The Korean Journal of Parasitology
Vol. 31, No. 3, 277-283, March 1993

Influence of heat shock, drugs, and radiation on karyotype of *Leishmania major*

Min Seo, Duk-Kyu Chun, Sung-Tae Hong* and Soon-Hyung Lee

Department of Parasitology and Institute of Endemic Diseases, Seoul National University College of Medicine, Seoul 110-799, Korea

Abstract: Leishmaniasis is one of the important tropical diseases in the world. Although it is not prevalent in Korea, imported cases have been recorded. The karyotype of *Leishmania* sp. has been observed to be variable by localities or by strains, but the karyotype of a strain is known to be stable. This study was performed to observe if the karyotype of a *Leishmania* sp. would be changed under some stressful conditions. The karyotype, analyzed by pulsed field gradient gel electrophoresis, was not grossly changed by heat shock, chemotherapeutics, UV illumination, and gamma irradiation. Radiation destroyed the chromosomes mechanically, but subcultured organisms after irradiation showed unaffected karyotype. The present findings suggest that the karyotype of a *Leishmania* strain is so stable that it is not altered by temporary stimulation with heat, drugs, and radiation.

Key words: *Leishmania major*, karyotype, heat shock, drugs, radiation

INTRODUCTION

Approximately, 22-28 chromosomes are known depending on the species of *Leishmania*. The resolved DNA bands of different strains vary in number, size and staining intensity within some ranges and there are distinct differences in band and gene position of the *Leishmania* species(Giannini *et al.*, 1990).

Chromosome size polymorphism, previously as observed in *Plasmodium falciparum*, is supposedly derived by amplification or deletion of the repetitive DNA sequences or physical translocations of DNA between chromosomes (Richard *et al.*, 1986). The karyotype of *L. major* is composed of 23 discrete chromosome-sized DNA bands in the size range from 250 to 2,000 kilobase pair (Kb) (Samaras & Spithell, 1987). Similarities in karyotypes of members of the same species made it possible to differentiate a species by karyotype analysis, and strain identification can be made by the unique pattern and stability of the karyotype in each strain.

The karyotype of *Leishmania* is known to be stable enough that it is not changed through animal passage or alternation of hosts and extensive subculture. This suggests that gross chromosomal rearrangements do not occur frequently by switching during developmental stages. The purpose of this study is to observe any karyotype alteration of *L. major* under various stressful or sublethal conditions, *e.g.* heat shock, chemotherapeutics, and ionizing radiation.

• Received July 3, revised July 22 1993.
• This study was supported by the grant from College of Medicine, Dankook University (1992).

* Corresponding author

MATERIALS AND METHODS

Parasites: *Leishmania major* was obtained from the parasitology laboratory, University of Toronto (December 1990), and continuously subcultured in primary N.N.N. medium containing the rabbit blood. The number of *Leishmania* reached maximum 6 days after subcultivation (Fig. 1). The 6-day cultured organisms were used for the experiment.

Preparation of samples: The promastigotes of *Leishmania* were collected and washed 3 times with 0.05 M EDTA solution. They were trapped in equal volume of 1.2% low-melting point agarose (BRL Co., U.S.A.) and were lysed overnight in 0.1 mg/ml solution of proteinase K (Boehringer Mannheim, Germany). The gel blocks were stored in refrigerated 0.5 M EDTA (pH 9.0) before use. Each gel block was trimmed to contain 1 × 10^8 cells.

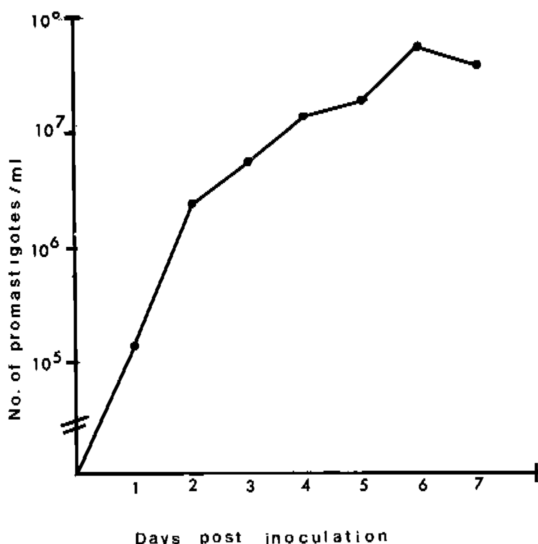

Fig. 1. Population growth of *Leishmania jamor* in N.N.N. media at 25° C.

