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Problems of Diagnosis of Trypanosomiasis *

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Diagnostic problems in trypanosomiasis arise from the inadequacy of current diagnostic procedures. This inadequacy deprives the clinician of essential data, hinders individual therapy and therapeutic control programmes, and makes suspect many conclusions drawn from previous experimental work. In this paper the author examines alternative methods of diagnosis, emphasizing cultivation and immunological procedures.

An independent standard for diagnostic evaluation is available with the culture method. Comparison indicates that conventional procedures are frequently in error and that the error is always an underestimation, which may reach 20%-25% or more. The culture method has proved to be sensitive, reliable, usable under African field and hospital conditions, and applicable to isolation of Trypanosoma gambiense *and* T. rhodesiense *from both blood and spinal fluid.*

Complement-fixation is the most widely used of the immunological reactions available. Procedures and results are reviewed and possibilities both with this method and others are discussed.

Continuing research on diagnostic methods is needed, and certain practical difficulties in such a continuing research programme are discussed.

THE RELATIONSHIP OF CLINICAL AND LABORATORY DIAGNOSIS IN AFRICAN SLEEPING-SICKNESS

Laboratory confirmation of the clinical diagnosis of sleeping-sickness is important both for the individual patient and for control of the disease; this is generally agreed upon. Confidence that laboratories can furnish the information desired and expected of them is far less universal. For example, in lower Guinea, Pautrizel et al. (1960) state that in 10%-20% of their sleeping-sickness suspects trypanosomes could not be demonstrated; in the former Belgian Congo 10% of infected suspects were reported as not having been diagnosed by conventional methods (Schoenaers et al., 1953). In Northern Nigeria, the Ministry of Health reported (1960) that in 1958 the over-all incidence of sleeping-sickness (0.14%) remained at about the same level as in 1957 (0.15%) and that " no further reduction is believed possible by present methods because mass diagnostic techniques are too crude to detect the very early cases which are infective to fly but have no lymphadenitis ". In certain areas of East Africa, Weinman (1960) estimated that, if one included non-clinical cases as well as suspects, the diagnostic failure rate might run as high as 20%-25%.

Diagnostic mistakes arising from laboratory inadequacy are almost invariably erroneous negative diagnoses. Such mistakes may cripple control procedures and, delaying treatment, are dangerous for the patient.

For the individual patient, the opposite error, to mistake another disease for sleeping-sickness and to institute treatment accordingly, may be disastrous. The clinician is today in an extremely difficult position. Knowing that the laboratory has given him a negative report on which he cannot rely, he is faced

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with a group of clinical suspects and with contradictory data concerning the advisability of treatment.

The situation arises with particular acuteness in patients showing central nervous symptoms but in whose spinal fluid the laboratory has failed to demonstrate trypanosomes. If the patient is known to have had trypanosomiasis and the cell and protein estimations are not typical of the " second stage ", the difficulty is extreme.

Thus, the painstaking work of Neujean (1950) has led him to conclude that spinal fluid findings are not a guide to therapy, *even when negative*, and consequently that treatment with compounds that do not cross the blood-brain barrier is useless and dangerous. The conclusion is expressed so emphatically that I quote:

> "Ceci revient à dire que dans la pratique courante le traitement de la maladie du sommeil avec des trypanocides purement organiques expose les malades, *quelle que soit la composition initiale du liquide rachidien* (prélevé et examiné par les méthodes habituelles), à la progression d'une invasion nerveuse qu'il n'a pas été possible de déceler.
>
> "Agir de la sorte, c'est exposer le malade à un retard inutile et dangereux dans l'application de la thérapeutique appropriée."

This expert then recommends the treatment of sleeping-sickness patients with organic arsenicals, even if the spinal fluid is normal. This is a procedure which can be satisfactory if trypanosomiasis is the only disease evolving in the patient, but that the etiology is single may be very difficult to establish. If encephalitis of another type is developing, treatment with arsenicals may be disastrous.

In sleeping-sickness areas, signs of encephalitis or meningitis or even persistent violent headache quite rightly make the patient a trypanosomiasis suspect. If trypanosomes are not found in the spinal fluid, this lack of laboratory support may be attributed to inadequacy of the methods employed and the decision made to proceed with treatment. Since the cosmopolitan virus diseases are present in sleeping-sickness areas and a number of these may involve the central nervous system, there are opportunities for error. Janssens et al. (1960), for example, describe a patient with normal spinal fluid in which trypanosomes could not be demonstrated. Because of meningitic symptoms and a previous history of treated early trypanosomiasis, Mel B therapy was none the less started. After three injections, acute encephalitic symptoms developed, the patient required immediate emergency treatment, for a long period his life was in danger, but after months of hospitalization the patient was restored to approximately normal condition in one year. The episode was interpreted quite plausibly as an acute encephalitis set off by Mel B in association with an agent, possibly viral, which had involved the central nervous system.

Just how commonly a " foreign " encephalitis develops in trypanosomal suspects is not known. That dual infection may not be rare is suggested by observations on an epidemic of Asian influenza which broke out in an endemic focus of trypanosomiasis. Dual infections gave a higher death-rate in untreated patients than expected; when the patients were already undergoing arsenical treatment the added virus infection proved a disaster. Thus, of 10 new patients in good physical condition and placed on Mel B therapy, all developed a violent encephalitis after contracting influenza, and nine died within 6-48 hours (Bezon & Ducasse, 1958; Richet & Ducasse, 1958).

