

## PROCEEDINGS OF THE ROUND-TABLE ON METHODS IN DIAGNOSIS AND EPIDEMIOLOGICAL STUDIES

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During the past ten years, a great deal of work has been done in malaria immunology. The majority of these studies have been devoted to the development of a malaria vaccine whereas the application of immunology has been greatly neglected for the diagnosis of malaria-disease, the detection of malaria infection or the identification of infected mosquitoes. It has certainly been a mistake to partially neglect these applications since there is a great deal we ignore in the epidemiology and pathogenesis of malaria and new tools are definitely needed to improve our surveys.

On the other hand, epidemiological studies have the considerable advantage of allowing easy and constant comparison between laboratory data and practical field observations. So, it should be possible to avoid the over theoretical and unrealistic positions due to narrow specialization, essentially based on technological considerations and without enough – or sometimes any contact with the malaria realities. Malaria is not just a model for chemical, immunological or bio-molecular studies. It is the main disease in the world and we must always bear this fact in mind.

It is therefore very fortunate that the International Symposium on Malaria, organized in Rio de Janeiro June 1-5, 1986, devoted a round-table session to the contribution of new technologies to the diagnosis and epidemiological surveys of malaria. Talks were given by A. Cochrane from the New York University Medical Center, by W. Ferreira and M. Arruda from the Instituto de Medicina Tropical de São Paulo e FIOCRUZ, by E. Franco from the Instituto Ludwig de Pesquisas sobre o Câncer de São Paulo, by L. Monjour of Hôpital de la Salpêtrière in Paris and lastly by L. Perrin from the Hôpital Cantonal de Genève – Centre OMS de Recherche et de Formation en Immunologie. A wellcome paper by Dyann Wirth, that unfortunately could not be present during the meeting, dealing with the diagnosis of the malaria infection using DNA probes was also included in this section. This review will be a synthesis of the results and discussions presented and an attempt will be made to maintain as wide a scope as possible. For this we will review first the diagnosis of human malaria infection by DNA recombinant technology followed by the detection of infested *Anopheles*, and, by that of human antibodies directed against sporozoites or asexual erythrocytic forms.

### DETECTION OF PLASMODIAL MATERIAL IN HUMANS USING DNA PROBES

The paper by Dr. Wirth drawn attention to the need of sensitive and rapid method for detecting parasites for the evaluation of future vaccine and drug trials. Since the determination of present malaria infection by thick smears can be time consuming, tiring and potentially subject to reader bias, specially when large numbers of slides must be read in a short time period, an alternative method could be the use of DNA probes specific for human malaria. According to the authors, the p<sup>PF14</sup> (a *P. falciparum* specific probe that does not react with *P. vivax*) has a sensitivity comparable to that of the routine microscope detecting parasite densities as low as 40 parasites per microliter of blood (approximately one parasite per 125 erythrocytes) and could detect *P. falciparum* infection in 129 out of 632 patients compared to 121 detected by routine thick smears. Since careful microscopic examination further confirmed the DNA probe diagnosis, one question that could arise is: concerning on one hand, the experience and efficacy that can be achieved by a well trained microscopist in endemic areas and, on the other hand, the relative low sensitivity/cost ratio of the DNA probe methodology so far recorded, is it the time for doing this replacement? Similarly to the detection of malarial antigens by monoclonal or polyclonal specific antibodies, the detection of plasmodial material by DNA probes seems to be a very promising approach of the malaria infection diagnosis that must be however, subject of further research and technical improvements before their use in routine screenings.

### IDENTIFICATION OF INFESTED ANOPHELES; SPOROZOITE CARRIERS OF ONE OF THE FOUR PLASMODIAL SPECIES THAT PARASITE HUMANS

In this area, the most important work and main applications have been done by the group headed by R. and V. Nussenzweig of the New York University Medical Center, particularly by F.

Zavala. Monoclonal antibodies were prepared against surface proteins (CSP or Circum Sporozoite Proteins) of *P. falciparum*, *P. vivax*, *P. malariae* and *P. brasilianum* and were used for the detection of *Anopheles* carriers of sporozoites of homologous or heterologous plasmodial species (A.W. Ferreira et al.). With an ELISA test and these monoclonal antibodies, the authors obtained specific results for each plasmodial species, the lower limit of detection being 5-10 sporozoites/30  $\mu$ l of mosquito extract. In certain cases at least, there is a definite correlation between the number of sporozoites and the absorbance measured in ELISA. The method can be applied to freshly caught mosquitoes as well as dried and stored ones. From a practical point of view, the method has a double interest in that it uses reagents that can be stored up to two years and that it permits the study of several hundred anopheles per day. It is considerably faster and more precise than the traditional techniques that involve the dissection of salivary glands and the subsequent microscopic observation of the sporozoites without allowing a species identification since the sporozoites are not morphologically different.

