

LEISHMANIA SPP.: COMPLETELY DEFINED MEDIUM WITHOUT SERUM AND MACROMOLECULES (CDM/LP) FOR THE CONTINUOUS *IN VITRO* CULTIVATION OF INFECTIVE PROMASTIGOTE FORMS

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Abstract. The elimination of serum or of serum-derived macromolecules that supplant the fetal calf serum requirement from *Leishmania* culture media could decrease costs and improve the feasibility of large-scale production of well-defined parasite material. We report a completely defined medium, without serum-derived protein and/or macromolecules as a serum substitute, of common, available, and inexpensive constituents that can be used in place of serum-supplemented media for the continuous *in vitro* cultivation of promastigote forms of various *Leishmania* species. Typical promastigote morphology was observed in Giemsa-stained smears, regardless of the strain analyzed. Electrophoretic analysis showed that the proteinase patterns of asexually grown promastigote forms were similar to those obtained in serum-supplemented RPMI 1640 medium for all *Leishmania* studied. Similar antigenic profiles were recognized in immunoblots by sera from hosts with visceral or cutaneous leishmaniasis after growing promastigotes in the two different culture media. For parasites causing both cutaneous and visceral leishmaniasis, the absence of serum and macromolecules in the culture medium did not markedly change their *in vitro* infectivity for resident mouse macrophages and their virulence in animals compared with parasites cultivated in nondefined medium. Serum-free technology will be increasingly important in providing stability and reproducibility as research using promastigote moves closer to therapeutic applications.

Parasites from the genus *Leishmania* cause a variety of disease states in humans and other mammals in tropical and subtropical areas, which include cutaneous, mucocutaneous, and visceral leishmaniasis. The parasite undergoes a digenic life cycle between a nonmotile intracellular amastigote stage parasitizing the mammalian phagocytic cells and a flagellated, motile promastigote stage in the midgut of its sandfly vector.¹ A similar promastigote form develops when parasites are cultured in cell-free medium.²

In vitro standardized cultivation of the members of the *Leishmania* genus is a useful approach for yielding amount of parasites suitable for diagnosis purposes to provide a better knowledge of host-parasite relationships and for the determination of biologic and immunologic characteristics of the parasite. One of the primary goals of culturists has been to achieve the long-term maintenance of active and dividing populations of different *Leishmania* species. The different media developed over the past 90 years can be classified in two major categories: semi-solid biphasic media and liquid monophasic media. *Leishmania* promastigotes were first grown on diphasic blood agar (NIN),^{3,4} which was later enriched with bacteriologic additives such as brain heart infusion,⁵ and is used today with various modifications of the liquid phase added to the solid one.⁶⁻⁹ These nondefined diphasic media are still used today for adaptation and cultivation of *Leishmania* strains directly isolated from both vertebrate and invertebrate hosts. However, they have some disadvantages. For example, they are complex to prepare and difficult to standardize. Moreover, they contained blood and bacteriologic additives as important factors for parasite replication, which complicate biologic and immunologic studies.

Further progress has been made with the use of serum-enriched liquid monophasic media. Nondefined glucose-lactalbumin-serum-hemoglobin medium, originally developed for African trypanosomes,^{10,11} liver infusion tryptose (LIT) medium,¹² Panmede medium,¹³ modified NIH medium using

hemolyzed rabbit blood,¹⁴ and semi-defined HOMEM's medium¹⁵ have been reported. Serum-enriched tissue culture media, initially formulated for the cultivation of mammalian or insect cells, have been successfully used to cultivate several Old and New World *Leishmania* species. These include 199H medium, RPMI 1640 medium, Dulbecco's minimal essential medium,^{5,16} Schneider's *Drosophila* medium, and Grace's insect tissue culture medium.¹⁶⁻¹⁸ However, all of these tissue culture media require addition of high concentrations of fetal bovine serum (10-30%) as an essential factor for long-term growth of *Leishmania* promastigotes.

Defined media were developed for the cultivation of certain *Leishmania*. *Leishmania tarentolae* has been cultured in a chemically defined medium;¹⁹ however, this medium could not support the growth of other *Leishmania* species. Attempts to replace serum by bovine serum albumin^{20,21} or mixture of purine bases, vitamins, and bovine albumin fraction IV²² were also made for cultivating *L. donovani* promastigote forms. An easily prepared, nearly defined medium containing salts, glucose, and tryptose (i.e., an LIT medium), to which was added concentrated RPMI 1640 and 199 media, was shown to be adequate for the cultivation of *L. chagasi* and *L. amazonensis* promastigotes²³ and other *Leishmania* species.²⁴ Media developed for serum-free growth of mammalian cells were adapted for the serial cultivation of different *Leishmania* species. These media were enriched with large concentrations of certain amino acids, vitamins, bovine albumin, hormones, peptide supplements,²⁵⁻³¹ or more recently, with human urine.^{32,33}

In the present paper, we report a completely chemically defined culture medium free of serum, macromolecules, proteins, and peptides that readily supports the growth and maintenance of promastigote forms of various *Leishmania* species without compromising parasite growth rates. Morphologic, biochemical, immunologic, and biologic properties of asexually grown promastigote forms are presented.



