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Infectivity of Salivarian Trypanosomes to the Mammalian Host

W. H. R. LUMSDEN

Introduction

In 1939, at the time of the outbreak of hostilities between Britain and Germany, consideration had to be given in Britain to the availability of antimalarial drugs, in case of a need to conduct military campaigns in malarious countries. The main source of supply of the gametocytocidal 8-aminoquinoline Plasmoquine was the German company of Bayer and this source was likely soon to be precluded. A drug believed to be of identical constitution was available from French sources (Praequine: May and Baker) but yields, following procedures defined by patent specifications, were reported to be lower than expected and, as the drugs were amorphous, it remained uncertain if they were of identical chemical constitution (WARRINGTON YORKE, personal communication). The writer, and his colleague D. S. BERTRAM, then working in the Liverpool School of Tropical Medicine, were requested, therefore, to compare the biological, i.e. gametocytocidal, activity of the two preparations. *Plasmodium gallinaceum* Brumpt, 1935, had, shortly before, been received in the Liverpool School of Tropical Medicine, by courtesy of Professor Werner Schulemann of the University of Bonn and offered as a suitable material for the experimental study of this matter.

The ways by which the efficacy of a gametocytocidal drug may conceivably be assessed are discussed by LUMSDEN & BERTRAM (1940b). They include:

a) Reduction in numbers or morphological changes in the gametocytes occurring in the peripheral blood. Clearly, any study of the gametocytocidal activity of a drug must be related to the concentrations of gametocytes which circulate in the peripheral blood of the host and are there available to be ingested by the vector. However, study of changes in gametocyte concentration alone is inadequate as a measure of the purely gametocytocidal activity of a drug as changes in numbers may be due not solely to direct effects of the drug on the gametocytes but also secondarily to effects of the drug on the asexual reproductive cycle from which the gametocytes are derived.

Further, no clear morphological criteria seemed to be available which could be correlated with non-viability on the part of the gametocytes. Among the authors describing the effects of gametocytocidal drugs on the morphology of the organisms: some mention changes in asexual parasite morphology and refer little to the gametocytes; different authors disagree as to the morphological changes induced in asexual as compared to sexual forms; some describe minor changes in staining, difficult to measure objectively (LUMSDEN & BERTRAM, 1940b). Thus neither observation of gametocyte concentrations alone, nor that observation taken together with study of morphological changes, were considered adequate for the assessment of the gametocytocidal activity of a drug, particularly as it was desired to determine whether small doses, below those inducing recognizable morphological damage, would render the sexual forms non-viable.

b) Prevention of exflagellation of male gametocytes. There was, however, evidence (LUMSDEN & BERTRAM, 1940b) that drug dosage levels insufficient to prevent exflagellation were, nevertheless, sufficient to prevent oocyst development.

c) Alteration of the infectivity of the host to mosquitoes fed upon it. Although many workers had experienced difficulty in obtaining hosts which would consistently infect mosquitoes before the administration of the test drug (LUMSDEN & BERTRAM, 1940b), this approach did appear to be that most likely to offer incontrovertible and quantitative evidence of the gametocytocidal activity of a drug. Although the presence of sporozoites in the salivary glands of the mosquito is the ultimate criterion of the viability of the gametocytes taken in by the mosquito, doubt has been cast on the validity of counts of sporozoites in the salivary glands (LUMSDEN & BERTRAM, 1940b) and the criterion lacks quantitative value. More useful quantitatively appeared to be counts of the numbers of oocysts generated on the midgut of mosquitoes *(Aedes aegypti)* fed on infected hosts and comparisons of these counts with the absolute concentrations of gametocytes existing in the peripheral blood of the same infected hosts at the time of feeding. A basic study on the biology of *Plasmodium gallinaceum* in the domestic fowl with special reference to gametocyte production and to oocyst development in *Aedes aegypti* was therefore instituted (LUMSDEN & BERTRAM, 1940a).

The main conclusions of this work were:

i) that gametocytes appeared in the peripheral circulation of the host at the beginning of the apparent infection, as early as did asexual forms,

ii) that gametocytes were produced in broods reaching maturity during the period of about 24 hours succeeding schizogony,

iii) that fowls are infective to *Aedes aegypti* from the beginning of the apparent infection. The highest average oocyst counts tended to *precede* the highest gametocyte count per unit volume. The curves of average oocyst counts tended to show peaks in each interschizogony period, presumably corresponding with the coming to full development of a brood of gametocytes.

Similar relationships have since been demonstrated by HAWKING, WORMS & GAMMAGE (1968) for *Plasmodium knowlesi* in monkeys but what most impressed the author in 1940 was the lack of close correspondence between the two parameters expected to be closely correlated – the concentration of gametocytes in the peripheral blood of the host and the numbers of oocysts developing on the midgut of *Aedes aegypti* fed on the hosts at the same time. Although there was a general correspondence between these two parameters over the course of the infection it was clear that the correspondence was not particular – large, but clearly significant, fluctuations in average oocyst counts were not reflected in corresponding fluctuations in concentrations of gametocytes in the peripheral blood (LUMSDEN & BERTRAM, 1940a).

The writer has never been able to forget this demonstration, early in his career, that "all that glitters is not gold" and has thenceforward been critical of assessments of functional capability made on morphological appearance.

This reserve was reinforced by 10 years association with virologists (1947–1957) working on the epidemiology of yellow fever and other arbovirus infections. At this time, in virology, the approach was dramatically different from that which would have been followed in protozoology or even in bacteriology. Unable to visualize the organisms causing the diseases in which they were interested, virologists concentrated studies on effects in defined experimental systems. The microscope was an instrument of minor importance, its usefulness more or less restricted to study of the histopathological effects of the virus infection.

During this time, also, the writer became accustomed to the concept of organismal particles of different potentiality – to the concept of particles of anti-

genic but not necessarily of infective potentiality – of the differentiation in, say, a lyophilized virus preparation, of dead, though still antigenic, virus particles from living particles, antigenic and also reproductive.

