Transfusion Malaria

Accidentally induced malaria as a result of blood transfusion is not uncommon in several countries and may become more frequent as the demand for blood increases. The available though incomplete records covering the period 1950-68 and based on data gathered in 35 countries gave the figure of 655 cases, of which over one half were due to *P. malariae*, with *P. vivax*, *P. falciparum*, and *P. ovale* in the decreasing order of frequency.[3] There is a definite relationship between the incidence of imported malaria and the number of cases due to blood transfusion.[13] In the United Kingdom only eight cases of transfusion malaria have been described over the past 30 years, but probably such occurrences were more common in this country and elsewhere and not diagnosed or not reported.[14]

Prevention of transfusion malaria depends on the elimination as a whole-blood donor of anyone who ever had malaria in the past or who has ever been exposed to malaria during a specified period prior to blood transfusion. This type of screening is quite effective when strictly applied. Detection of malaria infection in a suspected donor is notoriously difficult. Microscopic examination of a blood film is of little value, since asymptomatic parasitaemia is usually very scanty. Nevertheless, methods of indirect diagnosis of malaria by the use of immunofluorescence tests offer the best possibilities for routine screening of latent malaria infections.

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Laboratory Diagnosis

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A diagnosis of malaria can be made with certainty only in the laboratory, and, as with most other infective diseases, this can be done either directly, by demonstrating the presence of the infective organism, or by serological methods, which are chiefly of retrospective value in diagnosis.

Demonstration of Malaria Parasite

The surest and certainly the most convenient way of demonstrating malaria parasites is by the use of stained thin or thick blood films taken from the patient. Parasites are usually most numerous in blood taken during or soon after the paroxysm, though in urgent cases one should not delay taking blood films, as some parasites are often present at all stages of the cycle. Several examinations can, and preferably should, be made at different times, especially if it is desired to find different stages of the parasite to aid in identification of the species. Even experienced malariologists may have difficulty in species differentiation when the blood films show only the young ring stages. Nevertheless, it is important to remember that in primary infections with *Plasmodium falciparum* the fever is nearly always irregular and that even in fatal infections parasites are sometimes quite scanty in the peripheral circulation. The parasites may develop mostly in the viscera and only a few small ring forms may be present in the blood, which can be easily missed by the inexperienced. Similarly, if the blood films have to be sent to a distant laboratory for staining and microscopical examination treatment of an acutely ill patient should not be delayed if there is any suspicion of malaria from the clinical and geographical histories. There is little risk of toxic reactions from modern antimalarial drugs, nor of the masking of other infections to confuse the diagnosis, while a delay of a few hours in starting specific therapy in a patient with *P. falciparum* malaria may decide the difference between recovery and death.

MAKING BLOOD FILMS

The most important prerequisite to making blood films is to have scrupulously cleaned glass slides.

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New, unused slides are nearly always available, and it is usually sufficient to polish these shortly before use with a dry, dust-free cloth. If there is any suspicion that they may be greasy they may be rinsed briefly in alcohol, or better a mixture of equal parts of ether and ethyl or methyl alcohol, before polishing. Even a trace of grease will cause uneven spreading of a thin blood film and detachment of a thick film. Of almost equal importance is the need for a suitable slide tray in which the clean slides may be taken covered to the bedside and in which the blood films may be laid to dry horizontal and protected from dust and dirt. The presence of small amounts of foreign matter can greatly increase the difficulties of reading the films. The need for the provision of suitable slide boxes for sending unstained or stained films to other laboratories should also be mentioned here, as this detail is often neglected.

Blood films are usually made from capillary blood taken from the finger or ear lobe, but they may also be made from blood obtained by venepuncture if this is being done. A minimum of at least three thin films and three thick films should be made on each occasion the patient is examined: one of each for staining and examination as soon as possible, a pair to be kept in case the staining of the first is unsatisfactory and modification of the staining technique is indicated, and a pair to be sent unstained to a reference laboratory (see below) for checking or confirmation of the diagnosis. For making good thin films it is essential to use a *small* drop of blood, the size of a large pin head, placed at the end of a slide. As a spreader the clean smooth edge of another slide is used, held at an angle of about 45° to the first slide; the film is made by pushing the spreader *away* from the drop of blood. By this means a film should be made with the red blood corpuscles only one layer deep and almost touching but not overlapping each other. This is well illustrated in the book by Shute and Maryon,[15] which gives further details of techniques in malaria, and which should be in any laboratory concerned with its diagnosis.