As can be seen, a patient with encephalitis of undiagnosed cause, but possibly trypanosomal, poses a dilemma for the physician. If the encephalitis is not trypanosomal, then treatment of a damaged cerebrum with arsenicals is dangerous and unwarranted. Conversely, if the encephalitis is trypanosomal, delay itself may be imprudent. If laboratory assistance is not forthcoming, then the decision, whether to treat or not, has to be based on circumstantial evidence with its usual results: an unspecified number of unnecessary treatments, of " false cures " and of avoidable toxic encephalopathies. As knowledge of the other infectious encephalitides of tropical Africa accumulates, it is evident that there is geographical overlapping with sleeping-sickness and that symptomatology is not a satisfactory guide.

Methods of sleeping-sickness control depend on the reduction of either the tsetse fly population or of the mammalian reservoirs of the trypanosome, or both. Human treatment campaigns are used in many sanitation schemes. Plans which depend upon treatment to reduce the carrier frequency below successful transmission levels are themselves dependent on diagnostic procedures to detect the infected. If the elimination of trypanosomes from the human population is an essential part of a carefully planned control programme, then success of the programme will be endangered if a substantial reservoir of infected persons is left untreated.

Since in certain areas there is a suggestion that 20% or more of the infected are undetected by current techniques, if use of the routine diagnostic procedures is to be continued, then either more reliance should be placed on tsetse fly eradication, or the human population should be re-examined repeatedly in order to compensate for the essential insensitivity of the diagnostic methods; or, as a third choice, more precise diagnostic methods should be employed. The possibilities will be discussed.

CURRENT METHODS OF LABORATORY DIAGNOSIS

Anyone medically educated since 1910 needs no description of the usual laboratory procedures employed in the diagnosis of African sleeping-sickness; they have not changed in the last half-century. These methods depend upon visual recognition of a micro-organism in a small sample of body fluid. With few exceptions, such procedures are considered inadequate in the diagnosis of bacterial, viral, rickettsial or mycotic infections.

The fresh- or thick-drop examination of lymph fluid or blood is most used, and can succeed only when the sample contains at least one micro-organism. The sample here is one drop, estimated by Ross (1903) to vary from 1 mm³ to 20 mm³. Even if we assume this to have a volume as large as 0.1 ml, the methods are certain to fail unless the material contains approximately 10 micro-organisms per ml.

As to the examination itself, although the heavy infections are recognized readily, detection of light infections demands that the efficiency of the method be pushed to its maximum. Accordingly with one micro-organism in the sample, a 45-minute search can be and is required for its detection by a trained microscopist. This allotment of time may be satisfactory in individual cases but not usually in survey work, for if one deals with predominantly light infections or with an apparently negative population, then 10 examinations daily are all that should be demanded of a skilled technician if maximum efficiency of the method is to be obtained. In practice, the technician is usually required to examine more than 10 persons daily. Consequently, the method is not so employed as to render maximum sensitivity.

To improve on the obvious shortcomings of the above methods, two concentration procedures are used. The cerebrospinal fluid is usually examined after centrifugation; this is theoretically a satisfactory method, in practice its efficiency may be as low as 10% (see page 736, " Comparison of the culture method with conventional methods of diagnosis "). Triple centrifugation of the blood, a technique which requires much larger samples, is not widely employed.

To summarize, current diagnostic procedures are time-consuming, and extremely time-consuming if maximum efficiency is to be obtained. Further, current methods are inherently insensitive, and even at peak efficiency and maximum time-consumption, can be expected to fail as diagnostic procedures.

For an estimate of just how frequently they fail, it has been necessary to develop and test a completely independent diagnostic method.

IN VITRO METHODS OF DIAGNOSIS

THE CULTURE METHOD

History and problems

The history of the attempts to cultivate *Trypanosoma gambiense* and *T. rhodesiense* has been given elsewhere (Weinman, 1960).[1] Briefly, until about 1938, results were irregular. Isolations could not be obtained with any constancy, and when they were successful, subcultures frequently could not be maintained.

In retrospect, the difficulties probably arose from two main causes:

(*a*) The media employed contained blood. As reported by the present author in 1953, the blood of man and of all animals tested contains " natural " lytic substances which destroy culture trypanosomes *in vitro*. Until this was realized and the lytic substances inactivated, both the blood media and the inoculated blood contained inhibitory substances.

[1] Interested readers are referred to the following important publications: Laveran & Mesnil (1904) (first trial); Thompson & Sinton (1912) (detailed morphology); Ponselle (1924); Prates (1928); von Razgha (1929); and Reichenow (1932, 1934).

(*b*) Strains of trypanosomes passaged in animals were used as a source of inoculum, particularly in European and American laboratories. After repeated passage in animals strains become uncultivable on media that previously supported growth. It is clear, then, how later work failed to confirm earlier results even though the same strains and media were employed.

In 1938, Brutsaert & Henrard again attacked the problem with two liquid media and a special anticoagulant. Using 10 tubes of medium for each patient, their method proved sufficiently sensitive to be of value as a diagnostic procedure, probably because of partial inactivation of natural lytic antibodies.

This success stimulated investigations in two directions: first, the production of media of increased reliability and sensitivity when used for direct isolation from mammals (Weinman, 1944, 1946, 1960); and, secondly, the development of media suitable for the growth of *T. gambiense* and *T. rhodesiense* in large numbers for metabolic studies (Tobie, von Brand & Mehlman, 1950). Mass culture methods and sensitive isolation methods did not prove to be identical.

Procedure

In this paper, we are concerned only with methods showing maximum sensitivity in the isolation of trypanosomes directly from man. The isolation procedure here discussed was evolved from previous methods (Weinman, 1944, 1946, 1960) which had proved not completely satisfactory in field trials or laboratory experiments. Each modification introduced was adopted only after proof that it did indeed increase sensitivity. The isolation procedure has been reported elsewhere (Weinman, 1960).