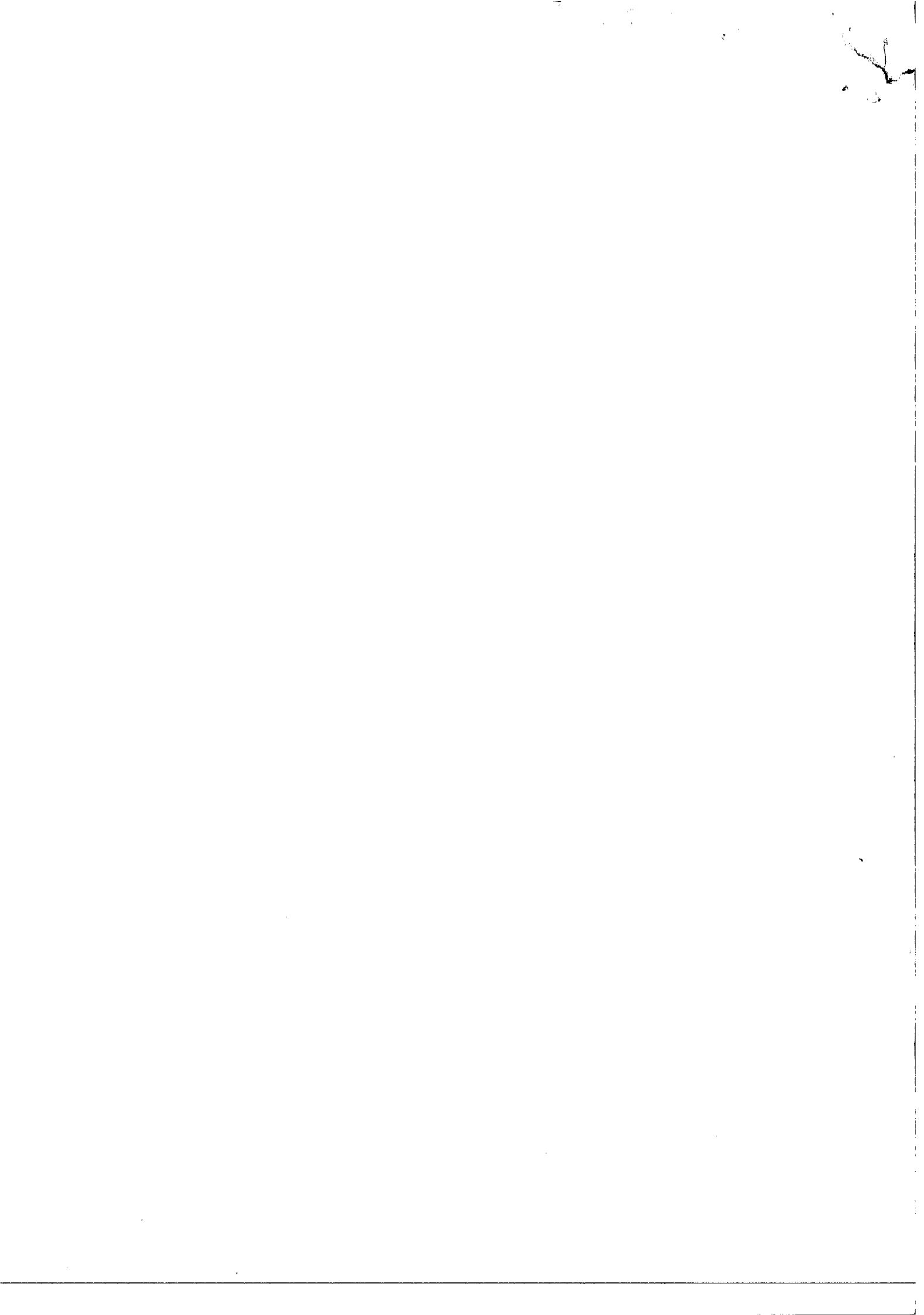


TABLE I

Composition of the completely defined medium (CDM/LP) that supports the continuous growth of *Leishmania* promastigotes

Vitamins		Nonessential amino acids	
L-ascorbic acid (C)	0.06 μ M	D ₁ -Alanine	0.11 mM
d-Biotin (H)	1.50 μ M	Asparagine	0.36 mM
Folic acid	3.00 μ M	L-Aspartic acid	0.26 mM
p-Aminobenzoic	9.00 μ M	L-Glutamic acid	0.34 mM
Nicotinic acid	0.04 μ M	Glycine	0.28 μ M
Nicotinamide	9.40 μ M	L-Proline	0.20 mM
D-Pantothenic acid	0.90 μ M	L-Serine	0.42 mM
Pyridoxine-HCl (B ₆)	5.40 μ M	Glutathione	3.60 μ M
Pyridoxal-HCl	1.00 μ M	Hydroxyproline	0.17 mM
Riboflavin (B ₂)	0.56 μ M		
Thiamine-HCl (B ₁)	0.34 μ M	Salts, sugars, and nucleotides	
Cyanocobalamin (B ₁₂)	4.00 μ M	NaCl	139.00 mM
Calciferol (D ₃)	0.05 μ M	KCl	7.00 mM
Menadione-3 H ₂ O (K ₃)	0.01 μ M	CaCl ₂	0.72 mM
L- α -tocopherol (E)	0.01 μ M	MgSO ₄ ·7 H ₂ O	0.61 mM
Retinol (A)	0.07 μ M	Fe(NO ₃) ₃ ·9 H ₂ O	0.36 μ M
Inositol	0.25 μ M	Na ₂ HPO ₄	6.22 mM
Choline	24.0 μ M	NaHCO ₃	24.00 mM
Cholesterol	0.10 μ M	KH ₂ PO ₄	0.09 mM
		Glucose	13.30 mM
Essential amino acids		Hemin	7.70 mM
L-Arginine-HCl	1.90 mM	HEPES	20.00 mM
L-Cysteine-HCl	0.12 μ M	Sodium acetate	0.12 mM
L-Cystine-H ₂ O	0.60 mM	D-ribose	0.66 mM
L-Glutamine	2.30 mM	2-deoxyribose	0.74 mM
L-Histidine-HCl·H ₂ O	0.13 mM	Tween 80	4 mg/l
L-Isoleucine	0.48 mM	ATP (Na ₂)	0.36 μ M
L-Leucine	0.60 mM	Adenosine sulfate	8.60 μ M
L-Lysine-HCl	0.32 mM	Guanine-HCl	0.32 μ M
L-Methionine	0.14 mM	Hypoxanthine	0.52 μ M
L-Phenylalanine	0.16 mM	Xanthine (Na)	0.50 μ M
L-Threonine	0.29 mM	Uracil	0.62 μ M
L-Tryptophan	46.00 μ M	Thymine	0.48 μ M
L-Tyrosine	0.17 mM		
L-Valine	0.27 mM		

MATERIALS AND METHODS

Parasites. Promastigote forms of *L. chagasi* (MHOM/BR/79/LI-01), *L. infantum* (MHOM/MA/67/IT-263, clone 2 and clone 7), *L. donovani* (MHOM/IN/83/H570, MHOM/IN(-)/61/L-13, MHOM/-/-/IT-2217, and MHOM/IN/80/DD8, clone 2), *L. mexicana* (MNYC/BZ/62/M-379), *L. amazonensis* (MHOM/VE/76/JAP78, MHOM/BR/76/LTB-012, MHOM/BR/73/M-2269, MHOM/BO/83/LPZ-155, and MPRO/BR/72/M-1841), *L. braziliensis* (MHOM/BR/72/1670, MHOM/BR/75/M-2904, MHOM/BO/90/CS, and MHOM/BO/90/AN), *L. panamensis* (MCHO/PA--M-4039, MHOM/PA/71/LS-94, and MHOM/EQ/91/A8044), *L. guyanensis* (MHOM/BR/78/M-5378 and MHOM/BR/75/M-4147), *L. peruviana* (MHOM/PE/85/FR-6), *L. major* (MHOM/SU/73/5ASKH), and *L. tropica* (MHOM/SU/60/OD) and epimastigote forms of *Trypanosoma cruzi* (Tehuentepec strain, zymodeme 12 and SO34 strain, clone 4, zymodeme 20) were routinely maintained in our laboratory by weekly passages of mid-log phase parasites into 10 ml of RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD) buffered with 25 mM HEPES, 2 mM NaHCO₃ (pH 7.2), and supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) at 25 \pm 1°C. Initial parasite concentrations were 10⁵ or 5 \times 10⁵ flagellates/ml. The *Leishmania* and *T. cruzi*

strains used in this work have been characterized by isoenzyme analysis.³⁴ All the cultured isolates were preserved in a cryobank research and reference collection.

Culture methods. The qualitative and quantitative formulation of the completely defined medium developed for the continued cultivation of *Leishmania* promastigotes (CDM/LP) is shown in Table 1. This medium contains common salts, sugars, hemin, HEPES, purines, and purine nucleosides required by the parasites, a high concentration of water-soluble vitamins, 23 amino acids, intermediates of amino acid metabolism, and Tween 80. After adjusting the pH to 7.2, the medium was sterilized by pressure passage through 0.22- μ m Millipore (Saint-Quentin Yvelines, France) filter. Ingredients used in the medium preparation are readily available at low cost. This medium could be stored at 4°C for at least one month without significantly altering its growth-supporting potential. The osmolarity value was 353 \pm 3 milliosmoles/kg of water. Gentamicin could be added to a final concentration of 25 mg/L to decrease the risk of bacterial contamination.

