Alternatives in the induction and preparation of phenobarbital/naphthoflavone-induced S9 and their activation profiles

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With the aim of optimizing the efficiency of S9 fractions used in in vitro mutagenicity assays, different schemes for the induction of liver enzymes in rats were tried and the amount of S9 fraction required was assessed. The activity of 2-anthramine (2AA), 2-acetylaminofluorene (2AAF), 3-methylcholanthrene (3MTCL) and benz[a]pyrene in bacterial mutagenicity tests was compared with the enzymatic activity in S9 fractions obtained from rats treated with either phenobarbital (NaPB), β-naphthoflavone (βNF) or combinations of both. Three pool systems prepared with either phenobarbital (NaPB), β-naphthoflavone (βNF) induction and preparation of the S9 fraction without using PCBs as inducers. We prepared and evaluated different enzymatic liver preparations according to the basic procedures of Ames et al. (1977) and Ong et al. (1980). The inductive protocols tested included individual or combined treatment with NaPB and βNF. Preparations with pooled fractions were also analyzed. The efficacies of the preparations in genotoxicity assays were compared with the biochemical characterization of each of the S9 fractions. A higher sensitivity in genotoxicity assays was obtained with a combined S9 induction regime.

Materials and methods

Chemicals
Sodium phenobarbital USP was obtained from Socram (Buenos Aires, Argentina). β-Naphthoflavone, 2-anthramine (2AA), 2-acetylaminofluorene (2AAF), 3-methylcholanthrene (3MTCL) and benz[a]pyrene (B[a]P) were obtained from Sigma (St Louis, MO), 2-Nitrofluorenone (2NF) was from Aldrich (St Louis, MO).

Induction protocols
Seven-week-old, 200 g weight, inbred Sprague-Dawley female rats were used. Animal treatment for enzymatic induction in the S9 fraction was as follows.

\textit{NaPB induction.} Single drug induction with NaPB, as used by Ong et al. (1980), was with three daily doses of 80 mg/kg i.p., given as a 16 mg/ml solution in DMSO, as suggested by Elliot et al. (1992).

\textit{βNF induction.} Three daily doses of βNF (oral gavage, 80 mg/kg, given as a 16 mg/ml fine suspension in 0.5% methylcellulose) were used according to Elliot et al. (1992). The oral gavage route was preferred for βNF, since using the i.p. route we observed, as Elliot et al. (1992), that the liver becomes encrusted, as with the chemical.

\textit{(NaPB+βNF) induction.} The dosing regime was three daily doses of NaPB (80 mg/kg i.p., as a 16 mg/ml solution in DMSO) and βNF (oral gavage, 80 mg/kg, as a 16 mg/ml suspension in 0.5% methylcellulose) (Elliot et al., 1992).

S9 fraction preparations
Following drug treatment, the animals were killed by cervical dislocation and the S9 fraction was obtained following the procedures of Ames et al. (1977). Uninduced S9 fractions were obtained from untreated rats. Pooled S9 fractions were obtained as follows.

\textit{Fixed NaPB-variable βNF \{F(NaPB)-V(βNF)\}.} A fixed amount of NaPB-induced S9 (the minimum amount that elicits a maximal response with the aromatic amine carcinogens 2AA and 2AAF) and increasing amounts of βNF-induced S9 (Table I). \textit{Variable NaPB-fixed βNF \{V(NaPB)-F(βNF)\}.} A fixed volume of βNF-induced S9, such that it elicits a good reverse mutation response with the polycyclic hydrocarbons 3MTCL and B[a]P, and increasing amounts of NaPB-induced S9 (Table II).

\textit{Fixed NaPB-fixed βNF \{F(NaPB)-F(βNF)\}.} Fixed volumes of NaPB-induced S9 and βNF-induced S9 were chosen such that individually they produced a maximal response. The pooled S9 fraction was prepared by mixing NaPB-induced S9 and βNF-induced S9 at a constant ratio of 1:1.5 (Table III).