This ELISA test was used for an epidemiological survey done between May 1983 and July 1985 in the Pará State of northwestern Brazil (M. Arruda). The area studied is of considerable interest because of the double transmission of *P. falciparum* and *P. vivax*. Based on a total of 4310 *Anopheles* studied, the authors established that global infestation rate is of 10.9% for *P. vivax* and of 16.7% for *P. falciparum*. The main vector species were definitely identified, confirming previous data. For *P. falciparum*, the most important vector is *Anopheles darlingi* and to a much lesser extent *A. oswaldoi*. For *P. vivax*, sporozoites were detected in five *Anopheles* species: *A. oswaldoi*, *A. albitarsis*, *A. triannulatus*, *A. nuneztovari* and *A. darlingi*.

A.H. Cochrane used the same monoclonal antibodies against CS proteins with the radio-immunological test IRMA. Microtitration plates are coated with the monoclonal antibodies. After an overnight incubation with the mosquito extracts, followed by washes, a solution of the same monoclonal antibodies labelled with  $I^{125}$  is added.

The results are read with a gamma counter and show a strict correlation with the ELISA test described above. Cochrane's study was also done in the Pará State where the ecology has recently been upset by the construction of hydro-electric dams and by an intense agricultural development that has provoked an immigration of non immune workers to areas in which a high incidence of *P. falciparum* and *P. vivax* malaria was recorded. More than 11.000 *Anopheles* were captured on volunteers, at 30 different sites, in six districts of the state. On the whole the results obtained with the IRMA test confirmed those obtained with ELISA by Ferreira and Arruda. The low specificity of *P. vivax* for its vector was noted again since it is found in five different *Anopheles* species; whereas for *P. falciparum* only *A. darlingi* was identified as a local vector.

Broadly speaking the immunological techniques using monoclonal antibodies have enabled considerable progress in the identification of the different vectors of the *Plasmodium* parasites in man. Recent works done in Brazil confirm the results obtained in West Africa namely in Gambia by F. Zavala and in Burkina Faso by P. Carnevale's group and by the Italian team of M. Coluzzi and A. Esposito. The value of these tests is well established for *P. falciparum* and, thanks to the Brazilian study, for *P. vivax*. On the other hand, field results are still lacking for *P. malariae* whose, in many aspects, mysterious epidemiology would benefit greatly from these methods. Studies with *P. ovale* do not appear to have been undertaken yet. These ELISA and IRMA tests, in a large measure, do not only give undeniable qualitative results but also give quantitative information on the number of sporozoites present in each *Anopheles*. It seems that these methods do not distinguish between *Anopheles* that have sporozoites in their salivary glands or only mature oocysts in the gut. If the tests were run with extracts of *Anopheles* heads and thoraxes rather than whole mosquitoes the distinction would be possible. Nevertheless, this would require a supplementary manipulation which would slow down the test and not always be absolutely accurate. As these methods exist now they do give a better identification of *Anopheles* infected by a certain plasmodial species rather than the detection of infestant *Anopheles* and therefore the real vectors. Lastly, from a strictly practical point of view, the radio-immunological test undeniably requires specialized equipment, that is expensive and fragile, and therefore its application is more limited than the ELISA tests.

#### DETECTION OF HUMAN ANTI-PLASMODIUM ANTIBODIES

**Anti-sporozoite antibodies:** The detection of anti-sporozoite antibodies can be done by immunofluorescence on whole sporozoites, with or without previous fixation. Recent techniques used to characterize sporozoite antigens have enabled first an isolation than a preparation of more specific antigens. This is the case for a synthetic peptide, NANP<sub>3</sub>, that corresponds to repetitive immuno-dominant peptide of the circum sporozoite progein (CS protein). NANP<sub>3</sub> consists of



three repetitions of the sequence Asparagine-Alanine-Asparagine-Proline and, since Zavala's study, it is known to be specific for species and evolutive stages but it can be detected in many isolates from a wide variety of geographic areas. A.H. Cochrane used this synthetic polypeptide for the serologies run on 58 samples from Gambia, in Western Africa. Technically NANP<sub>3</sub> is coupled to BSA with glutaraldehyde to coat the microtitration plates. With solutions of lysine then ethanolamine, all the free sites of the glutaraldehyde are then saturated. The radio-immunological IRMA test then proceeds in the usual manner; the specific antibodies fixed to the antigen are revealed with anti-human immunoglobulins labelled by I<sup>125</sup>. The results obtained show a high correlation with those from indirect immuno-fluorescence. There is a confirmation that the percentage of positivity increases with age since only 21% of children between 1 and 14 are positive whereas the proportion goes up to 85% for adults over 34.

