

Development of Monoclonal Antibodies to Assay for Circulating Antigen in Visceral Leishmaniasis

Tom Evans, M.D.
Division of Geographic Medicine
University of Virginia
Charlottesville, Va., USA

At present the detection of antibody to *Leishmania donovani* by IFA or ELISA is a highly accepted method to determine whether or not an individual has been infected with the organism. In acute disease the titers of specific antibody rise to enormous levels, and fall to background over the ensuing two to five years. The work of Badaro et al in Bahia^{1,2}, and confirmed by our work in Ceara, show that when using such testing to screen a population, many of the resulting positives will actually be asymptomatic or oligosymptomatic seroconverters who will go on to resolve their infection without the development of symptomatic disease. In addition, it appears that earlier recognition and treatment of disease portends a better prognosis and outcome.

In addition to the testing of human subjects antibody is determined in widespread national campaigns undertaken by SUCAM to identify and eliminate infected dogs which are felt to be a major reservoir and environmental factor in the spread of visceral leishmaniasis. In the state of Ceara alone there are tens of thousands of dogs tested each year with elimination of thousands of the IFA positives, requiring huge sums of financial and human resources. The time between collection of the earlobe blood sample, testing and then elimination can run to two to three months, in which time the disease could theoretically be spread to other mammalian reservoirs. In addition, we have now studied hundreds of seropositive dogs in the state of Ceara by autopsy, culture, and hybridization studies and find that only about one half of all seropositive dogs are culture or biopsy positive. We have not been able to tell these animals from the uninfected half by clinical criteria. The study of other mammalian reservoirs which may need closer public health scrutiny is now underway in ours and other studies.

Thus, the development of an accurate, fast, field test which can not only identify animals and humans previously infected, but can distinguish those with ongoing infection from those with self-resolving disease has been our goal. Since the illness is associated with a huge parasite burden within the reticuloendothelial system of the host, we hypothesized that one should be able to detect circulating antigen released from infected macrophages throughout the course of clinical

illness. Antigen is present on the surface of macrophages³ in some forms of leishmaniasis, and others have shown that there is likely circulating antigen in the blood and urine of patients with visceral leishmaniasis^{4,5}. If specific antigens could be detected, then monoclonals should be able to be manufactured. These monoclonals could then be used to develop a latex agglutination test similar to that used in cryptococcus or HIV antigen testing, or could be used in a double-sandwich ELISA to detect antigen. If the test could then be quantitated, the antigen detection system could also be used to document response to therapy.

A number of approaches bear mentioning in regard to speed and field applicability. These include 1) the fast-ELISA system of Jaffee⁶, which should be usable in the field and has had some success in initial field trials; 2) the dot-ELISA system developed at Walter Reed which is not widely used⁷; and 3) the direct agglutination test developed by Harith and colleagues⁸ which is now under study by the WHO in numerous localities throughout the world and may replace the IFA and ELISA.

In regard to antigen detection, little progress has been made. We and others have been unable to culture the parasite from the blood or buffy coat of asymptomatic seropositive individuals. However, one can hypothesize that using the present technology of polymerase chain reactions that have been useful in the detection of both HIV and toxoplasmosis DNA in human blood⁹, that one should now be able to identify the presence of leishmanial DNA in infected macrophages of almost all infected individuals by this technique. This would allow confirmation that the seroconverters that we recognize by antibody testing are true positives without resorting to invasive tests such as liver or bone marrow biopsy for definitive diagnosis. Another approach would be to use polyvalent purified anti-leishmanial sera from rabbits or another animal to screen human blood for circulating leishmanial antigens.

Instead of attempting to directly detect the circulating antigens in human sera we took an indirect approach. We chose to make monoclonals to any antigen that may be present in sera from patients with visceral leishmaniasis and then to use these monoclonals to determine the nature of the antigen. The sera of patients with visceral leishmaniasis contain immune complexes consisting of large amounts of antibody, rheumatoid factors, and circulating antigens^{10,11}. We have previously shown that these complexes contain antigens that are recognized only by anti-leishmanial sera, and that the control complexes do not contain these antigens¹². Interestingly, one of the strongest responses of anti-leishmanial sera in our patient complexes was against a 70 kDal antigen. Jaffee et al. have found that during production of monoclonals to *L. donovani chagasi*, that a specific monoclonal was produced against a 70 kDal antigen, and the binding of this monoclonal to

Leishmania was inhibited by sera from infected individuals¹³. Thus two completely different techniques yielded a similar result.

Antibody was produced against these putative leishmanial antigens in BALB/c mice by immunization with high molecular complexes purified from patient sera by precipitation of the sera in 2.5% polyethylene glycol. We were able to document by western blot that mice injected with these complexes developed antibody to a number of leishmanial antigens. Spleen cells from the mouse with the highest antibody titer to leishmania were fused to the sp/20 cell line by standard techniques. This resulted in 24 hybridoma lines that produced antibody to leishmania as evaluated by ELISA. These antibodies reacted minimally with control human IgG. 20 were of the IgM isotype, two IgG1, and two IgG2b.