Growth on isolation medium is detectable by microscopic examination after an interval which varies with the number of trypanosomes inoculated and the size of the sample examined. The generation time of trypanosomes taken from established cultures is approximately 1.25 days. Peak production is about 1×10^7 per ml of fluid, and counts are above 5×10^6 per ml for approximately a week. If examined during this period, a 0.1-ml sample furnishes 0.5-1 $\times$ 10^6 trypanosomes, each microscopic field (200-400 magnification) containing numerous organisms which occur singly, or as dividing forms, or in large and small rosettes.

With a " negative " inoculum by conventional methods, a 0.1-ml sample of the original cultures may show micro-organisms as early as three to five days. With this size of sample, cultures are more frequently positive by 10 days, and they may require up to 30 days to produce a sufficient concentration. If more rapid results are required, the effective sample size can be increased by centrifugation.

When growth is maximal, colonies may be seen on the surface of the slant. The colonies are colourless, translucent, round, with regular outline, and rarely exceed 2 mm in diameter; they are not completely distinctive on this medium, and identification should not be based on gross appearance alone. A detailed morphological study has been published elsewhere (Weinman, 1953).

Rationale

The fact that normal human serum lyses culture trypanosomes and the present requirement for blood in sensitive isolation media have dictated the major features of the present method.

Lysis of culture trypanosomes has taken place with all human serum samples tested. The majority of human sera give 100% lysis at dilutions of 1/10 at 25°C or 1/20 at 37°C. Five sera were specially studied for titre levels; the findings are given in the accompanying table.

Lysis has also been observed with all animal blood tested; it is pronounced with rat and rabbit sera, present but less marked with mouse sera, and,

LYSIS OF CULTURE TRYPANOSOMES BY NORMAL HUMAN SERA, EXPRESSED AS PERCENTAGE SURVIVAL [a]

	Serum dilution				
	1:2	1:10	1:20	1:40	1:100
Average of 5 sera					
At 25°C	0	3	38	48	80
At 37°C	0	0	0	17	60
Variations in the 5 sera at 25°C					
Serum DW	0	0	0	4	40
Serum LW	0	0	4	10	53
Serum RS	0	0	19	55	100
Serum AH	0	0	50	90	100
Serum AK	0	16	56	70	100

[a] All readings made at 4 hours. Serum dilutions made in Hanks's solution. Control suspension in 10% inactivated human serum in Hanks's solution. Titres are final serum dilutions.

interestingly, takes place with the sera of " germ-free " guinea-pigs,[1] five days old and fed sterile rations since birth.

It is clear that massive destruction of culture trypanosomes by normal serum could impede the development of a sensitive isolation procedure which used a blood-containing medium. Yet the use of serum or plasma has, thus far, been essential in any sensitive isolation method.

Lysis proved to be, in part at least, complement-dependent. Accordingly, blood in the medium is processed to remove complement from the red blood cells as far as possible by washing, and to inactivate complement in the plasma by heating. Heat treatment of the entire blood is simpler, but the resultant medium is less sensitive.

The problem of the natural antibodies in the specimen blood remained and was obviously not amenable to the same procedures. It has been known for some time that there is a class of substances which are anticomplementary, and some of which are also anticoagulants. We use polyvinyl sulfuric acid (PVSA), which Ecker & Pillemer (1941) found the most effective anticomplementary substance studied, being approximately 100 times as active as the reference standard, heparin.

A concentration of 0.5 mg per ml inactivates all the complement in 1 ml of fresh guinea-pig serum (Ecker & Pillemer, 1941). In the present method the final concentration is 0.5 mg per ml of blood or approximately 1 mg/ml of serum and is thus in excess of the amount estimated for total inactivation. PVSA inhibits complement by action on C′4, not by chelation of calcium and magnesium ions (Inoue et al., 1959).

At the concentration recommended, PVSA acts as an anticoagulant and thus combines two useful functions : it is anticomplementary and it acts as an anticoagulant. Most important, at effective anticomplementary and anticoagulant concentrations, it allows cultures to develop from very small inocula (see " Results " below).[2]

No extensive studies of other anticomplementary compounds have been made, but ethylene diamine tetra-acetate has been found to be lethal for trypanosomes at effective anticoagulant concentrations. Heparin, when used in high concentration to provide a solution comparable with PVSA in anticomplementary activity, is thick, viscous and difficult to use in many procedures.

Results

Previous discussion has centred on the suitability of procedures for cultivation. Whether human trypanosomes are cultivable under all circumstances must also be considered. It is known that strains which originally develop readily on media can no longer be grown after numerous passages in animals. Since prolonged residence in animals produces non-cultivable strains, the question whether prolonged residence in man would produce the same results immediately arose. Animal data did not distinguish between prolonged residence *per se* and the possible selective effect of repeated animal passage.

We have encountered no non-cultivable strains from man, and our trials included presumed relapse cases and what are almost certainly some long-term chronic infections. Indeed, the ease with which trypanosomes are isolated from both the blood and spinal fluid is a remarkable feature of the method.

Thus far, we have isolated trypanosomes in cultures 53 times. These isolations included both *T. gambiense* and *T. rhodesiense*, and were obtained from both blood and spinal fluid, from treated and untreated patients, and from " negative " suspects. In no case has the growth of trypanosomes in pure cultures failed to confirm laboratory diagnoses made by other means; in addition, cultures have revealed infections undiagnosed by conventional methods both in man and in test animals.