The concept of differing potentiality for further multiplication in specified environments is accepted in protozoology, as, e.g., with regard to the differing potentiality of the different morphological forms of the trypanosomes to develop in the vertebrate or the insect host or intra- or extra-cellularly. Differing potentiality of different populations of the same general form will also be accepted as, e.g., the non-infectivity to the vertebrate host of the trypomastigote forms occurring in the midgut of *Glossina* as compared with the trypomastigote forms found in the salivary glands of *Glossina* or in the blood stream of the vertebrate host. Differing potentiality between individuals of the same morphological form, and apparently of similar motility and viability, is less commonly recognized. Yet they occur, as is evidenced by the lack of close correspondence between the gametocyte counts in the peripheral blood of fowls infected with *Plasmodium gallinaceum* and the numbers of oocysts occurring on the midguts of *Aedes aegypti* fed upon the fowls (LUMSDEN & BERTRAM, 1940a), and by the occasionally expressed belief on the part of some workers on trypanosomes that the organisms might vary in "quality" (e.g., DESOWITZ, 1963). Partly the lack of recognition of the variability of potentiality between organisms of the same population and of similar microscopical appearance is due to the lack of experimental methods by which potentiality could be studied. Protozoology has developed until recently mainly as an observational rather than as an experimental subject. Nevertheless, methods are now becoming available by which the potentiality of protozoal populations for further development in defined situations may be studied, such as methods for measuring the infectivity of organismal suspensions to vertebrate hosts (LUMSDEN, CUNNINGHAM, WEBBER, VAN HOEVE & WALKER, 1963; WARHURST & FOLWELL, 1968; OVERDULVE & ANTONISSE, 1970a, b). This approach, quantitative as regards a main parameter of interest in experimental work – the infectiousness of the inoculum used – is, however, still in its infancy and most inocula in descriptions of protozoal experimentation are still defined simply as numbers of organisms. There is a large field of informative study accessible, now that the numbers of organisms in a suspension and the infectivity of that suspension may both be quantitated and the two parameters compared.

These general concepts and principles having been introduced, it is worthwhile to consider in their light some of the situations in which trypanosome populations may vary in, or lose and regain, their infectivity to the vertebrate host. The discussions which follow are not intended to be a comprehensive and exhaustive survey but simply a survey picking out some situations of interest to consider in these ways.

Definitions

There are some inconsistencies and indefinitenesses in the terms used by different authors which tend to confuse discussion. For instance, some authors use "virulence" and "infectivity" practically interchangeably. In the present review substantially the definitions given by WILSON & MILES (1966), based on bacteriological models, will be followed, with some modifications:

Infectivity. The capacity of an organism to establish a primary lodgement on arrival at the body's surface. "Lodgement" requires to be

further defined, perhaps, as a situation in which the introduced organism may reproduce, at least to some extent. And it is convenient to extend the use of the term infectivity to cultures as well as to vertebrate or arthropod hosts.

WILSON & MILES (1966) also include a more general definition of infectivity capacity to spread from one host to another under specified conditions of exposure to risk of infection. The first, more precise, definition will be used here.

Virulence. The capacity of an organism to cause disease, the host and conditions being specified or an average resistance (see below) implied. High virulence is most often, in protozoal contexts, applied to acute situations when organisms multiply to such an extent that they overwhelm the host as, e.g., in a trypanosome infection producing a fulminant parasitaemia. Low virulence strains are those which produce long term low intensity infections; though these might still in WILSON & MILES' (1966) definition cause disease, though probably secondarily, by immunological mechanisms rather than by the direct effects of the organisms.

WILSON & MILES (1966) use degrees of virulence to qualify individual cultures and strains and reserve pathogenic to apply to classes of bacteria. DAVIS et al. (1967) regard pathogenicity and virulence as synonymous.

Resistance. In statements regarding the resistance of a host, either the strain of parasite must be specified or a parasite of average infectivity and/or virulence is implied.

DAVIS et al. (1967) discuss also the concepts of invasiveness, comprising the capabilities of organisms to establish themselves intracellularly, to protect themselves from host attack by capsules, or to adapt to the host environment, and toxigenicity, comprising their ability to produce exotoxins and endotoxins which attack the host.

Infectivity of blood-stream forms

In in vitro *suspensions*

In experimental studies on the maintenance of infectivity *in vitro* it is of first importance that the performance of any diluent used, as regards maintenance of the infectivity of the organisms suspended in it, is known. For instance, THEILER (1933) in developing the yellow fever protection test in mice devoted a great deal of attention to proving the suitability of his diluents beforehand. He tested the infectivity of various dilutions of yellow fever virus, suspended in the test diluents and incubated at 37 °C, by their intracerebral inoculation to mice. He found ascitic and pleural fluids suitable and set up large quantities on whose performance he could rely.

LUMSDEN, CUNNINGHAM, WEBBER, VAN HOEVE, KNIGHT & SIMMONS (1965) adopted a similar approach with regard to trypanosomes. They point out that, although there was clear evidence of the importance of such factors as pH in determining the survival of trypanosomes outside their hosts, information of this sort had been, strangely, overlooked and a multiplicity of different, and often not clearly defined, solutions were in use to suspend trypanosomes – e.g., saline, physiological saline with 1% sodium citrate, citrated normal saline, 4%, 5% or isotonic glucose, Ringer's glucose solution, Alsever's solution, dilute serum. They point out, further, that even named solutions are of uncertain constitution unless formulae are given. Many differences were found in the formulae given for named solutions in different books of reference; for instance, no less than four different formulae for Alsever's solution were found.