Biochemical determinations
Total P450 cytochrome(s) activity was measured by differential spectrophotometry at 450–490 nm by the method of Omura and Sato (1964). Protein concentrations of the S9 fractions were determined according to Lowry et al. (1951) using bovine serum albumin as standard. Aminopyrine demethylase activity was determined by spectrophotometric quantitation of formaldehyde with Nash reagent (Nash, 1953; LaDu et al., 1955). 7-Ethoxyresorufin-O-deethylase activity was determined by the spectrophotometric method of Klotz.

Introduction

Many carcinogens and mutagens have to be metabolized by monoxygenase systems before their mutagenic activity can be detected. To induce liver enzymes prior to preparation of S9 fractions used in in vitro mutagenicity tests, animals are treated with agents such as the polychlorinated biphenyl (PCB) Aroclor 1254. In spite of PCBs being potent inducers of liver enzymes, these drugs are highly toxic and carcinogenic (Alvares et al., 1973) and have a detrimental environmental impact (Clare, 1989). Therefore, other alternatives to PCBs as inducers, such as the use of combined induction regimens with sodium phenobarbital (NaPB) and β-naphthoflavone (βNF), have been investigated, following Matsushima et al. (1976) and subsequent studies (Elliot et al., 1992; Callander et al., 1995; Paolini et al., 1996a).

In order to increase the sensitivity of the assays for testing the mutagenic activity of drugs, we have focused on optimizing the preparation of the S9 fraction without using PCBs as inducers. We prepared and evaluated different enzymatic liver preparations according to the basic procedures of Ames et al. (1977) and Ong et al. (1980). The inductive protocols tested included individual or combined treatment with NaPB and βNF. Preparations with pooled fractions were also analyzed. The efficacies of the preparations in genotoxicity assays were compared with the biochemical characterization of each of the S9 fractions. A higher sensitivity in genotoxicity assays was obtained with a combined S9 induction regime.
Table I. Composition of F(NaPB)-V(βNF) pooled S9 fractions

<table>
<thead>
<tr>
<th>Volume of S9 per plate (µl)</th>
<th>Protein (µg/plate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaPB</td>
<td>βNF</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>25</td>
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<td>50</td>
</tr>
<tr>
<td>10</td>
<td>75</td>
</tr>
<tr>
<td>10</td>
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Table II. Composition of V(NaPB)-F(βNF) pooled S9 fractions

<table>
<thead>
<tr>
<th>Volume of S9 per plate (µl)</th>
<th>Protein (µg/plate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaPB</td>
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</tr>
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<td>100</td>
</tr>
<tr>
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</tr>
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<tr>
<td>7.5</td>
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<td>12.5</td>
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</table>

Table III. Composition of F(NaPB)-F(βNF) pooled S9 fractions

<table>
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<th>Volume of S9 per plate (µl)</th>
<th>Protein (µg/plate)</th>
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</thead>
<tbody>
<tr>
<td>NaPB</td>
<td>βNF</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>25</td>
<td>37.5</td>
</tr>
<tr>
<td>50</td>
<td>75</td>
</tr>
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</table>

et al. (1984). p-Nitrophenol glucuronyltransferase activity was measured according to Pogell and Krisman (1960).

Salmonella mutation assays

Compounds were tested using the standard plate incorporation assay protocol with Salmonella typhimurium TA98 strain, according to Ames et al. (1977). Spontaneous revertants obtained in the negative control plates without drugs were 34 ± 12 (n = 6). In the presence of 2NF (1 µg/plate), used as a positive control without S9, 484 ± 167 (n = 6) revertants were obtained. The following addition sequence was used: 100 µl overnight (~16 h) bacterial culture, 500 µl S9 mix (or 500 µl mix where appropriate), 100 µl dosing solution and 2.0 ml top agar (containing 0.05 mM histidine and 0.05 mM biotin). Plate numbers were two per test dose, four for the solvent control and four for the bacteria control without S9 (2NF). Plates were read under a Quebec Colony Counter (American Optical, New York, NY) viewer after 72 h incubation (Gatehouse et al., 1990).

