BIOTECHNOLOGY AND VISCERAL LEISHMANIASIS IN THE WORLD HEALTH ORGANIZATION'S SOUTHEAST ASIAN REGION: RESEARCH AND REALITY

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INTRODUCTION

The Leishmania may well be the most enigmatical and perplexing of all the protozoan parasites of humans. They are obligate intracellular parasites within mononuclear phagocytes, the very cells that are paramount effectors in the destruction of pathogens. Under the microscope all leishmania look alike but in the human each species induces a different and characteristic disease accompanied by, and related to, its pattern of immunological response.

If Leishmania as a group is an enigma, then *Leishmania donovani* is an enigma within an enigma. Within its extensive geographical range there is a broad variation in zoonotic host restrictions: in South America, China, North Africa, most of tropical Africa, the Middle East and the Mediterranean Europe the dog and wild canines are reservoir hosts; in Ethiopia it is the hyrax; in the Sudan it is rodents; and in the Indian subcontinent humans are the only animal species that is infected. In the symptomatically affected human *L. donovani* causes a profound depression of cell-mediated immunity while concurrently inducing both high antibody titres and, possibly acting as a polyclonal B cell mitogen, a level of IgG globulinaemia so high as to be diagnostic for the infection. For many years the accepted clinical concept of visceral leishmaniasis was that it was invariably fatal if left untreated in all those who were infected. A more recent, but still cloudy, view proposes that there are humans who have either resolved the infection or have become asymptomatic carriers. Neither the immunological mechanism responsible for this phenomenon nor the epidemiological significance of the putative carrier state are, presently, understood.

Visceral leishmaniasis (kala-azar) in the WHO–Southeast Asia Region:

After a respite of almost 20 years visceral leishmaniasis, kala-azar (the black sickness), returned to the Southeast Asian region (using the WHO-defined geography of what constitutes Southeast Asia). It struck first in Bihar, India in 1971 and in the early stages of the epidemic the national and state health authorities, except for some older workers who had been uttering cautionary warnings over the years, were unfamiliar with the savagery of the disease. From 1971 onwards the infection has been spreading to recapture its ancient domain in the Indian subcontinent. Although the affected countries had been gradually developing a breadth and sophistication of their biomedical research establishment over the past 20 years, for all

practical purposes the health authorities - the workers in the field - have been less capable of controlling kala-azar than their predecessors of the 1950s. There were multiple reasons for this. Health professionals trained during the 20 years lull had no experience, and little education of, kala-azar's diagnosis, treatment and control. Vector control was in disarray; the once strong vector control units operated by the national malaria eradication programs had been mostly disbanded and decentralized as state/provincial responsibilities. Spraymen were less well-trained, budgets reduced, and DDT became difficult to obtain. Epidemiological services were not prepared to mobilize for extensive case-detection and their laboratory services were not prepared or trained to carry out large-scale serological and parasitologic support. Moreover, when cases *were* diagnosed there was difficulty in treatment. The drug of choice, sodium antimony gluconate, required a prolonged chemotherapeutic regimen to which a large number of patients mainly agricultural peasants would not or could not comply. At any rate, some of the national health services had no stocks of drugs to dispense at their primary health facilities and when the drug was available in the local market it was at a price beyond the reach of most of those who needed it.

Clearly, older proven methods had to be implemented and new methodologies invented if the disease was to be brought under lasting control. It was also believed, particularly by organizations concerned with international health issues, (such as the WHO) that biotechnology would contribute to the discovery of new, effective approaches to diagnosis and treatment. Thus, an assessment and review of the biotechnological research on visceral leishmaniasis should be done within the context and perspective of the natural history-history of the disease in the Indian subcontinent. It is with this in mind that I give the following synoptic account.

Although great and devasting epidemics of kala-azar have periodically swept through the India subcontinent, its early history is uncertain. It had long been confused with malaria and not clearly identified in ancient Hindu medical writings (Jaggi, 1979). The first outbreak, which epidemiologists and medical historians are of a concensus that it was kala-azar had its epicenter in Jessore in 1824 where it took some 75,000 souls. The epidemic progressed to the Parganas by 1832, to the Hooghly District by 1857 and Burdawan in 1862. A civil surgeon in Burdawan at that time writes of entire villages "in which not a healthy person was to be met with" and "the daily death, the loss of their children, the increasing depopulation, the absence of all hope for better times had so demoralized the population that they neglected to avail themselves of medical and other aid." In 1862 its presence was felt in Dacca of East Bengal where "The mortality was so great that the dead were left in their houses or thrown into the *beels* or rivers". From 1872 to 1882 it progressed to North Bengal, Bihar and Assam. This vast region, the Gangetic Plain was (and is) the landscape epidemiology of Indian kala-azar although there have been outliers of discrete foci in Tamil Nadu, Andra Pradesh, and Orissa.