The method is suited to African conditions. The results cited were obtained in the laboratory which served as base, from bleedings performed in hospitals and dispensaries visited by automobile, and under field conditions while performing mass surveys.

Influence of the species. Of the 53 isolations, 31 were of *T. gambiense* and 22 of *T. rhodesiense*; no difference in ease of cultivation was noted.

Influence of the source of the inoculum. Forty-two isolates were obtained from blood, 11 from the cerebrospinal fluid. Contrary to previous report (Van Hoof, 1947), no difficulty whatsoever was encountered in growing trypanosomes from the spinal fluid.

[1] These sera were kindly provided by Dr Bruce P. Phillips, National Institutes of Health, Bethesda, Md.

[2] Inoue et al. (1959) report that the activity of minimal amounts of PVSA can be reversed non-specifically with egg albumin. No reversal was observed using the much higher concentrations of PVSA and the amount of blood and the medium specified in this report.

Influence of the length of residence in man. Precise data are not available; patients once diagnosed are not left untreated. Among the patients tested were a few who had relapsed with *T. rhodesiense* infection weeks to months after treatment (Weinman, 1960). Also, many isolations of *T. gambiense* were made in 1957, during study of an epidemic in Lango District, Uganda. The local strain appeared particularly mild and produced few or no symptoms in many of the human hosts, who were continuing hard manual labour at the time of examination. The history of the epidemic suggests that the pool of infected humans had been built up over several years. Since our subjects were drawn from this pool, at least some of them could be expected to have had long-standing infections measured in months, if not years. Yet no particular difficulty in cultivation was noted.

To summarize from the limited data available, long-standing infections have readily yielded cultures, so that, if uncultivable strains exist in man, they have not yet been encountered.

Influence of concurrent treatment. A few of our patients were treated for malaria, suspected typhoid fever, postpuerperal sepsis, etc. with non-trypanosomicidal compounds either shortly before or during the time cultures were attempted; this non-specific therapy did not prevent successful isolation (Weinman, 1960).

Specific treatment, of course, produced different results. No isolations were made from patients undergoing Mel B (Arsobal) therapy. Relapse strains after pentamidine isethionate and tryparsamide therapy, after suramin and Mel B therapy, or after a course of suramin were readily cultivable. Also, during suramin therapy isolation in culture was made, not from the blood (which was negative), but from the cerebrospinal fluid.

Thus, on the observations so far available, no difficulty has been encountered in cultivating relapse strains after specific therapy, nor has non-specific antimalarial or antibacterial therapy, even when given concurrently, hindered isolation in culture.

Comparison of the culture method with conventional methods of diagnosis

Data on sensitivity in man. There is no perfect method of diagnosis so, *faute de mieux*, the culture method was compared with diagnoses made by the standard procedures executed by others. In the majority of cases the two methods agreed. Where the two methods differ, the culture method appears to be the more sensitive. Cultures have detected numerous cases which were not diagnosed by conventional methods or even in some instances by animal inoculation. In the total of 53 isolations by the culture method from blood and spinal fluid, conventional methods were negative with the same material 19 times (36%). The record was particularly striking with cerebrospinal fluid, conventional methods failing in nine of 12 cases (75%) where trypanosomes were demonstrated in the fluid by culture.

Of particular interest are six culture-proved infections declared negative by all conventional laboratory methods. One patient was under treatment as a suspected relapse, for another there was clinical suspicion of the disease, but for four there was no reason to suspect infection. Since these four asymptomatic or pauci-symptomatic persons were derived from a total group of 21 suspects, it is apparent that, in certain areas at least, an important proportion of the infected may go undetected by present means of diagnosis.

Data on sensitivity in animals. In a few instances the same material from patients was tested both in laboratory animals and in culture. In general the results were parallel; in a few instances the animals were "negative" and cultures positive. This included mice, usually considered the most sensitive of animals. Where we were able to test some of the "negative" animals, their blood yielded positive cultures, indicating that they were not refractory but had an infection so light as to be undetected by usual methods.

Evaluation of the culture method as a means of diagnosis

The culture method has for the first time furnished an independent standard by which other laboratory diagnostic procedures could be evaluated. It is clear that the conventional methods are frequently in error and that this error is always in one direction: that of underestimation.

It follows that certain statements based on classical diagnostic methods concerning the absence of trypanosomes in population groups, or in certain organs and tissues at certain times, or in animals, wild and domestic, will require reconsideration; for what was actually being measured was the limit of sensitivity of conventional methods, and only in part, as was supposed, the presence or absence of

micro-organisms. For epidemiological control, it is important to realize that 20% of apparently normal persons in endemic areas may carry undiagnosed infections.

There remain to be considered certain characteristics of cultures and possible sources of error.

Period required for reading. If the culture method is extremely sensitive, it is because small inocula grow out. Presumably, the smaller the inoculum, the longer the period before the cultures are recognized as positive by any single standard of examination. This delay is inescapable and an obvious corollary of the sensitivity itself.

As stated, the generation time for culture trypanosomes is slightly more than 24 hours with the method and materials here described. It is probable that the generation time cannot be changed very substantially by varying the procedures recommended. However, some speeding of the readings can be obtained by increasing the size of the sample examined, as by concentration through centrifugation.

Possible false-positive cultures. The only trypanosomes infecting man in tropical Africa are *T. rhodesiense* and *T. gambiense*, so far as is known. Therefore if trypanosomes do grow out in culture, they can be ascribed to one of these two species with complete assurance.[1]

The only source of error known to this author has been pointed out by Pinto (1954b), and resides in the choice of a blood donor for the medium. Trypanosome-infected donors can give rise to false-positive cultures ; with care and proper controls this source of error can be eliminated.