With these considerations in mind, LUMSDEN et al. (1965) investigated by a titration procedure (LUMSDEN et al., 1963) the maintenance of infectivity in suspensions of trypanosomes in a basic physiological salts solution adjusted to various hydrogen ion concentrations with citrate, phosphate and borate buffer systems, and maintained at 0–2 °C. Both cryopreserved and fresh materials were studied. Results may be summarized:

a) With cryopreserved materials kept at pH 8.0 numbers of organisms were little diminished over 24 hours and infectivity was well maintained for 8 hours. However, at pH 7.0 and below, both the numbers of trypanosomes in the suspension diminished rapidly and infectivity collapsed practically instantaneously. For instance, in one experiment all the experimental operations for the initial estimate of infectivity of the same suspension exposed to the different pH conditions were completed in 11 minutes. There was little difference discernible between the suspensions of pH 8.6 and pH 6.2 as regards numbers of organisms or as regards their motility. However, infectivities were dramatically different. The infectivity of the pH 8.6 suspension was antilog 8.0 $\pm$ 0.3 ID_{63} per ml of original suspension, while it was only 4.4 $\pm$ 0.5 ID_{63} for the pH 6.2 suspension. Thus in 11 minutes, although there was little perceptible alteration in the visual appearance of the suspension, infectivity was reduced by 3.6 logs, i.e., to about one four thousandth of the infectivity of the same trypanosome population maintained at pH 8.0.

b) With fresh suspensions of *T. (T.) brucei* similar effects were observed. In one experiment no significant difference was observed in the numbers or in the motility of a suspension up to 2 hours in hydrogen ion concentration conditions varying from pH 5.6 to 7.7; trypanosome concentrations were throughout between antilog 9.0 and 9.2 organisms per ml. On the other hand, estimates of infectivity showed dramatic differences. Even in the initial infectivity estimation there was a ten thousand-fold difference between pH 5.6 and pH 7.7, viz.:

	Initial infectivity estimate (Log no. mouse ID_{63}/ml)
pH 5.6	4.4 ± 0.5
pH 7.7	8.4 ± 0.5

These differences were maintained over the subsequent period and confirmed by other experiments (LUMSDEN et al., 1965).

That such large effects on infectivity can take place so swiftly, and in the absence of any indication of degeneration to microscopical examination, has been slow to be recognized. Even so likely to be deleterious a diluent as the modified Alsever's solution (pH 6.1) has been noticed recently to be still in use.

The mechanism determining these cataclysmic losses of infectivity in conditions of low pH are still uncertain. However, VICKERMAN (personal communication) has pointed out that at low pH levels trypanosomes lose their surface coat (VICKERMAN, 1971), the coat, believed to be composed of variant antigen, which normally covers the entire body and flagellum of the blood stream organism, outside its essential boundary, the 3-ply surface membrane. It would be instructive to investigate if other methods of denuding organisms of their surface coat, such as incubation with pronase, would affect infectivity.

In the vertebrate host

CUNNINGHAM et al. (1963) infected rats with *T. (T.) brucei* and, beginning on day 2 after their inoculation, estimated every 24 hours the concentration of organisms in the peripheral blood of the animals and at the same times the infectivity of the peripheral blood to mice, using the technique of LUMSDEN et al. (1963). Their study covered a first parasitaemic wave with a peak at day 4 after inoculation, a trough occurring at day 6 and a subsequent rise of level of parasitaemia which ended in the death of the animals on days 9 or 10. These workers distinguished long and short forms of the organism and their findings in this respect were classical – the long forms predominated in the crescendo stages of parasitaemic waves while the short forms occurred, even predominated, during the diminuendo stage and through the trough. However, neglecting these fluctuations in the representation of different forms and considering only the two main essential parameters, numbers of organisms and infectivity, the findings were as shown in Figure 1. Since the infectivity estimates, expressed as mouse ID_{63} per ml of rat blood, represent essentially the number of infective particles, i.e., trypanosomes, capable of further reproduction in a new host (LUMSDEN

