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RESEARCH BRIEF

On the Introduction of Genetically Modified *Leishmania* outside the Laboratory

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The recent development of DNA transfectional methods for *Leishmania* and other parasites has provided powerful tools for studying gene function and expression and altering the parasite genome (Kapler *et al.* 1990; Laban *et al.* 1990; LeBowitz *et al.* 1990; Lee and Van der Ploeg 1990; Ten Asbroek *et al.* 1990; Eid and Sollner-Webb 1991; Goonewardene *et al.* 1993; Soldati and Boothroyd 1993). The growing sophistication and utility of these methods has led many investigators to consider applications of genetically engineered parasites outside the research laboratory. One promising direction is the development of safe attenuated vaccine lines capable of inducing protective immunity. For example, by homologous gene targeting we have generated an attenuated line of *Leishmania major* that shows some promise in vaccination trials in mice (Cruz *et al.* 1991; Titus *et al.* in preparation). However, creation of genetically attenuated parasites typically requires the introduction of dominant selectable markers, and it is important to consider whether these could pose a potential problem in situations outside the laboratory.

As an illustration we consider the attenuated *L. major* vaccine line mentioned above. This parasite is a thymidine auxotroph (*thy*)$^-$ which is inviable in mouse infections, a phenotype arising from deletion of the bifunctional dihydrofolate reductase–thymidylate synthase (DHFR-TS). This was accomplished by two rounds of homologous gene replacement, first using a targeting construct containing the neomycin phosphotransferase gene (*NEO*) followed by one using the hygromycin B phosphotransferase gene (*HYG*; Cruz *et al.* 1991). These two markers are effective dominant selectable markers in *Leishmania* and many other organisms and have been used in double gene targeting protocols to generate a number of homozygous gene knockouts.

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Both the *NEO* and the *HYG* resistance markers originated in aminoglycoside-resistant prokaryotes, as do many other dominant selectable markers in common usage. Aminoglycosides are not usually considered to be effective as therapeutic agents for trypanosomatids (Berman 1988) and initially one might have little concern about the environmental introduction of engineered trypanosomatids expressing such resistance markers. However, transfectional studies of many pathogenic *Leishmania* have shown that these species are more sensitive to the aminoglycosides G418 and hygromycin B than mammalian cells (EC_{50}s less than 50 μg/ml vs >100 μg/ml; Southern and Berg 1982; Bernard *et al.* 1985), which suggested the possibility that therapeutically effective aminoglycosides could be found. In fact, several workers had shown previously that paromomycin (aminosidine) has clear leishmanicidal activity and is currently being considered as an alternative to antimonials for the treatment of visceral leishmaniasis (Chunge *et al.* 1990; Scott *et al.* 1992; Thakur *et al.* 1992) and as a topical medication against cutaneous leishmaniasis (El-On *et al.* 1988, 1992). It is well established from bacterial systems that aminoglycoside resistance genes can confer cross-resistance to other related compounds (Umezawa and Kondo 1982) NEO in particular is known to detoxify the aminoglycosides G418, kanamycin, and paromomycin, and we decided to investigate whether our transformed *Leishmania* lines expressing NEO and HYG were cross-resistant to paromomycin.

Three lines of *L. major* developed by homologous gene targeting were examined: one containing a replacement of a single allele of *DHFR-TS* with *NEO* (+/*NEO*; Cruz and Beverley 1990), one with a single replacement of *DHFR-TS* with *HYG* (+/*HYG*), and the *thy*$^-$ auxotroph described earlier which contains replacement of both *DHFR-TS* alleles with *NEO* and *HYG* (*NEO*/*HYG*; Cruz *et al.* 1991). Promastigotes of the wild-type and the single *HYG* replacement lines were inhibited similarly by paromomycin, with EC_{50}s of 12–18 μg/ml, comparable to those reported previ-