Each culture test was carried out in two experiments using three culture flasks. Mid-log phase promastigotes previously adapted in RPMI 1640 medium with 10% inactivated FCS were first transferred into an equal mixture of CDM/LP and

RPMI 1640 media supplemented with 5% FCS during 2–5 subcultures and were then grown in completely defined medium at $25 \pm 1^\circ\text{C}$. Cultures were initiated with 10^6 mid-log phase promastigotes (about four days)/ml in 25-cm² plastic culture flasks containing 10 ml of media mixture without shaking. Long-term continuous cultures of the different *Leishmania* and *T. cruzi* strains were maintained by successive passages every week of 5×10^5 flagellates/ml into 10 ml of CDM/LP medium. Larger numbers of organisms can be obtained in 72-cm² or 175-cm² plastic culture flasks that accommodated 50 ml and 200 ml of culture medium, respectively.

Parasite growth was assessed qualitatively and quantitatively by microscopic observations on the appearance and mobility of promastigotes and by enumeration of organisms in a hemocytometer. Twenty microliters of Vortex-homogenized culture samples were mixed with 20 μl of 0.01 M phosphate-buffered saline (PBS), pH 7.2 containing 0.2% glutaraldehyde. The flagellate concentration was determined daily after adequate dilution in PBS by counting fixed parasites in a Thoma counting chamber (Poly Labo, Strasbourg, France) at 400 \times magnification. Simultaneously, staining with erythrosin B was used to differentiate between living and dead cells. A 20- μl flagellate suspension and 20 μl of 0.4% erythrosin B staining solution previously cooled to 4°C were mixed. After incubation in ice for 5 min, one drop was examined at 400 \times magnification to determine the percentage of viability (stained parasites were nonviable).

Parasite-macrophage interactions. *In vitro* infection of mouse peritoneal macrophages with promastigote forms was performed as previously described.^{35,36} Briefly, 2.5×10^5 normal resident peritoneal exudate cells from BALB/c mice in RPMI 1640 medium (Gibco-BRL) with 10% heat-inactivated FCS, 5 units of heparin/ml, 25 mM HEPES, 200 units of penicillin/ml, and 200 μg of streptomycin/ml were allowed to adhere to 12-mm diameter glass coverslips placed into 24-well culture plates (Nunc, Roskilde, Denmark) at $36 \pm 1^\circ\text{C}$ in 5% CO_2 . After 4 hr, the nonadherent cells were removed by washing and the remaining macrophages were infected with stationary phase promastigote forms at an approximate 5:1 parasites:cell ratio. At the end of 4 hr, all wells were gently washed to remove free parasites and the cultures were left at 37°C in 5% CO_2 for additional periods. At various intervals (4, 24, and 48 hr) coverslips were rinsed in sterile PBS, fixed with absolute methanol, and stained with Giemsa. For each experiment, more than 500 cells were counted in duplicate to determine the percentage of infected macrophages.

Infection of animals. Stationary-phase promastigotes (6–7-days old) of *L. mexicana* (MNYC/BZ/62/M-379), *L. amazonensis* (MHOM/BR/76/LTB-012), *L. donovani* (MHOM/IN/80/DD8, clone 2), and *L. infantum* (MHOM/MA/67/IT-263, clone 2) were washed twice in fresh RPMI 1640 medium. For cutaneous leishmaniasis, 10^7 parasites (about the 20th subculture) were inoculated subcutaneously into the right hind footpads of female BALB/c mice (6–8-weeks old, 10 animals per group), with the left paw serving as a control. Footpads were weekly measured with a direct-reading vernier caliper. For visceral leishmaniasis, 5.0×10^7 promastigotes were injected intraperitoneally in five-month-old golden hamsters (groups of 10). The animals were killed at 2, 4,

8, 12, and 24 weeks postinfection. Spleens were removed and spleen tissue was homogenized in sterile physiological saline and inoculated either into RPMI 1640 medium or in CDM/LP to detect any live parasites in this tissue. Cultures were incubated at 25°C , passaged, and examined weekly over a five-week period.

Preparation of parasite lysates. Promastigotes were collected at different phases of growth by centrifugation at $2,000 \times g$ for 10 min at 4°C and washed three times by resuspension in PBS and further centrifugation. Pelleted promastigotes were solubilized in a lysis buffer containing 50 mM Tris and 0.1% Triton X-100, pH 8, and left for 20 min at 4°C . The detergent extract was then centrifuged at $5,000 \times g$ for 20 min at 4°C to remove cell debris, and the supernatant was used. Protein concentration was determined according to Bradford method (Bio-Rad Laboratories, Munich, Germany) using bovine serum albumin (Sigma, St. Louis, MO) as the standard.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis and immunoblotting. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out under reducing conditions in 1 mm-thick 10% polyacrylamide slab gels according to Laemmli³⁷ and subsequently stained with silver. Wells were loaded with equivalent amounts of protein from cells in the same growth phase. Western blot analyses were performed as previously described.³⁸ Protein samples resolved by SDS-PAGE were electrophoretically transferred to 0.45- μm pore size nitrocellulose membrane (Amersham, Buckinghamshire, United Kingdom) and subsequently blocked in Tris-buffered saline (TBS, 10 mM Tris, HCl, 150 mM NaCl, pH 7.2) containing 5% fat milk powder (TBSm). The membrane was cut into vertical strips and incubated at a fixed dilution (1:200) in TBSm with normal sera or immune human or dog sera obtained from hosts infected with *L. infantum* or *L. amazonensis*. Following a 1 hr-incubation with the primary antibody, the strips were washed three times in TBSm and incubated with either goat anti-human or anti-dog immunoglobulin G conjugated to peroxidase (Tebu, Le Perray-en Yvelines, France) for 1 hr. After three washings with TBS, the strips were treated with H_2O_2 and 4-chloro-1-naphthol. The reaction was stopped with deionized water.

Gelatin SDS-PAGE. Proteinase activities were examined after electrophoretic separation of total lysates under non-reducing conditions by SDS-PAGE in a 10% gel containing 0.2% (w/v) copolymerized gelatin as described.³⁹ After electrophoresis, enzyme activity was renatured by incubation of the gel for 1 hr at room temperature, with agitation, in acetate buffer, pH 5, containing 2.5% Triton X-100 and then for 2 hr in buffer alone. Activity against gelatin was revealed by staining the gel with Coomassie Blue R-250.

RESULTS

As shown in Table 2, continuous growth of 26 strains of various Old and New World cutaneous and visceral *Leishmania* species and two strains of *T. cruzi* isolated from humans or other vertebrate hosts can be achieved in CDM/LP medium. Different defined media combinations were tested but the best results, as judged by a faster rate of cell proliferation and higher final cell density, were obtained with

TABLE 2

Principal characteristics of the strains studied, including promastigotes of 26 strains of 11 *Leishmania* species and epimastigotes of two strains of *Trypanosoma cruzi*

