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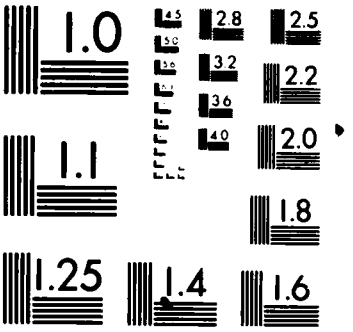

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ANTIGEN/ANTIBODY ANALYSES IN LEISHMANIASIS

Final Report

RAYMOND E. KUHN, Ph.D.

September 1983

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SUMMARY

Four major objectives were accomplished during the first year of these investigations. A simple modification of existing methods for growing culture forms of Leishmania spp. in larger numbers was determined. This modification was the addition of fetal bovine serum which had been selected for optimal ability to support the growth of mouse lymphocytes in cultures. Immunoblotting procedures were developed for the identification of reactions of antibodies in human sera with antigens of protozoan parasites. It was found that enzyme substrate reactions had distinct advantages over typical autoradiographic procedures.

Analyses of various sera identified a number of antigens of protozoan parasites which may be useful in discriminating infections caused by various species of Leishmania and between these parasites and Trypanosoma cruzi. These results, however, are complicated by the surprising observations that normal, uninfected persons contain antibodies in their sera which frequently show strong reactions to numerous antigens of Leishmania spp. and T. cruzi. These "natural antibodies" are the sources of confusing false-positive reactions in immunodiagnostic procedures. Whereas it has been known for some time that "natural antibodies" exist in the serum of many uninfected individuals, the apparent diversity of the specificities of these antibodies, as determined in the present study, was not known.

Future studies will examine the nature of these "natural antibodies" and attempts will be made to identify antibodies induced specifically as a result of active infections.

FORWARD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

For the protection of human subjects the investigator has adhered to policies of applicable Federal Law 45CFR46.

REPORT

This is a Final Report of studies completed during the first year of support for investigations analysing reactions of antibodies in the sera of patients and normal humans to antigens of Leishmania species. This report covers the period from 1 September 1982, to 31 August 1984.

Clinical manifestations of leishmaniasis are dependent on many factors, including the species of Leishmania, the geographic area in which human infections occur, and the genetic composition of the host. At present, it is not possible to make definitive serodiagnoses of the different possible forms of leishmaniasis because existing methods can not discriminate between species of organisms causing the infections, in some cases even when these organisms can be isolated from patients. It is extremely important, therefore, to development methods which will allow early and accurate identification of the species of Leishmania infecting persons in order to initiate appropriate approaches of chemotherapy.

The immunology of human and experimental leishmaniasis remains poorly understood (see reviews by Poulter (1) and Reed (2). In general, however, protective immunity is thought to be manifested primarily by the development of T-cell dependent, cellular mechanisms which, in some cases, wane (immunosuppression) as the infection progresses. Recently, however, studies in experimental systems revealed that antibodies in combination with cells augment the protective capacity of cellular mechanisms when both antibody and cells from convalescent animals are transferred to newly infected recipients (3).

Whether or not antibody is significant in providing protection to mammals infected with the various species of Leishmania, antibody in sera of humans is of primary importance in the diagnosis of these diseases. However, because several species and sub-species of the parasite can cause wide ranging clinical manifestations, the mere presence of titers against antigens of Leishmania spp. is insufficient in predicting the possible subsequent development of disease, except in cases where geographic considerations limit the choice of parasites to consider. An accurate and early identification of the species of Leishmania which infects an individual becomes especially important in the diseases in which apparent reactivation of the infection may appear years after clinical control or self-cure, such as in muco-cutaneous leishmaniasis (L. braziliensis braziliensis; 4,5). In addition, it has been reported that the success of therapy can be monitored by examining the titers of patients and that the successful treatment of leishmaniasis may be indicated by decreases in titers in patients during treatment (6).

There were four major accomplishments during the first twelve months of the current contract period.

