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The effect of schistosomiasis on the covalent binding of 2-acetylaminofluorene to mouse liver macromolecules in vivo and in vitro

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Aromatic amines such as 2-acetylaminofluorene (AAF) have been shown to be carcinogenic in many animal species including humans, rodents, rabbits and dogs [1]. AAF undergoes metabolic activation via N-hydroxylation [2, 3] to yield the compound N-hydroxy-2-acetylaminofluorene (N-OH-AAF), the proximate carcinogenic metabolite which is sulfated to form the ultimate carcinogen [4]. The cellular nucleophiles that react covalently with the active metabolite of AAF in vivo have been identified to be proteins [5, 6], DNA [7] and glycogen [8]. The binding of AAF to mammalian protein and nuclear DNA in vivo has also been demonstrated [9, 10]. It is the formation of the carcinogen-macromolecular adducts that is thought to lead to the toxic and carcinogenic effects of the compound.

The response of animals to carcinogens may be modified by parasitic infection. Infected animals develop increased susceptibility to toxins and neoplasia [11-13]. To investigate the effect of parasite infection on metabolic activation of chemical carcinogens, we monitored the binding of AAF to macromolecules in schistosome-infected and non-infected mice both in vivo and in vitro.

Materials and Methods
Treatment of animals. Eight-week-old BALB/c mice were randomly divided into two groups. One group was infected with 30 Schistosoma mansoni cercariae per mouse using the method of Moore et al. [14] while the other group served as the control. After infection, the two groups were

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Table 1. The effect of schistosomiasis on microsome-mediated binding of AAF to DNA and protein

<table>
<thead>
<tr>
<th>Assay</th>
<th>Non-infected§</th>
<th>Infected§</th>
</tr>
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<tbody>
<tr>
<td>DPM/mg DNA†</td>
<td>1674 ± 258‡</td>
<td>988 ± 95‡</td>
</tr>
<tr>
<td>DPM/mg microsomal protein†</td>
<td>1129 ± 418†</td>
<td>909 ± 134†</td>
</tr>
<tr>
<td>Cytochrome P450‡</td>
<td>0.73 ± 0.03§</td>
<td>0.49 ± 0.02§</td>
</tr>
<tr>
<td>Total microsomal protein/liver</td>
<td>32.1 ± 2.9‡</td>
<td>25.4 ± 2.3‡</td>
</tr>
</tbody>
</table>

* Data represents means of duplicate incubations each for N = 4.
† nmoles/mg microsomal protein.
‡ Difference statistically significant (Student's t-test, P = 0.01).
§ Data presented as mean ± SD.

kept under the same conditions and fed Purina Lab Chow and tap water ad lib. for 12 weeks.

Microsome-mediated binding of AAF to DNA and protein in vitro. Animals from each group were killed and hepatic microsomes prepared by high speed centrifugation [15]. Microsomes were stored at −70°C until used. The incubation medium and conditions for microsome-mediated binding to calf thymus DNA and protein were as described by Gurtoo et al. [16] except that 0.01 μCi [14C]AAF (0.44 mM AAF) was used and incubation was for 60 min at 37°C with shaking. Equivalent amounts of microsomal protein were used from control and infected animals at 0.9 and 2.0 mg/mL in incubations for binding to DNA and to protein, respectively. The [14C]AAF-alkylated DNA was isolated from the reaction mixture [17], redissolved in 0.5 mL of 0.015 M NaCl-0.0015 M sodium citrate (pH 7.0) and dialysed against four changes of 0.01 M Tris-HCl-1 mM EDTA, (pH 7.0). Duplicate 50-μL aliquots of each sample were counted whilst the remaining fraction was used for determination of DNA concentration [18]. The reaction to determine AAF-binding to microsomal protein was terminated by addition of 20% TCA. Unbound radioactivity was removed by four washings of the protein precipitate with ether-ethanol (1:1). The precipitate was then hydrolysed with 2 mL 1 M NaOH at 60°C for 1 hr after which 1.0-mL aliquots were taken for determination of radioactivity and protein [19]. Hepatic microsomal cytochrome P450 was determined according to the method of Omura and Sato [20].

