Promutagenic methylation damage in liver DNA of mice infected with *Schistosoma mansoni*

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Male and female BK-T0 mice were infected with different numbers of *Schistosoma mansoni* cercariae under standard environmental conditions. Promutagenic methylation damage (6-O-methyldeoxyguanosine; O6-MedG) was detected in liver DNA, but not in kidney, spleen or bladder DNA of infected animals. It was shown that levels of hepatic O6-MedG increased with increasing intensities of schistosomal infection. Possible mechanisms of action are discussed. These include the activating effects of schistosomes and their products on murine macrophages and subsequent endogenous formation of N-nitroso compounds by the activated macrophages.

Introduction

A large number of biological factors have been implicated in predisposing individuals to later development of cancer. These include viral and bacterial infections as well as plasmoidal and other parasitic infections (1). About 30,000 species of protozoa and a similar number of helminths are known. Of the latter, about 100 species of worms afflict man (2,3). An important group of trematodes are the blood vessel flukes (the schistosomes) which, among other pathological consequences, can cause cystitis, intestinal and vesical ulceration, hepatic fibrosis, portal hypertension, hepatosplenomegaly and bladder cancer (4,5).

One of the earliest cases of parasites being involved in the causation of cancer was the association between infection with the trematode *Gorgonolaima neeplopticum* and gastric carcinoma in rats (6). Although this was later refuted (7), there exists a well-documented relationship between infections with certain parasites, e.g. *Schistosoma haematobium* and *Opisthorchis viverrini* or *Clonorchis sinensis* and the development of particular types of cancer [e.g. bladder cancer (8) and cholangiocarcinoma (9) respectively]. More recently this association between *O. viverrini* infection and cholangiocarcinoma risk has been confirmed, but no involvement with aflatoxin intake, hepatitis B infection or dietary patterns was established (10). In many other cases, an association between cancer induction and parasitic infection has been presumed. For example, the association between infection with *Onchocerca volvulus* and induction of lymphangiosarcoma (11–13); *Toxoplasma gondii* and CNS neoplasia (14), *Babesia rodhaini* and *Linguatula serrata* and leukaemia (15,16) and infection with *Armillifer armillatus* has been linked with the development of colon cancer (17). The evidence implicating the participation of infection with *S. haematobium* in the development of bladder cancer is greater by far than that for any other parasitic infection (5,8). Relationships that exist between cancer and infection with schistosome species other than *S. haematobium* are considerably weaker (18); nevertheless, available evidence suggests that infection with *S. mansoni* can influence the development of carcinoma of the female genital tract (19) and intestine (20), splenic lymphoma (21–22) and breast cancer in males (23). No adequate epidemiological studies have been performed, however, to substantiate any of these associations, although infection with this parasite in mice was found to increase the risk of hepatic carcinoma associated with the administration of the liver carcinogen 2-amino-5-azotoluene. This was suggested to involve tumour promotion rather than initiation, since *S. mansoni* infection alone did not induce liver cancer (24). This protocol was also applied with other parasites. Subcarcinogenic doses of N-nitrosodimethyamine induced cholangiocarcinoma in Syrian golden hamsters previously infected with *O. viverrini* (25,26). Likewise, beef cattle infected with the liver fluke *Fasciola hepatica* were more sensitive to aflatoxicosis than uninfected animals (27) and *F. hepatica*-infected mice metabolized aflatoxin B1 into its carcinogenic form more efficiently than did uninfected mice (28). Low doses of N-nitroso compounds in the presence of *S. haematobium* infection induced neoplasia in the urothelium of four out of 10 infected baboons (29).

Various hypotheses have been proposed to explain the carcinogenic process induced by parasite infection, either by schistosomes (8,30) or others (31) and most concern has been directed towards the possible role of chemical carcinogens (2). N-Nitroso compounds are an important class of environmental carcinogens and their role as causative agents in the pathogenesis of some human cancers has been extensively reviewed (e.g. 32,33). These agents, or their breakdown products, can react with cellular DNA to form a complex spectrum of adducts (34) and it is thought that such alkylation of DNA can be a critical event in the induction of cancer (35,36). Of the lesions induced in DNA, probably the most significant is due to the alkylation of guanine at the O6-position (O6-alkylguanine; O6-RG*) as this is formed in significantly larger amounts than the other promutagenic base O6-alkylthymine (34). The persistence of O6-RG in different tissues and cells has been correlated with the cytotoxic, mutagenic, carcinogenic and other biological effects of this group of chemical carcinogens (34–37). Such damage has been detected in tissue DNA from different populations in the world, in individuals suspected of exposure either to environmentally derived (38–42) or to endogenously formed (42) alkylating agents. Consistent with these observations, the presence of N-nitroso compounds has been demonstrated in the urine of schistosome-infected subjects at levels significantly higher than that in normal individuals (43–48). It was suggested that this is mainly due to endogenous formation arising from the urinary bacterial infection that invariably accompanies schistosomiasis.
The recent discovery of O'-methyl-2'-deoxyguanosine (O'-MedG) in DNA obtained from those individuals (42) emphasizes the importance of understanding the role played by the parasite in the possible endogenous formation of N-nitrosocompounds in schistosome-infected subjects. The present work was undertaken to investigate whether schistosomal infection might be directly involved in the induction of promutagenic methylation damage in liver DNA using mice infected under controlled conditions.