Pulsed field gradient gel electrophoresis (PFGE): The contour clamped homogeneous electric field gel electrophoresis (CHEF) and field inversion gel electrophoresis (FIGE) were used for separation of *Leishmania* chromosomes. Most of the gels were 1% agarose, and running buffer was 0.5x TBE(0. 045 M Tris/0.045 M boric acid/0.001 M EDTA). Depending upon the size of the target chromosomes being separated, pulse frequency, voltage, and running time were controlled, and at the end of PFGE the gel was stained in 0.05 mg/ml ethidium bromide solution for 1 hour, then was observed and photographed through UV transillumination.

Heat shock: The N.N.N. media containg *Leishmania* were incubated at 37° C for diverse durations or methods(Table 1).

***In vitro* drug experiment:** Different concentrations of amphotericin B(Fungizone®) and trimethoprim-sulfamethoxazole(Bactrim®) were added to N.N.N. media(Table 2). Only the living worms were processed for PFGE.

Gamma irradiation: The source of gamma ray was MK 1-68 Cs-irradiatior(JL Shepherd and Associate Co., U.S.A.). *Leishmania* were exposed to radiation from 10 to 1,000 Gy(Table 3). Both of the worms immediately after irradiation and subcultured after irradiation were prepared for the experiment.

RESULTS

In the process of confirming the stability of the karyotype of *Leishmania* during long period

Table 1. Heat shock schedule for *L. major* in N.N.N. media

Group	Duration in		Remarks
	37° C	Room temperature	
H 1	1 wk	1 wk	
H 2	1 days	2 wk	
H 3		2 wk	
H 4	3 days	3 days	alternate daily
H 5	3 days	3 days	alternate daily and subculture
H 6	56 hrs		
H 7	100 hrs		

Table 2. The effects of chemotherapeutics on *L. major*

Agents	Concentration (μg/ml)	Remarks
Trimethoprim	0.1	dead, deformed, live
-Sulfomethoxazole	1.0	dead, deformed, live
	10	sluggish, small
	100	dead
Amphotericin B	0.1	dead, deformed, live
	1.0	active, live
	3.0	dead
	10	dead

Table 3. Gamma irradiation on *L. major* in N.N. N. media

Group[a)]	Irradiation dose(Gy)	Growth by subculture
I 1	10	yes
I 2	20	yes
I 3	30	yes
I 4	50	yes
I 5	60	no growth
I 6	100	yes
I 7	300	yes
I 8	500	no growth
I 9	1,000	no growth

[a)]: All of the group were used for karyotyping immediately after irradiation.

of subculture or after cryopreservation, the karyotype remained unchanged. Various conditions of heat shocks, administration of drugs, and UV illumination induced no changes of *Leishmania* karyotype in spite of deforming or death of parasites(Figs. 2, 3, 4, & Table 3).

However, several bands were lost by radiation. Until 30 Gy there happened no changes, but at 50 Gy the bands in the range of 900-1,000 kb began to disappear. The bands near 1,000 kb disappeared to be in smear pattern by 300 Gy radiation, but small bands under 700 kb was not changed. Though the large chromosomes disappeared, there were no new bands(Fig. 5).

The groups irradiated 500 Gy and 1,000 Gy, the bands were so definitely lost that it looked like smear patterns(Figs. 6 & 7). In the FIGE gels, there were no separated bands in the groups over 500 Gy radiation, and small fragmented molecules were hanged at the bottom of the gel. Subcultured organisms after irradiation showed the original karyotype(Fig. 8).

DISCUSSION

The *L. major* strain used in this study showed same size range of the chromosomes as previously recorded(Samaras and Spithill, 1987). *L. major* is known to have 23 discrete bands of chromosomal molecules from 250 kb to over 2,000 kb, but size polymorphisms in bands 2 and 3 were observed by strain. The present data dissolved only 16 bands from 250 kb to 2,000 kb. Further detailed analysis is