Designation and source	Species	Number of passages	Mean growth ($\times 10^7$ /ml)
MHOM/BR/79/LI-01	<i>L. chagasi</i>	189	6.6
MHOM/MA/67/IT-263	<i>L. infantum</i>	89	5.6
MHOM/MA/67/IT-263 clone 2	<i>L. infantum</i>	59	6.5
MHOM/MA/67/IT-263 clone 7	<i>L. infantum</i>	61	7.1
MHOM/IN/83/H 570	<i>L. donovani</i>	161	6.9
MHOM/IN(-)/61/L-13	<i>L. donovani</i>	99	7.8
MHOM/-/IT-2217	<i>L. donovani</i>	86	7.2
MHOM/IN/80/DD8 clone 2	<i>L. donovani</i>	89	7.3
MYHC/BZ/62/M-379	<i>L. mexicana</i>	249	7.9
MHOM/VE/76/JAP-78	<i>L. amazonensis</i>	96	6.5
MHOM/BR/76/LTB-012	<i>L. amazonensis</i>	192	6.9
MHOM/BR/73/M-2269	<i>L. amazonensis</i>	45	7.7
MHOM/BO/83/LPZ-155	<i>L. amazonensis</i>	62	5.6
MPRO/BR/72/M1841	<i>L. amazonensis</i>	34	6.4
MHOM/BR/72/1670	<i>L. braziliensis</i>	71	6.5
MHOM/BR/75/M-2904	<i>L. braziliensis</i>	59	7.1
MHOM/BO/90/CS	<i>L. braziliensis</i>	86	5.8
MHOM/BO/90/AN	<i>L. braziliensis</i>	49	6.1
MCHO/PA/-/M-4039	<i>L. panamensis</i>	67	6.6
MHOM/PA/71/LS-94	<i>L. panamensis</i>	96	5.3
MHOM/EQ/91/A8044	<i>L. panamensis</i>	98	6.8
MHOM/BR/78/M-5378	<i>L. guyanensis</i>	76	5.3
MHOM/BR/75/M-4147	<i>L. guyanensis</i>	73	6.2
MHOM/PE/85/FR-6	<i>L. peruviana</i>	69	5.5
MHOM/SU/73/5ASKH	<i>L. major</i>	85	5.0
MHOM/SU/60/OD	<i>L. tropica</i>	80	6.8
Tehuentepec clone 1 (Zymodeme 12)	<i>T. cruzi</i>	85	8.9
SO 34 clone 4 (Zymodeme 20)	<i>T. cruzi</i>	49	8.2

CDM/LP medium. If hemin was omitted, most of the *Leishmania* strains studied failed to show promastigote growth after about five subinoculations. Addition of 25 μ g of gentamicin sulfate/ml of medium did not affect the ability of promastigotes to grow *in vitro*. Cultures inoculated with an initial density of 5×10^5 flagellates/ml into 10 ml of CDM/LP medium showed intensive growth at 26°C, with final cell yields ranging from 5 to 8.9×10^7 promastigotes/ml in 6–7-day period. Using this methodology, it has been possible to serially maintain as promastigotes cultures of *L. chagasi* (MHOM/BR/79/LI-01), *L. donovani* (MHOM/IN/83/H 570), *L. amazonensis* (MHOM/BR/76/LTB-012), and *L. mexicana* (MNYC/BZ/62/M-379) continuously for a period of up to three years (involving more than 160 subpassages) without any macromolecule, protein, or peptide supplement (Table 2).

Figure 1 shows the growth curves of several strains of various *Leishmania* species and *T. cruzi* established in CDM/LP medium after at least 10 weekly serial subpassages. The following results were obtained by daily counting of the promastigote number of various *Leishmania* species in CDM/LP medium. Since standard deviations for each test point were less than 8% of the corresponding mean values, the data could be compared for significant differences. In parallel, staining with erythrosin B demonstrated that for all the species studied the percentage of mortality was less than 5%

during the exponential growth phase and reached 10–15% in the late stationary phase. Typical growth curves were obtained with an initial latent phase of growth, followed by logarithmic and stationary phases, and eventually cell death leading to a decrease in density. The doubling time during logarithmic phase of growth varied between 19 and 26 hr depending on the strain studied. As demonstrated for *L. amazonensis*, *L. braziliensis*, *L. chagasi*, and *L. donovani*, similarity was observed between the growth kinetics of asexually grown promastigotes and those of the corresponding parasites cultivated in nondefined medium (Figure 2).

Typical promastigote morphology was observed in Giemsa-stained smears for all strains analyzed. Asexually grown promastigotes appeared elongated and pointed with an anteriorly directed flagellum exceeding the body length. A bar-shaped, deeply stained kinetoplast was seen at the base of the flagellum. The nucleus appeared broadly ellipsoidal or spheroidal and occasionally, a central nucleolus surrounded by a clear zone was observed. In the logarithmic phase of growth, many promastigotes were observed in various stages of division.

We also investigated the proteinase activities using gelatin SDS-PAGE. Figure 3 shows the proteinase patterns of seven strains of *Leishmania* cultured in CDM/LP medium in comparison with those of the corresponding promastigotes cultured in RPMI 1640 medium containing 10% FCS. The proteinases of both types of promastigotes resolved similarly in the substrate gels, with mainly high molecular weight proteinase activities being detected in the range of 60–180 kD. Polypeptide profiles revealed by asexually grown promastigotes were very similar to those of the corresponding parasites collected from serum-supplemented cultures in terms of apparent molecular weight and relative intensity of individual bands for all species studied. Comparative study of the antigenic profiles of both promastigote lysates of *L. infantum* and *L. amazonensis*, respectively were carried out by immunoblotting using homologous sera of natural infections. As shown in Figure 4, similar antigenic recognitions were observed with sera from humans with visceral and cutaneous leishmaniasis, as well as with serum of dogs infected with *L. infantum*. Control reactivities with normal human and dog sera were all negative (Figure 4).

The *in vitro* interactions between promastigotes of various *Leishmania* species cultured in CDM/LP medium and taken from the stationary phase of their growth and normal resident mouse peritoneal macrophages were investigated at different incubation periods (Figure 5). Analysis of the *in vitro* infectivity showed that after 4 hr of incubation, about 40–70% (dependent on the species studied) had promastigote forms attached to their surface. The percentages of infected macrophages increased significantly at 24 hr to reach 90–100% after 48 hr depending on the species (Figure 5). These results demonstrated that asexually grown promastigotes were capable of infecting macrophages *in vitro*. The infectivity of promastigotes *in vitro* was predictive of their virulence in mice and in hamsters. A comparative study of the experimental infection curves of the asexually grown promastigotes and of the others revealed a close similarity. Characteristic lesions appeared at the point of inoculation for *L. amazonensis* and *L. mexicana* within four and six weeks, respectively (Figure 6). Their size increased rapidly over time and