Covalent binding of AAF to liver macromolecules in vivo. Each animal from both groups was treated with a single intraperitoneal injection of 0.1 μCi [14C]AAF (9 μg AAF in 10 μL ethanol). Three animals from each group were killed at each of the following time intervals after treatment with AAF: 1 hr, 6 hr, 18 hr and 1 week. The livers were removed, washed with chilled 0.9% NaCl and stored at −70°C until analysed. For analysis, each liver was homogenized in 0.15 M NaCl-0.015 M sodium citrate (pH 7.0) and DNA, RNA and protein isolated [21]. Aliquots of the macromolecules were counted and specific activities calculated after assay of DNA [18], RNA [22] and protein [19].

Results and Discussion
Pathology typical of S. mansoni infection was evident in infected mice, with livers showing enlargement secondary to the formation of granulomas around parasite eggs. Average mass of infected livers was 2.6 ± 0.1 g versus 1.6 ± 0.1 g for uninfected livers. Data on microsome-mediated binding of [14C]AAF to calf thymus DNA and microsomal protein are shown in Table 1. Liver microsomes obtained from infected animals demonstrated a 42% decreased capacity to mediate covalent binding of AAF to DNA. In contrast, the covalent binding of AAF to microsomal protein was unchanged in microsomes from infected compared to non-infected animals. If results are calculated in terms of binding to macromolecules (DPM) per nanomole of cytochrome P450 no statistically significant differences in binding to DNA or protein is observed. It appears that it is depressed levels of cytochrome P450 that are responsible for the decreased binding of AAF to DNA in vitro.

Figure 1 shows the specific activities of DNA, RNA and protein isolated from livers of S. mansoni-infected (○) and non-infected (●) mice at various times after treatment with [14C]AAF. Difference between infected and non-infected mice statistically significant at ** P < 0.001 and * P < 0.01.

Fig. 1. Specific activities of DNA, RNA and filter bound protein isolated from livers of S. mansoni-infected (○) and non-infected (●) mice at various times after treatment with [14C]AAF. Difference between infected and non-infected mice statistically significant at ** P < 0.001 and * P < 0.01.
protein isolated from livers of infected and non-infected animals at different time intervals after treatment with \([\text{[}^{14}\text{C]}\text{AAF}\]. The yields of DNA from infected and control livers was comparable but the yields of RNA and protein from infected livers were 50 and 75% of controls, respectively. Extent of binding of AAF to macromolecules was generally less in infected mice up to 18 hr after treatment. By 1 week the difference between infected and non-infected mice had disappeared. It is generally accepted that the binding of a chemical carcinogen to DNA is a critical step in tumour induction and that covalent binding of a compound \textit{in vitro} and \textit{in vivo} to DNA correlates well with its carcinogenicity. The results reported here for AAF are therefore in contrast to reports showing increased tumorigenicity of chemical carcinogens, including AAF [13], in schistosome-infected animals [12, 23]. Previous work in this laboratory, however, on the covalent binding of aflatoxin B\textsubscript{1} to DNA, RNA and protein in schistosome-infected animals is consistent with the results reported here [24]. In addition, the results are consistent with studies showing that \textit{S. mansoni} infection decreases levels of hepatic drug metabolizing enzymes [25]. It appears that there is no simple relationship between tumour induction and levels of carcinogen–DNA binding in schistosome-infected mice.

In the light of the current views of carcinogenesis [26] it is possible that, although the initiating event in chemical carcinogenesis (i.e., metabolic activation of the carcinogen) may be depressed in schistosomiasis, the later stages in the multistep process may be altered such that promotion and progression are enhanced in \textit{S. mansoni} infected animals.

In summary: the covalent binding of \textit{N}-\text{2-fluorenylacetamide} to liver macromolecules \textit{in vivo} was measured in \textit{Schistosoma mansoni}-infected and in non-infected mice. Liver microsomes from infected mice demonstrated a 42% decreased capacity to mediate covalent binding of AAF to DNA. In addition, the extent of binding of AAF to liver macromolecules \textit{in vivo} was generally less in infected than non-infected mice.

