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FURTHER STUDIES ON THE LYTIC AND NEUTRALIZING ACTION OF HUMAN SERUM ON *T. BRUCEI*

by

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Summary — The influence of several factors on the process of lysis and neutralization of *T. brucei* in human serum has been studied. Some of the results may contribute to a better methodology of serum incubation infectivity tests.

The trypanocidal activity of human serum was found to be not dependant on the intact complement system and not absorbable.

Poor activity is present in some newborn sera.

Resistance of *T. brucei* to the action of human serum could not be induced.

KEYWORDS : Trypanosomiasis, African; Trypanosoma brucei; Trypanosoma rhodesiense; Serums, Human; Neutralization Tests; Blood Incubation Infectivity Test; Trypanolysis.

Introduction

The only well defined criterion for distinguishing *T. brucei* from *T. rhodesiense* is its unability to infect man. It is highly probable that this difference depends upon the lytic and neutralizing action, exerted by human serum on the former but not on the latter. This phenomenon was repeatedly reported by earlier authors and its practical implications were revived some decennia later by the blood incubation infectivity test, proposed by Rickman and Robson (1970) for distinguishing *T. brucei* from *T. rhodesiense* without recourse to human volunteers.

However, some scepticism, whether or not justified, seems to persist in relation to the validity of such test. The controversy on this subject is favoured by some equivocal results and by the fact that many factors underlying the mechanism of lysis and neutralization of *T. brucei* in human serum remain obscure. Neither the trypanosome substrate(s), nor the serum factor(s) acting on it have been characterized. According to Awan (1971), the cellular sediment of human blood is not responsible for the trypanocide activity. Recently, by using indirect methods, Aaronovitch and Terry (1972) came to the conclusion that the trypanolytic factor is a serum IgM immunoglobulin.

It was toward obtaining better insight into practical and fundamental aspects of the lytic and neutralizing process that the present studies were undertaken. For the sake of completeness, some already well established conceptions were critically reexamined.

Materials and methods

A strain of *T. brucei* (EATRO, 1125), proven to be not infective to man, was used throughout experiments. From this strain tens of variant populations, isolated from infected rabbits, were available as stabilates. A stabilate of *T. rhodesiense,* recently prepared by inoculation of mice with patient blood, served for control purposes in all instances where irrelevant trypanocidal activity of reagents was to be excluded.

Suitable quantities of each trypanosome population were prepared by infection of mice or rats and collection of parasitized blood whitin 3 days after inoculation. Trypanosomes from long lasting parasitaemia in mice and in rats had sometimes been found to be particularly fragile. Whole parasitized blood mixed with a minimal amount of heparine was used for common incubation tests; in some instances pure trypanosome concentrates, obtained by column separation (Lanham, 1968), were used.

Freshly collected human serum was the source of trypanocidal activity. Normal guinea-pig serum, being an excellent medium for each trypanosome material studied, served for diluting and washing procedures. In some experiments where preexisting low grade activity or a relative loss of activity, due to any destructive process, were to be detected, a titration procedure was used.

Infectivity tests were done in mice which were highly susceptible to infection with minor numbers of intact trypanosomes. For reference tests, 10^6 trypanosomes per ml of serum were incubated at 37 °C for 5 hours; 0.3 ml volumes of the incubated suspension were then intraperitoneally inoculated into a series of 3 mice and subsequent infections traced by regular wet smearing of tail blood for at least 15 days. Mortality of mice showing no sign of parasitaemia was low and did not complicate the interpretation of the test results.

At phase contrast microscopy, *T. brucei* trypanosomes, incubated in human serum, firstly revealed a typical modification of the optical properties of the nuclear area and then became progressively lysed. Impaired trypanosomes however might remain motile for a long period and mobility in no respect was a measure for infectivity. On the other side, trypanosomes with altered refractive aspect of the nucleus were found to be no longer infective. This fact facilitated the orientation of some experiments. Nevertheless, in view of the possibility that even a single intact trypanosome might give rise to infection, the incubated suspension was inoculated whenever complete information on the infectivity of a population was needed.

Decomplementation of human serum was obtained by adding E. D. T. A. (0.01 M), by incubation with Zymosan (*) (20 mg/ml) or with purified Cobra

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(*) Nutritional Biochemicals Corporation.

venom factor (*) (25 U/ml) at 37 °C for 1 hour, or by incubation with Hydrazine (0.015m) at 37 °C for 90 minutes. The effectiveness of each procedure was verified by the absence of haemolytic complement activity. For Properdin depletion, serum was incubated with Zymosan (20 mg/ml) at 12 °C for 1 hour.

Results

Stability

Freshly collected human adult sera had neutralizing activity up to dilutions ranging from 1/10 to 1/40.

The trypanocidal activity of human serum is rather labile. Storage of the serum at 6 °C resulted in progressive decrease of activity and many samples became inactive after one week. Storage at − 20 °C and especially lyophilization ensured conservation of the activity with minor loss of titer. Heating of fresh serum at 56 °C for 30 minutes had little influence upon its neutralizing titer, but marked decrease of activity was caused by heating at 60 °C for 30 minutes and equally by sonification for 1 hour.