Statistics

Statistical analysis of the data was carried out by analysis of variance (ANOVA) and by the Dunnet (Tallarida and Murray, 1981) and Duncan (Bliss, 1967) tests.

Results

To determine conditions which allow increases in sensitivity of in vitro mutagenicity assays we tested S9 fractions obtained following three induction systems. In two of these schemes a single drug (NaPB or βNF) was used as inducer, in the third scheme a combined induction with both drugs was used. In addition, the S9 fractions obtained were pooled in three different ways in order to obtain S9 fractions of variable composition, such that a low enzymatic activity in one prepara-

tion could be enhanced by addition of another S9 fraction enriched in other enzymatic systems.

Figure 1 shows mutagenic assays for the four test drugs using different S9 fractions. In order to decide which is the most efficient S9 fraction, the region of 700–1000 µg protein was chosen because higher protein concentrations seem to interfere with the assay, as is evident from Figure 1a, with a lower number of revertants observed at protein concentrations >1000 µg/plate. Protein concentrations <800 µg/plate seem to give higher revertant numbers with 2AA (Figure 1a). For the other drugs tested there was very low activity at low protein concentrations (Figure 1b and c).

Using 2AA as test drug (Figure 1a) high activity (i.e. >200 revertants/plate) was observed for all S9 fractions at protein concentrations of 700–1000 µg/plate. The pooled F(NaPB)+V(βNF) fraction was the most active (>3000 revertants/plate). The other S9 fractions were still very active, yielding revertant numbers >500 at these protein concentrations.

Figure 1b shows that using 2AAF as test drug the NaPB+βNF-induced S9 fraction was the most active at protein concentrations close to 1000 µg/plate (>1000 revertants/plate), whereas the other S9 fractions generated between 200 and 1000 revertants/plate.

The lowest overall activity was observed using 3MTCL as test drug (Figure 1c). Although all the S9 fractions had enough activity to give clearly positive results for 2AA and 2AAF in mutagenicity tests (Figure 1a and b), only the NaPB+βNF-induced S9 fraction produced >200 revertants/plate at ~1000 µg protein/plate for 3MTCL. All NaPB+βNF activation levels were significantly higher (P < 0.05) than the corresponding values for NaPB and βNF. The NaPB+βNF-induced S9 fraction is the only preparation that yielded a positive result for 3MTCL in the in vitro mutagenicity test.

The activation profiles shown in Figure 1d for B[a]P suggest that only the pooled F(NaPB)+F(βNF) S9 fraction had negligible activity. All the other S9 fractions produced >200 revertants/plate in the protein concentration range 700–1000 µg/plate and the most active preparations were the βNF- and the NaPB+βNF-induced S9 fractions.

The enzymatic activities in NaPB-induced S9, βNF-induced S9, NaPB+βNF-induced S9 and uninduced control S9 are summarized in Table IV. Total cytochrome P450 was highly enriched in other enzymatic systems.

Discussion

Mutagenicity profiles seem to correlate positively with the amount of enzymatic fraction added, since in most cases high concentrations of S9 fractions yield an increase in the number of revertants. Only with 2AA was a peak of mutagenic activity observed at low protein concentrations followed by a decrease in activation at higher protein concentrations. In such cases the addition of high amounts of S9 fraction seems to interfere with the system. Murray and Reidy (1990) proposed that inhibition of oxidative drug metabolism may be interfering with S9 action by means of reversible inhibition, by complexation of an intermediate metabolite or by autocatalytic inactivation. Guengerich et al. (1982) proposed that cross-interference of
Fig. 1. Induction of *S. typhimurium* TA98 reverse mutation by four reference genotoxins. For each S9 preparation, prepared as indicated in Materials and methods, the number of revertants was determined as a function of the amount of S9 added to the medium. The genotoxins were (a) 2AA (0.2 µg/plate), (b) 2AAF (50 µg/plate), (c) 3MTCL (50 µg/plate) and (d) B[a]P (5 µg/plate). Results are means of two plates; standard deviations (SD) are also shown. Dashed lines indicate the proposed range of S9 amounts to be used in mutagenicity assays. *P* < 0.05 for NaPB and βNF for 3MTCL at every corresponding S9 amount (c).