The above epidemic began to wane in 1882 and the infection "smoldered" until 1920 when a new epidemic began in Jessore and peaked in 1935. The next epidemic began in Bengal in 1942 and peaked in 1949. Then in 1953, India embarked upon a malarial eradication scheme. By 1958 the National Malaria Eradication Programme was in full exercise with centralized direction, adequate funding and a remarkably efficient

and wide DDT spray coverage. By 1966 it was realized that for a variety of reasons eradication was not possible and the "great effort" was discontinued and decentralized to an operational level that was described earlier in this paper.

It is believed that the NMEP had as great, if not greater, impact on kala-azar as on malaria. The main vector, *Phlebotomus argentipes* was (and is) exquisitely sensitive to DDT and the domestic spray activities reduced their numbers to a degree that resulted in little or no transmission of kala-azar during the NMEP years (although the minority opinion has it that those years, 1951–71, coincided with the natural 20 year inter-epidemic interval). Then, as I noted earlier, a new epidemic announced itself in Bihar with its epicenter in Varshali, a village with holy associations to Bhuddism and Jainism. Accurate prevalence rates were never really known but from model village studies it is estimated that by 1977 there were at least 100,000 cases in Bihar State with at least 4,500 deaths. Cases first came to notice in West Bengal in 1975 and the incidence rose continuously until it is now believed to be holding steady at about 10,000 new cases per year in that State.

It is uncertain when kala-azar reappeared in Bangladesh (formerly East Bengal). Sporadic cases, chiefly occurring in Mymensingh District were reported during 1968 to 1970 and again from 1973-1980. The main outbreak began in 1981 with its epicenter in Shahajadpur thana of Pabna district. The majority of those affected have been children under 10 years of age. Cases continue to be reported and other districts now seem to be affected although the true extent and endemicity is not really known. Extensive active case detection has not been undertaken in Bangladesh. Epidemiological intelligence has chiefly derived from case reports to the national authorities via the district (thana) health officer. The district health officer receives his data chiefly from sub-district and primary health centres. When interviewed, the physicians at those primary health centres were candid. They have had no stocks of sodium antimony gluconate for several years (or ever). Their patient population are aware of this deficiency as well as being aware of the symptoms of kala-azar and no longer present themselves to the centers for diagnosis or treatment. Thus the number of cases illustrated on the Ministry of Health epidemiologists' graph, prominently displayed on the office wall, is undoubtedly an egregious under-representation of the true prevalences.

The status of kala-azar in Nepal is even less clear than in Bangladesh. Although the infection had long been present in the terai region an effective NMEP spray programme (1958–1964) had drastically reduced transmission. The reappearance of new cases was first noted in 1981. Between 1981 and 1985, 557 cases, of whom 47 died, occurring chiefly in the Eastern terai districts were reported to the national health authorities by the hospitals in the affected region.

The distance and difficult accessibility of the Eastern terai from Kathmandu, the shortage of trained epidemiologists, and lack of laboratory support for parasitological and serological diagnosis have hampered the epidemiological surveillance of kala-azar in Nepal.

The Application of Biotechnological Research

Despite the differences in epidemiological status and biomedical research resources between the three affected SEARO region countries there is a commonality in their problems to which biotechnology may help

provide the needed solutions. However, before embarking on a discussion/review of relevant biotechnological research I would invite the reader to keep the image of Fig. 1 in mind. This is a photograph of the laboratory in a district health care facility in Bangladesh. It is typical of the great majority of clinical laboratories in district hospitals throughout SEARO's kala-azar-endemic region. It is at these district/primary health facilities that the majority of cases are diagnosed and treated. The photograph of Fig. 1 is a reminder of the conditions under which biotechnology-derived methods may have their ultimate application.