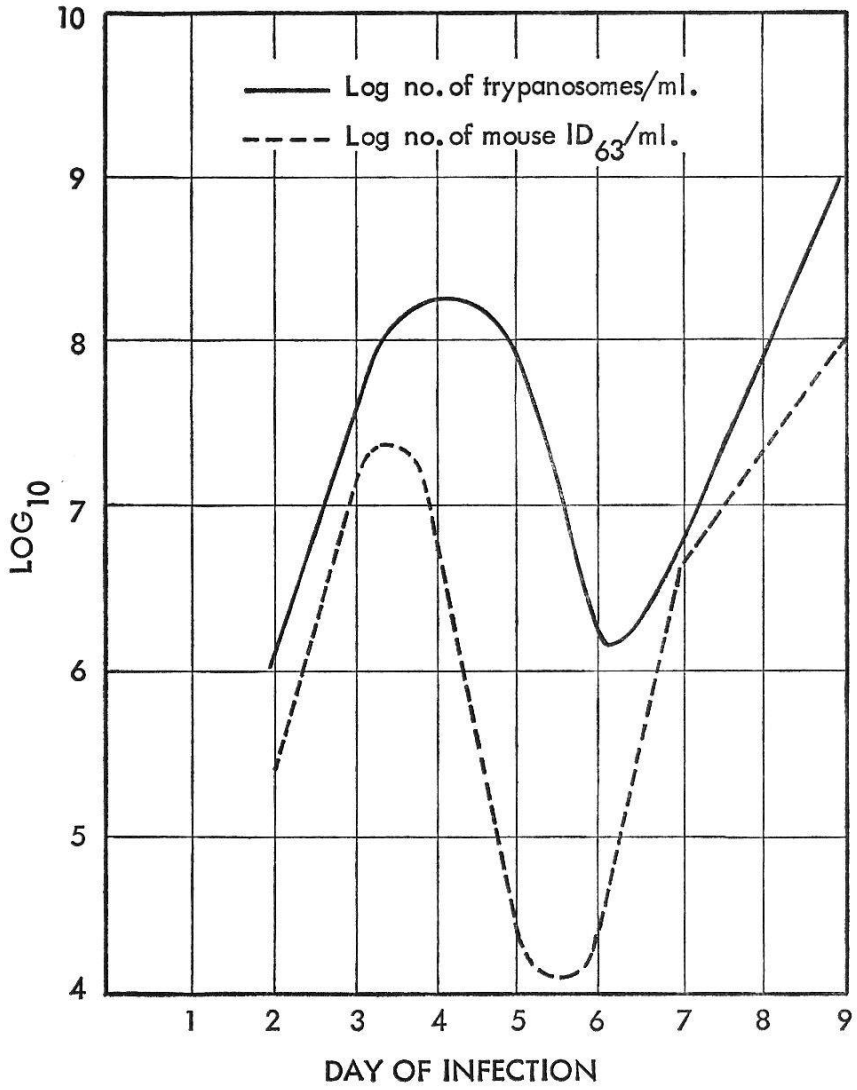

Fig. 1. The concentrations of organisms in and the infectivity (expressed as ID_{63} for mice) of, the cardiac blood of 2 rats infected with *T. (T.) brucei* stabilate EATRO-147 at 24-hour intervals from day 2 to day 9 after inoculation. Redrawn from CUNNINGHAM, VAN HOEVE & LUMSDEN (1963).

Table I. Concentrations of organisms in, and infectivity (as mouse ID_{63}) of, the cardiac blood of rats infected with *T. (T.) brucei* stabilate EATRO-147 from day 2 to day 9 after inoculation. Constructed from the figure given by CUNNINGHAM, VAN HOEVE & LUMSDEN (1963)

Log numbers per ml	Day							
	2	3	4	5	6	7	8	9
Organisms	6.2	7.4	8.3	8.1	6.2	6.7	8.0	9.0
ID_{63}	5.3	7.0	7.0	4.3	4.3	6.6	7.3	8.0
Difference	0.9	0.4	1.3	3.8	1.9	0.1	0.7	1.0
Proportion of organisms infective to mice	1 in 8	1 in 2.5	1 in 20	1 in 6,300	1 in 79	1 in 1.2	1 in 5	1 in 10

et al., 1963), they may be directly compared with the estimates of the total numbers of organisms present in the same blood. It is instructive to compare these values day-by-day through the course of the infection.

Unfortunately the actual values observed by CUNNINGHAM et al. (1963) are not recorded on their graph; Table 1, however, transcribes these as accurately as is possible. Through the course of most of the infection variations in the proportion of organisms infective to mice among the total number of organisms present are quite wide though still comparatively restricted – between 1 in 1.2 on day 7 and 1 in 20 on day 4. However, on days 5 and 6, differences of quite a different order of magnitude are evident. On day 5, coinciding with the beginning of the diminuendo phase of the trypanosomaemia, only 1 in some 6,000 organisms was infective; and on day 6 coinciding with the depth of the trough of the parasitaemia only 1 in about 80 organisms was adjudged infective.

Differences in infectivity of this order or magnitude, occurring over short periods of time, are obviously likely to influence the results of experimentation in which such variables are uncontrolled, such as those experiments of FAIRBAIRN (1933a, b) in which the infectivity of trypanosome suspensions to rodents was tested after exposure *in vitro* to human serum. Variations in infectivity of the trypanosome suspensions used may well account for the inconsistencies in the results of such experimentation (see below).

Although these variations in the relationship between the numbers of organisms present in the blood of the host, and their infectivity to mice, have been demonstrated, little is known of the mechanisms determining these effects. It is possible that the low infectivity of the organisms, during the diminuendo phase of the parasitaemic wave is due to the organisms at this time being coated with antibody specific for their particular antigenic type, so that they are immediately phagocytosed when they are introduced into a new host. But such suggestions are purely conjectural at present.

In suspensions containing human serum

The demonstration that human serum, unlike the sera of most other animals, had an adverse effect on the survival or multiplication of some *T. (T.) brucei* strains (LAVERAN & MESNIL, 1902) attracted a great deal of attention. Such an effect offered prospects for therapeutic and prophylactic application and for the differentiation of trypanosomal strains of different host-species-infective potentiality.

LAVERAN & MESNIL (1902) do not describe their experimentation in detail but it is interesting to recall their general conclusions:

a) In experiments in which mice or rats, infected with *Trypanosoma (T.) brucei,* were injected with sera from normal animals of several species (chicken, goose, horse, sheep, goat, pig, monkey, man) only human serum showed any effect. With human serum, however, injections of 0.5–1.0 ml into infected mice or 1–2 ml into rats, abolished the trypanosomaemia, at least temporarily. Usually animals remained aparasitaemic for 4–8 days, but sometimes for as long as 18 days. When the organisms reappeared they multiplied as in an untreated animal.

b) Sometimes animals were completely cured by serum injections but only if this result was secured after 1 or 2 injections. Although the life of infected animals could be prolonged for several months by oft repeated injections, such animals were never cured.

c) Morphological changes, including deformation of the body-shape, towards a spherical form, were observed in trypanosomes from 4 hours after the serum injection.

d) Serum heated to 56 °C for 1 hour retained most of its activity, but not serum heated to 62 °C.

e) Sera differed in their activity, some being effective in doses as low as 0.1 ml.

LAVERAN & MESNIL (1902) considered that antibody formed against the human serum, neutralizing its active principle, might be responsible for the progressive loss of its efficacy. However, they could not show that the serum of a rat in which injections of human serum had become ineffective, had any neutralizing effect on the anti-trypanosomal efficacy of human serum. However, this and other conclusions need to be accepted with caution as the experimentation was not quantitated.

LAVERAN & MESNIL's (1902) work touched off a large amount of study in the following decades and the state of knowledge three decades later is admirably reviewed by YORKE, ADAMS & MURGATROYD (1930). Controversy had centred around many questions, such as whether or not human serum exerted any effect on trypanosomes *in vitro,* whether the effect was exerted by plasma as well as serum, whether the effect was associated with the cellular or with the liquid components of the blood, which *Trypanosoma* spp. were susceptible to the action of which host sera; and so on. Many of the conclusions were discordant as was almost inevitable in an era when the organismal populations used for experimentation were strains maintained over long periods in the laboratory by serial passage, not stabilates – populations of more fixed characteristics preserved by cryopreservation (LUMSDEN, in press).

