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METABOLISM-MEDIATED CYTOTOXICITY OF CHEMICAL CARCINOGENS AND NON-CARCINOGENS

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Abstract—The results presented in this paper clearly demonstrate that a fibroblast suppression test system incorporating viable hepatocytes can be of value in studying the problems of metabolism-mediated cytotoxicity for those compounds and/or metabolites that are sufficiently stable to traverse cell membranes. The possibility that this test system can be used to distinguish carcinogenic and non-carcinogenic chemicals cannot be resolved at this time, however.

INTRODUCTION

We recently reported the development of a novel mixed cell culture system capable of detecting those chemicals that are not cytotoxic *per se* but become so after metabolism by the liver[1]. The model compound used was cyclophosphamide (CPA)* which is known to require liver metabolism for its full toxicity to be realized[2]. Briefly, the protocol for the system involved a short incubation of rat liver fibroblasts with the CPA in the absence and presence of viable rat hepatocytes and the fibroblasts were then cultured until confluency was attained. Cytotoxicity was determined as inhibition of fibroblast growth and if cytotoxicity was greater in the presence of the hepatocytes this was interpreted as indicating that metabolism of the CPA by the hepatocytes activated its cytotoxic properties. A limitation to this assay system is that it can only detect those chemicals for which the active metabolites are sufficiently stable to leave the hepatocytes and then enter the fibroblasts.

We have now submitted a range of chemicals to this system in order to study the general applicability of this approach in assessing the importance of metabolism in chemically-mediated cytotoxicity. As it has been suggested that water-soluble direct-acting carcinogens can be detected by the manner in which they inhibit cell growth[3] we have concentrated our attentions on certain classes of carcinogens and compared wherever possible carcinogenic and non-carcinogenic isomers. The results of this study are presented in this paper.

EXPERIMENTAL

Cell isolation, incubation and culture details were essentially as described previously[1]. The chemicals were dissolved in either buffered saline (pH 7.4) or dimethylformamide which was present in the incubations at a final concentration of 0.2% v/v. This concentration of dimethylformamide produced no overt signs of toxicity to either the hepatocytes or the fibroblasts. Wherever possible the concentrations of the chemicals used were chosen so as not to cause overt toxicity to the hepatocytes and so confuse interpretation of the results. At least five concentrations of each compound were studied and four culture flasks were set up at each concentration. At the end of an 8-day culture period, nuclei counts were performed as described previously[1] and results were calculated as ID_{50} values, i.e. the concentration of the chemical that inhibited fibroblast growth by 50 per cent. In a series of seven experiments the ID_{50} value for CPA in the presence of hepatocytes was $16.3 \pm 1.3\ \mu g/ml$ (mean ± S.D.) thus attesting to the reproducibility of this method.

RESULTS AND DISCUSSION

It is evident from the results presented in Table 1 that a variety of chemicals of widely differing chemical structure require metabolism by hepatocytes for their full toxicity to be realised. In general, it appears that the toxicity of the three pairs of compounds follows the order dimethylaminoazobenzenes > aminochrysenes > acetylaminofluorenes irrespective of the isomers studied. The similarity in the ID_{50} values for the aminochrysene isomers in the presence of the hepatocytes suggests a common metabolic activation step or a similar cytotoxic metabolite, whereas the big (20-fold) difference in the hepatocyte-mediated cytotoxicity between the methyl-DAB isomers suggests different activation steps or cytotoxic metabolites. Perhaps surprisingly there is evidence that mitomycin c also requires hepatocyte metabolism for its cytotoxicity to be fully expressed whereas with 4-NQO hepatocyte metabolism decreases its cytotoxicity (Fig. 1). With CCl_4, a well-documented liver toxin, the fibroblast toxicity is the same whether or not hepatocytes are present and, because this high level of CCl_4 is also extremely toxic to the hepatocytes themselves, it is probable that this toxicity

*Abbreviations used: AAF, acetylaminofluorene; AC, aminochrysene; CPA, cyclophosphamide; DBA, dimethylaminoazobenzene; DMN, dimethylnitrosamine; 4-HAQO, 4-hydroxylaminoquinoline N-oxide; 4-NQO, 4-nitroquinoline N-oxide.

Table 1. ID_{50} values for the toxicity of various chemicals to fibroblasts incubated in the presence or absence of rat hepatocytes

Chemical	Carcinogenicity (+/−)	ID_{50} values (μg/ml):- (+ Hepatocytes)	(− Hepatocytes)
Cyclophosphamide	+	16	> 500
Dimethylnitrosamine	+	150	> 1000*
3'-methyl 4-dimethylaminoazobenzene	+	0.1	> 125*
2-methyl-4-dimethylaminoazobenzene	−	2.0	> 250*
2-acetylaminofluorene	+	140	> 250*
4-acetylaminofluorene	−	75	> 200*
6-aminochrysene	+	11	> 200*
2-aminochrysene	−	16	> 30*
4-nitroquinoline N-oxide	+	3.50	0.63
Mitomycin c	+	0.63	2.50
Carbon tetrachloride	?	2,000	2,000

*These values represent the limits of solubility in the culture medium.