Possible false-negative cultures. We have no proved case of trypanosomiasis from which trypanosomes failed to grow in pure culture.

In three instances it was impossible by any means to confirm the diagnosis made by dispensary workers or field teams. These three patients were sent to the hospital, where examinations were repeated with negative results. In two of these patients, 0.5-ml samples of blood failed to infect mice. Since the correctness of the original diagnoses could not be established, no conclusion could be drawn as to the efficiency of the cultures, which also did not show trypanosomes.

Inconclusive cultures. One complication of the culture method is the effect of bacterial and fungal contaminants which almost invariably prevent the growth of trypanosomes. This is the most serious disadvantage of the method as it has been practised, giving rise as it does to inconclusive cultures.

Contamination is a negligible factor in a relatively dust-free laboratory or suitable hospital room, but in field work or in rural dispensaries, the avoidance of contamination will require special precautions. The problem is a technical one, and essentially soluble; by combining mechanical and chemical means which are already available, it should be possible to obtain pure trypanosomal cultures even under unfavourable conditions.

Cultivability of different strains. The data which have been presented are based on isolations from man of both *T. gambiense* and *T. rhodesiense* in East Africa (Uganda and Kenya). Whether these observations will apply to the human trypanosomes of other areas has not yet been tested extensively. However, Pinto (1952, 1954a) in West Africa has reported success in cultivation from patients in Portuguese Guinea. Furthermore, a variety of fresh human strains, obtained from widely different areas, were readily cultivated by the present author from infected laboratory animals. These strains include *T. gambiense* from Liberia, from Senegal and from the Congo (Léopoldville) and two *T. rhodesiense* strains, one from Ruanda-Urundi and one forwarded from South Africa. This uniform success with recently isolated strains originating from a wide geographical area suggests equally wide applicability of the method.[2]

Summary

An independent standard for the evaluation of current diagnostic procedures used in African sleeping-sickness is now available with the culture method. This assessment shows that current procedures are frequently in error and that this error is always in one direction; that of an underestimation, which may reach 20%-25% or more.

The culture method has proved to be sensitive, to be reliable, and to be applicable under African field and hospital conditions. These data have been obtained for *T. gambiense* and *T. rhodesiense* in East Africa and there is suggestive evidence for

[1] For one possible but unproved case of *T. congolense* infection in man, see Lapeyssonie (1960) and Hawking (1960).

[2] It is a pleasure to acknowledge the aid received from Dr M. Vaucel, Mme Henrard-Peel and Dr H. J. Heinz, who furnished some of these strains.

assuming that the method may prove equally valuable in West Africa and other areas.

The chief present drawback to the culture method is the frequency of inconclusive cultures following contamination of the medium with other micro-organisms, particularly in field trials. This is essentially a technical difficulty; possibly the means for its solution are already available.

IMMUNOLOGICAL METHODS

General considerations

If one compares the culture method with immunological methods, next to be discussed, it is seen that each has advantages. A positive culture is diagnostic; there are no false-positives. Immunological methods are independent of the presence of micro-organisms at the time of examination, and they can be faster, giving results in hours rather than days. However, an immunological result is never final, it must always be interpreted in the light of the specificity of the reaction, and general experience has shown that the longer and more widely an immunological procedure is used, the more exceptions to a rigid diagnostic specificity are found.

The unusual character of certain immune reactions of trypanosomes (e.g., antigenic variation) and the great number of tests which demonstrate antigen-antibody reactions in trypanosomiasis have led to an enormous literature on the immunology of trypanosomiasis. Only a small proportion is concerned with evaluating immunological procedures as a diagnostic tool.

Methods available

Methods and results of workers prior to 1929 are detailed in Taliaferro's comprehensive review (1929). Classical reactions studied by some recent workers include: agglutination (Cunningham & Vickerman, 1962; Inoki, 1960; Soltys, 1957b), precipitation (Brown, 1961; Gray, 1960; 1961a; Seed [1]), complement-fixation (Neujean & Evens, 1958; Pautrizel et al., 1959, 1960), and neutralization (Soltys, 1957a).

In addition to the more usual procedures the following have been described: auto-agglutination of red blood cells (Yorke et al., 1911), hetero-agglutination of chick red blood cells (Gear, 1959), adhesion of platelets (Rieckenberg, in Taliaferro, 1929) or red cells to trypanosomes (Duke & Wallace, 1930; Wallace & Wormall, 1931), immuno-conglutination (Ingram & Soltys, 1960), respiratory inhibition (Desowitz, 1961), agglutination and agglutination-inhibition of tanned red cells (Lee-Jones, 1961), agar-gel precipitation (Brown, 1961; Gray, 1961a). Probably the fluorescent antibody technique used successfully with *Trypanosoma cruzi* (Fife & Muschel, 1959) would be also applicable to the African trypanosomes. Recently Seed [1] has shown that blood and culture trypanosomes possess common precipitating antigens and Cunningham and co-workers [2] have adapted the agglutination reaction to the identification of trypanosomal antibodies in insect blood meals. Also, the interference effect with certain species of *Borrelia*, which has been demonstrated by Galliard et al. (1958), may have an antibody basis.

The short list given above is a sample to illustrate the diversity both of procedures and of ends in view. It is not surprising that the findings, as well as the interpretations, in these studies are not always in agreement. However, certain general statements appear justified.