YORKE et al. (1930), besides reviewing previous work, contributed a careful series of illuminating experiments based on a technique elaborated by them earlier (1929) by which trypanosomes could be maintained alive, and observed, for extended periods at 37 °C. They showed clearly that normal human serum, and also normal human

plasma, exerted a pronounced trypanocidal action *in vitro* at 37 °C on a number of strains of salivarian trypanosomes, as judged by microscopical observation of survival in suspensions. Such trypanocidal action could be demonstrated with some strains only in undiluted serum (e.g., with a recently isolated *T. rhodesiense*-like strain from Nigeria) with others to very high dilutions (e.g. to log dilutions $\bar{4}.3$–$\bar{5}.6$ for old laboratory strains of, respectively, *T. equiperdum* and *T. rhodesiense*). On the other hand, normal human serum had no appreciable trypanocidal effect on an old laboratory strain of *T. gambiense*.

These workers established various other relationships: that the process of trypanolysis by serum proceeds much more rapidly at 37 °C than at 15 °C, that heating of serum to 62 °C abolishes the trypanocidal property of the serum, and that this cannot be restored by adding complement (fresh rabbit serum), that some sheep sera (a component of the basic medium used for the experiments) inhibited the trypanocidal activity of the human serum, that the sera of different human individuals did not differ much in activity. They were puzzled by the difference in susceptibility to human serum shown by two man-infecting strains – *"T. gambiense"* and *"T. rhodesiense"* – the former unaffected, the latter rapidly killed, when exposed to human serum. They explained this by postulating that "serum-resistance" was in *T. gambiense* a fixed, and in *T. rhodesiense,* a labile character. They proposed, then, that *T. rhodesiense* epidemics in man are likely to be initiated by infections in unduly susceptible individuals – perhaps those with such pathological conditions as amoebic liver abscess or obstructive jaundice, or from some dietary deficiency, whose plasma loses its trypanocidal power.

FAIRBAIRN (1933a) carried the same approach a step further by adding the experiment of inoculating the trypanosome-human-serum mixtures into rats at the end of the 24-hour incubation period. By these means FAIRBAIRN (1933a) studied 64 strains of *T. (T.) b. rhodesiense* freshly isolated from human subjects in the Kibondo District of Tanganyika (Tanzania), in February and March, 1932. Although most of the strains were resistant to human serum as indicated by their surviving up to 24 hours incubation, some were not. FAIRBAIRN found, however, with some of those that appeared sensitive, in that trypanosomes appeared to be abolished from the suspension after 24 hours incubation with human serum, that these suspensions were still infective to rats on inoculation. FAIRBAIRN urged, therefore, that the criterion of sensitivity to human serum to be preferred was the inoculation of rats rather than simple observation of the number of organisms in the suspensions.

FAIRBAIRN (1933a) also tested each of 17 strains repeatedly in the course of laboratory serial passage. Most of the strains were tested at each serial passage and most up to passages 8 to 17. Of the 164 ex-

perimental inoculations involved in the study of these 17 strains 96 were "negative", i.e., the rats were not infected by the inoculated suspension. FAIRBAIRN comments with regard to this result that it shows "how very frequently, when a strain is carried on by syringe, the resistance to human serum disappears as early as the 2nd rat passage, to reappear in subsequent passages. It would therefore appear that, when any strain is isolated from man, game or tsetse, its resistance to serum should be tested in the 1st rat passage, as subsequent passages might give misleading results (unless a long and consecutive series of passages were tested)." In this comment, and elsewhere, FAIRBAIRN gives the impression that the loss of resistance to human serum is a progressive loss, according to the level of passage. Inspection of his tables, however, gives the impression that the distribution of the negative results through the passage series is rather at random, though some strains were found to produce more negative results than were some others. Not all the strains were tested at their first passage but among those that were, two were negative at that test and only became positive at later passages. It seems more likely to the writer that the inconsistency of the results with any given strain is due to the lack of standardization of the test. The suspensions used must have been of unmeasured and uncontrolled infectivity. Suspensions were prepared from rats at very varying times after their inoculation – from 8 to 33 days –, and the degree of dilution of the rat blood in making the trial suspension also varied widely – from $^1/_2$ to $^1/_{44}$. With such large variables in the experimental system irregularities in result of the sort observed are not surprising.

That these variables are the explanation of the irregularities of the results seem to be confirmed by another experiment of FAIRBAIRN's (1933b). One of the strains mentioned above (FAIRBAIRN, 1933a) – No. 26 – was inoculated into a human subject. The strain had been found to be infective to rats after incubation with human serum at passage 8. However, at passages 9, 10, 11, 12, 14, 15 and 16, rats were not infected by inoculation of the suspension and organisms were not seen in the suspension later than after 2 hours incubation (except 1 on one occasion at 6 hours). The inoculation into man was made at passage 19. Despite the indication from the 7 closely-preceding passages that the strain had become sensitive to human serum the human subject became infected, and so did the test rats at the same passage.

ADAMS (1933) quotes another case of the infection of a human subject by the inoculation of a "human-serum-sensitive" strain of *T. (T.) b. rhodesiense* and contributes some more experimental work on the same lines as that by YORKE, ADAMS & MURGATROYD (1930).