(1) Initial efforts involved growing a sufficient quantity of promastigotes of several American species and strains of Leishmania. In collaboration with Captain Patrick McGreevy of WRAIR, isolates of parasites were brought or sent to Wake Forest University and cultures initiated in NNN and Schneider's medium. After testing and selecting several lots of fetal bovine sera (for use with the Schneider's medium), it was found that those pre-tested for ability to support mammalian lymphocytes were best for growth of promastigotes. Of

these, however, some lots of HyClone sera were clearly superior and large amounts were purchased to assure availability. Using these sera, a sufficient quantity of each isolate of parasites was grown and stored in liquid nitrogen or used immediately for assays.

(2) As parasite material became available, various methods for separating parasite antigens on SDS-PAGE were tested and the appropriate method chosen. It was found that, as with T. cruzi, distilled water lysis and freeze/thaw provided a suitable extract of parasites for electrophoretic preparation of antigenic components. Various concentrations of acrylamide were tested for optimal separation of antigens and one with a 5 per cent stacking gel and 10 per cent running gel proved most appropriate. Gradient gels were of no advantage nor were gels of higher or lower concentration. The concentrations chosen will resolve antigens with molecular weights between 200,000 and 14,500 daltons which includes the majority of bands.

(3) The basic protocol for identification of antibodies of patients which bind to antigens of Leishmania spp. is the Western Blot procedure. Parasite antigens are separated on SDS-PAGE, transferred electrophoretically to nitrocellulose paper, a patient's serum reacted on the nitrocellulose, a second ^{125}I-labeled or peroxidase conjugated goat anti-human Ig added, and, after extensive washing, assayed using X-ray film or addition of appropriate substrate. Most of our experiments were done using ^{125}I but three realizations dictated discontinuation of the use of radiolabeled indicator antibody: (a) precise development times are difficult to predict using X-ray film; (b) development times are generally greater than 24 hours causing unnecessary delays in data analysis; and (c), most importantly, any field test for diagnosis of leishmaniasis will not use an autoradiographic procedure. After a number of experiments, it was found that indicator antibodies conjugated with peroxidase provided the speed, sensitivity, and reproducibility for not only our experimental approach but for future diagnostic procedures. The substrate most suitable for both uses was determined to be chloronaphthol rather than DAP (3-3'-diaminobenzidine).

(4) When these first three objectives were fulfilled, sera from soldiers who had become infected while on manuevers in Panama were tested for reactivity to antigens of five species/strains of Leishmania. All of the sera were run against strains of Leishmania obtained from Cpt. McGreevy and included numbers WR 111, 225, 183, 424, and 063. In addition, some of the sera were run against these isolates of Leishmania and the Brazil strain of T. cruzi. It was noted that there exists a region of high molecular weight bands in all of the reactions against Leishmania which are absent in the reactions to antigens of T. cruzi. It is entirely possible that these antigens of Leishmania spp. can be used to discriminate between American leishmaniasis and Chagas' disease. Several experiments must be performed using different strains of T. cruzi, however, before this can be confirmed. Only the Brazil strain of T. cruzi was used in these experiments.

Careful analysis of the banding patterns of antigen/antibody

reactions revealed differences in reactivity between the various sera and species of Leishmania. This analysis, however, will not be fruitful until results of our most recent experiments are evaluated and compared to the reactivity of sera of uninfected people, including sera obtained from soldiers prior to infection. Of particular interest are bands formed as a result of reactions of antibodies in sera of uninfected people to the various antigens of Leishmania spp. This was a most unexpected set of results. We and others had previously thought that immune responses against parasites were de novo. It is probable on the basis of these results that protozoan parasites (and we have evidence for metazoan parasites also) are confronted immediately by, and induce activation of, previously sensitized clones of immunocytes. There are reports in the literature that various normal sera contain "natural antibodies" against promastigotes of leishmania which are capable of agglutinating and lysing these organisms (7,8). Our results confirm these reports of "natural antibodies" and demonstrate their heterogeneity in reacting with many antigens of Leishmania promastigotes.

The presence of "pre-existent antibodies" may not complicate the immunodiagnosis of the various forms of leishmaniasis and our future studies may reveal the nature of the antigens which are relevant for both diagnosis and protective immunization. It is also possible, however, that the majority of protective a host develops against Leishmania (and other protozoan parasites) results from the stimulation of previously sensitized immune cells. Also, this may explain how immunity can develop against parasites when profound immunosuppression is manifested in hosts during the course of protozoan infections (9,10).

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