**Table IV.** Hepatic S9 cytochrome P450 levels and enzyme activities for different induction systems

<table>
<thead>
<tr>
<th>Induction</th>
<th>Phase I</th>
<th>Phase II</th>
<th>Protein (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cyt P450 (nmol/mg)</td>
<td>Aminopyrine demethylase</td>
<td>Glucuronyl transferase (nmol/min/mg)</td>
</tr>
<tr>
<td>Control</td>
<td>0.177</td>
<td>3.7</td>
<td>61.5</td>
</tr>
<tr>
<td>NaPB</td>
<td>0.370 (209%)</td>
<td>4.2 (114%)</td>
<td>101.5 (165%)</td>
</tr>
<tr>
<td>βNF</td>
<td>0.110 (62%)</td>
<td>2.4 (64%)</td>
<td>169.8 (276%)</td>
</tr>
<tr>
<td>NaPB+βNF</td>
<td>0.618 (350%)</td>
<td>6.1 (166%)</td>
<td>153.3 (249%)</td>
</tr>
</tbody>
</table>

Values in parentheses are percent of control.

enzyme activation may be masking the mutagenic response. Accordingly, in the present study a higher limit of 1000 µg protein/plate was chosen in order to minimize these interfering effects.

The S9 fraction prepared following simultaneous induction with NaPB+βNF was the only one to produce a significant response with the four drugs studied. The oral gavage route, rather than i.p., was preferred for NaPB treatment to avoid liver encrustation that could lead to equivocal results in the activation profiles. Most S9 preparations were active with 2AA, 2AAF and B[a]P, however, only the NaPB+βNF-induced S9 fraction gave a high response with 3MTCL. Pooling single drug-induced S9 fractions was not as effective as simultaneous induction with both drugs. A pooled S9 fraction was more active than the NaPB+βNF-induced S9 only when testing 2AA. Therefore, according to our results, the induction method of choice to obtain the most active S9 fraction for mutagenicity assays is simultaneous treatment with NaPB (i.p.) and βNF (per os).

The high activity of the S9 fraction obtained from rats treated following the combined induction protocol may be explained by its enzymatic activities. Both Phase I and Phase II enzymes were highly induced in this preparation, whereas Phase I enzymes were not increased following single induction with βNF and the Phase II enzyme marker was poorly induced by single NaPB treatment. The induction of Phase I activities (cytochrome P450, aminopyrine demethylase and 7-ethoxyresorufin-O-deethylase) bears certain similarities to the pattern obtained by other authors (Elliot *et al.*, 1992; Callander *et al.*, 1995). As previously reported by Paolini *et al.* (1996b), Phase...
II enzymes (i.e. glucurononyltransferase) may also be involved in the activation of certain promutagens.

We consider that the choice of the combined induction protocol is not enough to ensure optimal conditions in mutagenicity assays. Due to batch-to-batch variability, total amount of protein to be used has to be determined for each preparation. Therefore, we suggest as good practice the testing of different amounts of the S9 fraction with reference drugs such as 3MTCL and 2AA, which are sensitive to variations in S9 composition, prior to the use of an S9 preparation in an in vitro assay. Further studies about these systems should be carried out in order to obtain information that would allow better standardization and validation of in vitro mutagenicity assays.

Acknowledgements

We acknowledge Dr F. Berard for providing the animals, Dr R. Baistrocchi for his contribution in statistical analysis, Mr B. O. Serrano for his excellent technical and secretarial assistance, Dr E. Ortí for his careful revision of the manuscript and Dr R. C. Combès for his encouragement and support.

References


Hantzsch reaction.


Received on October 9, 1998; accepted on February 2, 1999