Since about that time, perhaps because of the irregularity of the results obtained and their lack of constant correspondence of its results with the characteristics of infectivity or non-infectivity to man, little

further study of the system has been conducted. Recently, however, RICKMAN & ROBSON (1970a, b) have introduced a basically similar test – the blood incubation infectivity (BII) test – which consists of incubating the test trypanosome strains, as represented by the blood of infected rats, with whole human blood for 5 hours at 37 °C and then testing the residual infectivity of the suspensions for rats by inoculation. The test has given generally consistent results with given strains: negative, non-infectivity to rats of the suspension after incubation, considered to correlate with *T. (T.) brucei brucei,* or positive, infectivity to rats of the suspension after incubation, considered to equate with *T. (T.) b. rhodesiense.*

The correspondence of negativity in the BII test with *T. (T.) b. brucei,* and positivity with *T. (T.) b. rhodesiense,* i.e., respectively non-infectivity and infectivity to man has been studied both by RICKMAN & ROBSON (1970b) and by TARGETT & WILSON (in preparation). Although a broad correspondence between these two characteristics clearly exists, there are inconvenient inconsistencies. Authors, in general, naturally are attracted to the immediately practically useful relation of the results of tests to the characteristic of infectivity to man. Broadly results of the BII test appear to be so related but such experimentation is essentially only narrowly based as there is a paucity of materials fully critically tested for infectivity to man by inoculation into human subjects. Although a certain number of strains are available which have reputedly been shown to be non-infective to man by experimental inoculation, few (or even none) of these experiments will withstand really critical examination. For instance, in such experimentation it is often found that such, usually considered essential, information as the provision of prior evidence of the susceptibility of the subject, the measurement of the infective potentiality of the inoculum administered, the performance of the material on repeated trial, and so on, are missing. Experimentation on man will always be subject to restrictions in its scope and difficult to bring up to fully critical levels. It seems most useful at present to concentrate on showing the BII test, a laboratory test without these drawbacks, is consistent, or can be made consistent, with any given trypanosome material. Thereafter, consideration may be given to the study of the concordance of the test with the characteristic of infectivity to man. Ethically it seems unjustified to inoculate into man any materials believed to be infective to man on the basis of positivity in the BII test; this concordance may be established by the test of strains isolated from naturally-infected human cases in the laboratory test. Only the opposite concordance would need to be established by inoculation into man. When a representative selection of trypanosome materials, clones not possibly heterogenous "strains" (LUMSDEN, in press), had been established as negative in the BII test (or in a more sophisti-

cated test developed from it), and consistently so, there would then appear to be a case for the test of these materials by human inoculation. Such trials should be fully controlled, as e.g., by the comparison of the antibody titres to trypanosome antigens, and of the serum and cerebrospinal fluid IgM content, before and after the experiment, by measurement of the infectivity of the inoculum given; and so on.

There is no doubt, however, that the phenomenon of differential susceptibility to some component of human blood, demonstrated by this work, is worthy of further study. That tests so uncontrolled as those of FAIRBAIRN (1933a, b) and of RICKMAN & ROBSON (1970) give such a degree of consistency of result is in itself encouraging. The matter requires, however, intensive further study to isolate and standardize the active principle at work, to study its mode of action and to develop a test consistent enough to use for epidemiological studies in the field.

Reacquisition of infectivity to the vertebrate host by forms from extra-vertebrate cycles of development

By forms in the cycle of development in Glossina

The cycle of development of *T. (T.) brucei* sspp. in the gut of the vector insect *Glossina* has been described repeatedly in textbooks and elsewhere. It is one of the articles of faith of protozoology that the developmental forms of the trypanosomes in the insect are not infective to the vertebrate and that infectivity to the vertebrate is only reacquired by the "metacyclic" trypomastigote forms which appear in the salivary glands. It is somewhat surprising, therefore, when one turns to such recent and authoritative textbooks as MULLIGAN (1970) and HOARE (1972) to find little or no experimental evidence quoted to support the belief. HOARE (in MULLIGAN, 1970, p. 14) states that "the intermediate host is incapable of transmitting the infection to the mammalian host until this cycle (from blood trypanosome through epimastigote stage to metatrypanosome) is completed". But this result might be due to the other forms not being accessible to be injected with the saliva, not necessarily to their intrinsic incapability to infect. Later (p. 44), the proof of the fact is ascribed to BRUCE and his colleagues (1911). Nor does the review of the subject by FAIRBAIRN & CULWICK (1950) cite specific experimental evidence to show that pre-metacyclic trypanosomes are never infective to the vertebrate host.

BRUCE and his colleagues (1911) allowed *Glossina palpalis* to feed on monkeys on 29 occasions, varying from 1 to 56 days after their infective blood meal. Up to day 20 no monkeys were infected by the bites of the flies and neither were any organisms seen in the salivary glands. On day

25, the monkey on which the fly (? flies) fed was not infected but organisms were seen in the salivary glands. This result was ascribed to an accident "such as this particular fly not biting the monkey, or the invasion of the salivary glands only taking place after the fly had bitten, or to the fact that the blood-type of trypanosome was not present". Thereafter monkeys became infected on 8 of 12 occasions and on each of these organisms, including "blood-types", were found in the salivary glands of the *G. palpalis*. On the four occasions on which monkeys were not infected, organisms were not seen in the salivary glands. These "blood-types" are described by BRUCE et al. (1911) as being very similar to the short forms found in the blood of the vertebrate host. They did not find them in parts of the fly other than the salivary glands and suggested that the coincidence of occurrence of these forms with renewed infectivity of the fly was more than a coincidence. They do not, however, in this paper (1911) report their results in detail; more than one fly appears to have been used on occasions and details of the numbers of flies found infected and of the character of the infection in each is not given. The conclusion regarding the significance of the "blood-type" is, essentially, based only on the one occasion, on day 25, on which although organisms occurred in the glands, the infection was not transmitted by bite. No experimental inoculations of suspensions of organisms from the glands appear to have been made.

According to ROBERTSON (1912) proventricular forms, when inoculated into clean monkeys, do not produce infection. WENYON (1926) states "as the glands are not infective when injected into animals till the final trypanosome forms appear, the latter are the actual infective metacyclic forms". On the other hand, some recent evidence indicates the possibility that proventricular forms may sometimes be infective. WARD & BELL (1971), working with *T. (T.) brucei* and *Glossina morsitans* obtained 2.2 % of 138 flies with salivary gland infections in laboratory experiments. However, when these flies were fed individually on mice the infection rate was five times higher. They considered it possible that this was due to regurgitation of proventricular forms; if this is confirmed then it indicates that infectivity is not confined to the trypomastigote forms appearing in the salivary glands after the epimastigote multiplication there (HOARE, 1972).