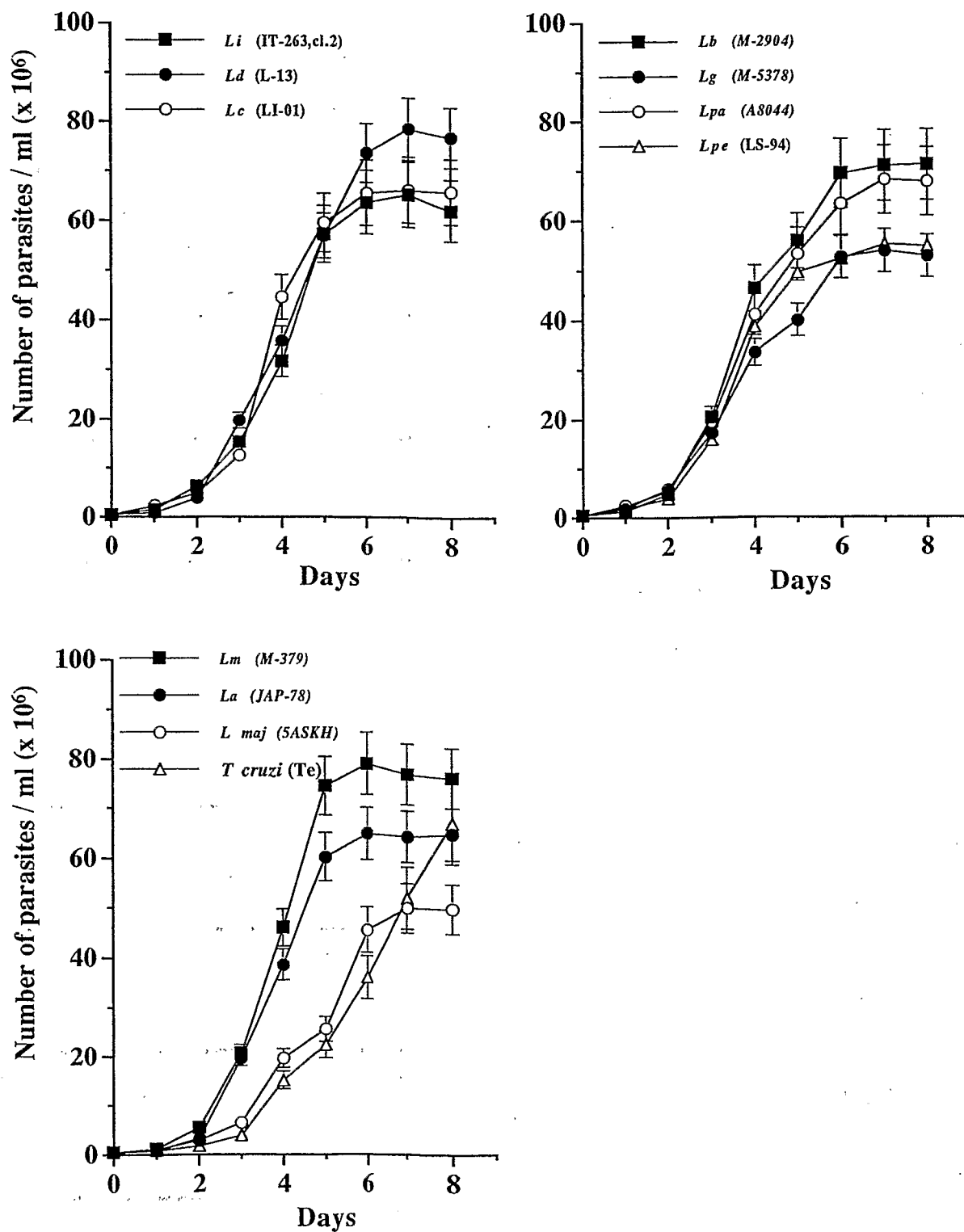


FIGURE 1. Growth curves of 10 *Leishmania* species and *Trypanosoma cruzi* established in CDM/LP medium after at least 10 weekly serial subpassages of 5×10^6 flagellates in 25-cm² flasks containing 10 ml of CDM/LP medium at 25°C. *Li* = *L. infantum*; *Ld* = *L. donovani*; *Lc* = *L. chagasi*; *Lb* = *L. braziliensis*; *Lg* = *L. guyanensis*; *Lpa* = *L. panamensis*; *Lpe* = *L. peruviana*; *Lm* = *L. mexicana*; *La* = *L. amazonensis*; *Lmaj* = *L. major*. Bars show the mean \pm SD.

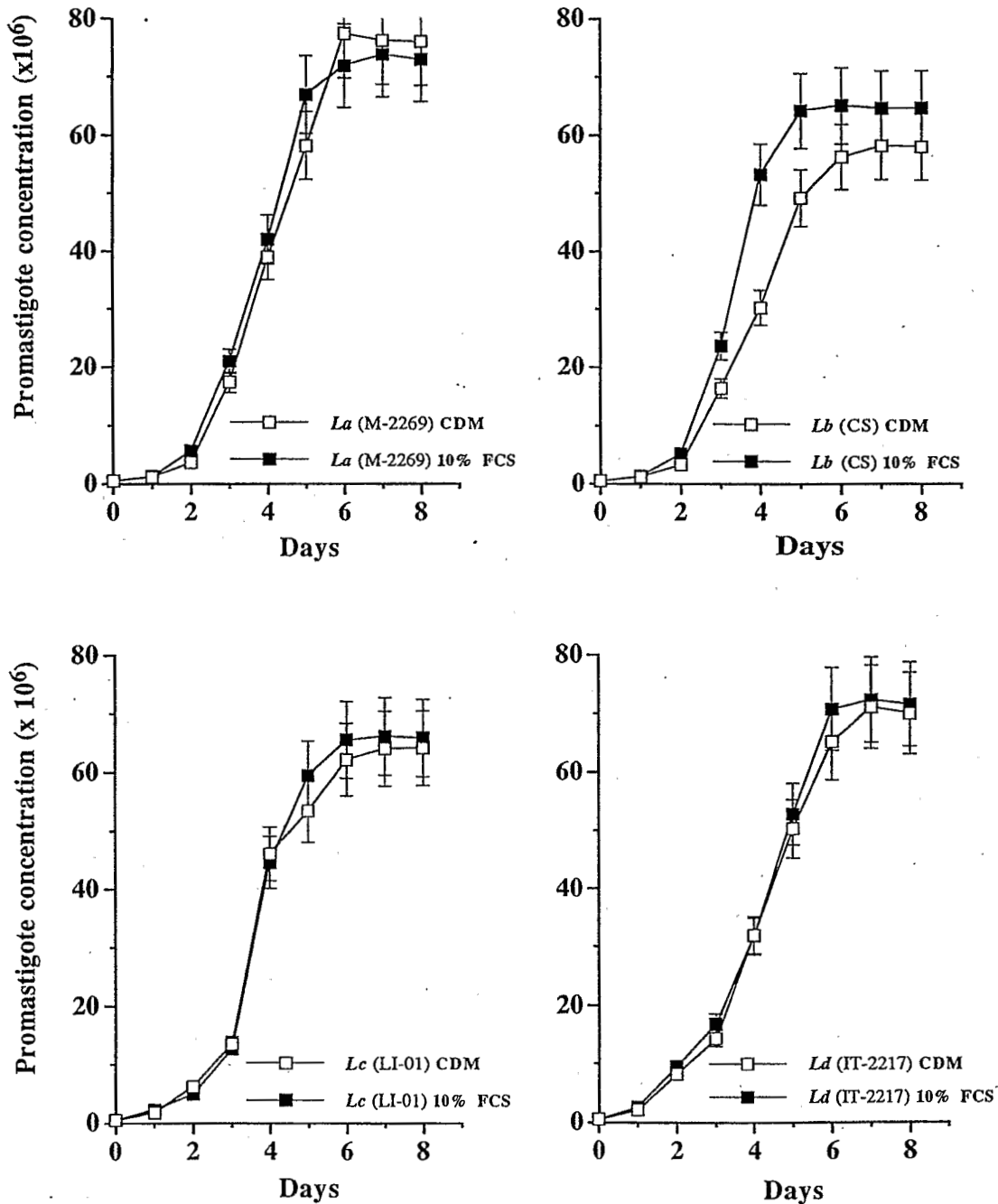


FIGURE 2. Comparative study of the growth kinetics of asexually grown promastigotes (CDM) of *L. amazonensis* (*La*), *L. braziliensis* (*Lb*), *L. chagasi* (*Lc*), and *L. donovani* (*Ld*), and those of the corresponding parasites cultivated in nondefined medium (10% fetal calf serum). Bars show the mean \pm SD.

reached 5–7 mm by the 14th week. For *L. amazonensis*, the footpads became ulcerated and necrotic from the eighth week onward. Inoculation of 5.0×10^7 promastigotes of *L. donovani* and *L. infantum* at the stationary phase of growth caused infection, as demonstrated by the presence of severe disease after 12 weeks of infection and promastigotes in cultures made from spleen samples within four and eight weeks postinfection, respectively. It was noteworthy that amastigote forms isolated from infected spleen tissue were able to differentiate into proliferative promastigotes in CDM/LP medium.