**Detection of antibodies directed against asexual endo-erythrocytic plasmodial forms:** Using endo-erythrocytic plasmodial forms or culture supernatants, more than 50 different antigens have been described. Their characterization and purification vary considerably. In many cases, they have only been examined from an immunochemical stand point oriented towards a possible vaccination against malaria. Nevertheless, several serological surveys have been done with these antigens. The most important difficulty encountered has been cross reactions. To get around this E.L.F. Franco et al. have suggested competition ELISA using monoclonal antibodies. For these studies, the authors have used ten monoclonal antibodies against the different intra-erythrocytic plasmodial stages: ring forms, trophozoites, schizonts or merozoites. A series of control sera and samples from carriers of *P. falciparum* or *P. malariae* from Western Africa or Peru were studied. The practical aspect of this test can be debated nevertheless, since the test seems to be specific using only a relatively crude antigen (ultrasonicated *P. falciparum* from *in vitro* cultures).

A similar application can be foreseen for the monoclonal antibodies of human origin, presented by L. Monjour, obtained by collaboration with C. Desgranges et al. These are antibodies obtained by Epstein Barr virus "immortalized" human B lymphocytes. A total of 24 lymphoblastoid cell lines have been established; 15 of them secrete anti-*Plasmodium* antibodies, IgG in six cases and IgM in the other nine. The specificity of these antibodies was determined by immunoblotting or by SDS-PAGE autoradiographies run with solubilized precipitates obtained from radio-labelled cultures. Antibodies from the different clones recognize very different antigens; 230-240KD in two cases and 115KD in two other clones. All the other cell lines produce antibodies directed against trophozoite antigens. These techniques for immortalizing human lymphocytes are still too recent to estimate all the possible applications to malaria. Nevertheless, in serology, an interest would be for a competition test and for the identification and isolation of particular antigens.

A very complete comparative study has been done in ELISA by L. Perrin using four different antigens: normal red blood cell extracts, schizont extracts from *in vitro* cultures, 41KD antigens from rhoptries, and lastly a 31-1 fusion polypeptide. Strong cross reactions were observed with the crude schizont antigen probably due to contamination by the red blood cell cytoskeletons. The specificity was "perfect" for the 41KD and 31-1 antigens. In terms of sensitivity, the serology was positive in 68% of the cases with the schizont antigen and, respectively, 92% and 98% for the 41KD and 31-1 antigens. On the other hand, there is a progressive serological response with these antigens as the age of the patients increase. If there is a high correlation among the results obtained with normal red blood cell, schizont and 41KD antigens there is none with the 31-1 antigen. This antigen contains a repetition of the sequence Ser-Gly-Gly-Ser-Val-Ala and seems to be very particular. In adults with the same malaria background and the same immunological status, sometimes this antigen reveals very high levels of antibodies and other times there is only a very weak serological response.

This study — as well as the one presented by G. Brown during another session (Parasite Antigens and Molecular Biology) of the International Symposium on Malaria — is of great interest since it applies to the serology of malaria recent results obtained with molecular biology in the search for a vaccinating antigen. Nevertheless, as L. Perrin mentioned himself, many ambiguities and uncertainties remain in studies devoted to the serology of malaria. The main question is to determine if the aim of this serology is to detect a past or still evolving malaria (serodiagnosis) or determine an anti-malaria immunity. In the second of these applications, the problem is all the more difficult since, clinically, there is no means to detect objectively, for each individual, his degree and his type of anti-malarial immunity. Indeed malaria-immunity can be directed against the infection (parasitemia) or against the disease. It can only reduce the parasitemia or avoid main complications (cerebral malaria) or the normal clinical manifestations giving an asymptomatic parasitemia. In each case, the results are rarely absolute and usually only relative modifications

are noted. Under these conditions, it is easy to imagine how difficult it will be to find an antigenic reagent that would be able to characterize such a poorly defined immunological state. In fact, this can probably only be solved when truly efficient vaccines will be available and when it will be possible to positively identify reagents that are involved in acquired immunity.

For the serodiagnosis of malaria, the situation is almost as confused. With the relative standardization of serological methods and the advent of chemically definite antigens, the purely technical problems should soon be solved. On the other hand, it is essential to better determine what the exact use of the serology should be and to precisely define the conditions of use in the different diagnostic and epidemiological situations. This should include of course, in the different epidemiological aspects, the date when the antibodies appear, their maximum level, their persistence after cure, or their variation in areas of seasonal transmission. Surveys are done now mainly with batches of stored sera that, at the most, give an evaluation according to the age of the individuals and do not meet any of the above criteria. The solution can only be found by a very careful individual and longitudinal follow up on persons living in different epidemiological situations. This implies an excellent collaboration with the population under study. The multiplication of certain badly defined "serological surveys" might provoke reluctance in populations that are constantly being tested without usually receiving any concrete results, and this would seriously compromise any future surveys.

Based on conclusions reached during International Symposium on Malaria in Rio de Janeiro, it would seem extremely desirable to organize a multilocus survey implicating, on the one hand, epidemiologists who would precisely define the problems to be solved by serology and suggest, in each case, a framework for each corresponding study and, on the other hand, laboratories specialized in immunology of malaria who would implement the different serological techniques with the plasmodial antigens that are presently available.