Perhaps this matter would be profitably reexamined experimentally taking into account the need to establish the suitability of the diluents used to prepare suspensions of developmental forms for inoculation into test animals as discussed above in relation to blood forms. FAIRBAIRN & CULWICK (1950) refer to "a number of flies ... which only extruded proventricular forms".

By forms in in vitro *culture*

The development of the salivarian trypanosomes *in vitro* in blood-agar cultures has generally been considered to follow part of the cycle of development of the organisms in the insect vector, though stopping short of the production of infective forms (HOARE, 1972). Nevertheless, infectivity to the vertebrate host has sometimes been observed in such cultures and valuable studies have been made of this occurrence, particularly by GEIGY and his collaborators. It is interesting to consider these.

WEINMAN (1957), using a human-blood-agar culture medium described by him earlier (1946), noticed cultures of *T. (T.) b. rhodesiense* which were infective to mice. Full details of the experimentation are not given but it appears that organisms were maintained in culture over a period of 30 days by 3 passages and then passage was maintained in two separate lines, one using the same culture medium alone and the other the medium with the addition of the sugar trehalose. The concentration of this sugar in the medium is unstated although GEIGY, HUBER, WEINMAN & WYATT (1959) refer to the amounts as "minimal". Mice were inoculated from both culture lines. Mice became infected with inocula from the trehalose-containing cultures on two occasions at 51 and 69 days *in vitro* maintenance (i.e., after 21 and 39 days maintenance in the trehalose-containing medium). Mice inoculated with corresponding inocula from the other, non-trehalose-containing cultures, did not become infected.

WEINMAN's (1957) work led to a study being made of the content of trehalose in *Glossina morsitans* (whole flies, intestines and salivary glands) (GEIGY, HUBER, WEINMAN & WYATT, 1959). Trehalose is known to occur in considerable amounts in the blood of insects but not in mammalian blood (GEIGY et al., 1959). However, this study was inconclusive in that, although trehalose was recognized in *Glossina morsitans* and *G. brevipalpis,* the trehalose content of whole flies was found to be trivial as compared to that of a non-blood-sucking muscid – *Sarcophaga bullata.* Also, the trehalose content of salivary glands was found to be extremely low, if it occurred there at all; only in the intestine were large quantities found. GEIGY et al. (1959) suggested, therefore, that if trehalose was influential as regards the acquisition of infectivity, the effect was likely to take place in the intestine, rather than in the salivary glands, of *Glossina.*

Various authors followed up the line of thought of the determinant effect of these sugars. WYSS-HUBER, STRIEBEL, WEISS & GEIGY (1961) found four saccharides – trehalose, saccharose, glucose and arabinose – in *Glossina morsitans* and *G. brevipalpis* by means of paper chromatography. GEIGY & KAUFFMANN (1964) found 1 of 8 cultures of *T. (T.)*

rhodesiense, treated with arabinose, became infective for mice but did not succeed with cultures treated with trehalose. The prepatent period in the mice becoming infected was very long, about 40 days. Similarly, 3 of 12 cultures treated with inositole became infective for mice; prepatent periods were of the same order, about 42 days. Results with *T. (T.) brucei* were similar.

AMREIN, GEIGY & KAUFFMANN (1965) cited other evidence to the effect that inositol, arabinose, alanine, taurine and trehalose occurred in "biologically interesting concentrations" in *Glossina palpalis* and *G. morsitans,* mainly in the salivary glands and saliva. They found that in cultures of *Glossina* tissues in tissue culture medium (TC 199) *T. (T.) brucei* organisms showed tropisms for muscle, midgut and salivary glands, not for malpighian tubules, gonads or fat cells. The attachment of organisms to preferred tissues declined after about 7 days and, despite the association with tissues none of the organism-plus-tissue cultures tested between day 3 and day 7, were infective to mice by inoculation. Further trials were carried out, adding to the culture medium extracts of salivary glands of *Glossina morsitans,* and alanine, taurine, arabinose and inositol. Occasional cultures were infective to mice on inoculation but were sporadically distributed in the experiment, some occurring among controls, some in cultures with arabinose and several in cultures with inositol.

Further experiments (AMREIN, GEIGY & KAUFFMANN, 1965) gave similar results. One series of 60 control and 60 experimental cultures (with addition of alanine, arabinose, inositol and taurine) were uniformly non-infective. In a second similar series 15 of the 60 control cultures were infective and 1 of the 60 experimental ones. All the infective episodes related to cultures tested 11–23 days after inoculation. According to the diagram given, cultures tested outside these limits were not infective. A further series of 80 controls (i.e., without addition of these sugars) were tested for infectivity to mice, 10 each day from day 17 to day 24. In all 25 cultures were infective, the largest proportion, 9 out of 10 tested, on day 18; however, there were wide variations from day to day, e.g., on day 20, none was infective.

Since the occurrence of infectivity sporadically both in control and in experimental cultures discounted the significance of these sugars in determining the genesis of infectivity, these authors then turned their attention to other variables in the system – differences in the donors providing the blood used for the preparation of the medium and differences in the age of the culture medium at the time of its being inoculated. Five batches of medium were prepared, differing only as regards the donor of the human blood used in their preparation. Culture vessels containing medium were stored at 4 °C and groups of 10 of each batch were inoculated after 4, 21, and 62 days storage at 4 °C. Cultures were

tested for infectivity by being inoculated into mice on day 18. Results were complex but may be summarized:

Donor No. 3. None of the 30 cultures prepared from the blood of this donor became infective to mice.

Donor No. 5. Only 1 of the 30 cultures prepared from the blood of this donor became infective, 1 of the 10 stored for 63 days.

