Arh hig rada toksikol 1989;40:231 – 239

Review
UDC 613.63:615.21

BIOCHEMICAL APPROACH TO OCCUPATIONAL NEUROTOXICOLOGY*

M. Lotti, A. Moretto and S. Caroldi

Institute of Occupational Health, University of Padua, Padua, Italy

Received November 10, 1988

Dose-effect and dose-response relationships in occupational neurotoxicology are rarely studied by means of biochemical methods. Some biochemical markers are however available to extrapolate from animal to man and to use in monitoring human exposures. They might be framed in three categories exploring: the delivery of chemicals to the site of action, the modifications of the molecular target induced by chemicals, the biochemical consequences of these modifications.

Estimation of absorbed doses in man is possible for virtually every neurotoxic chemical by means of analytical chemistry of body fluids. Protein adducts, as measured in cellular and other blood components, might assess more closely the delivery *in vivo*, to the site of action. In this way also *in vivo* comparisons across species will be more precise. Examples include haemoglobin adducts, plasma pseudocholinesterase inhibition etc. In addition measurements of blood enzymes involved in the detoxification (e.g. A-esterases and organophosphorus esters) might contribute to assess metabolic capabilities.

Once the molecular target of neurotoxicity is known, extrapolations across species are easy to make. Biochemical markers reflecting *in vivo* the effect at the site of action are available in very few cases, when the same target is accessible in body fluids. In such circumstances the biochemical marker represents an integrated dose/effect index. Examples include Red Blood Cell Acetylcholinesterase and Lymphocyte Neuropathy Target Esterase for acute and delayed neurotoxicity of organophosphorus esters.

The understanding of the pathogenesis of a neurotoxic effect might lead to markers reflecting biochemical consequences of the interaction of the chemical with the target. The specificity of the test will dissect the chain of pathogenetic events from secondary consequences. For example, changes of catecholamine metabolism induced by carbon disulphide.

* Presented at the meeting held on the occasion of the 40th anniversary of the Institute for Medical Research and Occupational Health, University of Zagreb, Zagreb, November 9 – 10, 1988.

In conclusion the development of biochemical markers to be used in occupational neurotoxicology requires the understanding of the mechanism of action. In this way it will be possible to rationalise the toxicokinetics and toxicodynamics of neurotoxic chemicals both in animals and man.

It is widely accepted that the initiation of toxicity occurs at molecular targets which are selectively modified by chemicals. Subsequently, a chain of biochemical and functional changes leads to the morphological and clinical expression of toxicity (1).

The determination of dose-effect and dose-response relationships represents the ultimate aim of toxicology. These relationships can be studied at levels of increasing complexity from single molecules, as in biochemical toxicology, to human populations as in occupational medicine. The assessment of such relationships in groups of workers is in fact very difficult, not only because of biological complexity but also because of interindividual variability.

Nevertheless, whenever a dose-response at a molecular level can be established, biomonitoring procedures in exposed workers can be more accurate. This »molecular approach« in occupational biomonitoring includes:

a. The measurement of the »target dose«, which is defined as the dose, expressed as time integral of concentration of the ultimate agent, which evades metabolic detoxification and penetrates a biologically significant site (2). Methods have been introduced to determine the amount of chemical which undergoes covalent interaction with macromolecular targets (proteins and DNA).

b. Whenever the mechanism of action of a given chemical is known and therefore the toxicologically significant target identified, effects can be measured quantitativcly and a threshold precisely established (3).

c. When the consequences of such interactions with targets are understood, it is possible to detect markers of specific biochemical and functional changes (4).

It is clear that biochemical approaches to occupational toxicology and, in particular, neurotoxicology are hampered by difficulties in the access to suitable tissues. Nevertheless, the blood is used in several circumstances to mirror the conditions in target organs (5). We will discuss in this paper examples where it is possible to use biochemical markers in blood to exploit the above mentioned conditions for the biomonitoring of occupational exposures to neurotoxicants.

THE »TARGET DOSE«

The estimation of the absorbed dose of a chemical in man is possible for virtually every neurotoxic chemical by means of analytical chemistry of body fluids. Nevertheless the wide variations among individuals in the ability to activate proximal toxins, in their delivery to the active site and in the timing of the whole toxicokinetic process, can be explored only partially by measuring either the compound or its metabolites in body fluids. However, a more reliable, specific and sensitive detection of molecular markers reflecting target doses is now possible.