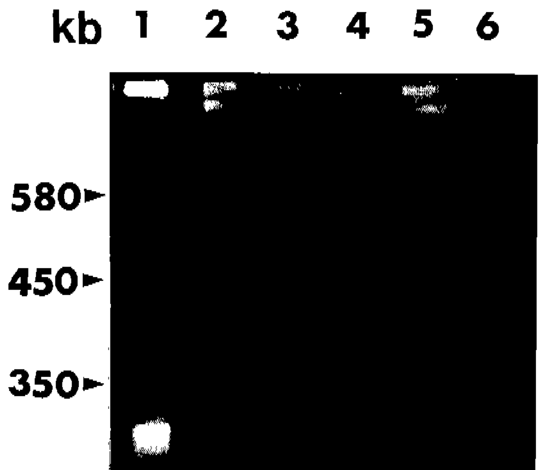

Fig. 2. Field inversion gel electrophoresis (FIGE) gel run under the parameters of 1% agarose, 100 seconds forward and 50 seconds backwards, 105 V, 72 hours, 1/2 × TBE, 14°C. 1) *Saccharomyces cerevisiae* AB972: 2-6) *L. major*, 2) primarily cultured; 3) subcultured after 72 hours heat shock at 37°C; 4) 100 hours heat shocked; 5) 72 hours heat shocked, every other day; 6) 56 hours heat shocked. The karyotype is constant under various heat shock cultivations.

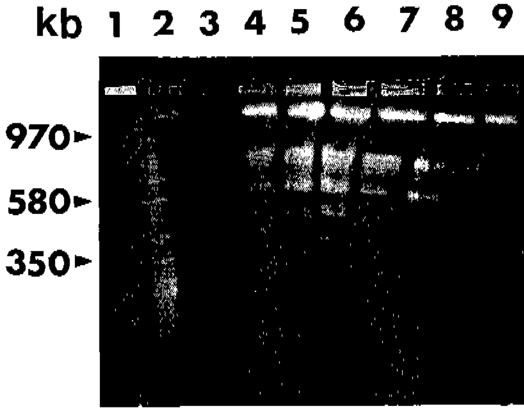

Fig. 3. Contour clamped homogeneous electric field electrophoresis (CHEF) gel run in 1% agarose under 90 V for 115 hours. Initial A time 50 seconds and final A time 300 seconds, A/B ratio 1, 1/2 × TBE buffer at 15°C. 1) *Saccharomyces cerevisiae* AB972; 2-9) *L. major*, 2) 37°C 1 week & room temperature (RT) 1 week; 3) 37°C one overnight & RT 2 weeks; 4) RT 2 weeks; 5) cultured in media with trimethoprim-sulfamethoxazole (Bactrm ®, B) 10 mg/ml for 1 week; 6) cultured in media containing amphotericin B (Fungizone ®, F) 0.5 mg/ml 1 week; 7) culture in F 1 mg/ml for 48 hours & normal media 1 week; 8) control cultured at RT; 9) 1 week heat shock by alternate day. All lanes display the same band pattern.

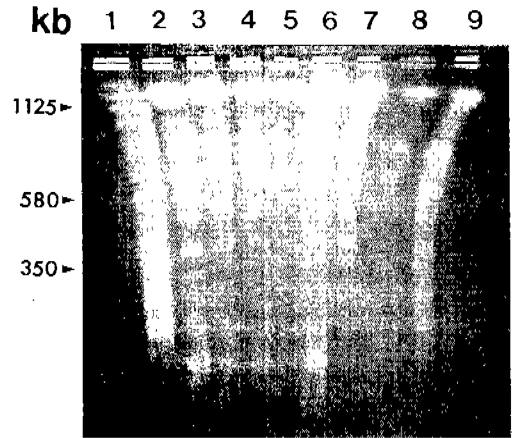

Fig. 4. CHEF gel run in 1% agarose under 90 V for 115 hours. Initial A time 50 seconds and final A time 300 seconds, A/B ratio 1, 1/2 × TBE buffer at 15°C. 1) *Saccharomyces cerevisiae* AB972 size marker; 2-9) *L. major*, 2) control; 3) room temperature (RT) 2 weeks; 4) one week heat shock alternately; 5) 37°C one overnight & RT 2 weeks; 6) cultured in media with trimethoprim-sulfamethoxazole (Bactrim ®) 10 mg/ml for 1 week; 7) amphotericin B (Fungizone ®) 1 mg/ml for 48 hours & normal media 1 week; 8) UV overnight in a clean bench; 9) control. All samples are in the same pattern of bands.