Materials and methods

Infection of animals

Infection of male and female BK-TO mice was carried out using a Puerto Rican strain of S.mansoni cercariae maintained by passage in laboratory-reared Biomphalaria glabrata snails and random-bred TO strain mice. Percutaneous infections of S mansoni cercariae (50-400 per animal) using the ring technique, and portal perfusions to determine worm burdens, were performed according to the method described by Smithers and Terry (51). The required number of cercariae (suspended in water) was placed in a ring attached to the shaved abdomen of anaesthetized mice for 30 min, after which the water was removed and the mice allowed to recover. In general, the number of cercariae remaining in the water was <10%. Based on worm burdens determined at the time of sampling (30 days) the infection rate for males and females using 200 cercariae was 48.3 ± 14.7 and 63.3 ± 14.5 on two separate occasions. Previous experience (52) shows that only an occasional worm and a few eggs remained after perfusion. At 30 days post-infection only minimal hepatopathological changes have been noted (52). Examination of the infected livers during this study showed the presence of worms in the radical vessels and minimal pathological changes, with variable degrees of lymphocytic infiltration.

Materials

Pancreatic DNase I (type IV, 1900 Units/mg), snake venom phosphodiesterase (type VII) and Escherichia coli alkaline phosphatase (type III) were supplied by Sigma Ltd, Poole, Dorset. Aminex A7 was purchased from Bioral Laboratories Ltd, Hemel Hempstead, Herts and 2'-deoxycoformycin was a gift from Professor D.Crowther. O'-MedG and [3H]O'-MedG (32.4 Ci/mmol) were synthesized as previously reported (53). All other reagents were of analytical grade.

DNA isolation and analysis

All the extraction and RIA procedures have been described elsewhere (42,53,54) and are only outlined here. Batches of DNA were prepared from thawed tissue (3-5 g) using a modified phenol extraction procedure (55). The DNA (3-5 mg; 2 mg in the case of the worm DNA) was dissolved in 2.0 ml 50 mM Tris-HCl, pH 7.0 containing 5 mM MgCl₂, 3 mM NaN₃ and digested enzymatically to nucleosides overnight at 37°C using DNase I (200 μg), snake venom phosphodiesterase (0.03 units) and alkaline phosphatase (5 units) in the presence of 2'-deoxycoformycin (1 μM), which is used to inhibit adenosine deaminase activity. The resultant hydrolysates were chromatographed on Aminex A7 (25 x 1 cm) maintained at 50°C and eluted with 10 mM ammonium carbonate, pH 8.0 at a flow rate of 0.75 ml/min. The four major deoxynucleosides were separated from O'-MedG by this procedure and quantitation of the normal purines in the DNA sample was achieved by rechromatography of a small portion of the pure fraction and peak area integration. The elution position of O'-MedG was identified in preliminary runs by tracer [3H]O'-MedG. The putative O'-MedG-containing fraction and control fraction (i.e., a similar volume of buffer from a blank region of the column elution profile) were further purified by solid phase extraction using C18 BondElut columns (D.P.Cooper, G.-F.Yao, Y.-H.Qu and P.J.O'Connor, in preparation) and analysed by radioimmunoassay (RIA) using a monoclonal antibody to O'-MedG (53). Calibration curves (0.01 - 0.50 pmol O'-MedG) showed a linear response when percentage inhibition of antibody-antigen binding (probit) was plotted against the amount of O'-MedG (log₁₀); typically 50% inhibition of tracer antibody binding (ITAB) was obtained with 0.11 pmol O'-MedG (see 42). Extensive control digestes of DNA containing known levels of O'-MedG indicate that the adduct was not being degraded by adenosine deaminase or by contaminating enzymes. All column blank fractions produced ≤2% ITAB in the RIA and any samples producing >5% ITAB were considered positive. The positive results presented here are based on ITAB values ranging from 7 to 78% compared with values of 0-4% in controls. Results are expressed as pmol O'-MedG/mg parent base; the lower limit of detection was ~0.01 pmol O'-MedG/mg 2'-deoxyguanosine (dG) and at these low levels of alkylation, the reproducibility of the analysis is ±35% (see also ref. 42).