A number of chemicals are electrophilic or produce electrophilically active metabolites and form covalent adducts with nucleophilic centres in macromolecules. For example these chemicals bind covalently cysteine, histidine and the terminal valine of haemoglobin forming adducts that are stable throughout the lifespan of erythrocytes and accumulate upon repeated exposures (6). It has been demonstrated that a constant ratio exists between the binding with tissue macromolecules and that with haemoglobin (7). Consequently, the extent of haemoglobin binding measured in accessible erythrocytes represents a relative measure of the dose in target tissue. More accurate information about absorbed dose, individual's capacity to activate the chemical and reactivity of the ultimate chemical species with macromolecular targets is therefore obtained. An example is the active metabolite of n-hexane, 2,5-hexanedione. It has been shown that n-alkanes which produce metabolites with two oxygen groups separated by four carbons (1,4 diketones) but not metabolites with another diketone spacing will cause neuropathy (8). This requirement was used to formulate the hypothesis that involves reaction of 1,4 diketones with primary amino groups to form a stable pyrrole ring adduct (9). The pathogenesis of n-hexane neuropathy is therefore explained in terms of formation of pyrrole adducts to neurofilaments and subsequent auto-oxidation to form a cross-linking centre (10). Such adducts are formed also in haemoglobin and they can be determined (11). It was suggested to use these measurements to establish dose-response relationships both in animals and exposed subjects (3). Monomeric acrylamide is an electrophile which interacts *in vitro* and *in vivo* with erythrocytes and brain proteins and was found to form adducts bound to cysteine residues (12). The adduct formed on acid hydrolysis yields a compound with chromatographic properties identical to those of S-(2-carboxyethyl)cysteine. This chemical can then be analysed by capillary gas chromatography / mass spectrometry and used for the biomonitoring of acrylamide exposure (7).

In conclusion, the possibility of measuring adducts in haemoglobin will enable an estimation of the dose of electrophiles closer to »the target«. A further advantage of measuring such adducts is the possibility to extrapolate dose more precisely across species. Once the number of adducts and their persistence in man, after exposures to a given chemical are known, it will be possible to compare them with data from animals dosed with a known amount of electrophilic chemicals and therefore to extrapolate toxicity data more precisely.

THE INTERACTION OF CHEMICALS WITH TOXICITY TARGETS

In certain circumstances the targets of toxicity are easily accessible in blood and it is therefore possible to assess their modifications, as induced by toxic chemicals, both qualitatively and quantitatively. Once the relationship between effects on the target organ and those on the mirror, i.e. the blood, are known, a given toxic effect can be quantitatively graded and threshold established. An example of this is given by organophosphorus esters (OP).

OP pesticides cause acute effects both in humans and experimental animals by inhibiting acetylcholinesterase (AChE) at nerve endings and when acetylcholine

accumulates at toxic levels (13). The effects on the enzyme are correlated to signs and symptoms of poisoning. After phosphorylation of AChE, two further reactions are possible: a) reactivation of the inhibited enzyme or b) aging of the phosphoryl-enzyme complex which involves the cleavage of an R-O-P bond and the formation of a charged monosubstituted phosphoric acid residue attached to the protein. Therefore the level of AChE inhibition is the result of several rate constants (inhibition, reactivation and aging) and is characteristic of a given OP (14).

It has long been known that an enzyme which is biochemically identical to nervous system AChE is present in the outer membrane of red blood cells (RBC) and OP exposure is usually monitored by measuring RBC AChE activity because this activity is correlated with that in the nervous system (15).

Besides the well known cholinergic effects and the possibility to monitor this toxicity, some OPs can also induce a quite different syndrome known as organophosphate-induced delayed polyneuropathy (OPIDP). Characteristic of OPIDP is the axonal degeneration of longer axons of spinal cord and of peripheral nerves which is evident 1 – 3 weeks after single doses (16, 17). The mechanism of initiation of OPIDP has been reviewed in depth (16). Briefly, OPIDP is initiated by phosphorylation and aging of a protein called neuropathy target esterase (NTE). In the hen, the animal model for OPIDP studies, 70% of NTE must be affected within hours of dosing to trigger the cascade of events which leads to the morphological/clinical expression of OPIDP about two weeks later. It has been shown that the critical target is axonal and not cell body NTE (18, 19). A further event in the pathogenesis of OPIDP has been also identified: a progressive deficit of retrograde axonal transport which develops well before the clinical expression of OPIDP (20).

