SY-3 NATURALLY-OCCURRING GENE AMPLIFICATIONS CONFERRING DRUG RESISTANCE IN *LEISHMANIA TARENTOLAE*

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Our laboratory has engaged in the study of specific gene amplification in the protozoan parasite *Leishmania* (summarized in 1). Specific gene amplification is distinguished from changes in chromosomal ploidy, and consists of an increase in gene copy number of a limited segment of the chromosome, often in the form of extrachromosomal circular DNA in *Leishmania* (1). Amplification leads to overproduction of certain proteins which confer resistance. Gene amplification has been observed in promastigotes of *L. major* selected with five different drugs, of diverse structures and mechanisms of action (1-4), and *L. mexicana* selected with tunicamycin (5), suggesting that this molecular mechanism of drug resistance is at least not uncommon. It is known that the amplifications present in methotrexate-resistant *L. major* (6) and tunicamycin-resistant *L. mexicana* can be passed through the amastigote stage following infection of susceptible mice (5). An unanswered question of considerable interest is the contribution of these molecular mechanisms to the biology of the parasite.

One form of amplified DNA observed in natural isolates of *Leishmania* is the occurrence of small, multi-copy DNAs in *L. donovani* and *L. braziliensis* (7-10). These DNAs have certain features suggestive of a transmissible DNA, such as a viral genome. Definitive characterization of these elements and their biological roles have not been reported, although they are probably unrelated to drug resistance.

We have studied an apparently natural DNA amplification in *L. tarentolae*, a parasite of lizards (11). When total genomic DNA of the clonal MG-C2 line of this parasite was digested with restriction endonucleases and examined by electrophoresis, several fragments were observed to be amplified to high levels (about 30-fold). These fragments were isolated and molecularly cloned, and used to examine other strains of *L. tarentolae* by

Southern blot hybridization. We found that one other strain contained this amplification, while four other strains lacked it. The amplified DNA appeared to be stably maintained, and the parental MG isolate as well as other clonal derivatives contained the amplified DNA as well.

A recombinant lambda phage library of the MG-C2 DNA was made in the vector Charon 4A (3×10^6 independent recombinants), from which clones encompassing the entirety of the amplified DNA were isolated. Restriction mapping of these recombinant DNAs revealed that the amplified DNA consisted of about 42 kb of unique DNA. Interestingly, the restriction map revealed that the amplified DNA was organized in the form of an inverted repeat, i.e., with a "head-to-head" kind of organization:

$$(\text{-------}\rangle*\langle\text{-------})_n.$$

We had previously observed this kind of sequence organization in the amplified H DNA of methotrexate (MTX)-resistant L. major (2), and wondered whether the amplified DNA in L. tarentolae could be related. To our surprise, blot hybridization experiments revealed that the L. tarentolae amplification was indeed an amplification of the H region homolog of this species. Though the restriction map was quite different due to the evolutionary divergence of the two species, every hybridization probe from the H region of L. major identified amplified sequences in L. tarentolae. Additionally, the organization of these sequences was the same in the two species.

Using pulsed field electrophoresis, we examined the chromosomal structure of the amplified DNA. We employed a new method for demonstrating the presence of circular DNAs, limited cleavage with gamma irradiation, which yields a discrete linear derivative of circular DNAs. Our analysis revealed that the amplified DNA existed mostly (if not entirely) as a 140 kb circular extra-chromosomal DNA. Comparison of the size of this DNA with the size of the restriction map suggested that the circular DNA consisted of an extra-chromosomal tetramer, whose repeats were arranged in an inverted manner. This kind of structure also resembles the H region amplification of L. major, which exists primarily as an inverted dimer repeat (2).

We observed that all lines of L. tarentolae contained a

similarly-sized chromosome that was identified by H region probes (both internal or external), in addition to the amplified sequences evident in the two lines. This chromosome presumably contains the wild-type H region locus. Current chromosomal and restriction mapping data suggest that the amplification is conservative, defined as the retention of the wild-type chromosomal structure and copy number. Other kinds of amplification, in which some wild-type sequence is removed from (non-conservative) or duplicated (integrational) have been observed in amplifications of other loci in L. major (1,10).

These observations prompted us to examine whether the strains of L. tarentolae bearing H region amplification were MTX resistant. Significantly, the two strains bearing H amplification were 20-fold resistant to MTX, a value consistent with results with other lines of L. major we have examined which bear exclusively H region amplification (4). Thus, the H region amplification of L. tarentolae appears to be functionally homologous to the L. major H amplification.

Currently, we are not sure of the role of the H amplification in the biology of L. tarentolae. The two lines bearing this amplification have not undergone any drug pressure in the laboratory, so the forces leading to the emergence of a drug-resistance amplification in this species are unknown. In L. major H region amplification has recently been observed in two lines selected for resistance to two drugs unrelated to methotrexate, and moreover, that lines bearing H amplification exhibit cross-resistance to these three drugs. Thus, the H region amplification may mediate a form of multi-drug resistance, although our data indicate that the mechanism in Leishmania is distinct from that observed in amplified DNA mediating multi-drug resistance in cultured mammalian cells (4). Hypothetically, it is possible that the amplified lines of L. tarentolae have seen pressure from agents such as secondary plant compounds, or pesticides, and that H amplification was effective in alleviating this force. Alternatively, H amplification may confer some property that we have not examined, other than drug resistance. It is also possible that for some reason H amplification occurs spontaneously without selective pressure, although our studies of lines of L. major lacking prior amplification do not confirm support this view (12).

Our data demonstrate the occurrence of functional gene amplifications in lab stocks of a natural population of Leishmania. Drug resistance in natural populations of human Leishmania has been reported, although it has been questioned by some workers and the biochemical and molecular basis of resistance in any presumably resistant line is unknown. We have not observed amplified DNA in several different lines of Leishmania suspected to be resistant to antimonials, although the methods we have employed would not detect amplifications of less than 10-fold. At this time, the occurrence of amplified DNA mediating drug resistance in natural populations remains unknown.

Interestingly, strains of T. cruzi can exhibit widely varying DNA contents (13). As amplified DNA in methotrexate-resistant Leishmania can comprise 10% of the total cellular DNA (14), it will be interesting to examine natural isolates of this species for the occurrence of amplified DNAs.

References

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