshly isolated and cultured hepatocytes and chicken brain cells is suitable. In order to find out more about the stability of liver-derived metabolites, co-cultures are appropriate. In order to determine how metabolites enter the brain, a combination of an in vitro system which mimics the blood-brain barrier and choroid plexus is proposed.

Keywords: brain, chicken, liver, rat, neurotoxicity, xenobiotics, co-culture, organ-specific cell culture, reduction, replacement

Background Information

Cellular neurotoxicity model systems

Model systems are chosen because of similarities to humans but are defined by their limitations in mimicking the original. Estimating the neurotoxicity of chemicals or drugs must be based on the fact that a compound which has entered the body passes through the liver and reaches the brain cells via the blood supply. When this situation is translated into an in vitro approach the set-up includes a sequential exposure of cells from liver and from brain tissue. The specific choice of system is defined by the type of test compound and its use.

Ideally the cell system should be based on human cells with organ-specific functions. Genetic differences between humans and laboratory animals (in most cases rodents) introduce a degree of uncertainty when toxicity data are extrapolated from animal experiments to humans. The use of human cell lines of neuroblastoma cells would therefore be an alternative for neurotoxicity tests. The genetic amenability of these neoplastic cells is not defined, however, and the functional capacity is not comparable to that of normal human brain cells. Primary cells from human nervous tissue would therefore be the best choice. But human tissue as source is limited because of ethical and legal considerations. Adult human tissue can only be obtained occasionally and if it can be obtained each sample will come from donors of different ages, different sexes and from a different brain area. Furthermore, the survival of adult nerve cells is reduced, especially in the case of dissociated cells as required for initial cultures. This would not be the case with isolated primary fetal brain cells which, in humans, can only be obtained only from therapeutic abortions. An alternative that is still being developed involves cells from embryonic human stem cell lines (Schrattenholz and Klemm, 2007). An already established and reliable alternative is nerve cell cultures obtained from embryonic chicks; a number of research groups have demonstrated a similar sensitivity to a variety of drugs in such embryonic chicks and humans (e.g. Bruinink et al., 1998).

Assessment of neurotoxicity

The identification of a potentially neurotoxic activity (damage to nervous tissue cells) of chemicals and drugs is an important task within the framework of the assessment of the toxicity of chemicals. Current guidelines recommend the use of animal tests because of the possibility that a compound might be converted by xenobiotic metabolism to metabolites that are neurotoxic. Current risk assessment guidelines for neurotoxic organophosphorus compounds (OPs) are based on the *in vivo* hen model, owing to its high sensitivity. Initially it was thought that OP toxicity could be estimated by measuring its effect through cholinesterase inhibition in a cell-free system and the consequent cholinergic hyperstimulation. Recent data suggest that the OP-induced neurotoxic effects are achieved by an interaction with cellular processes that are unique to brain development, however (Slotkin, 2004). This implies that OP toxicity can only be correctly measured using living nerve cells.

Conversion of non-toxic chemicals into neurotoxic metabolites

A chemical may exert its toxicity directly or indirectly, following metabolism in the liver. Stable metabolites can be released from liver cells into the blood stream and transported to the brain

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after crossing the blood-brain barrier. This pathway has been well established for some OPs, but cannot be reproduced in conventional human or animal brain tumour (neuroblastoma) cell cultures. Accordingly cell culture systems based on one single organ fail to identify "indirectly acting" neurotoxic compounds (compounds that need bioactivation) (Bruinink, 2008). The metabolic activation or detoxification of chemicals can be clearly detected in a variety of established cell culture systems from different species. Accordingly the possible interactions between organs require a combination of representative cell types from the organs involved. Hepatocyte cultures or co-cultures of different cell types from the liver are suitable for detecting liver-specific toxicity (Milosevic et al., 1999), while in order to detect neurotoxicty the liver cell cultures have to be combined with brain cell cultures and the blood-liver-brain pathway has to be taken into account.