To make a thick film a drop of blood, about three times the size of that used for a thin film, is placed on a slide and, using the corner of another clean slide, spread to cover about three times its original area. This must be done rapidly without excessive stirring to avoid the formation of strands of fibrin in the film. The traditional advice about the thickness of the film is that it should just be possible to read small print through it when dry. With these criteria the volume of blood which can be examined in a unit of area is about 10 to 20 times that of a thin film. Though the staining and interpretation of thick films is more difficult than for thin films, it is certainly worth making them on each occasion, if only to be sent to a reference laboratory.

Because their subsequent treatment is different it is preferable to make thin and thick films on separate slides, though if many slides are being made as in a survey they are sometimes made on one slide. Finally, each slide should at once be labelled legibly with the name or number of the patient and

the date, using a diamond glass writer or indelible marker or, for thin films, by writing on the film with a pencil.

Examination of films of the bone marrow, as has been recommended by some, has no advantage for finding parasites.

STAINING

While ignorance of the correct methods of making blood films is a frequent cause of poor results, the subsequent staining is of equal importance. This is done with a stain of the Romanowsky type. There are several varieties but the best reproducible results are probably to be obtained by the use of Giemsa stain, especially if staining of blood films for malaria parasites is done only occasionally in the laboratory. Unfortunately even with Giemsa stain there is considerable variation between the products of different manufacturers, and even between different batches of stain produced by one manufacturer, so that some initial experimentation is usually needed to find a good batch. Details for the handling of Giemsa stain, and for its preparation oneself, are given by Shute and Maryon.[15]

The best staining of the malaria parasites, and of the associated changes in the red blood cells which are often important for species differentiation, are obtained if the films are stained as soon as they are dry. Thin films dry almost instantaneously at room temperature; before staining they are fixed with a drop of pure methyl alcohol, which is allowed to run over the whole surface of the film and then to dry. Thick films are kept horizontal and covered to dry. This takes at least an hour at room temperature if detachment of the film during staining is to be avoided. However, the drying may be accelerated by *gentle* heating, such as by placing in an incubator at 37°C or over a radiator. They should *not* be heated over a flame, as this will usually cause fixation and prevent solution of the haemoglobin during staining. Similarly, thick films are *not* fixed with methyl alcohol.

If it is intended to send films away to a reference laboratory the thin film should also not be fixed, and this should be noted on the accompanying form giving clinical details of the patient. For staining the slides are placed vertically in a staining trough or, better horizontally and with the films downwards in a staining dish, and the correct dilution of Giemsa stain gently added. For thin films a dilution of from 5 to 10% in buffered distilled or deionized water is used, while for thick films a weaker dilution of from 2 to 5% is usually preferred. The exact concentration of stain which gives optimal results depends on the make and the batch of stain used (see above) but a certain amount of latitude is usually possible.

The reaction of the water used for diluting the stain must, however, be more accurately defined. Most haematological laboratories use water buffered to give a pH of 6·8 for dilution of Romanowsky-type stains for showing blood cell morphology. For the best staining of malaria parasites a neutral (pH 7·0) or slightly alkaline (pH 7·2) solution is better. This is achieved by adding an appropriate phosphate buffer to the water, which can be made up in the laboratory from the well-known formulae, or tablets may be bought commercially. For staining old films, particularly thick films, which have been kept for more than a few days after being made improved results can often be obtained by diluting the stain with isotonic saline buffered as above. Even so, in an emergency quite good results with thin or thick films can quite often be obtained by the use of tap water alone as a stain diluent.

After the addition of the diluted stain to the trough or dish the slides are left undisturbed for about 30 minutes and are then gently flushed with tap water to remove excess stain and the scum that will have formed, and are stood up vertically in a rack to dry.