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ously (El-On *et al.* 1991; Fig. 1A). In contrast, the single and double replacement lines bearing *NEO* were more than 150-fold resistant, showing little or no growth inhibition at 3 mg/ml of paromomycin (Fig. 1A). Interestingly, the resistance of the *NEO* replacement lines to the common selective aminoglycoside G418 was only about 10-fold higher than the wild-type or *HYG* replacement line (6–9 μg/ml vs. 0.8–1.1 μg/ml; Fig. 1B). This showed that the *NEO* transfectants are more efficient in the detoxification of paromomycin than they are for G418. This may arise from differences in the catalytic efficiency of NEO, the sensitivity of the *Leishmania* aminoglycoside target(s), or the pharmacokinetic properties of the drugs (uptake, compartmentalization, and excretion). Interestingly, these data suggest that paromomycin could be considered an alternative to G418 for use in routine laboratory transfections, as it is less expensive and shows more discrimination between sensitive and resistant parasites.

The fact that NEO confers cross-resistance to the clinically relevant anti-leishmanial paromomycin would seem to argue strongly against its use in parasites outside the laboratory. One could argue that genetically attenuated parasites expressing NEO would pose little hazard, since, being designed to perish, there would be little opportunity to encounter natural virulent lines. Even if co-infection occurred, several workers have found that sexual exchange is uncommon or even absent in *Leishmania* (Tibayrenc *et al.* 1990; Panton *et al.* 1991). However, other workers have noted the existence of occasional hybrid parasites, which implies that exchange is possible (Kelly *et al.* 1991; Momen *et al.* 1992). Thus, one cannot absolutely rule out the possibility of transmission of *NEO* into natural populations, although in our opinion this would be extremely unlikely.

Fortunately, the rapid evolution of DNA transfection technology in parasites suggests numerous alternatives for generating attenuated parasites that would bypass the concerns raised above. The first possibility is using alternative dominant selectable markers for which there is little concern about the consequences of environmental release. For *Leishmania*, these may include the *PHLEO* marker, which mediates resistance to bleomycin and its analogs (Freedman and Beverley 1993), or the *Leishmania N*-acetyl glucosaminyl transferase, which mediates tunicamycin resistance when overexpressed (Liu and Chang 1992). Alternatively, transfection strategies which ultimately remove the unwanted selectable marker, such as "hit-and-run" (Hasty *et al.* 1991) and "tag-and-exchange" schemes (Askew *et al.* 1993), or other procedures exploiting gene conversion and/or chromosomal segregation, could be used. By carefully considering the properties of the available selectable markers and their intended application outside the laboratory prior to their release, it seems likely that concerns about the safety of genetically engineered parasites can be eliminated, permitting the full potential of this emerging technology to be applied to these deadly tropical diseases.

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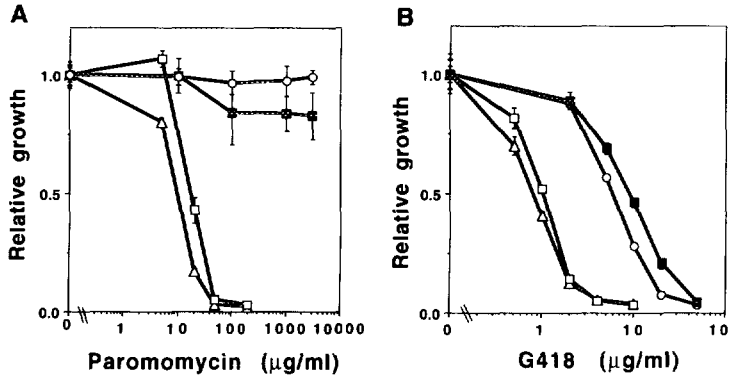

FIG. 1. Growth inhibition of wild-type and transfected *L. major* by paromomycin (A) or G418 (B). Promastigotes were seeded at 10^5 cells/ml in M199 medium (Kapler *et al.* 1990) containing the indicated concentrations of drug and periodically counted with a model Zf Coulter counter. The percentage of growth relative to the wild-type control lacking drug, counted at the time it had attained late log phase, is shown. For the *thy*$^-$ *NEO/HYG* line, thymidine was added to a concentration of 10 μg/ml. The average and standard deviations for triplicate points in one experiment are shown; two other experiments gave similar results. +/+ (clone CC-1), △; +/*NEO* (clone E2-7D2), ○; +/*HYG* (clone E8-5C5), □; *NEO/HYG* (clone E10-5A3), ■.

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