Donors Nos. 2, 4. Cultures prepared from the blood of these donors behaved similarly to one another; 2 to 5 out of each 10 cultures became infective, after all three storage periods. Prepatent periods in inoculated mice were long, 2–6 weeks.

Donor No. 1. None of the 20 cultures prepared from the blood of this donor and stored for 3, or for 21, days became infective for mice. However, all 10 of those stored for 62 days did so and the prepatent periods in inoculated mice were short, 1–2 weeks.

These results are of great interest and appear to indicate:

a) That the donor blood used in the medium is a determinant of the appearance of infectivity.

b) That substances in the medium inhibiting the appearance of infectivity may be degraded on long term storage of the medium.

c) That infectivity may be gained in cultures about day 10 and lost about day 23 after inoculation.

It would be of interest to know if this is a general characteristic of *T. (T.) brucei* organisms; unfortunately the particular stabilates used for each experiment are not designated. It would be of interest to compare the behaviour of the strain in mice before and after its sojourn in culture and regaining of infectivity. The long prepatent periods found after culture in media made up from the blood of Donors No. 2 and No. 4 are interesting. One would like to know if similar long prepatent periods occur in mice with minimal infective doses of blood-forms or whether this is a new characteristic, perhaps induced by the culture passage. One should perhaps not place too much emphasis at this stage on the differences found between culture medium of different ages as, although the media were prepared at the same time, the inocula used, being administered at different times, may have differed. And it would be of value to examine and compare the morphology of the organisms occurring and administered to the mice. The study is, however, a most interesting and thought-stimulating one and the same approach should be applied to a wide range of trypanosome species and materials.

AMREIN & HANNEMAN (1969) investigated a wide range of animal bloods, as constituents of the medium, with regard to their influence on the reacquisition of infectivity to mice of cultures of *T. (T.) b. brucei.* Usually some 18, but sometimes as many as 40, cultures were tested for each batch of medium, the batches differing in the identity of the

donor of the blood component, or in details of the treatment of the blood. Most cultures were inoculated after being stored at 4 °C for at least 17 days. Cultures were tested for infectivity to mice between days 14 and 22 after inoculation, mostly on days 17 to 19. Blood from 2 of 3 horses and 1 of 2 pigs generated infective cultures but only very few. For the horses only 1 out of 30 in 1 case became infected, and only 2 out of 34 in the other; for the pig only 1 of 40 cultures became infective. Blood from 4 rabbits was tested and 2 generated infective cultures, 1 of 18 in one case and 6 of 18 in the other. None of 36 cultures prepared with blood from the 2 other rabbits became infective and neither did any of 17 cultures prepared from a pool of the blood of "infectivity generating and non-infectivity generating" rabbits. Three of 32 cultures prepared with blood of *Giraffa reticulata* generated infectivity. Quite extensive studies were made on cultures prepared with cattle blood. 375 cultures, prepared from the blood of precolostral and other calves and of cows (10 animals in all) were tested. Only one of these animals failed to generate infectivity in any culture; this was a "newborn" calf for which none of 36 cultures were infective. Results with the other animals were very variable ranging from one cow whose blood generated only 1 infective culture out of 48 to a precolostral calf and a "heifer" for which the infective cultures were respectively 27 of 36 (72 %) and 22 of 30 (71 %). These authors emphasize the importance of the individual donor providing blood for the preparation of the medium in determining the generation of infectivity in the cultures but were sensible of the need, still, to study the long term performance of individual donors in this respect – to determine if the matter is a permanent character of the individual or variable depending perhaps on some physiological condition.

GRAINGE (1961) records another instance of the recovery of infectivity to the mammal host by trypanosomes in culture. He worked with *Trypanosoma (Trypanozoon) brucei rhodesiense,* using a strain originally isolated from *Tragelaphus scriptus* by HEISCH, MCMAHON & MANSON-BAHR (1958), using a culture medium essentially similar to that described by WEINMAN (1946). The first cultures were infected with heparinized cardiac blood of infected guinea pigs and the strain was maintained in culture for some 9 months, involving 17 passages. Rabbit blood was substituted for human in the culture medium for the passage 2 cultures and thereafter bovine blood was used. No attempts were made to test infectivity to mice until passage 15, when 3 mice were each inoculated i.p. with 0.25 ml of culture fluid. These mice became parasitaemic on day 10 after inoculation, the trypanosomes being pleomorphic with a high proportion of short and posteronucleated forms. Attempts to infect mice with organisms from passages 16 and 17 failed. Considering this episode GRAINGE (personal communication) considered

that infectivity was perhaps related to age of culture; the infective cultures were old cultures which were kept in reserve and used for a second passage when the first passage from them in culture had failed to grow. They were, thus, some 20–28 days old at passage as compared to the usual age of just over 14 days.

Summarizing the results of this line of research it can be said that there are two main factors which seem to be of determinant importance – a component existing in some of the bloods used to prepare the culture medium, and the age of the culture at the time of infectivity trial. More work, however, still requires to be done on this obviously fertile field of research fully to define the factors operative and their mode of action.

Conclusion

It was, of course, a pleasure to be invited by Dr. Freyvogel to contribute a paper to the seventieth birthday "Festschrift" for so illustrious a contributor to the field of tropical medicine as Professor Rudolf Geigy. The pleasure was accentuated as one progressed in thinking of the subject selected, finding it so germane to the development of thought in relation to experimental and epidemiological aspects of African trypanosomiasis at the present time. It was particularly stimulating to have the opportunity to study in greater depth the field to which Professor Geigy has so conspicuously contributed – the factors determining the generation of infectivity in *in vitro* cultures.

There was perhaps, also, in a document of this sort, an opportunity to be less formal, more sceptical and speculative, than is usual in review articles. If the speculations and disbeliefs, even heresies, included in the present review usefully stimulate thought then the credit should attach to Professor Geigy for attracting a "Festschrift" and to Dr. Freyvogel for inviting me to contribute to it.

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