DISCUSSION

Many different media have been used for the cultivation of hemoflagellates. Promastigotes of all *Leishmania* can be axenically cultured by serial subpassages in a large variety of media. Parasites produced in this way have been extensively used in studies on the biochemistry, immunology and molecular biology of *Leishmania*. Promastigote forms can grow or can be adapted to grow in culture media that are qualitatively and quantitatively different from each other. Parasites of some species taken from primary lesions require

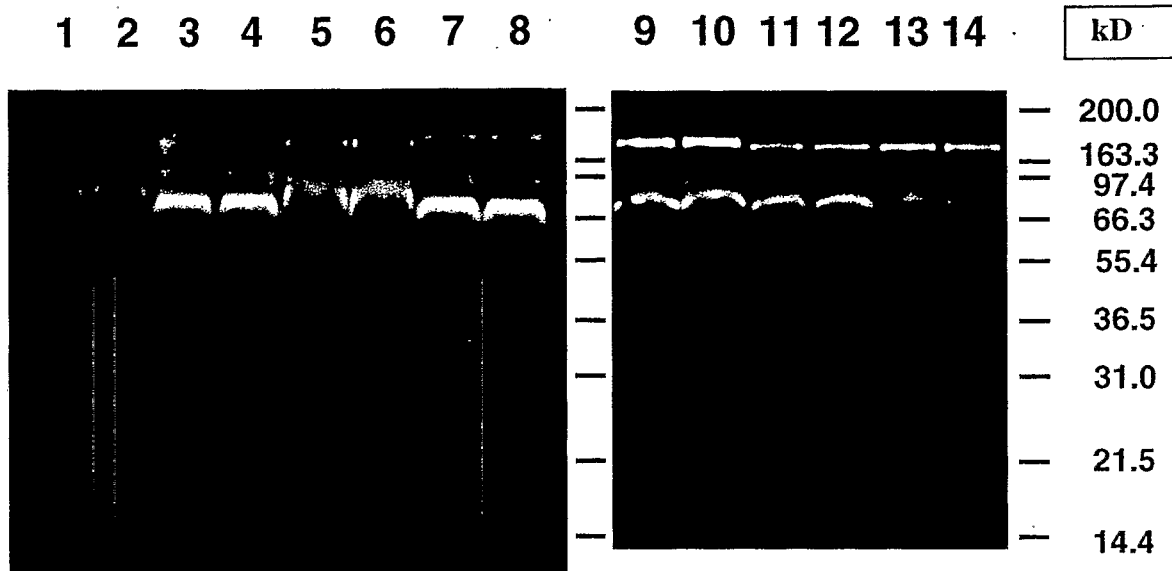


FIGURE 3. Comparative study of the protease activities in gelatin-containing acrylamide gels. A total of 5 μ g of protein was loaded/well. Lysates of mid-log phase promastigotes of *Leishmania amazonensis* strain JAP-78 (lanes 1 and 2) and strains LTB-012 (lanes 5 and 6), *L. mexicana*, parental (lanes 3 and 4) and clone 3 (lanes 7 and 8), *L. donovani*, strain IT-2217 (lanes 9 and 10), *L. chagasi*, strain LI-01 (lanes 11 and 12), and *L. infantum*, strain IT-263 (lanes 13 and 14) cultured in RPMI 1640 medium containing 10% fetal calf serum (lanes 1, 3, 5, 7, 9, 11, and 13-) and in CDM/LP medium (lanes 2, 4, 6, 8, 10, 12, and 14). kD = kilodaltons.

rich, multi-component media for continuous cultivation, such as Schneider's *Drosophila* medium supplemented with 30% FCS. Other strains or species have been adapted to continuous growth in less rich media containing serum or with serum replaced by bovine serum albumin,^{20,21} bacteri-

ologic media, or more recently, human urine.^{32,33} The complexity of the media used to cultivate members of the genus *Leishmania* has complicated the feasibility of comparative studies on nutritional requirements, metabolism, and other characteristics of these organisms, especially those isolated from mammals.

The current report describes a relatively simple formulation using common, available, and inexpensive ingredients that can be used in place of serum-supplemented media for the *in vitro* maintenance and mass cultivation of *Leishmania* promastigote forms. Different formulations were tested but the best results were obtained with CDM/PL medium, as judged by a faster rate of proliferation, higher final cell density, and the ability to culture most *Leishmania* species. This completely defined medium without serum-derived protein and/or macromolecules as a serum substitute supports the continuous growth of 26 strains of 11 *Leishmania* species at rates comparable with those obtained with serum-supplemented RPMI 1640 medium. The hyperosmolarity of CDM/LP medium due to the addition of high concentrations of various compounds did not affect parasite growth and viability. The inability to synthesize heme, a well-known metabolic defect of trypanosomatid protozoa, accounted for their growth requirement for heme compounds *in vitro*. Hemin, which provides the heme requirement of actively multiplying promastigotes, was found to be essential for continuous growth. Hemin-free cultures could only be maintained for a limited number of passages. Moreover, CDM/LP medium can be adapted for serum-free culture of other parasites, such as *T. cruzi*. This medium was also suitable for transformation of isolated amastigotes from infected animal tissue into proliferative promastigotes and could be used to isolate strains directly from hosts.

Morphologic, biochemical, immunologic, and biologic criteria have been applied to determine the similarity of as-

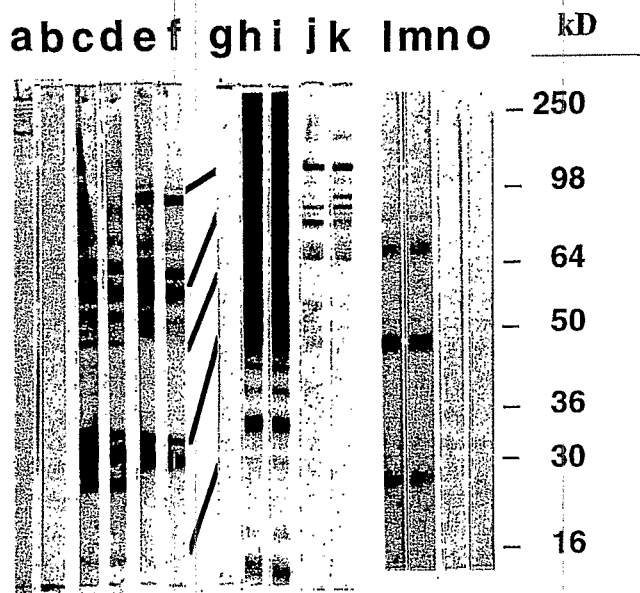


FIGURE 4. Immunoblotting of promastigote antigens of *Leishmania infantum* strain IT-263 (lanes a-k) and *L. amazonensis* strain LTB-012 (lanes l-o) cultured in RPMI 1640 medium containing 10% fetal calf serum (lanes a, c, e, h, j, l, and n) and in CDM/LP medium (lanes b, d, f, g, i, k, m, and o) recognized by dog (lanes a and b) and human (lanes g, n, and o) control sera, sera from two dogs (lanes c, d, e, and f) and two patients (lanes h, i, j, and k) with visceral leishmaniasis and serum from patient infected with *L. amazonensis* (lanes l and m). kD = kilodaltons.