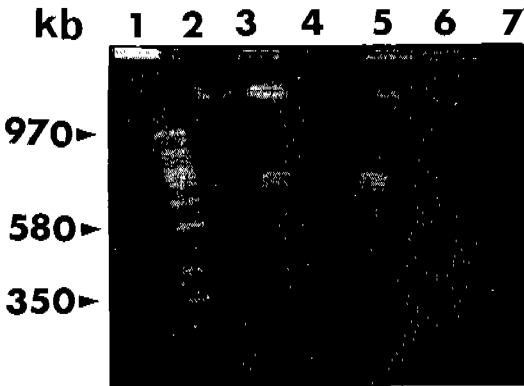

Fig. 5. CHEF gel run in 1% agarose under 115 V for 96 hours. Initial A time 50 seconds and final A time 400 seconds, A/B ratio 1, 1/2 × TBE buffer at 15°C. 1) *Saccharomyces cerevisiae* AB972 size marker; 2-7) *L. major*, 2) control; 3) 30 Gy irradiated; 4) 60 Gy irradiated; 5) 100 Gy irradiated; 6) 200 Gy irradiated; 7) 300 Gy irradiated. The lane 6 shows decreased amount of DNA in large bands and bands over 800 kb are missing on the lane 7.

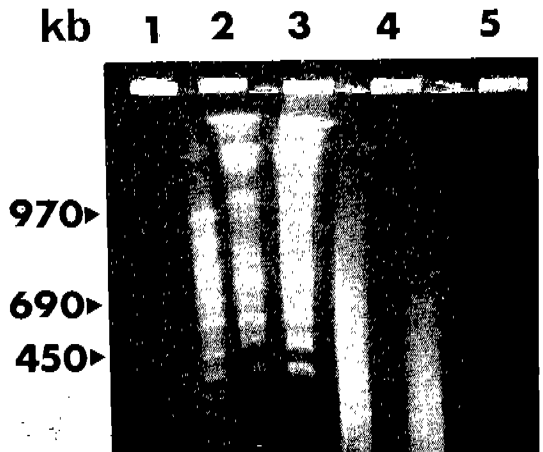

Fig. 6. CHEF gel run in 1% agarose under 115 V for 96 hours. Initial A time 50 seconds and final A time 400 seconds, A/B ratio 1, 1/2 × TBE buffer at 15°C. 1) *Saccharomyces cerevisiae* AB972 size marker; 2-5) *L. major*, 2) control; 3) subcultured after cryopreservation; 4) 500 Gy irradiated; 5) 1000 Gy irradiated. All of the bands irregularly degraded to be thick smear on lanes 4 and 5.

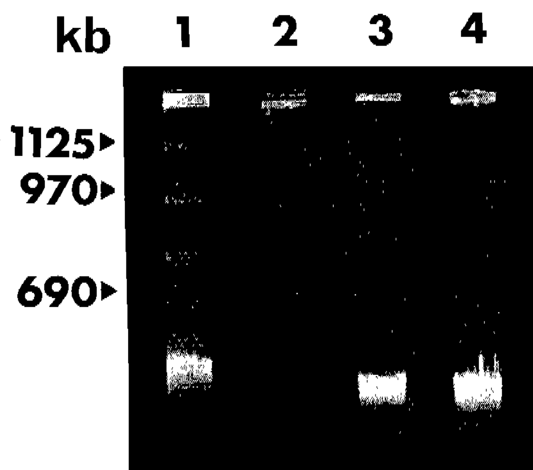

Fig. 7. FIGE gel run under parameters of 1% agarose, 100 seconds forward and 50 seconds backwards, 95 V for 64 hours in 1/2 × TBE at 14°C. 1) *Saccharomyces cerevisiae* AB972 size marker; 2-4) *L. major*, 2) control; 3) 500 Gy irradiated; 5) 1000 Gy irradiated. All of the irradiated specimens show no discrete chromosomal bands.

