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Identification of Arsenite-resistance in Trypanosomes by Isozyme Characterization

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ABSTRACT

Drug-resistance is one of the major problems in the treatment of trypanosomiasis. If drug resistant strains could be identified earlier, different combination of drugs could be used for better results. Arsenite containing drugs are still the most widely used drugs so far. This study intended to correlate arsenite-resistance with isozyme profiles in trypanosomes. Tulahuen (TL) and Corpus Christi (CC) strains of *T. cruzi*, *Trypanosoma conorhini* isolated form *Triatoma rubrofasciata* in Taipei, as well as a chiropteran trypanosome isolated from Kenting were cultivated at 27 C and used for comparisons. Their survivals in different concentrations of sodium arsenite were monitored every 6-12 hours. The bat-trypanosomes were identified as arsenite-sensitive species since they died at low concentration, while CC strain of *T. cruzi* was considered as an arsenite-resistanst strain as the trypanosomes survived even at a very high concentration. The electrophoretic morbilities of glucose-6-phosphate dehydrogenase (G6PD) and phosphoglucose isomerase (PGI) isozymes were also compared. The results revealed as isozyme polymorphism in different strains of *T. cruzi* and TL strain produced more than one isozyme bands in both isozyme cases. Similar PGI isozyme pattern was observed in *T. conorhini* and the bat-trypanosome. Uniquely, there was not any isozyme bands of G6PD displayed by CC strain of *T. cruzi*. The correlation between arsenite-resistance and G6PD deficiency required more investigation and G6PD deficiency could be considered as an easier method for the identification of arsenite-resistance in trypanosomes.

Key words: Trypanosome, Arsenite-resistance, Glucose-6-phosphate dehydrogenase.

Introduction

Members of the genus *Trypanosoma* include a variety of different organisms, which are parasites of all classes of vertebrates. Among them, *Trypanosoma cruzi* is the causative agent of Chagas' disease which affects up to 20 million people in Latin America (WHO, 1990). Chemotherapeutic agents have been sought and suggested by many investigators (Doyle and Weinbach, 1989; Gadelha, *et al.*, 1989). However, due to its complex life cycle, *T. cruzi* does not respond to chemotherapy as well as other trypanosomes of humans. Drug-resistance of the parasite is one of the major problems in the treatment of *T. cruzi* infections but the detailed mechanisms are still not clear (Grogl, *et al.*, 1989;

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Kaminsky, *et al.*, 1989).

The drugs used to treat trypanosomiasis including pentamidine, diminazene, suramin, eflornithine, nifurtimox and melarsoprol (Mel B) are often toxic to the hosts (James and Gilles, 1985; Pepin and Milord, 1994). Among them, melarsoprol, an organic arsenicals, remains the most active trypanocidal drug available (Pepin and Milord, 1994). The limited success and liability of these treatments had led to research on the basic metabolic processes of the parasites; the evaluation of significant distinctions between the biochemistry of the host and parasite would hopefully lead to the development of logical approaches to chemotherapy (Walsh, *et al.*, 1991).

This investigation was designed to compare the relationship between arsenite-resistance and the isozymes of these trypanosomes would hopefully lead to look for some genetic markers for the arsenite-resistance of trypanosomes.

Materials and Methods

Parasites

Two strains of *Trypanosoma cruzi* employed in this study were obtained from Dr. D.G. Dusanic, Department of Life Sciences, Indiana State University, Indiana, U.S.A. These are Tulahuen (TL) strain originally isolated from a Chilean *Triatoma infestans* in 1945 and Corpus Christic (CC) strain originally from a human case in U.S.A. in 1955. A bat-trypanosome was isolated from *Miniopterus schreibersii fuliginosus* by Dr. Chao in 1996 (Chao, *et al.*, 1996). They were routinely subcultured in a liquid metacyclic culture (LMC) medium (Dusanic, 1980) supplemented with 7.5% (v/v) heat-inactivated fetal calf serum every 10 days. *Trypanosoma conorhini* employed in this study was isolated from *Triatoma rubrofasciata* captured in Taipei (Chao, *et al.*, 1993). It was maintained in a modified diphasic blood-agar culture medium with an overlay of LMC medium without serum at 14-day intervals. Cultured parasites were collected during late log phase of growth. The number of trypanosomes were about 1×10^7 in each treatment.