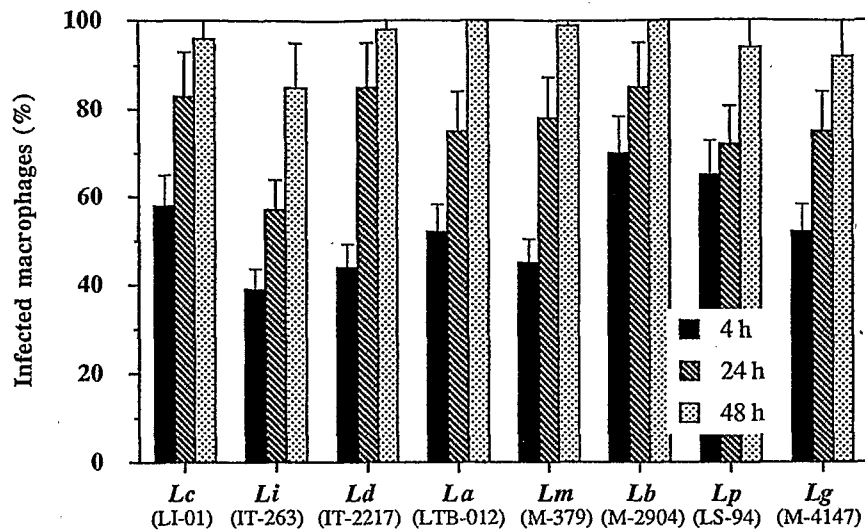


FIGURE 5. *In vitro* interactions between promastigotes of various *Leishmania* species cultured in CDM/LP medium and taken from the stationary phase of their growth and normal resident mouse peritoneal macrophages. Attachment (4 hr) and survival (24 and 48 hr) were studied. At each time point, more than 500 cells were counted in duplicate to determine the percentage of infected macrophages. Lc = *L. chagasi*; Li = *L. infantum*; Ld = *L. donovani*; La = *L. amazonensis*; Lm = *L. mexicana*; Lb = *L. braziliensis*; Lp = *L. panamensis*; and Lg = *L. guyanensis*. Bars show the mean \pm SD.

eric promastigotes to others. Typical promastigote morphology was observed in Giemsa-stained smears, regardless of the strain analyzed. Electrophoretic analysis showed that the polypeptide profiles and patterns of proteinases for asexually grown promastigotes of all *Leishmania* studied were similar in terms of apparent molecular weight and relative intensity of individual bands with those obtained in serum-supplemented medium. Immunoreactivity of promastigote lysates of two *Leishmania* species was also compared by immunoblotting using sera from different hosts with visceral and cutaneous leishmaniasis. These studies demonstrated that antigenically the asexually cultured promastigotes were closely related to those cultured in nondefined medium. For species that cause cutaneous and visceral leishmaniasis, the absence of serum, proteins, and peptides in culture medium did not markedly change their *in vitro* infectivity and virulence of promastigote forms in animals compared with parasites cultivated in nondefined medium. These findings clearly indicate that promastigotes produced by this technology were able to transform into amastigote forms, survive, multiply intracellularly, and infect other macrophages.

Most of the studies on promastigote forms of *Leishmania* have involved *in vitro* experiments using serum-supplemented media. The use of animal sera such as FCS in cell culture creates many obstacles, such as lot-to-lot performance variability, presence of adventitious agents, and fluctuations in price and availability. Serum is a complex, highly variable, and difficult to characterize reagent. It is particularly expensive when used to produce large cultures of cells for protein purification. The use of serum involves a risk of exposure to infectious agents and animals must be killed to provide serum for culturing of protozoan parasites. In certain biochemical and/or immunologic experiments, the presence of serum components in medium interferes with results. The elimination of serum or of serum-derived macromolecules that supplant the FCS requirement suppresses host factors in parasite experiments and minimizes interlaboratory varia-

tions in results that often have been attributed to culture conditions, especially to variability of serum batches. To obtain a high yield of well-defined material and easily purify cellular and/or naturally excreted antigens for the production of economical vaccines, the cultivation of parasites in completely defined medium is important since proteins have been found that are associated with *Leishmania* membranes,^{40,41} as well as with cytoplasmic structures in other parasites.⁴² Analysis of such soluble, released molecules is facilitated by the use of chemically defined macromolecule-free culture medium. Also, unlike membrane-bound components, detergent solubilization is not required. Access to a serum-free system for culturing promastigote forms could provide information leading to a better understanding of the nutritional requirements and metabolism of these organisms. It will also facilitate investigations of factors that promote growth and/or differentiation of promastigote forms, and will be a valuable tool for studies on metabolic processing of anti-leishmanial drugs.

The present work clearly demonstrates that a completely defined culture medium for the maintenance and mass culture of these important pathogens is a reality. All the advantages described here will be particularly important for researchers working in parts of world where FCS is expensive and difficult to purchase and transport, and where the facilities for cryopreservation are not present. In addition, serum-free technology will be increasingly important in providing stability and reproducibility as research using promastigote forms moves closer to therapeutic applications.

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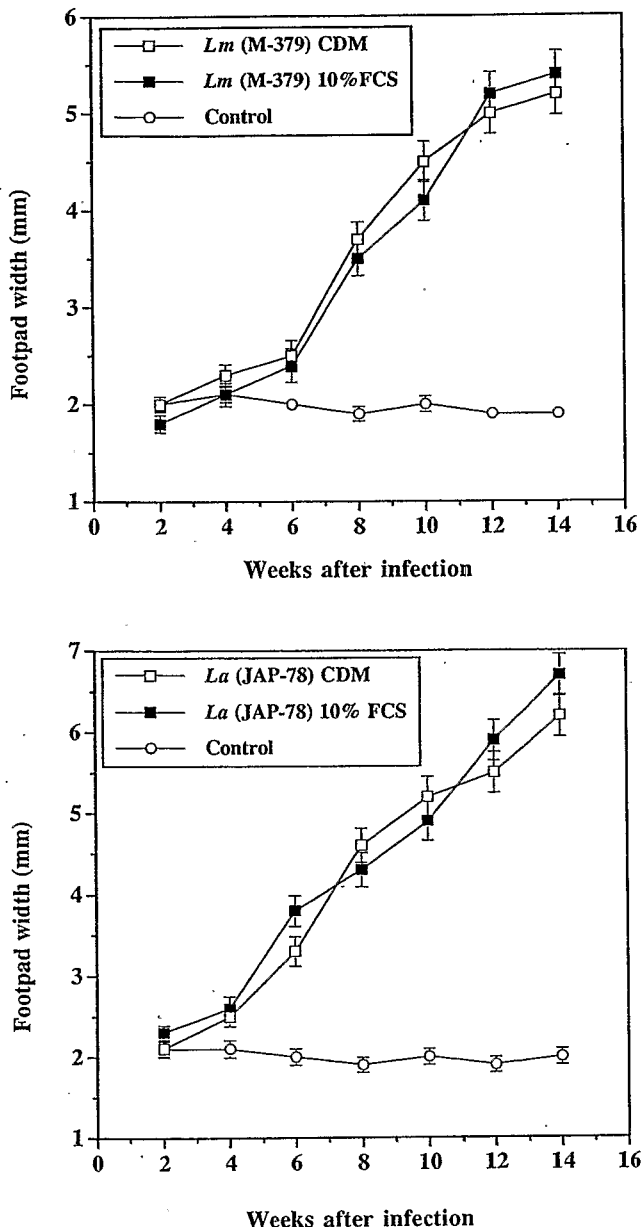


FIGURE 6. Comparative study of the kinetics of *in vivo* infectivity of asexually grown promastigotes (CDM) of *L. mexicana* (*Lm*) and *L. amazonensis* (*La*) and those of the corresponding parasites cultivated in nondefined medium (10% fetal calf serum). A total of 10^7 stationary phase parasites were inoculated subcutaneously into the right hind footpads of female BALB/c mice, with the left paw serving as a control. Footpads were weekly measured with a direct-reading vernier caliper. Values are the mean \pm SD for 10 animals per group.

