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# GENETIC COMPLEMENTATION ANALYSIS OF TWO INDEPENDENTLY ISOLATED HYCANTHONE-RESISTANT STRAINS OF *SCHISTOSOMA MANSONI*

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*The objective of this study is to determine whether various hycanthone resistant strains of schistosomes which have been independently isolated are all affected in the same gene. A strain obtained from a Brazilian patient was compared with a strain of Puerto Rican origin selected in the laboratory. If the mutation conferring resistance involved two different genes, one would expect that the progeny of a cross between the two strains would show complementation, i.e. it would be sensitive to the drug. We have performed such a cross and obtained $F_1$ hybrid worms which were essentially all resistant, thus suggesting that the mutation conferring resistance in the two strains involves the same gene.*

Key words: *Schistosoma mansoni* – hycanthone resistance – genetic complementation

A number of hycanthone (HC) resistant schistosome strains have been described over the last 20 years (Rogers & Bueding, 1971; Dias et al., 1978; Katz et al., 1973; Coles & Bruce, 1987). It would be of interest to determine whether all these resistant "mutants" carry the same mutation or whether they are mutated in different genes. If different mutations were shown to confer resistance, this would imply that several worm functions are involved in the mechanisms of drug action and drug resistance, and it would be obviously of interest to unravel and define such functions. We have examined two HC resistant strains which were isolated in widely different settings, and we report here on some experiments showing that the same genetic function is involved in the resistance of both strains.

The first resistant strain examined in our work was isolated in 1971 during laboratory experiments carried out in Baltimore by Dr. Ernest Bueding (Rogers & Bueding, 1971). The strain was subsequently established in our laboratory in Rome and was subjected to 3 cycles of drug pressure and selection of the survivors (Cioli & Pica-Mattoccia, 1984). It will be referred to as BRR, or Baltimore Rome Resistant. HC resistance of this strain is very high and it is absolutely stable. The strain is also remarkably resistant to the related drug oxamniquine (OXA), and – based on this cross-resistance and on other pieces of evidence – we have proposed that the two drugs probably have a similar mechanism of action (Pica-Mattoccia & Cioli, 1985).

The other resistant strain we examined was isolated by one of us in the field (Dias et al., 1978), from the feces of a treated uncured patient of the State of São Paulo. It was shown that the strain is resistant to both HC and OXA, while it is sensitive to niridazole and praziquantel. This strain (called MAP, from the initials of the patient) was also established in the Rome laboratory where it is currently being subjected to subsequent cycles of drug pressure and selection.

As a reference drug-sensitive (wild-type) strain we used a Puerto Rican (NIH) strain obtained thru the Mill Hill laboratory (Cioli, 1976). It must be stressed that this sensitive strain (referred to as SEN) is also remarkably stable in its HC sensitivity throughout the generations.

Table I reports the drug sensitivity of the various strains employed. The experimental technique we currently use to test for resistance can be summarized as follows. Adult schistosomes can be kept *in vitro* in serum-supplemented media, and in our hands they survive almost indefinitely showing normal morphology, normal pairing and some egg laying. If worms are exposed for a short time (minutes) *in vitro* to HC or OXA, they show no immediate signs of damage, but in the

subsequent 10-20 days – depending on the dose – they will die if sensitive or will survive indefinitely if resistant. We routinely expose worms to the drug for 1 h, we wash them repeatedly, and we keep them in drug-free medium for about a month. At this point we simply count the survivors and obtain a quantitative estimate of resistance or sensitivity. The advantages of this *in vitro* assay, compared to the traditional *in vivo* testing, are quite obvious: we don't have to rely on a separate group of untreated parasites to establish our control value; the time of exposure can be finely controlled and the drug concentration is not subjected to the possible pitfalls of in vivo treatment

TABLE I

Percentage of worms surviving *in vitro* to Hycanthone and Oxamniquine (1-hr exposure)

| | SEN | | MAP | | BRR | |
|---|---|---|---|---|---|---|
| Hycanthone (M) | M | F | M | F | M | F |
| $2 \times 10^{-7}$ | 16 | 100 | 100 | 100 | 100 | 100 |
| $10^{-6}$ | 0 | 0 | 100 | 100 | 100 | 100 |
| $10^{-5}$ | 0 | 0 | 34 | 51 | 100 | 100 |
| $10^{-4}$ | 0 | 0 | 29 | 32 | 100 | 100 |
| $4 \times 10^{-4}$ | 0 | 0 | 0 | 0 | 0 | ND |
| Oxamniquine ($\mu$g/ml) | | | | | | |
| 25 | 0 | 52 | 100 | 100 | 100 | 100 |
| 100 | 0 | 0 | 100 | 100 | 100 | 100 |
| 250 | 0 | 0 | 100 | 100 | 100 | 100 |
| 500 | 0 | 0 | 100 | 100 | 100 | ND |

Using this approach, we have collected the data reported in Table I. SEN worms are already partially sensitive to $2 \times 10^{-7}$M HC and to 25 µg/ml OXA. MAP schistosomes are totally resistant to $10^{-6}$M HC and partially resistant to higher doses. BRR worms, on the other hand, are totally resistant up to $10^{-4}$M HC. With OXA, both MAP and BRR strains show solid resistance at very high doses. Thus, we can conclude that BRR worms are resistant to higher HC doses than MAP worms, while the resistance of both strains is very high towards OXA. As usual, females are more resistant than males towards this class of compounds.