Co-cultures *versus* sequential cultures

The goal of the project was to assess the possibility of identifying chemicals which are neurotoxic only after bioactivation by the liver, using a method based on cell culture. Co-cultures or sequential cultures were established between primary rat hepatocytes (Ohno et al., 1995) and chicken brain cells isolated from total brain of Tetra SL embryonic chicks at stage 29 (Bruinink et al., 1998). The hepatocytes (Fig. 1a) represent the liver, the embryonic brain cells (Fig. 1b) the ultimate target. Stable hepatic metabolites in the supernatant have a high chance of reaching central (e.g. brain) and peripheral nervous tissue via the blood circulation. The test compound can be added either directly to the co-cultures (Fig. 2) or initially to hepatocyte cultures, after which the supernatant is transferred to the brain cells (Fig. 2). A comparison between the two results provides information on the stability of the hepatocyte-derived metabolites.

Brain cell toxicity was assessed by measuring the bioreductive capacity (conversion of the dye MTT), the lysososmal activity (neutral red uptake) and the brain-specific acetylcholinesterase (AchE) activity of the cells. A reduction of the latter activity may be provoked by a direct effect on AChE or indirectly by impairing cellular processes of the nerve cells (Slotkin, 2004).

Hepatocytes release neurotoxic metabolites

Two chemicals were compared: cyclophosphamide (CP), known to be converted in the liver to a metabolite which is cytotoxic for extrahepatic tissues but without a specific brain toxicity, and isofenphos (Bayer AG), a representative organophosphate known to be neurotoxic in humans after bioactivation.

Brain cells were exposed directly, in co-cultures or with the supernatant collected from exposed hepatocytes (exposure time

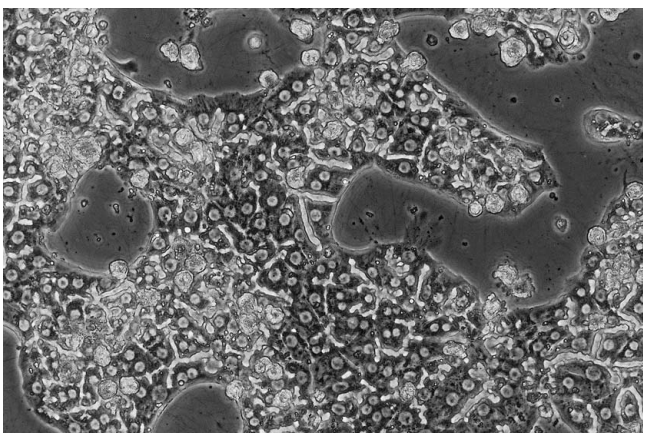

Fig. 1a: Rat hepatocytes (4-day culture)

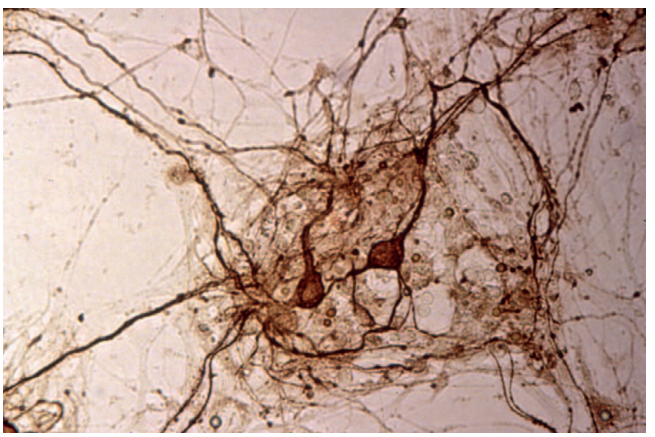

Fig. 1b: Chicken brain cells (8-day culture)