Chemical treatment

Since regular drug-sensitivity test determined in vitro based on growth inhibition of trypanosomes requires at least 5-10 days, arsenite-resistance is probably induced in the so-called wildtype trypanosomes at the end of this period. A modified crucial sensitivity test was performed to determine arsenite-resistance in trypanosomes within the first one or two cell division cycles to avoid the effect of acclimatization. Sodium arsenite (Sigma) stock solution at the concentration of 1000μM was prepared in LMC medium without serum. Trypanosomes were collected during late log phase of growth, washed twice in phosphate buffered saline (PBS, pH 7.2), and grown in LMC media containing different concentrations of sodium arsenite. Survival of parasites was monitored with a hemacytometer every 6-12 hours. The survival of trypanosomes was

examined microscopically by using trypan blue exclusion test. The 50% minimal lethal concentrations of sodium arsenite at 24h for each parasite species and strains used in this study were measured as MLC_{50}-24h.

Zymodeme analysis

Each trypanosomes culture containing 1×10^7 parasites was washed three times with PBS by centrifugation at 2,400*g* at 4℃ for 15 minutes. Pellets were resuspended in the solution containing 1.0mM EDTA, e-aminocaproic acid, and dithiothreitol (DTT). The suspensions of pellets were disrupted by nine cycles of repeated freezing and thawing and centrifuged at 11,000*g* at 4℃ for 30 minutes. The soluble protein in the supernatant was absorbed with several pieces of filter papers (Whatman No.3, 2mm× 10mm) and stored at -70℃. Electrophoresis was performed using 12% starch gel containing 85ml of gel buffer (0.05M histidine-HCl, 1.4mM EDTA, at pH 7.0 with 1M Tris and diluted fourfold to use), 10.2g of starch, 0.06g of EDTA, and 1g of urea. The parasite samples were loaded on the gel by inserting the pieces of filter papers once rinsed with soluble protein of trypanosomes. The filter papers rinsed with 2% bromophenol blue were inserted in two sides of the gel for the indicator. The samples were electrophoresed in 0.125M Tris buffer, pH 7.0 with 1M citric acid, at 20mA for 0.5h, then 50mA for 2.5h. The staining of the enzymes were performed using the method of Cheliak and Pitel (1984). In our preliminary study, among 20 kinds of enzymes of trypanosomes displayed clearly in our laboratory, 6 of them exhibited differences among strains or species of trypanosomes (our personal data). Glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49) and phosphoglucose isomerase (PGI; EC 5.3.1.9) were further studied in this investigation. G6PD of trypanosomes was assayed in a standard reaction mixture containing 25ml of 0.2M Tris-HCl (pH 8.0), 100mg of glucose-6-phosphate, 0.5ml of 1% $MgCl_2$ (w/v), 0.5ml of NADP, 0.5ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), and 0.5ml of phenazine methosulfate (PMS). The staining recipe of PGI was 25ml of 0.2M Tris-HCl (pH 8.0), 12.5mg of fructose-6-phosphate (disodium salt), 5 unit of G6PD, 0.5ml of 1% $MgCl_2$ (w/v), 0.5ml of NADP, 0.5ml MTT, and 0.5ml of PMS. The gels were incubated in the dark at 37℃ until dark blue bands appeared and destained with methanol-acetic acid-water (4 : 1 : 10) solution.

Results

Figure 1 shows the viability of trypanosomes at different concentrations of sodium arsenite. Most typanosomatid protozoa survived in media containing 50μM sodium arsenite for a comparably long time as the controls except the bat-trypanosome. All bat-trypanosomes died at 50 μM sodium arsenite concentration within 24h and died at 100μM within 12h. *T. conorhini* died at 100μM within 36h and died at 200μM within 12h. The parasites of TL strain of *T. cruzi* died within

36h in media containing 200μM sodium arsenite. However, CC strain of *T. cruzi* survived even at the high concentration of 200μM for at least 60h. The MLC_{50}-24h of sodium arsenite for *T. cruzi* (CC), *T. cruzi* (TL), *T. conorhini* and the bat-trypanosome were 107μM, 74μM, 35μM and 19μM, respectively. Therefore, the bat-trypanosome was considered as arsenite-sensitive, and CC strain of *T. cruzi* was arsenite-resistant.