Results

The effect of schistosomal infection on the formation of promutagenic methylation damage was observed initially in Balb/c mice maintained on an uncontrolled diet and pre-infected at various intervals from 15 to 45 days with S.mansoni cercariae obtained from snails collected from waterways in and around the Alexandria region of Egypt, an area hyperendemic for schistosomiasis (data not shown; 5). O'-MedG was found in the DNA of several tissues, including the liver from the control group of animals. Significantly higher levels of alkylation were observed...
unexpectedly in the tissue DNA of the schistosome-infected animals. In the case of hepatic DNA, these were as high as 2–3 μmol O\(^6\)-MedG/mol dG in some groups.

In an attempt to confirm whether or not S. mansoni infection alone might be responsible for the induction of such DNA damage, the levels of the promutagenic lesion were assayed in BK-TO mice maintained on a controlled diet and infected under standard laboratory conditions, i.e. from in vitro culture cycles of S. mansoni. Male and female mice were thus infected with different intensities of S. mansoni cercariae (100 and 300 per mouse) and after 30 days tissues were taken for DNA isolation and analysis. O\(^6\)-MedG was found, but in this case only in liver DNA and at much lower levels that ranged from 0.042 to 0.392 μmol O\(^6\)-MedG/mol dG. Subsequent assays, therefore, were carried out only on the liver DNA.

In a further series of experiments five male and five female mice were infected with 300 cercariae per animal and O\(^6\)-MedG was again detected in the liver DNA (Table I). The level of O\(^6\)-MedG was higher in females than males (0.262 versus 0.088 μmol O\(^6\)-MedG/mol dG), although both received the same number of cercariae (300/animal). The corresponding control groups showed no detectable levels of O\(^6\)-MedG (i.e. <0.01 μmol/mol dG).

When groups of male mice were given S. mansoni cercariae at different intensities of infection (i.e. from 50 to 400/animal) 30 days prior to tissue collection, a dose–response relationship was observed between the intensity of infection and the levels of O\(^6\)-MedG in hepatic DNA (Figure 1).

The above-mentioned analyses were all performed on DNA from livers that also contained the growing worms, so it was possible that this damage might exist in the worm DNA rather than in the DNA of the tissue itself. Thus, in order to determine the location of the methylation damage, 30 days after infection DNA was extracted and assayed from livers perfused to remove the worms themselves and the non-perfused livers of male mice infected with S. mansoni cercariae (300/animal). O\(^6\)-MedG was detected in the liver DNA of both of the infected groups, regardless of the perfusion (Table II). Neither the livers of the control groups (whether perfused or not), nor the worms showed any detectable levels of the DNA adduct.

In view of the differences observed in the levels of alkylation between different experiments, a further experiment was performed to compare the hepatic DNA of two groups of animals infected using cercariae from the same batch of patent snails, but the second group was infected with fresh cercariae 1 week after the first. Liver DNA from the two groups was analysed simultaneously. In this experiment using male mice (six per group) infected with S. mansoni cercariae (300/animal), similar levels of DNA alkylation were detected in the two groups (i.e. 0.082 and 0.120 μmol O\(^6\)-MedG/mol dG).

**Discussion**

Schistosomiasis-induced promutagenic methylation damage in tissue DNA of S. mansoni-infected animals has been observed for the first time in the present study. The levels of O\(^6\)-MedG detected in the liver DNA of infected animals were initially thought to be due to the experimental conditions and possible concurrent exposure to environmental N-nitroso compounds, rather than an effect of schistosome infection itself. As the S. mansoni cercariae used in the initial experiments were obtained from snails collected from waterways in and around the Alexandria region, the cutaneous surface of the developing worms may be colonized with different species of nitrate-reducing bacteria. These might exist in a symbiotic relationship with the schistosomes (56,57) and generate nitrosation reactions (30) leading to the formation of methylating species capable of inducing DNA damage. However, we also considered that the schistosomes themselves had a possible role in inducing or contributing to the levels of DNA damage observed in the infected animals, compared with the control group in the first experiment.

To try to confirm these data and to ascertain the mechanisms involved, male and female BK-TO mice maintained on a controlled diet were infected with S. mansoni cercariae obtained from laboratory-maintained life cycles. In the preliminary experiments O\(^6\)-MedG was found in mice of both sexes and to varying degrees, but it was detected only in liver DNA and the levels observed were much lower than those found in the Balb/c mice used in Alexandria. The data shown in Table I and Figure 1 demonstrate that the alkylation damage in hepatic DNA can arise due to the presence of schistosomes in infected animals. No evidence for the damage was observed in the worm DNA (Table II) indicating that the DNA damage detected after schistosomal infection exists primarily in the liver, which is the main habitat of the worms at that stage of infection. Alkylation of the worm DNA, however, cannot be excluded due to the small amount of schistosome DNA available and the sensitivity of the analyses (~0.01 μmol O\(^6\)-MedG/mol dG). It is evident, however, that the majority of the observed DNA damage occurs in hepatic DNA as a result of schistosome infection.