The foreseeable major applications of biotechnology-derived research would be, first, a better understanding of the immune responses to provide a logical basis for immunotherapeutic methods to cure the disease and immunization to prevent it. There has been a relatively long history of research on immunization against the leishmaniases in experimental animals and humans. An account of that research would require an extensive separate review and artificial immunization will not be considered here except to note that a practical, effective vaccine to protect humans against visceral leishmaniasis has yet to be discovered. The second application would be for better, more sensitive serological tests for both diagnosis of the individual patient and epidemiological surveillance. For all practical purposes, the time-honoured combination of a positive formal gel reaction and clinical history has served primary health care physicians quite well in diagnosing the patients with established manifestations of the disease. It is for the diagnosis of early visceral leishmaniasis and for the putative carrier state that better serological methods are needed. It may also be that high-tech serology, in respect of absolute species-strain specificity will not be as necessary in the SEARO's endemic region where there is little chance of human exposure to other haemoflagellate antigens as is the case in other endemic regions where the cutaneous leishmania, trypanosomiases, and haemoflagellates of wild and domestic animals may be coendemic.

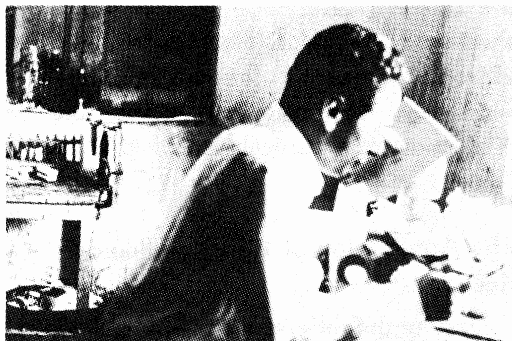

Fig. 1–A typical laboratory of a health facility located in the Kala-azar endemic area of SEARO, the ultimate user of biotechnologically-derived research.

For reasons discussed earlier it is generally conceded that the present chemotherapeutic armamentarium for the treatment of visceral leishmaniasis is far from ideal. An alternate approach would be to induce an immune response that would lead to self-cure as occure in uncomplicated cutaneous leishmaniasis. Many years ago it was recognized that an immunological defect peculiar to kala-azar (as contrasted to the cutaneous disease) existed in which there was abundant detectable antibody concomitant with the failure of leishmania antigen to elicit a delayed skin test response. This anergy is now recognized as a sign of immunodepression/immunounresponsiveness of cell mediated immunity (CMI) and that CMI is essential in killing the parasites to resolve the infection.

The recent advances in immunology made possible in large part by the development of

biotechnological techniques (particularly the hybridoma methodology to produce monoclonal antibody probes), has allowed an inquiry into *Leishmania donovani*-host relationships that will, hopefully, lead to a logical means for inducing immunologically-mediated cure and/or prevention. The logical entry into the quandry has been a search for the mechanism(s) responsible for the permissiveness whereby the amastigotes are able to exist within the phagocytes.

When all goes right the activated macrophage destroys the amastigote by both a superoxide, oxygen-dependent mechanism and an oxygen-independent mechanism (Murray and Cartelli, 1983) modulated by lymphokines, especially interferon-γ. There is also evidence that killing, when it does occur, is also accomplished by a unique process in which a *Leishmania donovani*-specific T cell population of the Ly $^{-}1^{+}2$ phenotype in a MHC class II restricted reaction does its lethal work by non-lymphokine mediated direct contact with the macrophage (Panosian *et al.*, 1984; Wyler *et al.*, 1987). The underlying mechanism of this antileishmanial defense has not, as yet, been elucidated.

However, in infections of *L. donovani* of man and mouse and of *L. tropica major* in Balb/c mice (which visceralizes to produce a disease similar to that of kala-azar in humans) all does not go right and the parasites survive and multiply within the phagocyte. The parasite appears to employ a remarkable variety of different strategies to obtain this host-cell permissiveness:

(1) By some unknown mechanism the parasite induces a greatly increased myelopoiesis which in turn results in elaboration of large numbers of immature phagocytes. These promonocytes can be invaded by the parasites but are physiologically incapable of making a kill even in the presence of lymphokines (Mirkovich *et al.*, 1986).

(2) Promastigotes activate complement (probably through the alternate pathway). The promastigote has a receptor which binds C_3 without participation of immunoglobulin. The promastigote is phagocytized and transforms to the amastigote. For some unknown reason the C_3-coated promastigote/amastigote inhibits the respiratory burst which produces the superoxides and other oxygen products which would kill the parasite (Mosser and Edelson, 1987). Oxidative metabolism of the infected phagocyte is, in general, impaired (Buchmuller-Rouiller and Mauel, 1987).