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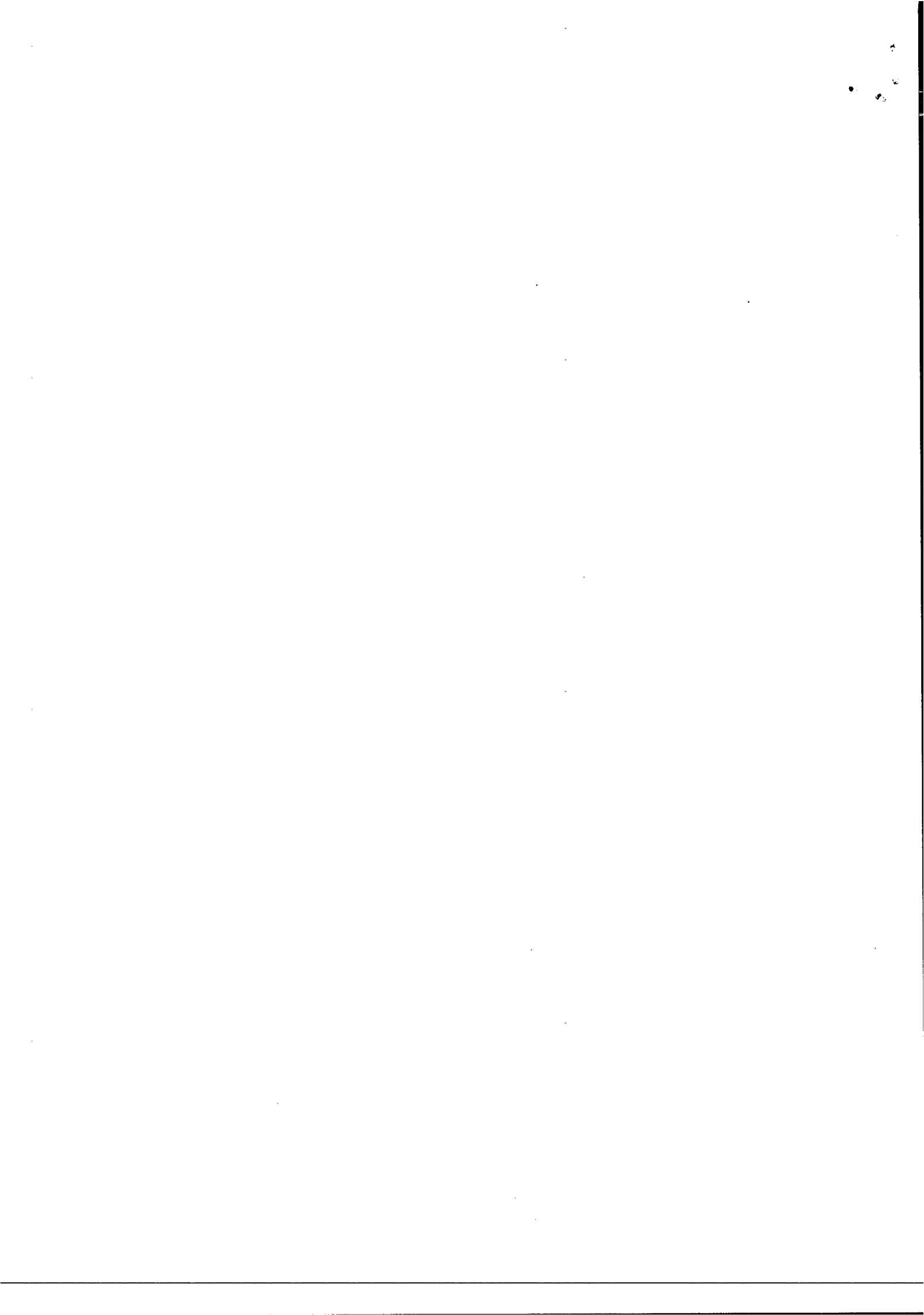
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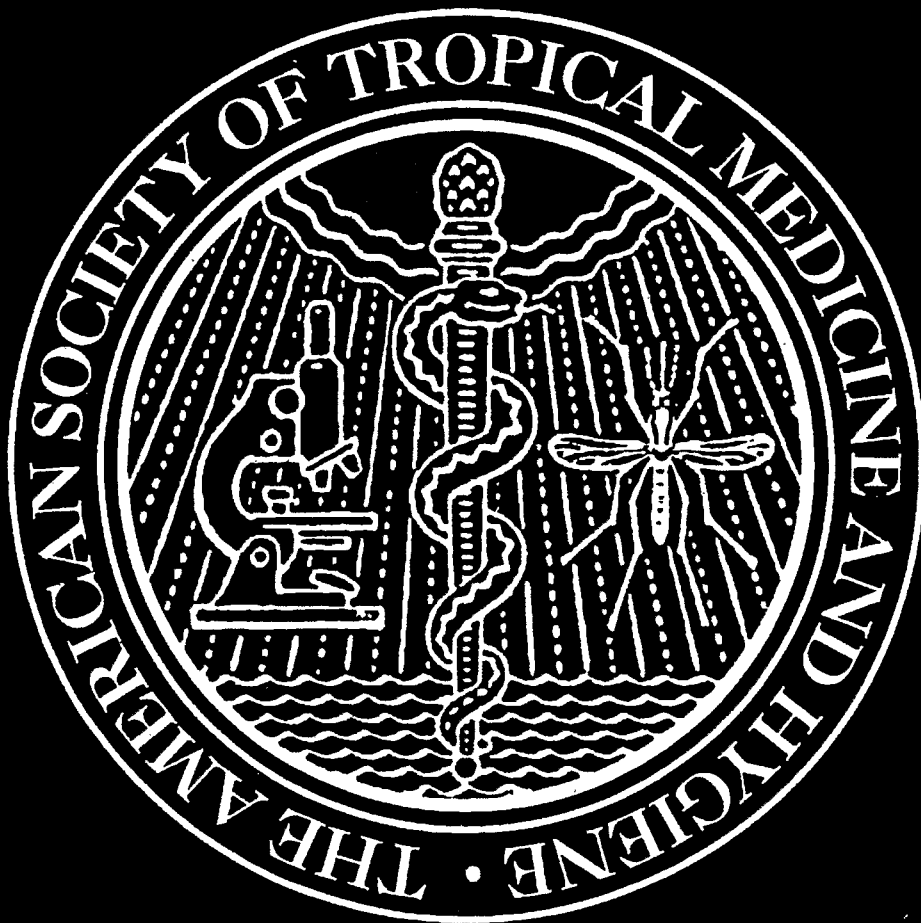
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