It was shown that NTE activity is present in hen lymphatic tissue and in blood lymphocytes (21). Conflicting results have been reported in correlation studies between the inhibition of blood lymphocyte NTE and that of nervous system NTE after OP treatment (22, 23). The measurement of lymphocytic NTE activity to mirror the effect on nervous system NTE needs therefore more knowledge on the correlation between NTE inhibition in the two organs. It is possible that the brain/lymphocyte ratio of NTE inhibition changes with the compound and with time after treatment as it has already been shown for some compounds (23).

However, since NTE activity was also detected in human blood lymphocytes (24) the possibility of measuring NTE activity in lymphocytes to monitor OPIDP in man was actively explored (25, 26). Recently, it has been shown that inhibition of lymphocytic NTE predicts the development of OPIDP in man (27). In a case of unsuccessful suicide attempt with chlorpyrifos, high inhibition of lymphocytic NTE was found when the patient recovered from the acute cholinergic symptoms. At that time clinical and electrophysiological signs were negative for peripheral neuropathy. Two weeks later the patient developed clinical signs of OPIDP and the diagnosis was confirmed by the biopsy of the sural nerve.

Measurements of lymphocytic NTE soon after poisoning may become important if an antidote for OPIDP becomes available and, when the threshold of NTE inhibition is established in man, it may become a test to be used in occupational medicine to monitor exposures to neuropathic OPs, as it was already attempted (28).

In conclusion, the mechanisms of OP toxicities, cholinergic toxicity and OPIDP are now largely understood and the target molecules are easily accessible, AChE in RBC and NTE in lymphocytes. It is therefore possible to assess quantitatively the effects of potentially neurotoxic exposures, to establish rational thresholds and to extrapolate more confidently from animal data.

THE BIOCHEMICAL CONSEQUENCES OF TARGET-TOXIN INTERACTIONS

The molecular targets of several neurotoxic chemicals are unknown, but some insight into the pathogenesis of the disease might offer a rational frame to the development of biomonitoring tests.

This might be the case of carbon disulphide (CS_2) poisoning. Clinical manifestations include psychiatric symptoms, parkinsonism, polyneuritis, early atherosclerosis leading to encephalopathy and metabolic disorders. More recently, higher rates of coronary heart death, hypertension, depression and suicide have been also reported in exposed workers (29).

In spite of the great variety of symptoms, they might basically be referred to as neurological and cardiovascular disorders.

Endogenous catecholamines are a family of related compounds which are synthesized step by step from tyrosine. At least two neurotransmitters (dopamine and noradrenaline) and a hormone (adrenaline) are known to exert an important role in the modulation of both cardiovascular and central/peripheral neurological functions. Disorders as parkinsonism, depression, hypertension and coronary heart disease as well as some metabolic alterations have often been ascribed to malfunction of catecholaminergic system and drugs which modify catecholamine metabolism are commonly used in such diseases. As a consequence, the sympathoadrenal system seems to offer a single plausible target to explain as a whole most symptoms of CS_2 intoxication.

CS_2 changes catecholamine content both in the brain and adrenal glands of exposed rats, namely dopamine is increased and noradrenaline is decreased, suggesting that CS_2 could inhibit dopamine-β-hydroxylase (DBH), the enzyme which converts dopamine to noradrenaline (30). DBH contains about 2 micromoles of Cu^{++} per micromole of enzyme and is inhibited by metal chelators as disulfiram and diethyldithiocarbamate (31). CS_2 reacts *in vivo* with nucleophilic compounds which possess free amino groups to form dithiocarbamates (32) and similarly inhibits DBH by copper chelation (33). Inhibition was also studied *in vivo* in rat adrenals: at 4 mg CS_2/l air dopamine conversion to noradrenaline is completely abolished in 30 minutes (34). However, because of fast clearance, (CS_2 half-life in rats is about 30 min (35), and perhaps because of enzyme reactivation due to copper redistribution, the hydroxylation of dopamine is back to pre-exposure levels within two hours after the end of exposure. Moreover, 24 hours later, the rate of catecholamine synthesis is significantly higher than in controls (36).