There were marked differences in the levels of alkylation measured in the different experiments described above in which *in vitro* cultured worms were used. In a subsequent experiment, therefore, the hepatic DNA of animals infected at two different times were analysed at the same time and in this case very similar results were obtained. We concluded that for a given intensity of infection with S. mansoni cercariae, the resultant level of alkylation is somewhat unpredictable although in all the experiments carried out, alkylation of hepatic DNA was consistently observed. Much greater differences in the levels of alkylation of hepatic DNA are seen when the results from experiments using cultured *S. mansoni* are compared with those in which snails collected from the waterways of Alexandria were used. These greater differences could be due to a combination of factors, e.g. the source of infectious material which could be contaminated with different bacteria (see above), strain differences between the host animals, or to a less well controlled laboratory diet or to a different geographic isolate of *S. mansoni*.

The possible presence of carcinogenic and/or toxic substances in schistosome eggs, schistosomula and adult worms has not been greatly studied (58). These experiments were terminated at 30 days at which time the infection would be as adult worms in the portal system, virtually no eggs would be produced (52) and hepatopathology was minimal. It seems therefore, that the presence of the worms can induce the alkylation of DNA and, contributing to the levels of DNA damage observed in the infected animals, compared with the control group in the first experiment.
macrophages are stimulated in vivo during the course of S. mansoni infection (59,60). It is now generally recognized that macrophages are capable of inducing nitrosamine formation under physiological conditions (61,62). Since there is a possibility that N-nitroso compounds may be formed endogenously via activated macrophages, the infected liver could possibly be considered as a site of endogenous nitrosation and the DNA alkylation damage observed in the schistosome-infected liver DNA could be due to the activating effects of schistosomes on murine macrophages.

Although the role of liver cell proliferation in chemically induced hepatic carcinogenesis is not precisely defined, considerable evidence is available indicating that the liver is more susceptible to the development of putative preneoplastic lesions induced by several carcinogens when liver cells are exposed during the early S-phase of the cell cycle (63,64). This S-phase sensitivity possibly reflects differences in susceptibility via the metabolic transformation of chemical carcinogens or via DNA replication (35,68). Generally, data using a fluke-infested definitive host (e.g. humans infected with schistosomes) suggest that net production of carcinogenic metabolites is intimately dependent upon the balance between activation and detoxification in the host organisms. It is tempting to speculate that schistosom-infected subjects either have an induced enzyme profile in selected organs (66) or have elevated levels of enzymes that detoxify carcinogenic metabolites (67). Clearly, the presence of parasites and transient changes in enzyme levels coupled with the possible diffusion of highly reactive carcinogens may lead to a complex sequence of events. This situation is better understood in the case of S. haematobium infection (8,42,68) but still has to be investigated in the case of S. mansoni infestation.

Apart from its role in the initiation of cancer, O2-MedG is demonstrably a toxic DNA lesion (37). It is possible therefore, that schistosome-induced DNA alkylation may play some role in the development of hepatopathology associated with schistosomiasis e.g. in the development of portal fibrosis. Alternatively, the observed alkylation of DNA could be a secondary effect of tissue toxicity or even of senescence and cell death caused by physical or toxic chemical damage in areas adjacent to the worms, if this leads to the generation of reactive alkyllating intermediates. Hepatopathology at 30 days, however, is limited (see above), but increases significantly by 40 days (52).

Although as yet we are unable to determine the significance of schistosome-induced hepatic DNA damage, it is evident that damage of the kind normally associated with exposure to alkylling carcinogens is found as a result of schistosome infection. It is of interest to note that the levels of DNA alkylation observed in this study (0.04–0.40 μmol O2-MedG/mol dG) are comparable with those detected in human DNA as a result of environmental exposure. In the latter studies samples were obtained from various geographical locations e.g. oesophagus and stomach from Lin Xian, N. China (38), stomach from Shanghai (41), stomach and colon from S. Manchester, UK (40) and bladder tissues from the Nile Delta (42). The mechanisms indicated in the present study, therefore, could have direct relevance for the development of bladder cancer in areas such as the Nile Delta where urinary schistosomiasis is widespread. These observations could also be of wider significance for other parasitic infections implicated in the development of neoplasia.

Acknowledgements

This work was supported in part by the Egyptian Government (Cultural and Educational Bureau), in part by the Cancer Research Campaign and in part by the Welcome Trust (UCNW). Facilitating exchange visits between Alexandria and Manchester were sponsored by the British Council and the Egyptian Government.

References