(3) *L. donovani* stimulates the macrophage to produce E-series prostaglandins (PGE_2) (Reiner and Malamud, 1985). The intracellular accumulation of PGE_2 down-regulates the expression of MHC gene products (Snyder *et al.*, 1982) and, in fact, Reiner *et al.*, (1987) have demonstrated that mouse macraphages parasitized by *L. donovani* have 50% to 90% less class II MHC gene products (I−A and I−E) on their surface after interferon-γ stimulation than do similarly stimulated macrophages from an uninfected mouse. In the cascade of effects it has been shown that the macrophage needs class II MHC antigen expression for production of interleukin-1 (I1−1) (Gelman *et al.*, 1983). Thus, I1−1 production is inhibited in the *L. donovani*-infected phagocyte (Reiner, 1987). Less I1−1 = decreased T cell proliferation = CMI immunodepression.

From the foregoing discussion (which is by no means, comprehensive) it is obvious that an impressive body of knowledge has been, and is being accumulated on the mechanism(s) concerned with immunity (or lack of it) to

leishmanial infection, particularly as it pertains to the response of certain inbred strains of mice. The human picture, however, is clouded by conflicting observations. Moreover, the immunological research on kala-azar in humans has been of a somewhat less biotechnologically sophisticated nature than that on murine visceral leishmaniasis. While there is agreement that there is a CMI anergy in active cases there is a difference of opinion as to whether this anergy is specific or generalized. A parasite-specific anergy was reported for Indian patients (Haldar *et al.*, 1983; Sacks *et al.*, 1987) and an American case who had acquired the infection in Greece (Wyler, 1982). In these patients there was a normal DTH skin test to such antigens as PPD and candida but not to leishmania. Similarly, for the *in vitro* correlate, the lymphroproliferative response, there was a normal blastogenic response to the mitogens but not to *L. donovani* antigen. However, in active visceral leishmaniasis cases from Kenya, Ho *et al.*, (1983) found there to be a generalized anergy with depressed DTH and *in vitro* lymphoproliferation responses not only to leishmania but also to other antigens such as PPD, and candida and to mitogens. A serum factor from a kala-azar patient was found to suppress autologous T cell, leishmania-specific proliferation (Wyler, 1982) and Petersen *et al.*, (1982) reported that adherent suppressor cells inhibited leishmania-specific lymphocyte proliferation in diffuse cutaneous leishmaniasis (in which there is a similar parasite-specific CMI anergy). In contrast, Koech *et al.*, (1987) found that neither patient's serum or adherent cells were participating to any degree in the antigen (leishmania, PPD) or mitogen- (PHA, Con A) depressed blastogenic response of lymphocytes from Kenyan patients with active visceral leishmaniasis. The study of Sacks and his colleagues (1987) is of particular interest in that they showed (1) that family members of kala-azar patients frequently produced a leishmania-specific lymphoproliferative response suggesting "that the frequency of infection within an endemic area in Bihar is very high" and (2) that the antigen-specific lymphoproliferative response of kala-azar patients was not restored by depletion of supressor Leu-2^{+} (OKT8^{+}) T cell subpopulation.

The new knowledge on the nature of immunodepression in some leishmaniases, notably the visceral, mucocutaneous, and diffuse cutaneous forms, has begun the consideration of novel immunotherapeutic approaches. Gorczynski (1987) has shown that *L. mexicana* infections in BALB/c mice can be modulated by an anti-idiotype (receptor epitope) "vaccine" prepared from T cell hybridomas. Lymphokine therapy has not yet moved to extensive *in vivo* trial although the *in vitro* leishmaniacidal activity of natural and recombinant IFN-γ makes it a promising form of immunotherapy (Nacy *et al.*, 1981; Passwell *et al.*, 1986, 1987). Humans with 'Indian' kala-azar are yet to be treated immunotherapeutically. Mucocutaneous *(L. braziliensis)* leishmaniasis has been so treated and may serve as a guide for the immunotherapy of kala-azar. Injections of dialyzable lymphocyte extract prepared from drug-cured cutaneous leishmaniasis, DHT-positive patients was reported to cure *L. braziliensis* patients with an early stage of the disease but did not induce cure in patients with advanced disease (Delgado *et al.*, 1981).