Pertinent results obtained concerning antigens

As trypanosomes develop in the animal body, multiple antigens are liberated, some of which can be detected in the serum (Weitz, 1960a, 1960b). Trypanosomal antigens vary as to specificity, and the corresponding antisera may react only with the same strain, or only with the same species, or may have inter-specific reactivities. The reactivities displayed depend upon the kinds of antigen, how they are prepared, and the nature of the test employed. With the solvents thus far utilized (serum, saline, etc.), there is evidence that the common antigens which determine inter-specific reactivity are more firmly bound than the strain-specific antigens.

The foregoing suggests that if a diagnostic test is to be evolved which is applicable to all the strains of *T. gambiense* and *T. rhodesiense* which are encountered in tropical Africa, the procedure should be based on the use of a common antigen with as broad a reactivity as possible, and the antibody tested for should be the corresponding broadly reacting one. Since man is not infected by any of the animal trypanosomes,[2] the latter can serve, and have served, as antigens. A recent personal communication from Seed that the culture forms of *T. rhodesiense* contain an antigen common to them and to the blood forms suggests interesting new possibilities in the production of antigenic material.

[1] Personal communication.

[2] Possible exception already cited.

Methods in use and results obtained

Of all the specific antigen-antibody reactions available, the complement-fixation reaction has been the most thoroughly tested and widely used, and has given the greatest satisfaction in diagnostic use. The results of two groups of workers are illustrative. In both reports, antigens were prepared by disruption of trypanosomes, suggesting that both bound and superficial components were utilized.

Neujean & Evens stated in 1958 that approximately 95% of new cases had given them a positive complement-fixation reaction with few or no false-positives. Out of 282 sera from proved cases at different stages 263 were positive, 18 negative and one anticomplementary. Following experimental inoculations in man, the reaction became positive between the seventh and the fifteenth day and reached maximum intensity about a week later. After treatment, fixation ceased in 12-18 months, negative reactions being reached more rapidly following Mel B and pentamidine and more slowly after Bayer 205 and tryparsamide. One hundred and eighty-two controls, including 20 with a positive Bordet-Wassermann test and 75 with tuberculosis, were negative and nine were doubtful and subsequently negative on repeat examination (Evens et al., 1953, 1954). Thirteen suspects with a positive reaction were considered on the border-line; despite repeated examinations trypanosomes were never found, nevertheless these persons could not be considered negative controls. The antibody which is present in the serum does not appear in the cerebrospinal fluid. Fewer positives are found both when the nervous system is involved and in post-treatment relapses, in which positive reactors fell to 80% (of 65 cases) (Neujean & Evens, 1958; Evens et al., 1953).

These workers used a heterologous antigen, *T. equiperdum*, harvested from rats, disintegrated by freezing, and not entirely purified of rat red cell debris. Results were expressed qualitatively (Schoenaers et al., 1953). Approximately 10% of 125 cases would have been missed by other diagnostic procedures but were detected with the complement-fixation reaction (Schoenaers et al., 1953).

Pautrizel and co-workers (1959) addressed themselves to improved specificity, sensitivity and stability of the antigen, and used quantitative reporting methods. *T. equiperdum* in rats was also employed, followed by treatment of the trypanosomal blood with rabbit antiserum to rat red blood cells. Antigen, prepared in early studies by mechanical grinding and cutting of trypanosomes, was later improved by substituting ultrasonic disintegration. With this improved antigen, laboratory testing indicated practically no anticomplementary activity; higher fixation titres could be obtained; and, when glycerinated, the antigen was stable at −25°C for at least 12 months. The Kolmer low-temperature, long-fixation technique was used and final serum titres determined by successive dilution, the end-point being the highest dilution at which any fixation could be detected (Pautrizel et al., 1960).

Tested in man, the reaction gave no fixation in 116 control sera, including 48 from syphilitics, and fixation with all of 25 human sleeping-sickness sera. There was no significant titre difference in the two stages of the disease. In general, titres decreased three weeks after Arsobal; a few "second-stage" patients showed a titre increase at this time. The complement-fixation reaction was considered of particular value in the 10%-20% of clinical suspects in whom trypanosomes could not be demonstrated.

Summary and considerations concerning future work

Complement-fixation can be a highly sensitive test, capable, in purified systems, of detecting as little as 0.75-2.5 μg of antibody protein (Treffers, 1958). Corresponding quantitative data in regard to trypanosomal systems are not known to the reviewer, but it may be suspected that difficulties with the test arise, not from inherent insensitivity, but from other sources such as interfering substances or inactivation of components. If this view is correct, then fixation may prove to be an even more valuable procedure than has yet been demonstrated and well worth the additional labour that a perfected system may require. It is encouraging that thus far there is no proof of antigens shared with other micro-organisms. The problem of providing stable complement for field use is perhaps soluble with the aid of freeze-dried preparations.

In this section only complement-fixation has been considered, because it is the one immunological procedure employed on a scale substantial enough for evaluation.

Whether any of the many other tests available may be of equal value and have certain practical advantages over fixation remains to be determined. Certainly a sensitive, specific, rapid method using stable reagents and capable of being used in the field would be highly desirable. For any such future work, the following appear to be important points at this time.

The test material should contain antigens common to all strains and species of trypanosomes infecting man in tropical Africa and not specific antigens only. This does not restrict the material of origin to *T. rhodesiense* and *T. gambiense*, for common antigens have now been found in the human African trypanosomes and in *T. brucei* (Cunningham & Vickerman, 1962), *T. brucei* and *T. vivax* (Gray, 1960, 1961a) and *T. equiperdum* (Neujean, Evens and coworkers, *loc. cit.*; Pautrizel el al., 1959, 1960). Seed [1] has observed that the blood and culture forms of *T. rhodesiense* also share antigens in common.