Other methods of staining for malaria parasites give good results in competent hands, such as the use of Leishman's, Wright's, or Field's stains. The last, which is used only for thick films, has the advantage that the staining process is completed in a few minutes. These methods, and variations of them, are given by Shute and Maryon.[15]

MICROSCOPIC EXAMINATION

When they have completely dried the slides are ready for microscopical examination unmounted, using a × 100 oil immersion objective. For those experienced in reading them the thick film is usually examined first and the thin film used for confirmation of the species diagnosis. For those less experienced it is recommended that a prolonged examination of the thin film, of not less than 30 minutes, should be done, and only if no parasites are seen should the thick film be attempted.

This is not the place to describe the morphology of malaria parasites as seen in stained thin and thick blood films. Descriptions, of varying accuracy, may be found in all textbooks of parasitology and in most textbooks of microbiology. Some which may be recommended are those by Maegraith and Leithead[16] and by Swartzwelder *et al.*[17] and the less easily available but excellent monographs by Wilcox[18], Field[19], and Field and Shute.[20]

To guide the clinician an attempt should be made to assess the degree of parasitaemia, and this is obviously of importance when following the effects of specific treatment. This can be done by giving the number of parasites per 50 microscopical fields of the thick or thin film or, more accurately, the number per 100 or 500 white blood cells in the thick film. If the white blood cell count is known the number of parasites per cubic millimetre of blood may be calculated. In heavy infections the percentage of parasitized red blood cells may be given from the thin film.

Serological Diagnosis

Like that with any other foreign organism, an infection with malaria parasites causes a complex immunological response, including the production of many sorts of humoral antibodies.[21] Measurement of these antibodies has recently been shown to be a useful tool in individual diagnosis and, more particularly, to be of value in studying the epidemiology of malaria in places where it is endemic. By comparing the immunological profiles of different populations the amount of malaria to which they are exposed can be measured or the progress of an eradication scheme assessed.

The presence of complement fixing antibodies in malaria was first shown over 30 years ago. Subsequent studies of this test[22] showed that non-specific reactions were frequent, probably owing to difficulties in preparing a sufficiently purified antigen, so that in its present form the test is of dubious value. Similar defects also occur with other of the earlier serological tests such as the precipitin and melano-flocculation tests. Much valuable research has been done with a gel diffusion test for precipitating antibodies, and for soluble antigens, in human malaria, but, chiefly because of difficulties in preparing the antigen, the test has been little used outside its laboratory of origin.[23] One of the latest developments is an indirect haemagglutination test for malaria antibodies.

In this, tanned red blood cells, usually human group O, are coated with a concentrated extract of malaria parasites. Because of strong group antigenic relationships simian malaria parasites may be used to test for antibodies in human malaria. When the sensitized cells are mixed with sera containing malaria antibody agglutination occurs. The test is rapid and convenient, and allows the titration of many sera; it has been used for survey purposes as well as for testing clinical sera.[24] The chief difficulties at present seem to lie in preparing reproducible batches of antigen for sensitizing the red cells, and in the occasional presence of heterophile antibodies in sera, giving false-positive compound reactions. Probably these difficulties will be overcome.

INDIRECT FLUORESCENT ANTIBODY TEST

The serological test which has been most widely used in malaria is an indirect fluorescent antibody test, stemming from the work of Tobie *et al.*[25] on direct fluorescent staining of malaria parasites.

In this test human serum in incubated with a blood film heavily infected with malaria parasites, of which the schizont forms are the most reactive. After appropriate washings an antiserum

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prepared in animals against human serum and labelled with a fluorescent dye, usually fluorescein isothiocyanate, is applied. After further incubation and washings the film is examined under an ultraviolet fluorescence microscope. If antibodies were present in the serum they will have adhered to the parasites and the labelled antihuman serum will then have adhered to these, which will be shown by their fluorescence. If there is no antibody in the serum there will be no fluorescence. The antibody in sera can readily be titrated and, depending on the sensitivity of the parasites used as antigen, very high titres may be obtained. The antibodies detected by this test are not necessarily immunologically-active neutralizing antibodies; in fact, only a very small proportion of them probably are.