Taxonomy and Specific Diagnosis

Under the microscope all *Leishmania* look alike making taxonomic assignments by classical morphological methods impossible. Older workers used clinical manifestations,

serological (mainly agglutination test) type, geographical and host range as characters in classifying the *Leishmania*. This has been a somewhat equivocal process, particularly in respect to clinical manifestations which is not necessarily a fixed character from host to host. For example, *L. (tropica) major* a cutaneous infection of humans visceralizes in BALB/c mice (as if it were *L. donovani* of humans). More recent work has employed biochemical and genetic tools to refine the genus' taxonomy and to seek genetic confirmation of the validity for categorizing the human Leishmania into 4 major complexes *(L. donovani, L. tropica, L. mexicana and L. braziliensis)*. These methods have also provided important new insights into subspecific relationships although the data are not yet sufficient for their elevation to specific status. My remarks here are chiefly concerned with the findings for the *L. donovani* complex *(L. donovani donovani, L. donovani infantum, L. donovani chagasi*.

Chance *et al.*, (1979) used a combination of DNA buoyant density and malate dehydrogenase (MDH) isoenzyme pattern to identify species and subspecific differences of a very large array of isolates (promastigotes) from many parts of the tropical world. They made the theoretical assumption that a buoyant density difference of 0.001 g/ml indicates a 10% difference in genetic constitution, i.e. nucleotide sequences. Difference of that magnitude was present between the Indian strains of *L. donovani* and strains from Brazil *(L.d. chagasi)*, Mediterranean Europe *(L.d. infantum)*, and Ethiopia *(L.d. donovani)*. Differences were also seen in some MDH patterns but this was not as consistent as that of the DNA buoyant densities. Interestingly, although the Indian strains were biochemically distinct, the buoyant densities and MDH patterns of the strains from South America and East Africa were very similar.

Gene mapping on chromosomes has been a useful modern means in making species diagnoses. However, for *Leishmania* (and most other parasitic protozoa) their chromosomes do not condense during the cell's reproductive cycle. Recently, several biotechnological methods have been applied in analyzing the relationships amongst the *Leishmania*.

Beverly et al., (1987), extracted the DNA, fragmented it by digestion with six different restriction enzymes and then analyzed it by blot hybridization using several probes including that for *B-tubulin*. In this way a "molecular divergence/evolutionary tree" was constructed for thirty-one strains and species (as defined by the World Health Organization, 1984). There was a distinct divergence of DNA fragment pattern relationships between the major group-lineages. Using the timetable developed for other animal species the authors make the interesting conjecture that the magnitudes of difference (13–25%) indicate that these groups of *Leishmania* diverged 10–80 million years ago – a time (at the more distant end) when dinosaurs still walked the earth. Of the *L. donovani* complex, the Old World *L. donovani donovani* were all found to be similar (although it is not clear if an Indian isolate was included for analysis). However, *L. d. donovani* had a different DNA fragment pattern than that of *L. d. infantum* and *L. d. chagasi*. Isolates of the latter two subspecies were found to have almost identical DNA hybridization patterns and the authors believe that *L. d. infantum* gave rise to the New World *L. d. chagasi* in the recent past (since European colonization).

Within the past two years a powerful new method, pulsed field gradient gel electro-

phoresis (PFGGE) which separates DNA into chromosome-sized fractions has been applied to the molecular karyotype relationship analysis of the *Leishmania*. Gene mapping hybridization with such 'housekeeping' probes as that for the tubulin genes has added further analytical refinement to PFGGE. By these methods it was possible to distinguish *L. donovani* from the other species (Giannini *et al.*, 1986; Samaras *et al.*, 1987) but from the limited PFGGE/hybridization analyses carried out so far strain-subspecies difference have not, or cannot, be defined.

These biochemical/genetic findings have confirmed the older concept of speciation amongst the Leishmania infecting humans. It almost axiomatically follow that if there is a diversity of true species then, ideally, serodiagnosis should yield species-specific results. As I noted earlier, for all practical purposes a highly specific antigen/methodology is less urgently required in the SEARO kala-azar endemic zone than in other parts of the tropics because there being little or no exposure to Leishmania other than *L. donovani*. Even the 'distant' antigens produced from *Trypanosoma cruzi* and *T. brucei* work very well for the serodiagnosis of kala-azar by counterelectrophoresis (Desowitz *et al.*, 1975; Khan and Desowitz, 1985). The first step toward that objective has been accomplished through biotechnology species-specific monoclonal antibodies have been raised following immunization with antigens from a variety Leishmania. They specifically distinguish, by several serological methods, antigens of one Leishmania parasite species from another those of another species (McMahon-Pratt, and David, 1981; Handman and Hocking, 1982).