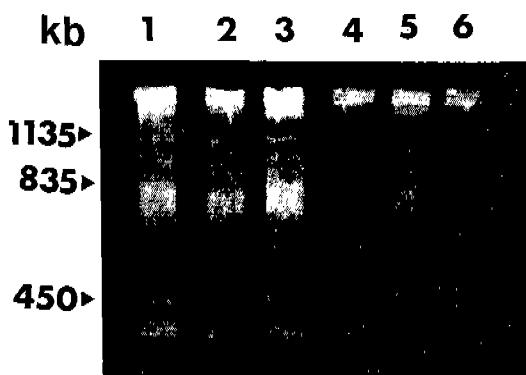

Fig. 8. FIGE gel run under parameters of 1% agarose, 100 seconds forward and 50 seconds backwards, 95 V for 64 hours in 1/2 × TBE at 14°C. All lanes were loaded with *L. major* samples, 1) 50 Gy irradiated; 2) subcultured after 50 Gy irradiation; 3) 100 Gy irradiated; 4) subcultured after 100 Gy irradiation; 5) 300 Gy irradiated; 6) subcultured after 300 Gy irradiation. All of the irradiated specimens show same pattern of chromosomal bands. The plugs on lanes 4 and 6 include less DNA amount than others.

necessary for exact karyotype comparison of the present *L. major* strain with other recorded strains.

Heat shock, drugs, or radiation were found to be of no definite roles in induction of any gross change of *L. major* chromosomes, but radiation was observed to destroy the chromosomes mechanically.

The heat shock response is one of homeostatic mechanisms that protect cells and the entire organism from the deleterious effect. The physiological response of organism to heat, termed heat shock response, is characterized by a rapid induction of a specific set of genes. The induced product of the gene, called heat shock protein (HSP), is approximately 11 kDa. The synthesis of HSP provides organisms with thermotolerance at otherwise lethal temperatures and protects them from lethality and heat-induced developmental defects. Promastigotes of South American *Leishmania*, causing mucocutaneous leishmaniasis, become morphologically and antigenically amastigote-like form when incubated at 37°C. It is interesting that both axenic amastigotes and heat-shocked promastigotes are more infective than untreated promastigotes to labaratory animals (Maresca & Carratu, 1992). *L. major* has two developmentally regulated genes, P100/11E and P101/10. The clone P100/11E is expressed predominantly in promastigotes and located on the chromosome band 20. Clone P101/10 is located on the chromosome band 22 and enriched in amastigotes. Since the expression of P101/10 is thermally inducible at 37°C *in vitro*, induction of this gene is mediated by heat shock *in vitro*. So, heating plays a role on the expression of not only HSP 70 and HSP 83 genes but also developmentally regulated genes (Kidane *et al.*, 1989).

The worms undergo morphological changes by heating as well. In this experiment, transfer of promastigotes from room temperature to 37°C made them amastigote-like form after 2 days, and at 4th day nearly all have lost motility of the flagellum. There were no differences of the karyotypes between the control and heat shocked organisms under various conditions. This result showed that heat shocked condition was not associated with any change of the karyotype.

The experiment with amphotericin B and trimethoprim-sulfamethoxazole in the media was also not related with the karyotype variation. The toxic effect of the drugs brought

about morphological deformation and death of the organisms, but it did not make any anomalous change of the chromosomal molecules. However, induction of drug resistant strain may be related with appearance of aberrant chromosomes. It must be a subject of further studies.

The most important effects of radiation on protozoa are inhibition of proliferation and decrease of infectivity. Male ixodid ticks exposed to 160 Gy radiation developed chromosomal aberrations such as bridges and rings. In addition to this, the testes were often underdeveloped, abnormal cells are prominent, and production of spermatids was slowed or halted (Oliver & Stanley, 1987). Radiation has been known to cause structural changes of DNA. The present results revealed that 900-1, 000 kb sized chromosomal bands were lost at 50 Gy, and the disappearance became more conspicuous by the increase of doses. In the groups irradiated 500 Gy or 1,000 Gy, the chromosomal bands ranging from 700 to 1,500 kb disappeared and the DNA amount widely increased under 550 kb. This suggested that the chromosomes in the range of 700-1,500 kb had been irregularly broken to small fragments under 300 kb. By summarizing, the large bands near 1,000 kb began to disappear over 50 Gy, and over 500 Gy all of the chromosomal bands disappeared. In this process there was no appearance of new discrete bands. The present karyotype change observed after irradiation was not by the karyotype variation, but by mechanical destruction of the chromosomes. If the experiment groups of more detailed radiation dose are employed, better interpretation will be available for destruction process of the chromosomes.