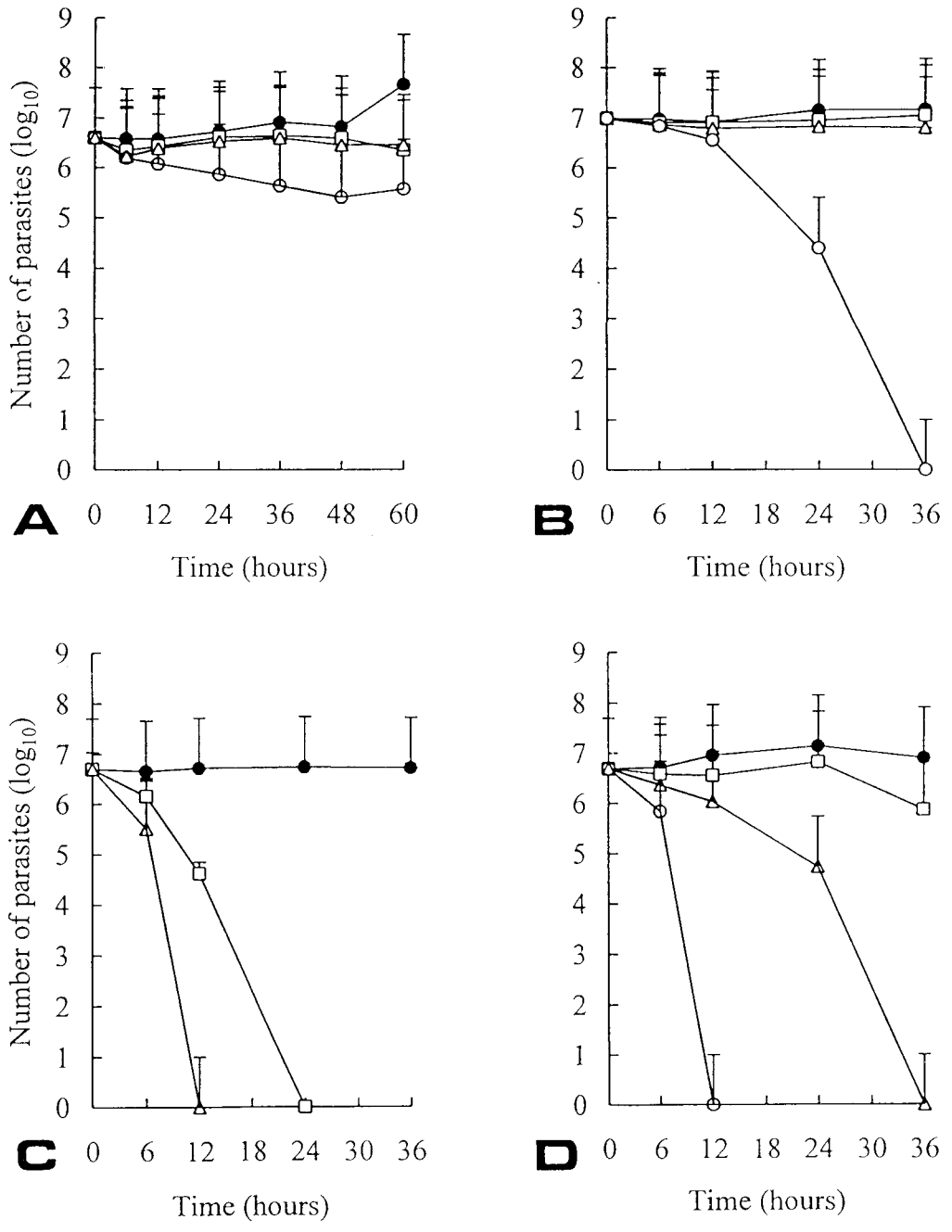

Figure 1. Survival of trypanosomes in the media containing different concentrations of sodium arsenite: 0μM control (solid circles), 50μM (squares), 100μM (triangles), and 200μM (open circles). Vertical lines represented standard errors of the mean. N=6. A, *T. cruzi* (CC); B, *T. cruzi* (TL); C, bat-trypanosome; D, *T. conorhini*.

Figure 2 shows the electrophoretic patterns of G6PD of different trypanosomes, while Figure 3 shows PGI isozyme patterns. The results indicated that these two isozyme patterns of Tulahuan strain were different from CC strain of *T. cruzi*, the isozyme patterens of bat-trypanosomes were similar to *T. conorhini*. *T. cruzi* (TL) produced more isozyme bands than the others, not only G6PD but also PGI. Uniquely, there was no isozyme band of G6PD displayed by *T. cruzi* (CC). It suggested that there was a G6PD deficiency in *T. cruzi* (CC).