When using this reaction considerable antigenic overlap is found among the four different human malaria parasites, and between these and simian parasites. For detecting antibodies in human malaria simian parasites are therefore often used, but for maximum sensitivity it is certainly better to use human parasites. Though *P falciparum* cross reacts with the other human parasites, it has been claimed that in single infections the highest serum titre is found by using the homologous parasite as antigen.[26] Failing human patients, parasites can be obtained only by infecting splenectomized chimpanzees or *Aotus trivirgatus* monkeys. For this reason the test is done in only a few specialized laboratories. Tests can be done using thin films of parasites,[27] but sensitivity is probably increased by employing unfixed thick films.[28]

It is only recently that steps have been taken to attempt some standardization of the indirect fluorescent antibody test, in terms of the sensitivity of the different antigens and details of technique, so that comparison of results from different laboratories is difficult. However, sufficient observations have been made on man to allow some generalizations to be given. Following single infections antibodies are usually detectable after about a week, rise to a maximum titre after several months, and then slowly decline, but may persist for years. As well as the sensitivity of the test these variables depend on the amount of antigenic stimulus, which is related to the species of the infecting parasite, to the occurrence of relapses, and to how much and how soon drugs were given. Nevertheless, a laboratory which is regularly doing these tests, and which has established its own reproducible standards, can say with certainty whether an individual has been infected with malaria in the fairly recent past, and may be able to identify the parasite, be more precise about the time, and indicate whether active infection is possibly still present.

The introduction of refinements, such as measuring the relative amounts of antibody in the IgG, IgM, and IgA immunoglobulin classes may give greater precision in the future.[29,30] In clinical practice the test is therefore of value in retrospective diagnosis—for example, in a person who has been exposed to malaria, has suffered a febrile illness, but in whom antimalaria treatment has made conventional parasitological diagnosis impossible. It has also been used in identifying the responsible donors in cases of transfusion malaria, in whom the parasitaemia may be low and intermittent.

The serological picture in those who have been repeatedly exposed to malaria, such as those living in highly endemic areas, is far more difficult to interpret on an individual basis, though the test is proving of value in epidemiological studies.

Reference Laboratories

Blood films for the diagnosis of malaria should be sent to the nearest competent laboratory for examination. For confirmation of the diagnosis, films may be sent to: The Malaria Reference Laboratory, Horton Hospital, Epsom, Surrey; The Hospital for Tropical Diseases, 4 St. Pancras Way, London N.W.1.; Dept. of Parasitology, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool 4; Tropical Diseases Unit, Eastern General Hospital, Edinburgh 6.

Sera for testing for malaria antibodies may be sent to: Ross Institute, London School of Hygiene and Tropical Medicine, Gower Street, London WC1E 7HT; The Hospital for Tropical Diseases, London. (a minimum volume of 0·5 ml of clean serum is required, sent by first-class mail).

Chemoprophylaxis and Chemotherapy

W. PETERS

British Medical Journal, 1971, **2**, 95–98

Man may be infected by four species of *Plasmodium.* Infection with *P. vivax*, *P. ovale*, or *P. malariae* is readily prevented and the treatment of acute attacks with these parasites, once the diagnosis is made, presents no problems. *P. falciparum,* on the other hand, may produce a more severe illness and complications that necessitate the management of the patient as a medical emergency. Furthermore, in certain parts of Brazil, Colombia, Panama, Thailand, Laos, Cambodia, Vietnam, Western Malaysia, Singapore, or the Philippines individuals may become infected with strains of this parasite that exhibit a reduced response to one or more of the synthetic antimalarial drugs[31,32] (and in rare cases even to quinine[33]). Drug resistance has been the major stimulus for the large volume of research that has been undertaken during the '60s,[34-36] and over 140,000 compounds have been screened in the United States army programme during the last seven years.

Though the current concepts of antimalarial prophylaxis and the treatment of the nonimmune individual have changed little from those of 15 years ago,[37] the prevention and management of drug-resistant falciparum malaria are new aspects of the present situation.

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Individual Prophylaxis

Proguanil (Paludrine) and pyrimethamine (Daraprim) are excellent casual prophylactics against *P. falciparum* and good suppressive drugs against the other three species. Both compounds (also referred to as "antifols") interfere with the parasites' metabolism by binding to their dihydrofolate reductase, which has a much stronger affinity for these drugs than the corresponding enzymes of the mammalian host.[38] Trimethoprim, another antifol, is relatively less effective than pyrimethamine in binding the parasite enzyme but has an exceptionally high affinity for that of certain bacteria.

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