In this study the 300 Gy irradiated promastigotes of *L. major* were succeeded in proliferation by subculture in spite of gross destruction of chromosomes, but 500 Gy irradiated groups failed to grow. The inhibition of proliferation by irradiation must be an outcome of destruction of the chromosomes. However, the organisms subcultured after 300 Gy irradiation kept the original karyotype. The DNA of the surviving worms may be recombined after irradiation cleavage or undamaged ones. Furthermore, failed cultivation in 500 Gy radiation dose suggested severe cleavage of the chromosomes by incomplete or abnormal recombination.

In malarial parasites, karyotype variant is known to be generated by mutation and genetic recombination(Janse *et al.,* 1992). And also one of karyotype mutants was related with drug resistance(Babiker *et al.,* 1991). In this context, we can expect experimental induction of karyotype variants of *L. major* by repeated cleavage and recombination of chromosomes.

REFERENCES

Babiker HA, Creasey AM, Bayoumi AL, Walliker D, Arnot DE (1991) Genetic diversity of *Plasmodium falciparum* in a village in eastern Sudan. 2. Drug resistance, molecular karyotypes and the *mdr1* genotype of recent isolates. *Trans Roy Soc Trop Med Hyg* **85:** 578-583.

Giannini SH, Curry SS, Tesh RB, Van der Ploeg LHT (1990) Size-conserved chromosomes and stability of molecular karyotype in cloned stocks of *Leishmania major. Mol Bioch Parasit* **39:** 9-22.

Janse CJ, Ramesar J, van den Berg FM, Mons B (1992) *Plasmodium berghei: In vivo* generation and selection of karyotype mutants and non-gametocyte producer mutants. *Exp Parasit* **74:** 1-10.

Kidane GZ, Samaras N, Spithill TW (1989) Cloning of developmentally regulated genes from *Leishmania major* and expression following heat induction. *J Biol Chem* **264**(7): 4244-4250.

Maresca B, Carratu L (1992) The biology of the heat shock response in parasites. *Parasit Today* **8:** 260-265.

Oliver JH, Stanley MA (1987) Effects of gamma radiation on spermatogenesis and fertility of male *Amblyoma americanum. J Parasitol* **73** (2): 309-313.

Richard PB, Michael AM (1987) Chromosome size polymorphism of *Leishmania donovani. Mol Bioch Parasit* **24:** 263-272.

Samaras N, Sphithill TW (1987) Molecular karyotype of five species of *Leishmania* and analysis of gene locations and chromosomal rearrangements. *Mol Bioch Parasit* **25:** 279-291.

=국문초록=

열대리슈마니아 핵형에 대한 열쇽, 약제 및 감마선 조사의 영향

서울대학교 의과대학 기생충학교실 및 풍토병연구소

서 민, 전덕규, 홍성태, 이순형

세계적으로 중요한 열대풍토병 병원체의 하나인 열대리슈마니아의 핵형에 영향을 줄 것으로 기대되는 인자와 그 효과를 관찰하고자 하였다. 토끼의 혈액을 포함한 N.N.N. 배지에서 유지하고 있는 열대리슈마니아(*Leishmania major*)의 promastigote를 열쇽, 약제 첨가, 자외선 조사 및 감마선을 이용한 방사선 조사를 여러 가지 방법으로 시행하고 주기변동전기영동(pulsed field gradient gel electrophoresis)을 이용하여 핵형의 변동 여부를 관찰하였다. 그 결과 여러 방식에 의한 열쇽과 약제 처리 및 자외선 조사에 따른 핵형의 변화가 없었다. 그러나 방사선 조사군에서는 50 Gy 이상 조사한 군에서 1 mega base pair(Mb) 크기에 있는 염색체부터 소실되기 시작하여 방사선 조사량이 증가함에 따라서 250-500 Kb의 작은 염색체도 파괴되어 500 Gy 이상 군에서는 뚜렷한 염색체 분획이 없이 겔 하단 200 Kb 크기 아래 부분에 몰려 있었다. 이러한 소견은 방사선에 의하여 염색체가 불규칙하게 파손된 것을 의미한다고 하겠다. 방사선을 300 Gy까지 조사한 충체는 계대 배양이 가능하였고, 이들은 원래의 핵형을 유지하였다. 방사선 조사 후에 배양된 충체는 염색체가 파괴되지 않았거나 부분적인 손상 후에 DNA 재결합에 의해 원상회복된 것으로 판단된다. 열대리슈마니아의 핵형은 일시적인 자극에 의하여 쉽게 변형되지 않는 안정된 것임을 확인하였다.

〔기생충학잡지 31(3): 277-283, 1993년 9월〕