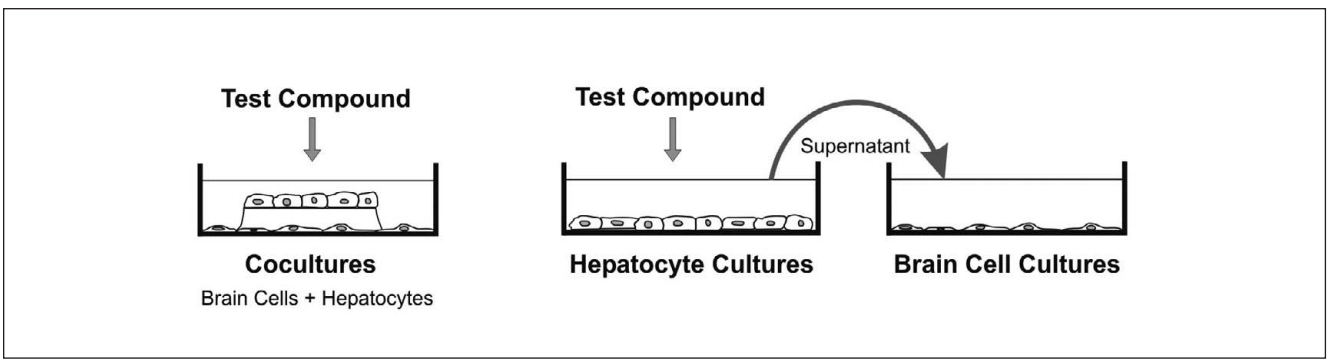

Fig. 2: Exposure of brain cells to liver-cell-mediated metabolites in co-cultures or via a supernatant (sequential cultures).

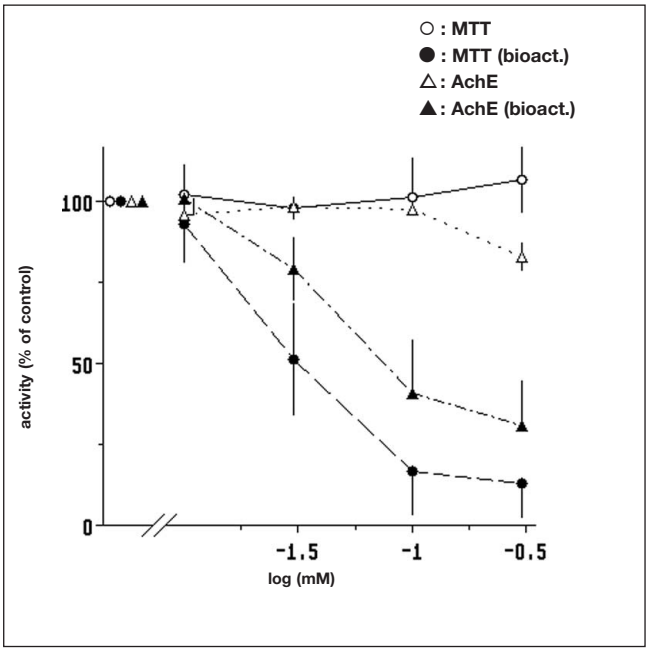

Fig. 3: Dose-response curves with cyclophosphamide: brain cells are exposed directly (△○) or to the supernatant (▲●) from hepatocyte cultures treated with CP.