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The importance of divalent cations on opioid receptor binding was first demonstrated prior to the identification of the various different receptor subtypes [1]. In these studies divalent cations, especially manganese, enhanced the binding of agonist ligands. This action was most pronounced in the presence of sodium chloride, which greatly reduces agonist binding [2]. Since the identification of receptor binding subtypes [3-8], the issue of divalent cations has been explored by a number of laboratories [9-12]. These reports confirmed the earlier report and extended the findings to both \( \mu \) and \( \delta \) receptors. Recently we examined the effects of magnesium on \( \mu \) binding using a selective assay [13]. In this study we also observed a significant potentiation of \( \mu \) binding in the presence of magnesium.

Most of the previous studies examined \( \mu \) and \( \delta \) binding sites but did not address the issue of multiple \( \mu \) receptors. One report noting the ability of magnesium to increase both \( \mu_1 \) and \( \mu_2 \) binding did not study other cations [11]. In the present study we have examined the sensitivity of \( \mu_1 \), \( \mu_2 \) and \( \delta \) binding to magnesium, manganese and calcium.

Materials and Methods

All radioligands and Formula 963 scintillation fluid were purchased from the New England Nuclear Corp. (Boston, MA). Fresh calf brains were obtained locally, dissected into the appropriate brain region, and prepared as previously reported and frozen [13]. Stored at -70°C, tissue binding is stable for at least 1 month. All binding was performed in potassium phosphate buffer (50 mM; pH 7.0) for 150 min at 25°C and assays were filtered over Whatman B glass fiber filters using a Brandel Cell Harvester. \( M_0 \) binding was determined in thalamic homogenates (2 mL; 15 mg wet weight tissue/mL) using \([\text{3H}]\text{D-Ala}^2,\text{D-Leu}^5\text{enkephalin} \) (DPDPE) (10 nM). The inclusion of DPDPE eliminates the non-specific binding of \( \mu \) and \( \delta \) receptors.

**Results and Discussion**

First, we examined the effects of increasing concentrations of the different cations in the three binding assays. Magnesium sulfate at concentrations ranging from 10 to 50 mM increased \( \mu \) binding over 2-fold (Fig. 1A), a result similar to that previously reported [13]. Although magnesium sulfate also increased the binding in the \( \mu_2 \) and \( \delta \) assays, the increases were far less (approximately 50%). Half-maximal increases by magnesium sulfate were similar for all the assays examined, approximately 1 mM. Previous work has established that magnesium chloride and magnesium sulfate affect opioid binding in a similar manner [1, 13].

Manganese chloride had a similar effect, increasing the binding in the \( \mu \) assay by over 2-fold with a maximal effect between 0.1 and 1 mM (Fig. 1B). A concentration less than that seen with the magnesium sulfate. However, the increase produced by manganese chloride was far less than those elicited by magnesium ions. Manganese chloride had a far less pronounced action on \( \mu_2 \) and \( \delta \) binding than on \( \mu \) binding. Calcium chloride also increased binding with results quite similar to those observed with manganese chloride (Fig. 1C). Little effect was seen on either the \( \mu_2 \) or the \( \delta \) assays, whereas we observed approximately a 75-80% increase in the \( \mu \) assay.

We next examined the effects of these ions on the affinity (\( K_D \)) and the \( B_{max} \) of the three assays (Fig. 2). Manganese and magnesium both increased \( \delta \) binding predominantly through increases in the \( B_{max} \). In contrast, the effects of the different cations were more complicated in both of the \( \mu \) assays. Of the different ligands examined, manganese produced the largest increase in \( B_{max} \) and affinity.

Manganese ions greatly prolonged the dissociation of \([\text{3H}]\text{DADL} \) in the \( \mu \) binding assay [13]. In the present assay, we examined the effects of calcium and manganese ions on the dissociation of \([\text{3H}]\text{DADL} \) in a \( \mu \) selective...