Discussion

Our data showed that difference in electrophoretic patterns between the arsenite-resistant strain of *T. cruzi* and other trypanosomes was seen with the enzyme G6PD and there was a G6PD deficiency in *T. cruzi* (CC). G6PD controls the first and regulated step in the pentose phosphate pathway. The pentose phosphate pathway is sometimes called the pentose shunt, the hexose monophosphate pathway, or the phosphogluconate oxidative

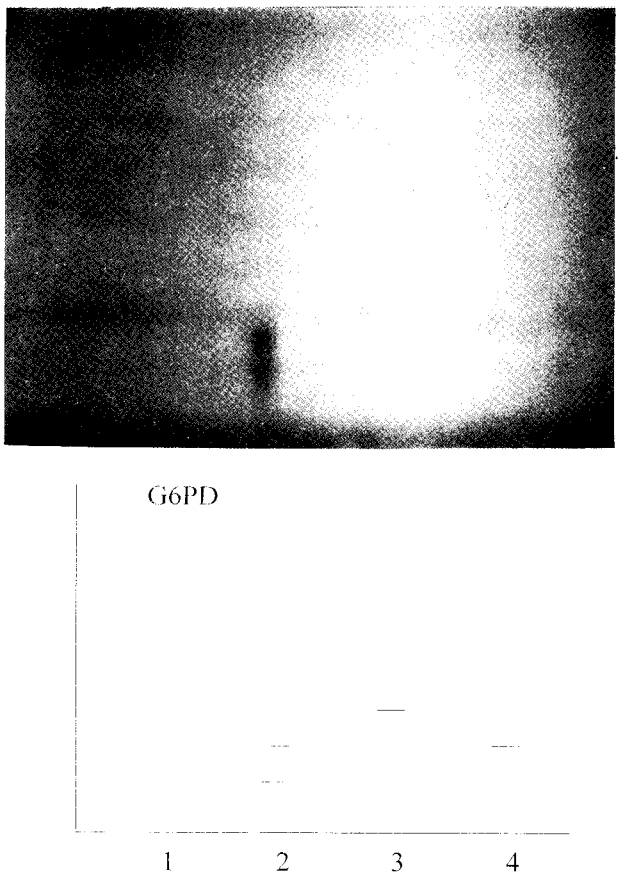

Figure 2. Electrophoretic mobility and diagrammatic representation of G6PD isozymes of different trypanosomes. Lane 1, *T. cruzi* (CC); Lane 2, *T. cruzi* (TL); Lane 3, bat-trypanosome; Lane 4, *T. conorhini*.

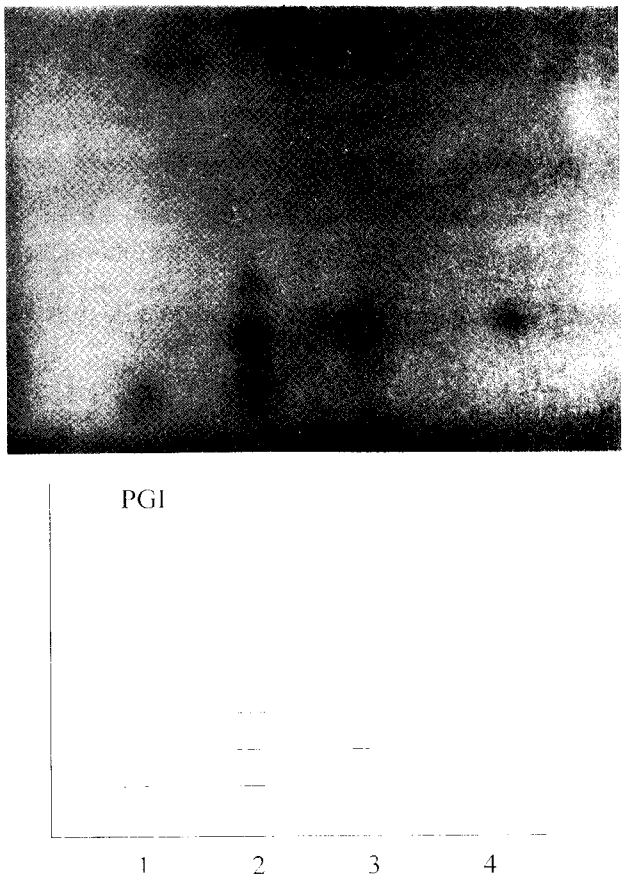

Figure 3. Electrophoretic mobility and diagrammatic representation of PGI isozymes of different trypanosomes. Lane 1, *T. cruzi* (CC); Lane 2, *T. cruzi* (TL); Lane 3, bat-trypanosome; Lane 4, *T. conorhini*.