The antigenic material should be purified to the point that inhibitors and substances giving false-positive reactions are eliminated. Source material for these antigens are homologous and heterologous species maintained in animals and possibly the hitherto unexploited culture forms of *T. gambiense* and *T. rhodesiense*. Reagents, including antigenic material, should be stable and, if field use is important, they should not become inactivated for short exposures at temperatures up to 100°F (38°C); in this respect, a test which did not use complement would be advantageous. Methods and apparatus used in antigen *preparation* can be as complicated as necessary since this phase could be carried out in central laboratories; conversely, *in use* the methods and apparatus should be as few and simple as possible so as not to require highly trained personnel. Finally, results should be available within hours so that treatment can be undertaken at once.

These are a very difficult set of requirements, and they may never be met. Of one thing we can be certain—they will certainly never be met unless an attempt is made, and the failure to meet the requirements hitherto may indicate more clearly a lack of effort and correct criteria than any inherent insolubility of the problem.

NEWER STAINING METHODS

Two general types have become available; both involve preferential staining with fluorescent dyes and require special microscopic equipment. The methods are either elective or specific.

Elective methods

Elective methods involve direct staining of trypanosomes by fluorescing dyes such as acridine orange R. Stained micro-organisms stand out against a background of non-fluorescing erythrocytes. Other stains and procedures used for this purpose are given by Gurr (1960). Methods of this type require the presence of trypanosomes in the sample examined and, when applied to material obtained directly from patients, are a refinement of the usual procedures. More rapid than the latter under optimal conditions, direct staining by fluorescent dyes still requires for success a high concentration of micro-organisms in the sample.

Specific method

The specific method involves the use of fluorescent antibody and has been used with *T. cruzi*, where it proved more sensitive but somewhat less specific than complement-fixation (Fife & Muschel, 1959). No reference to use with the African trypanosomes has been encountered. *A priori* the method should be applicable; the working out of methods applicable to large-scale diagnostic procedures remains to be done. A recent critical review and a description of newer simplified methods are available (Goldman, 1961).

NON-SPECIFIC CHEMICAL AND PHYSICAL METHODS

The formol-gel serum test (Gaté-Papacostas reaction) carried out in areas with no coincident leishmaniasis was positive in 85% of new trypanosomiasis infections, in 70% when the nervous system was included, and fell to 25% in relapses. Sera from leprous patients gave false-positives (Neujean & Evens, 1958). Griffiths (1956-57) found the colloidal gum mastic reaction so non-specific as to be unsatisfactory; positive reactions were obtained not only in trypanosomiasis, but following the inoculation of a variety of foreign protein: egg albumin, viral and bacterial vaccines and saliva of uninfected *G. morsitans*.

A suggestion of specificity of locus, if not of substance, derives from the work of Mattern et al. (1961) and Mattern (1962), who recently reported on β-2 macroglobulins detected by electrophoresis. In a preliminary study (Mattern, 1962), the striking finding was a large amount of the substances in the spinal fluid of two second-stage patients. According to Mattern this is unique, being the only condition known in man in which the spinal fluid contains large amounts of these globulins.

In serum, β-2 macroglobulins are increased in a

[1] Personal communication.

variety of conditions, including sleeping-sickness, in both primary and secondary stages (39 of 41 cases). The macro β-2 fraction fixes complement and is therefore presumably an antibody.

Possible use of Mattern's finding for large-scale diagnostic work awaits the extensive confirmation of his spinal fluid findings and the development of simplified identification procedures.

IN VIVO METHODS OF DIAGNOSIS

The inoculation of animals, particularly the smaller rodents such as mice, has been used as a means for evaluating standard diagnostic methods and reveals a certain number of missed infections. Basically, this is an unsatisfactory procedure for it is not an independent means of evaluation, requiring as it does conventional methods for detecting infection of the animal. It is not surprising therefore to find that animals as well as men, described as negative by standard methods, are in actuality infected, as can be proved by the cultivation of trypanosomes from their blood.

Use of animals in the field on a large scale is rare because of the expense, the personnel and buildings ultimately required, the general cumbersomeness of the method and the unrewarding nature of the results obtained. Use of the developing chick embryo and of tissue cultures has not seemed diagnostically more advantageous (Weinman, 1953; Demarchi & Nicoli, 1960).

Finally, xenodiagnosis, used so fruitfully in the diagnosis of *T. cruzi* infection, has not proved applicable in African trypanosomiasis because of low infection rates in *Glossina* offered an infectious feed.

CONCLUSIONS

Present diagnostic procedures fail to detect from 10% to 25% of all persons infected with African trypanosomes.

With this residue of unknown infections, control procedures which depend for effectiveness upon treatment of all infected persons will prove to be inadequate.

The inadequacy of current diagnostic procedures, long suspected by clinicians, has led to certain unsatisfactory practices, of which two seem particularly objectionable.

(*a*) The treatment of non-trypanosomal cases with potentially toxic drugs. Aside from the failure to combat the disease agent which is actively responsible, this is a dangerous and unjustified procedure in encephalitis of unproved origin, since it exposes the patient to a needless risk of crippling or death. The danger is frequently compounded, for failure to respond to treatment is often regarded as an indication to increase the dose or to prolong the treatment, thus increasing the opportunity for toxic reactions.

(*b*) The application of chemoprophylaxis to persons actually infected. The puzzling diagnostic picture which then results is well known, and the dangers inherent in this procedure have been considered great enough to prevent the use of chemoprophylaxis in areas where it could otherwise render valuable services.