We next asked the question whether HC resistance is a dominant or a recessive character, since the molecular implications of either result are quite relevant. In order to perform schistosome crosses, we have developed a simple technique which has allowed us to investigate a number (certainly in the hundreds) of genetic combinations over the last few years. A single male worm and a single female worm of the strains to be tested are surgically introduced into the mesenteric veins of mice, usually when they are about 25-27 days old (this is an age at which the two sexes are morphologically quite distinct, but fertilization has not started, thus avoiding possible carryover of sperm in the females). About 4 weeks later, the liver of the recipient mouse is loaded of hybrid eggs, which are passeged into snails to finally give the adult worms to be tested for resistance with the in vitro technique described above.

We have previously shown that the progeny of SEN worms is always sensitive, while the progeny of BRR worms is always resistant (Cioli & Pica-Mattoccia, 1984). We infer from this observation that the two strains are probably homozygous for the gene conferring, respectively, sensitivity or resistance. The $F_1$ hybrids are always sensitive, and from this critical result we infer that resistance is carried by a single, autosomal, *recessive* gene. This is confirmed by the results of the $F_2$ generation, where resistance from the two phenotypically sensitive parents reappears with a frequency close to the expected 25%. It is also confirmed by the backcrosses, where resistance parent, with a frequency close to the expected 50% (unpublished results).

We have performed the same basic experiment with the MAP strain, and although we have only the $F_1$ data so far, we have strong evidence that resistance is recessive in this strain as well (results not shown).

Thus, the sensitivity of schistosomes to HC is strictly dependent on the presence (at least on one chromosome) of a dominant gene, which is obviously critical for the production of a worm *factor* required for drug activity. We have proposed (Cioli et al., 1985) that such a *factor* could be an enzyme capable of converting HC to a reactive ester (possibly a sulfate or a phosphate). The reactive ester would spontaneously dissociate, thus producing a strong electrophile capable of alkylating the DNA and other schistosome macromolecules. Resistant schistosomes, in this hypothesis, would be lacking the esterifying enzyme and would thus

TABLE II

HC resistance of hybrids MAP x BRR

| Exp. No. | HC concentration (M) | Male | | Female | |
|---|---|---|---|---|---|
| | | No. tested | % resistant | No. tested | % resistant |
| F1 generation | | | | | |
| 1 | $10^{-4}$ | 66 | 42 | 25 | 60 |
| 2 | $10^{-4}$ | 38 | 63 | 40 | 78 |
| 3 | $10^{-4}$ | 120 | 89 | 96 | 94 |
| 4 | $10^{-4}$ | 91 | 88 | 95 | 99 |
| 1 | $10^{-6}$ | 25 | 100 | 15 | 100 |
| 4 | $10^{-6}$ | 74 | 92 | 72 | 100 |
| F2 generation | | | | | |
| 1 | $10^{-4}$ | 110 | 96 | 110 | 99 |
| | $10^{-6}$ | 80 | 100 | 80 | 100 |
| OXA 200 μg/ml | | 40 | 100 | 40 | 100 |

escape alkylation and death. At least two pieces of experimental evidence strongly support our scheme. First: an artificial, preformed, ester of HC was shown to kill both sensitive and resistant schistosomes (Cioli et al., 1985). Second: radioactive HC was demonstrated to become covalently bound to the DNA of sensitive worms, but not to the DNA of resistant worms (Pica-Mattoccia et al., 1988).

We next tackled the original question of whether BRR and MAP resistance is due to the same mutation, or to say it better, to a mutation affecting the same genetic function. This question can be answered by performing a genetic complementation test. If MAP and BRR are affected in the same gene, the expectation is that the $F_1$ hybrid of the two resistant strains should also be resistant, since no functional copy of the relevant gene would be present in the progeny. In the case that MAP and BRR are mutated in two different genes (the products of which are both necessary for HC to exert its activity), the $F_1$ hybrid should be sensitive, since each parental chromosome would carry a functional copy of one of the two genes required.

The results of such a complementation experiment are shown in Table II.

The MAPxBRR $F_1$ generation was largely, but incompletely, resistant when tested with $10^{-4}$M HC. Resistance, however, was complete at $10^{-6}$M HC. This is exactly the same behavior as the parental MAP strain shown in Table I and it is just what one would have expected from a hybrid carrying a gene with the level of *sensitivity* of the MAP strain. The $F_2$ generation gives strong reinforcement to the results, leading to the conclusion that the hybrid between the two resistant strains is again resistant.

The fact that the hybrid is resistant (e.i., there was no complementation) demonstrates that the same "gene" is affected in both strains. One could probably venture to speculate that the same *gene* is likely to be mutated in different parts or in different ways, since the resistance exhibited by MAP and BRR strains is not identical. The precise nature of the mutation cannot, obviously, be assessed until more information is available on the nature of the gene product (i.e., on the postulated activating enzyme). The data presented here show that the two mutants are affected in the same function, thus providing indirect support to our contention that a single critical activation step is required for HC activity.

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