pathway. In this pathway, NADPH is generated when glucose-6-phosphate is oxidized to ribose 5-phosphate. The major role of NADPH in cells is to reduce the disulfide form of glutathione to the sulfhydryl form. This reaction is catalyzed by glutathione reductase which is important in maintaining an intracellular reducing environment. Parasites such as *Plasmodium falciparuma* require reduced glutathione and the products of the pentose phosphate pathway for optimal growth. G6PD deficiency in red cells seems to protect a person from falciparum malaria (Stryer, 1988).

So far as we know all trypanosomatid parasites obtained an unusual derivative of glutathione termed trypanothione, a macrocyclic N^1,N^8-bis-glutathionyl spermidine (Fairlamb, *et al.*, 1989; Fairlamb and Cerami, 1992). Trypanothione represents over 80% of glutathione in trypanosomes and is thought to be unique to Family Trypanosomatidae (Fairlamb, 1990). It is the target of sodium arsenite, a trivalent arsenical and classic sulfhydryl-binding agent cytotoxic to both mammalian cells and parasites, to which it binds irreversibly to form a compound called Mel T, blocking the hydrogen acceptor sites. Trypanothione was the primary target for arsenical drugs against African trypanosomes. Increased synthesis of trypanothione produced resistance to arsenicals in *Leishmania* had also been reported (Mukhopadhyay, *et al.*, 1996). Secondary targets of arsenicals were thought to include trypanothione reductase (Fairlamb, *et al.*, 1989).

Trypanosoma conorhini is a cosmopolitan flagellate found in the gut of *Triatoma rubrofasciata* (Morishita, 1935). Compared to *T. cruzi*, its persistency in the mammalian hosts is relatively short and limited in both adult (Deane and Deane, 1961) and neonatal (Lu, *et al.*, 1993) stages of mice. This parasite actually provided a safe model for the study of trypanosomes.

One unique feature of trypanosomatid metabolism which would appear to fulfil these criteria was the trypanothione system and in particular the enzyme trypanothione reductase, an NADPH-dependent FAD-containing oxidoreductase (Shames, *et al.*, 1986; Henderson and Fairlamb, 1987). On the other hand, studies also indicated that resistance to arsenicals was not associated with changes in the concentration of trypanothione, glutathione, or related molecules (Fairlamb *et al.*, 1992; Yarlett, *et al.*, 1991). The properties of trypanothione reductase isolated from sensitive and resistant isolates were also indistinguishable (Fairlamb *et al.*, 1992). There were two high-affinity adenosine transport systems in melarsen-sensitive trypanosomes: a P1 type, which also transported inosine; and a P2 type, which also transported adenine and the melaminophenyl arsenicals. Melarsen-resistant trypanosomes lacked P2 adenosine transport, suggesting that resistance to these arsenicals was due to loss of uptake (Carter and Fairlamb, 1993).

The probable pathways for G6PD deficiency engaged with arsenite-resistance is proposed in Figure 4. Direct correlation between arsenite-resistance and G6PD