From the point of view of over-all trypanosomiasis control, the importance of diagnostic inadequacies has been overshadowed by the remarkable reduction in the total number of cases. That this reduction could be effected despite a relatively high rate of diagnostic failures is due to a variety of factors, *some of which may not be operative in the future*. Of these factors, one of the most important has been the use of quarantine measures; first, to force evacuation of the population from endemic areas and, secondly, to prevent re-entry. The evacuated areas have included some which were particularly desirable because of fertility, the abundance of the fish catch, etc. We now see a greatly increased African population, with every expectation that the increase will continue. There has always been in the past pressure for the re-occupation of the " forbidden areas "; now, with increased population, this pressure will also increase. And this pressure will be felt by new governments whose very survival will depend in the last analysis on the approbation of a satisfied citizenry. Whether these new governments will be able to render the forbidden areas sanitary

before they are occupied is unknown, but it is a task whose difficulties and expense were sufficient to deter the much wealthier European governments from undertaking it. If, as seems possible, land-use requirements lead to the re-occupation of unsafe areas, then we may well see a resurgence of the trypanosomiasis problem to a major health hazard. If it cannot be combated by quarantine measures, then dependence on diagnosis and treatment and on chemoprophylaxis will probably become more important. And as such measures do become more important, reliance on a perfected diagnostic method itself becomes more essential.

There is a present need, which soon may become more urgent, for improved methods of diagnosis of human trypanosomiasis. Two of these methods have already yielded results: the culture method and complement-fixation. Research should be continued until as nearly perfect a method as possible is obtained, the criteria being sensitivity and specificity.

Suitability to African conditions, particularly to use in the field, should be an eventual objective, but this objective should not be used to discriminate between research projects in their early stages. Development of the best possible method should be the prime objective of the sccientists concerned; adaptation of this method, once evolved, to the realities of African communications, equipment and personnel is a different problem, perhaps best entrusted to a different set of workers with the requisite engineering skills.

RÉSUMÉ

Les méthodes de laboratoire utilisées actuellement pour le diagnostic de la trypanosomiase africaine, et qui sont les mêmes depuis un demi-siècle, reposent essentiellement sur la constatation oculaire d'au moins un trypanosome dans un petit échantillon de liquide organique. Un tel procédé est presque toujours erroné lorsqu'on cherche à diagnostiquer une infection bactérienne, virale ou mycosique. Il semble bien qu'il soit également erroné dans le cas particulier de la trypanosomiase africaine.

Le pourcentage d'erreur des méthodes courantes est de 10 à 25% chez les malades; ce pourcentage est même plus élevé en cas d'invasion du système nerveux central. Ces erreurs sont préoccupantes pour les chercheurs, les cliniciens et les hygiénistes.

En ce qui concerne l'homme de laboratoire, il est de fait que de nombreuses conclusions, éparses dans la littérature consacrée à la trypanosomiase, doivent être soumises à une sérieuse critique. Des opinions basées sur les méthodes classiques de diagnostic et portant sur l'absence de trypanosomes dans certains groupes humains ou dans certains organes ou tissus à certaines époques ou chez des animaux sauvages et domestiques, doivent être reconsidérées, car ce que l'on établissait de façon précise, c'était la limite de sensibilité de la méthode et non (ou tout au moins de façon très parcellaire) la présence ou l'absence de micro-organisme.

Les cliniciens soupçonnent depuis bien longtemps l'imperfection de ces méthodes usuelles de laboratoire. Aussi, lorsqu'ils reçoivent un résultat négatif en lequel ils ne croient guère, sont-ils placés en face du dilemme suivant: ou bien administrer à un malade atteint d'une affection des médications trypanocides pouvant être dangereuses, ou bien s'abstenir de tout traitement spécifique et laisser la maladie évoluer de façon sévère.

Les méthodes thérapeutiques dont l'efficacité repose sur le traitement de toutes les personnes atteintes seront toujours imparfaites lorsque subsiste un lot substantiel d'infections non reconnues. Dans le même ordre d'idées, la chimioprophylaxie peut être appliquée à des sujets déjà infectés, avec pour résultat le tableau clinique trompeur bien connu. Ou bien au contraire, la crainte d'aboutir à un tel résultat va détourner les autorités d'entreprendre des campagnes de chimioprophylaxie dans des régions où elles seraient cependant extrêmement utiles.

La méthode de la culture offre à présent un moyen d'évaluer les méthodes courantes de laboratoire pour le diagnostic de la maladie du sommeil africaine. L'analyse des faits montre que ces méthodes sont souvent en défaut et que l'erreur ne se produit que dans un sens: celui de la sous-estimation, et ceci selon un pourcentage qui peut atteindre 20-25% ou davantage.

La méthode de la culture s'est avérée sensible, fidèle, utilisable en Afrique dans la brousse comme à l'hôpital, et applicable à l'isolement de *T. gambiense* et de *T. rhodesiense* du sang et du liquide céphalorachidien. Le problème pratique de la contamination des échantillons en brousse est discuté.

Il existe un grand nombre de réactions immunologiques. La réaction de fixation du complément a été la plus généralement utilisée à des fins diagnostiques. Dix à 20% des sujets suspects, chez lesquels aucun trypanosome n'avait pu être mis en évidence, ont eu une réaction positive avec l'antigène trypanosomial. La valeur diagnostique de la réaction obtenue à partir du liquide céphalorachidien n'est pas encore certaine. L'auteur passe en revue les résultats des méthodes de fixation du complément et discute leurs possibilités, ainsi que celles d'autres méthodes.

De nouvelles recherches s'imposent dans le domaine des méthodes de diagnostic de laboratoire. L'auteur discute des difficultés pratiques que rencontre la réalisation d'un tel programme.

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