The next step in the development of highly specific serological techniques for application to infections in humans has not, as yet, met with similar success. The research logic has been to use the species-specific monoclonals as ligands in immunoaffinity chromatography to capture the species-specific antigens. The eluted "pure" antigens have been used, chiefly in the ELISA but the results so far have been relatively disappointing. Although genus specificity has been good (there is little cross-reactivity to antibody in "Chagas" disease patients, for example) there has been little or no specific or subspecific serological attributes provided by these antigens (Sacci *et al.*, 1987). In fact, the monoclonal antibody-affinity chromotography-isolated antigens have not provided greater specificity (beyond being genus-specific) than whole promastigote antigens used in the ELISA with serum antibody at high dilution (Badaro *et al.*, 1986). SDS-PAGE of *L. donovani chagasi* antigen followed by immunoblotting with sera from visceral leishmaniasis, cutanaeous, and tuberculosis (Leishmania and mycobacteria share a number of common antigens) have revealed species-specific reactions with antigens of the 32-35 kd range (Reed *et al.*, 1987) giving indication that a species specific sero-diagnostic method may be possible. But whether such a test, when it is devised in the laboratories practicing the biotechnological arts, will be capable of being used by practitioners in the field is very doubtful. For the moment, the direct agglutination test as it has been modified by Harith *et al.*, (1986; 1987) is probably the only serology practicable for the conditions illustrated in Fig. 1. In fact, serum at high dilutions used as the threshold for positivity the DAT is of equal, if not better, specificity and sensitivity than the ELISA using 'crude' or 'pure' antigens.

However, biotechnology may provide for parasitological diagnosis what it has not yet been able to do for serological diagnosis. The

difficulties in obtaining spleen or bone marrow biopsy material, processing the tissues, and finding the parasites in kala-azar patients are well known. For exact species identification, an important consideration in therapeutic management when differentiating between *L. braziliensis* and *L. mexicana* for example, the not always successful isolation by culture is necessary. Recently, DNA probe methodology has been developed for the direct species-specific diagnosis of patients with leishmaniasis (see review by Wirth *et al.*, 1986). The probes have been prepared from the kDNA of the mitochondrial minicircle. So far, the DNA probe technique has shown the most promise in differentiating the New World species. It also seems to allow a direct diagnosis even when parasite density is below the threshold usually needed for microscopic diagnosis. Unfortunately, there is a minicircle kDNA sequence homology between the Old World leishmania species. Research is now being directed to developing, by such means as cloning restriction fragments, that will hybridize specifically with Old World species and subspecifically with New World species. DNA probes and labelled monoclonal antibody techniques should also be highly useful for identification of infection in the sandfly.

In assembling this review one cannot fail to be impressed and excited by the vitality and innovativeness of this large and diverse body of current research on the leishmaniases. There is no doubt that the knowledge from research using the methodologies of the biotechnological frontier delights the intellect of all students of host-parasite relationships. Yet, my thoughts keep returning to the scene in Fig. 1 as well as to the primary health care clinics in remote areas of endemic kala-azar where patients are diagnosed and (too often) inadequately treated. There is that nagging doubt that there is too great a discontinuity between research and reality; between the energies and resources directed to what is intellectually satisfying and to the worldly needs of the sick and those who attend them.

REFERENCES

BADARO, R., REED, S.G., BARRAL, G. and JONES, T.C., (1986). Evaluation of the micro enzyme-linked immunosorbent assay (ELISA) for antibodies in American leishmaniasis: Antigen selection for detection on infection-specific responses. *Am. J. Trop. Med. Hyg., 35* : 72.

BEVERLY, S.M., ISMACH, R.B., and PRATT, D.M., (1987). Evolution of the genus *Leishmania* as revealed by comparisons of nuclear DNA restriction fragment patterns. *Proc. Natl. Acad. Sci. USA, 84* : 484.

BUCHMULLER–ROUILLER, Y. and MAUEL, J., (1987). Impairment of the oxidative metabolism of mouse peritoneal macrophages by intracellular *Leishmania* spp. *Inf. Immun., 55* : 587.

CHANCE, M.L., GARDENER, P.J. and PETERS, W., (1979). Biochemical taxonomy of Leishmania as an ecological tool. *Coll. Int. C.N.R.S.,* No. 239.