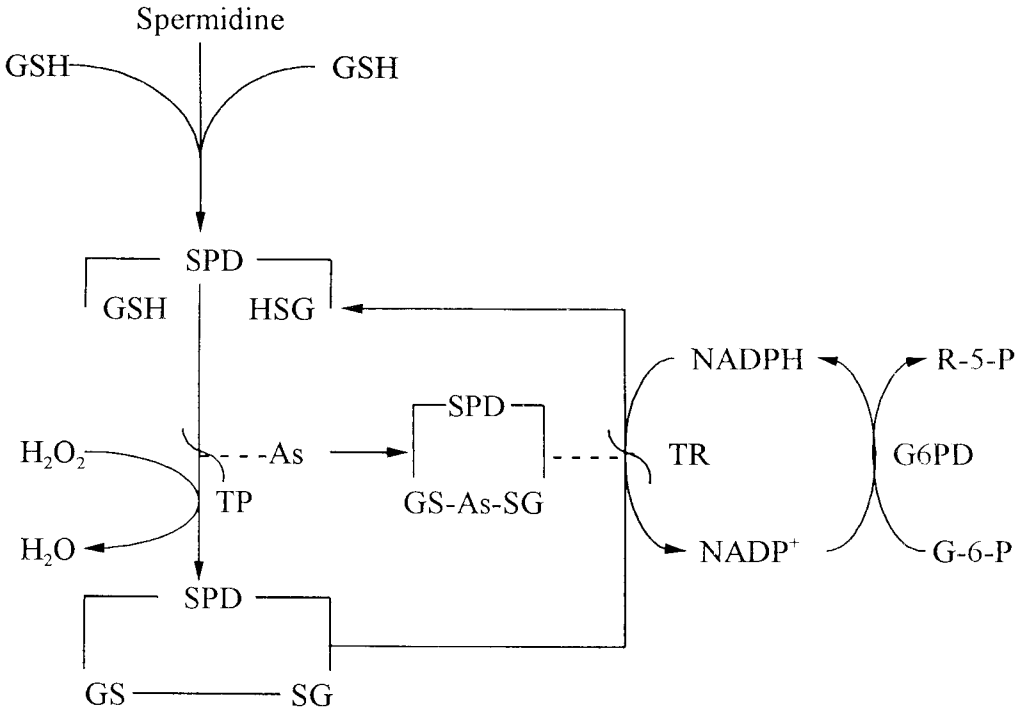

Figure 4. A proposed scheme for the indirect relationships between arsenite-resistance and G6PD deficiency in trypanosomes. Dashed lines represent inhibitary reactions caused by arsenical drugs. Abbreviations: As, arsenical drugs; G-6-P, glucose-6-phosphate; GSH, reduced glutathione; GSH(SPD)GSH, reduced trypanothione; R-5-P, ribose 5-phosphate; SPD, spermidine; TP, trypanothione peroxidase; TR, trypanothione reductase.

deficiency requires further study. However, G6PD deficiency could be considered as an easier method for the identification of arsenite-resistance in trypanosomes.

Acknowledgments

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錐蟲對砷化物抗藥性的異構酶分析

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摘 要

治療錐蟲病的主要困難之一就是錐蟲的抗藥性。如果能夠儘早辨認出錐蟲的抗藥株，即可使用不同藥物的組合而達到最好的治療效果。治療錐蟲病最主要的藥爲砷化物。本研究在探討錐蟲缺乏葡萄糖-6-磷酸去氫酶與其對砷化物抗藥性的關係。實驗所用的錐蟲培養在 27℃的培養液中，包括 Tulahuen (TL) 株及 Corpus Christi (CC) 株的枯西氏錐蟲 (*Trypanosoma cruzi*)、自台北紅帶錐鼻蟲分離出的錐蟲 (*Trypanosoma conorhini*)、及自墾丁分離出的蝙蝠錐蟲。將錐蟲培養在不同濃度的砷化鈉中，每隔 6 至 12 小時的間隔計算存活錐蟲的數目。結果蝙蝠錐蟲在低濃度下即迅速死亡，對砷最爲敏感；而 CC 株枯西氏錐蟲則因其在高濃度的砷化鈉培養液中仍可維持長時間的生存，爲對砷抗藥性的蟲株。比較葡萄糖-6-磷酸去氫酶及磷酸葡萄糖異構酶的電泳圖譜，顯示不同蟲株的枯西氏錐蟲具有多樣性的異構酶，並且 TL 蟲株的異構酶電泳圖譜都比 CC 蟲株多二條色帶。紅帶錐鼻蟲錐蟲及蝙蝠錐蟲具有相似的磷酸葡萄糖異構酶，且只有枯西氏錐蟲的 CC 株沒有任何葡萄糖-6-磷酸去氫酶的異構酶。錐蟲抗砷性與葡萄糖-6-磷酸去氫酶的缺乏之間的關係值得探討，而葡萄糖-6-磷酸去氫酶的缺乏可作爲錐蟲抗砷性確認的簡易指標之一。

關鍵詞：錐蟲、抗砷性、葡萄糖-6-磷酸去氫酶