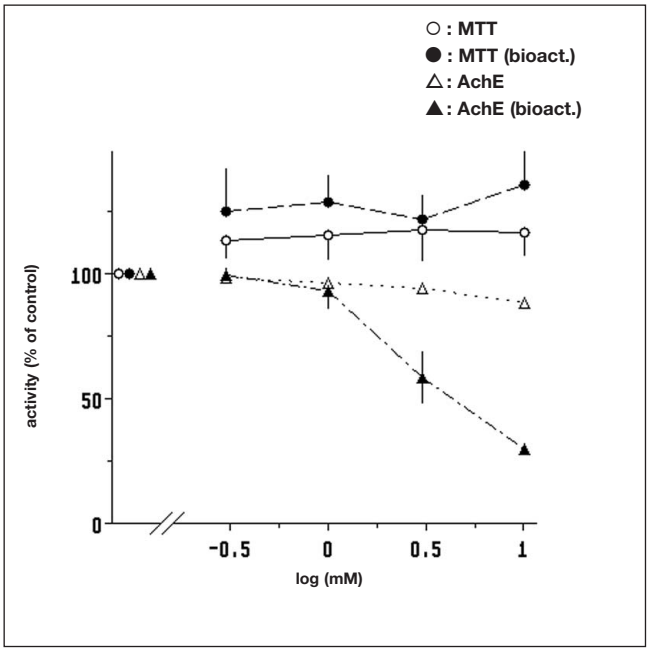

Fig. 4: Dose-response curve with isofenphos; brain cells show inhibition of AchE only after exposure to the supernatant (▲) from hepatocyte cultures treated with isofenphos at doses without reduced cell activity (MTT).

6-24 hours). Cultured chicken brain cells died only after CP had been in contact with hepatocytes (supernatant or co-cultures) (Fig. 3). This indicates that the metabolic competence of the hepatocytes is preserved and comparable to the situation in the living organism. Furthermore it indicates that the intermediates might have a sufficiently long half-life to reach the brain.

Brain-cell-specific functions can be impaired without cell death

As an overall rule, organ-specific toxicity should not be based on basal toxicity data. In most cases organ-specific toxicity is due to failure of proper functions. This should be mirrored in a corresponding choice of read-outs. In the range of concentrations used here, isofenphos inhibited AchE activity without affecting cell activity (MTT conversion), again only after contact with hepatocytes (Fig. 4). This inhibition (in this case the cholinergic nerves) prior to an overall cytotoxicity contrasts with the response to CP metabolites (Bruinink et al., 2002). Accordingly the present experimental model allows a distinction to be made between the induction of general cytotoxicity (MTT activity) and specific neurotoxic activity (inhibition of AChE activity without cytotoxicity) by chemicals and drugs.

Potential of co-cultures and sequential cultures

Co-cultures involving different cell types from the same tissue (e.g. nerve cells and glial cells or hepatocytes and Kupffer cells) allow intercellular interactions to be identified (including intercellular signalling) which take place within an organ and might affect cellular toxicity or the pharmacological behaviour of drugs. Individual cell populations can be pretreated with modulators of xenobiotic metabolism before being used in co-cultures. Such an approach enables specific aspects of pharmacological pathways to be investigated (Milosevic al., 1999).

Co-cultures of cells from different organs, e.g. the addition of hepatocytes to cultures of chicken brain cells or other extrahepatic tissue, provides a metabolic activation system comparable to that of the liver. Sequential testing of the cytotoxicity of the supernatant from hepatocytes treated with the parent compounds is a way of measuring the stability and half-life of the reactive metabolites under investigation. Combining the different set-ups helps to elucidate interactions which may be responsible for divergent results in toxicity and pharmacological tests *in vitro versus in vivo*.

Concerning actual exposure of the brain

Clearly the sequential and the co-culture approaches do not provide a complete answer as to the on-going processes in an intact organism. In the case of organophosphates, it is already known that this group of chemicals is potentially neurotoxic. In the case of pharmaceuticals, it may be important to know whether the metabolites reach brain cells or whether their access is inhibited by the well established blood-brain barrier (BBB) and choroid plexus (CP). This question can be addressed using *in vitro* cell culture systems in which the specific transporters and activities of

the BBB (e.g. Cecchelli et al., 2007) or the CP (Baehr et al., 2006) are expressed. Either the original compound or its metabolites can be tested and new information can be obtained about molecular structures which allow or prevent access to the brain.

In conclusion, the *in vitro* set-up can be adjusted depending on the requirements for safety assessment. Even the most simple sequential test involving liver hepatocytes and chicken brain cells might be reliable enough to replace the hen test as proposed by an OECD guideline.

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