The stimulation of this enzymatic pathway in response to the reversible inhibition of a key step, is sustained in CS_2 exposure by the increased activity of two enzymes involved in catecholamine synthesis: thyrosine hydroxylase (37), which in physiologic

conditions is the rate limiting enzyme and DBH. Furthermore, measurement of copper reactivable DBH soon after the end of repeated CS_2 exposures (up to 9) showed that stimulation of DBH correlates to the number of exposures (38).

DBH is released from the synaptic vesicles of postganglionic synaptic neurons and from chromaffine granules of adrenals along with catecholamines (39). Catecholamines and DBH can be measured in plasma and both of them increase during sympathetic activation. A new approach is therefore offered by the knowledge of the events which follow CS_2 interaction with its target and by the opportunity to use DBH determination in the blood of exposed workers as a marker for these effects. However, DBH levels vary widely between individuals and such variations are better related to genetic factors than to sympathoadrenal activation (40). Therefore, preliminary results of DBH determinations in the blood of CS_2 exposed workers in a Belgian factory were difficult to interpret (personal communication from L Magos). Only a careful follow-up study in which the results of subsequent measurements will be compared in the same subject at different time, may offer the final answer.

CONCLUSIONS

This paper was aimed to stress that the understanding of the mechanisms of action of chemicals is likely to permit a biochemical approach to the monitoring of occupational exposures. The identification of »secondary targets«, such as haemoglobin, might enable the assessment of the dose of electrophiles more precisely. The identification of accessible »primary targets« such as RBC AChE and lymphocytic NTE and the understanding of specific steps in the pathogenetic chain, such as changes in catecholamine metabolism, might enable the assessment of toxic effects. Certainly, such a biochemical approach is helpful for a rational definition of thresholds.

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Sažetak

BIOKEMIJSKI PRISTUP NEUROTOKSIKOLOGIJI U MEDICINI RADA

U neurotoksikologiji medicine rada rijetko se biokemijskim metodama ispituje odnos doza-učinak kao i doza-odgovor pojedinih spojeva. Pa ipak, neki se biokemijski pokazatelji mogu ekstrapolirati sa životinja na čovjeka te upotrijebiti u praćenju izloženosti ljudi. Oni se mogu podijeliti u tri skupine istraživanja: a) doprema spojeva do mjesta djelovanja, b) modifikacija ciljne molekule uvjetovana spojem, c) biokemijske posljedice ovakove modifikacije.

Određivanje apsorbirane doze u čovjeka moguće je za doslovno svaku neurotoksičnu kemikaliju pomoću metoda analitičke kemije tjelesnih tekućina. Spojevi koji se vezuju za proteine, a mjere se u staničnim i drugim dijelovima krvi, mogu dobro ukazati na njihovu pristupačnost *in vivo* na mjestu djelovanja. Tu spadaju spojevi koji se vezuju na hemoglobin, zatim oni koji inhibiraju pseudokolinesterazu, itd. Uz to mjerenje enzima koji sudjeluju u detoksifikaciji (npr. A-esteraze i organofosforni esteri) može pridonijeti razumijevanju metaboličkih svojstava.

Kad je već poznat molekularni cilj neurotoksičnog djelovanja, lako je ekstrapolirati rezultate s drugih životinjskih vrsta. Biokemijski pokazatelji koji odražavaju učinak *in vivo* na mjestu djelovanja spoja rijetko su dostupni, i to onda kada je ista ciljna molekula prisutna i u tjelesnim tekućinama. U takvim slučajevima biokemijski pokazatelj predstavlja integrirani indeks doza-učinak. Pri-

mjeri su za takve pokazatelje acetilkolinesteraza u eritrocitima i NTE (Neuropathy Target Esterase) u limfocitima za akutnu i kasnu neurotoksičnost organofosfornih spojeva.

Razumijevanje patogeneze neurotoksičnog djelovanja može dovesti do otkrivanja markera (pokazatelja) koji odražavaju biokemijske posljedice vezivanja spoja sa ciljem. Specifičnim testom moglo bi se razlučiti patogenezu od sekundarnih posljedica. Primjer su za to promjene metabolizma kateholamina djelovanjem ugljik disulfida.

Za utvrđivanje biokemijskih pokazatelja koji bi se mogli koristiti u neurotoksikologiji medicine rada potrebno je razumijevanje mehanizma djelovanja. Na taj način bilo bi moguće razriješiti problem toksikokinetike i toksikodinamike spojeva u životinja i u čovjeka.

Institut za medicinu rada Sveučilišta u Padovi, Padova, Italija