CONVIT, J., CASTELLANOS, P.L., RONDON, A., PINARDI, M.E., ULRICH, M., CASTES, M., BLOOM, B. and GARCIA, L., (1987). Immunotherapy versus chemotherapy in localized cutaneous leishmaniasis. *Lancet. i* : 401.

DELGADO, O., ROMANO, E.L., BELFORT, E., PIFANO, F., SCORZA, J.V. and ROJAS, J., (1981). Dialyzable leukocyte extract therapy in immudodepressed patients with cutaneous leishmaniases. *Clin. Immunol. Immunopathol., 19* : 351.

DESOWITZ, R.S., DRAPER, C.C., and PHILLIPS, T.M., (1975). Preliminary observations on countercurrent immunoelectrophoresis of *Trypanosoma cruzi* and Leishmania. *Trans. Roy. Soc. Trop. Med. Hyg., 69* : 430.

GIANNINI, S.H., SCHITTINI, M., KEITHLY, J.S., WARBURTON, P.W., CANTOR, C.R., and VAN DER PLOEG, L.H.T., (1986). Karyotype analysis of *Leishmania* species and its use in classification and clinical diagnosis. *Science, 232* : 762.

GILMAN, G.C., ROSENBERG, J.S., and FELDMAN, J.D., (1983). Inhibition of interleukin synthesis and T cell proliferation by a monoclonal anti-Ia antibody. *J. Immunol., 130* : 1236.

GORCZYNSKI, R.M., (1987). Immunization with *Leishmania*-specific T cell not B cell lines or hybridomas can modulate the response of susceptible mice infected with viable parasites. *J. Immunol., 139* : 3070.

HALDAR, J.P., CHOSE, S., SAHA, K.C., and GHOSE, A.C., (1983). Cell-mediated immune response in Indian kala-azar and post kala azar dermal leishmaniasis. *Infect. Immun., 42* : 702.

HANDMAN, E. and HOCKING, R.E., (1982). Stage-specific, strain-specific and cross reactive antigens of *Leishmania* species identified by monoclonal antibodies. *Infect. Immun., 37* : 28.

HARITH, A.E., KOLK, A.H.J., KAGER, P.A., LEEUWENBURG, J., FABER, F.J., MUIGAI, R., KIUGU, S., and LAARMAN, J.J., (1987). Evaluation of a newly developed direct agglutination test (DAT) for serodiagnosis and sero-epidemiological studies of visceral leishmaniasis: comparison with IFAT and ELISA. *Trans. Roy. Soc. Trop. Med. Hyg., 81* : 603.

HARITH, A.E., KOLK, A.H.J., KAGER, P.A., LEEUWENBURG, J., MUIGA, R., KIUGU, S., KIUGU, S., (1986). A simple and economical direct agglutination test for serodiagnosis and sero-epidemiological studies of visceral leishmaniasis. *Trans. Roy. Soc. Trop. Med. Hyg., 80* : 583.

HO, M., KOECH, D.K., IHA, D.W. and BRYCESON, A.D.M., (1983). Immunosuppression in Kenyan visceral leishmaniasis. *Clin. Exp. Immunol., 51* : 207.

JAGGI, O.P., (1979). Western Medicine in India: Epidemics and Other Tropical Diseases. Atma Ram & Sons pubs., Delhi.

KHAN, N.I. and DESOWITZ, R.S., (1985). A comparison of counter-immunoelectrophoresis and indirect haemagglutination tests for the immunoepidemiological investigation of kala-azar. *Southeast Asian J. Trop. Med. Pub. Hlth., 16* :1.

KOECH, D., IHA, D.W., HO, M., and WAMACHI, A.N., (1987). Contribution of adherent cells and serum components to immune suppression in Kenyan visceral leishmaniasis. *Am. J. Trop. Med. Hyg., 36* : 501.

KOECH, D.K., (1987). Subpopulations of T lymphocytes in Kenyan patients with visceral leishmaniasis. *Am. J. Trop. Med. Hyg., 36* : 497.

MCMAHON–PRATT, D., and DAVID, J.R., (1981). Monoclonal antibodies that distinguish between New World species of *Leishmania, Nature, 291* : 581.

MURKOVICH, A.M., GALELLI, A., ALLISON, A.C., MODABBER, F.Z., (1986). Increased myelopoiesis during *Leishmania major* infection in mice: generation of 'safe

targets', a possible way to evade the effector immune mechanism. *Clin. Exp. Immunol., 64* : 1.