ID_{50} values were derived from experiments in which at least five concentrations of each chemical were used and 4 culture flasks set up at each concentration.

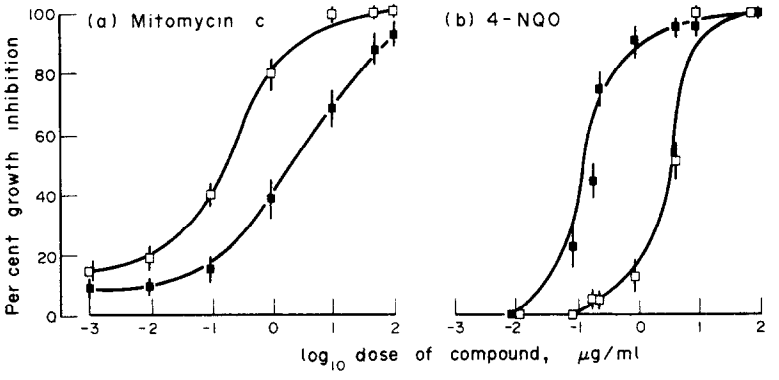

Fig. 1. Toxicity of (a) mitomycin c and (b) 4-NQO to fibroblasts in the absence (■) or presence (□) of viable rat hepatocytes. Rat fibroblasts were incubated with various levels of each compound in the absence or presence of viable rat hepatocytes for 1 hr at 37°C. The samples were then diluted and cultured at 37°C for 6–8 days after which time nuclei counts were performed. From these counts (using 4 flasks at each concentration) the per cent inhibition of cell growth was calculated. Each point is the mean of 4 determinations with the S.D. as the bar.

of CCl_4 is related to its general membrane-damaging properties rather than to a specific metabolic event. It is also probable that the active metabolite responsible for the hepatotoxicity (believed to be a free radical[4]) would be too reactive to leave the hepatocytes and so would not produce fibroblast toxicity in this system.

The nature of the metabolite believed responsible for the cytotoxicity is in most cases uncertain. For CPA there is some evidence that the cytotoxic agent is also the carcinogenic agent[5]. Similarly, it appears that the metabolism-mediated cytotoxicity and mutagenicity of aflatoxin B_1, benzo[a]pyrene, dibenz[a,c]anthracene and nitrosamines in mammalian cell culture systems are closely related[6, 7]. If this phenomenon is generally true the present results obtained with DMN, methylDABs, aminochrysenes and acetylaminofluorenes can be interpreted in light of current ideas on the metabolic activation of these various carcinogens, involving reactions such as *N*-hydroxylation and sulphation, *N*-demethylation and epoxidation (see ref. 8). This may, however, not hold true for every compound tested in this fibroblast suppression system in view of the fact that only metabolites sufficiently stable to pass out of the hepatocytes and enter the fibroblasts would provoke a cytotoxic response and that these metabolites by virtue of their stability may not be the ultimate carcinogen.

Enzyme reduction via an NADPH-dependent reductase is required for the cytotoxic activity of mitomycin c, but most cell types are apparently capable of carrying out this reaction[9] This probably explains the marked toxicity of mitomycin c to fibroblasts even in the absence of hepatocytes and the increased toxicity in the presence of hepatocytes (Fig. 1) which are known to have very high reductase levels. It is probable that the results obtained with 4-NQO can also be explained in metabolic terms. Thus, the metabolism of 4-NQO is known to involve an initial reduction by a cytoplasmic diaphorase to produce 4-HAQO (the postulated carinogenic and cytotoxic agent) which can then be further reduced by a microsomal enzyme to give 4-aminoquinoline N-oxide which is not carcinogenic[10]. The initial reduction by diaphorase appears to be common to most cell-

types and explains the marked toxicity of 4-NQO to fibroblasts in the absence of hepatocytes (Fig. 1). The decreased toxicity of 4-NQO to fibroblasts in the presence of hepatocytes is probably due to the high level of microsomal reductase present in the hepatocytes which will lower the level of 4-HAQO available to cause cytotoxicity. This is substantiated by a recent report which indicated that 4-NQO produced chromosomal damage in human fibroblasts in culture but that this damage was eliminated by the addition of a liver microsomal fraction as a metabolising system[11].

If the postulate that carcinogens can be detected by their ability to inhibit cell growth were true it would be expected that carcinogenic isomers would be more toxic in this fibroblast suppression test system than would the non-carcinogenic isomers. Whilst this holds true for the methylDABs it does not hold true for the aminochrysenes or the acetylaminofluorenes where the non-carcinogenic isomer is more toxic than the carcinogenic isomer. This argument requires some reservation, however, as the status of the non-carcinogens is not wholly resolved. Thus, although 2-methylDAB is not usually carcinogenic it can be so in newborn animals[12] or partially hepatectomised rats[13] and is positive in the *Salmonella*/microsome mutagenesis assay[14], whilst 4-AAF although not shown to be carcinogenic in animals, does produce a positive result in the *Salmonella*/microsome mutagenesis assay[14]. It may be possible that carcinogenic and non-carcinogenic isomers may be differentiated by analysis of the pattern of growth inhibition as described by Hooson and Grasso[3] and this work is currently in progress.

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