We selected five lines for cloning based on initial Western blot patterns of these antibodies to *L. donovani chagasi* electrophoresed in SDS-PAGE. The five clones had the same isotypes as the parent lines, and all bound to leishmanial antigens as evaluated by Western blotting. Three of these five monoclonals gave complex banding patterns that could not be eliminated by using a variety of protease inhibitors. Monoclonal 6H12 of IgM isotype detected a single 60 kDal antigen on reduced gels that migrated to approximately 40 kDal under non-reducing conditions; however, this monoclonal also reacted weakly to control human serum. Periodate treatment reduced binding indicating that it probably has an important carbohydrate residue. Monoclonal 11C10 was of IgG2b isotype and recognized one antigen strongly at approximately 140 kDal and another weakly at 45 kDal, and showed no reaction with control human serum as assayed by western blot. Periodate treatment at 10 mmol eliminated binding to the 140 kDal antigen on western blotting. Monoclonal 6C4 had the highest anti-leishmanial titer, was of the IgG2b isotype, and bound to a complex series of bands by blotting. Further attempts with variety of protease inhibitors and electrophoresis conditions were not able to eliminate the multiple bands seen by western blot. However, treatment of the blot with periodate essentially eliminated binding to the leishmania, and there was minimal binding of the monoclonal to human sera by either ELISA or western blotting.

We have gone on to study these monoclonals by IFA and ELISA. They all appear to recognize surface components of leishmania. From these initial results we believe that the antigens that are circulating probably contain antigenic carbohydrate groups, and that the carbohydrates in some instances may be bound to a number of different surface proteins of *Leishmania donovani chagasi*. We will soon evaluate the binding of these monoclonals to a variety of possible cross-reactive antigens including those from *M. tuberculosis*, Chagas disease, leprosy, and toxoplasmosis. We also plan to document the specificity of the carbohydrate involved by using blocking studies with specific inhibitors.

In initial studies we have found that the three monoclonals mentioned all bind more strongly to infected sera than to that of controls, although the background remains high by our present technique and will require modification. Thus far we have documented the presence of specific leishmanial antigens in infected human sera, that monoclonals can be raised against them, and that they hold promise in future use in the development of diagnostic testing to detect circulating antigen in both patients and animals, and to monitor response in patients undergoing treatment.

¹Badaro R et al. A prospective study of visceral leishmaniasis in an endemic area of Brazil. J.Infect.Dis. 1986;154:639-649.

²Badaro R. et al. New perspectives on a subclinical form of visceral leishmaniasis. J.Infect.Dis. 1986;154:1003-1011.

³Ridley, RJ, Ridley, DS. Monocyte recruitment, antigen degradation and localization in cutaneous leishmaniasis. Br.J.Exp.Path. 1986;67:209-218.

⁴Kohanteb, J, Ardehali, SM, Rezai, HR. Detection of *Leishmania donovani* soluble antigen and antibody in the urine of visceral leishmaniasis patients. Trans.Roy.Soc.Trop.Med.Hyg. 1987;81:578-580.

⁵Rassam, MB, Al-Mudhaffar, SA. The micro-ELISA sandwich technique for the quantification of *Leishmania donovani* soluble antigen. Ann.Trop.Med.Parasit. 1980;74:591-595.

⁶Jaffee CL, Zalis M. Use of purified parasite proteins from *Leishmania donovani* for the rapid serodiagnosis of visceral leishmaniasis. J.Infect.Dis. 1988;157:1212-1220..

⁷Pappas MG et al. Development of an antigen conservative enzyme immunoassay (Dot-ELISA) for the rapid diagnosis of visceral leishmaniasis. Trans.Roy.Soc.Trop.Med.Hyg 1983;77:425-426.

⁸Harith AE, et al. Evaluation of 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. 1987;81:603-606.

⁹Ou, C-Y et al. DNA amplification for direct detection of HIV-1 in DNA of peripheral blood mononuclear cells. Science 1988;239:295-297.

¹⁰Carvalho EM et al. Circulating immune complexes and rheumatoid factor in schistosomiasis and visceral leishmaniasis. Am.J.Trop.Med.Hyg. 1983;32:61-69.

¹¹Pearson RD et al Humoral factors during South American visceral leishmaniasis. Ann.Trop.Med.Parasit. 1986;80:465-468.

¹²Evans TG, Pearson RD. Identification of leishmanial antigens in the sera of patients with visceral leishmaniasis. In press.

13 Jaffee CL, Zalis, M. Purification of two *Leishmania donovani* membrane proteins recognized by sera from patients with visceral leishmaniasis. Mol.Biochem.Parasitol. 1988;27:53-62.