MOSSER, D.M. and EDELSON, P.J., (1987). The third component of complement (C_3) is responsible for the intracellular survival of *Leishmania major*. *Nature, 327* : 329.

NACY, C.A.M., MELTZER, M.S., LEONARD, E.J. and WYLER, D.J., (1981). Intracellular replication and lymphokine-induced destruction of *Leishmania tropica* in C_3H/He-N-mouse macrophages. *J. Immunol., 127* : 2381.

PANOSIAN, C.B., SYPEK, J.P. and WYLER, D.J., (1984). Cell contact-mediated activation of macrophages for anti-leishmanial defense. I. Lymphocyte effector mechanism that is contact dependent and non-cytotoxic. *J. Immunol., 133* : 3358.

PASSWELL, J.H., SHOR, R. and SHOHAM, J., (1986). The enhancing effect of inteferon-β and -γ on the killing of *Leishmania tropica major* in human mononuclear phagocytes *in vitro*. *J. Immunol., 136* : 3062.

PASSWELL, J.H., SHOR, R., TRAU, H., SHOHAM, J. and JAFFE, C.L., (1987). Antigen-stimulated lymphokines from patients with cutaneous leishmaniasis induce monocyte killing of *Leishmania major* intracellular amastigotes. *J. Immunol., 139* : 4208.

PETERSEN, E.A., NEVA, F.A., OSTER, C.N. and DIAZ, H.B., (1982). Specific inhibition of lymphocyte-proliferation responses by adherent suppressor cells in diffuse cutaneous leishmaniasis. *New Eng. J. Med., 306* : 387.

REED, S.G., BADARO, R. and LLOYD, R.M.C., (1987). Identification of specific and cross-reactive antigens of *Leishmania donovani chagasi* by human infection sera. *J. Immunol., 138* : 1596.

REINER, N.E. and MALAMUD, C.J., (1985). Arachidonic acid metabolism by *Leishmania donovani* infected murine peritoneal macrophages: *in vitro* evidence for parasite-induced alterations in cycloxygenase and lipoxygenase pathways. *J. Immunol., 8* : 134.

REINER, N.E., (1987). Parasite accessory cell interactions in murine leishmaniasis. I. Evasion and stimulus-dependent suppression of macrophage interleukin 1 response by *Leishmania donovani*. *J. Immunol., 138* : 1919.

REINER, N.E., NG, W. and McMASTER, W.R., (1987). Parasite-accessory cell interactions in murine leishmaniasis. II. *Leishmania donovani* suppresses macrophage expression of Class I and II major histocompatability complex gene products. *J. Immunol., 138* : 1926.

SACCI, J.B., CHRISTENSEN, H.A., VASQUEZ, A. and ANTHONY, R.L., (1987). Serodiagnosis of New World lesihmaniasis by using a genus-specific antigen in enzyme linked immunosorbent assays. *Diag. Microbiol. Infect. Dis., 6* : 229.

SACKS, D.L., LAL, S.L., SHRIVASTAVA, S.N., BLACKWELL, J. and NEVA, F.A., (1987). An analysis of T cell responsiveness in Indian kala-azar. *J. Immunol., 138* : 908.

SAMARAS, N. and SPITHILL, T.W., (1987). Molecular karyotype of five species of *Leishmania* and analysis of gene locations and chromosimal rearrangements. *Mol. Biochem. Parasit., 25* : 279.

SNYDER, D.S., BELLER, D.I. and UNANUE, E.R., (1982). Prostaglandins modulate

macrophage Ia expression. *Nature, 299* : 163.

WIRTH, D.F., ROGERS, W.O., BARKER, R., DOURADO, H., SUESABANG L. and ALBUQUERQUE, B., (1986). Leishmaniasis and malaria: New tools for epidemiologic analysis. *Science, 234* : 975.

WHO., (1984). World Health Organization Expert Committee. *The Leishmaniases,* WHO, Geneva.

WYLER, D.J., (1982). Circulating factor from a kala-azar patient suppresses *in vitro* antileishmanial T cell proliferation. *Trans. Roy. Soc. Trop. Med. Hyg., 76* : 304.

WYLER, D.J., BELLER, D.I. and SYPEK, J.P., (1987). Macrophage activation for antileishmanial defense by an apparently movel mechanism. *J. Immunol., 138* : 1246.