The role of complement and properdin

Decomplementation or properdin depletion had no notable effect upon the lytic and neutralizing activity. These findings and the minor influence of heat-inactivation at 56 °C, indicate that the classical complement system is not involved in the trypanocide action of human serum. This is in contrast to the characteristics of the analogous lytic activity against culture forms of Leishmania (Schmunis and Herman, 1970).

Incidence of the activity in adults and infants

The question whether the trypanocidal serum activity might be deficient in some individuals has been examined in a double series of experiments.

A first approach was made by the qualitative testing of a large number of adult sera from very heterogeneous origin; all sera were found to be quite effective at the undiluted state, thus confirming earlier reports on this subject (Lester, 1933). However, it should be noted that some of the earliest investigators found relative low trypanocidal activity in some patients with liver disease. For detecting such quantitative differences titration experiments would be required.

In a second series of attempts, particular interest has been given to the situation in newborns and infants. Rosenthal and Kleemann (1915) reported that the serum of a six months fœtus had no, and a cord serum sample only low trypanocidal activity, as estimated by mouse protection tests. In relation to infantile Leishmaniasis, it has been suggested (Taub,

(*) Cordis Laboratories.

1956) that *L. infantum* can establish itself more readily in an infant than in an adult because of the absence of a serum factor, lytic for leptomonads of Leishmania in the former and its presence in the latter, this hypothesis however being discussed by Ben Rachid (1967). Furthermore, in view of the observations of Aaronovitch and Terry (1972), it was thought that the low serum IgM level in newborns might have some consequences. In total, 23 cord sera, 4 corresponding venous samples, 3 venous samples from other newborns and 13 sera from infants between 4 and 24 months of age have been titrated on their neutralizing activity and on their IgM level. IgM levels in venous and cord serum from newborns were found to be 2 to 16 times lower than the normal mean adult level. All newborn sera had trypanolytic activity but in many samples this was markedly lower than that observed in adult serum. The undiluted sera of 7 out of 26 newborns did not neutralize all incubated *T. brucei;* a minority of trypanosomes remained intact and inoculated mice became infected. The mean neutralizing titer of the newborn sera was lower than 1:5. In the group of 13 infants, all serum IgM levels were comparable to the normal adult level. Only one undiluted serum sample, derived from a 5 months old baby, failed to neutralize all trypanosomes. Upon analysis of all individual results, no clearbut correlation between serum IgM level and neutralizing titer could be detected.

The question remains unanswered whether the low lytic and neutralizing titer of some infant sera may have any real importation in natural conditions.

Attempts for adaptation

Considering the accentuated ability of trypanosomes for adaptive variation upon contact with drugs or antibodies, a series of attempts were undertaken in order to induce resistance of *T. brucei* to human serum.

Previously it had been observed that trypanosomes, appearing in mice after inoculation with trypanosomes, incubated in uncompletely neutralizing undiluted newborn or diluted adult serum, remained fully susceptible to the action of undiluted adult serum. The most perseverant attempts for inducing progressive adaptation were based on the following systems. Varying concentrations of trypanosomes, combined with varying serum dilutions were incubated for varying periods, and suspensions, whether or not after washing, were inoculated into mice. From the resulting parasitaemias, the most promising trypanosome population was selected and the system continued. A somewhat analogous system was based upon the intraperitoneal administration of different amounts of human serum into parasitized mice, thus inducing relaps parasitaemia; some infected mice were repeatedly injected with increasing volumes of serum. By no method trypanosomes could be obtained having significantly higher resistance.

This observation should be taken in account in interpreting earlier reports concerning so-called human serum resistant *T. brucei*, obtained in mice, but which had not been tested *in vitro* (Lester, 1932).

Antibodies against many human serum proteins could be detected by immunoelectrophoresis in the serum from mice, injected with human serum. Consequently, the possible interference of mouse antibodies against the trypanocidal factor should perhaps better be taken into account in further attempts to obtain *T. brucei* types resistant to the action of human serum. Anyway, such resistance has never been convincingly demonstrated, nor induced.

A quite remarkable observation has been reported by Soltys and Woo (1970); on comparing the biological characters of two substrains derived from the same original strain of *T. brucei,* they found that the so-called rabbit-adapted strain, in contrast to the mouse-adapted one, was not lysed by human plasma. Unfortunately, infectivity tests have not been done, but, supposing that no infiltration of other trypanosome types had taken place during the numerous mechanical passages in rabbits and mice, the observation remains difficult to explain.

The present investigation did not reveal any difference in susceptibility to the action of human serum between different antigenic variant populations. It should be noted however that repeated passage in rabbits with terminal variants was not systematically continued.

Effect of anti-trypanosoma sera

The possible connection between the antigenic composition of *T. brucei* and its susceptibility to human serum was examined by indirect methods too. Natural antibacterial effect of normal human and cattle serum against *S. typhimurium* has been shown to be inhibited by variant specific antibacterial antibodies (Normann, B. E., 1972). In an analogous way, the natural anti-*Candida albicans* activity of normal rabbit serum is inhibited by specific antibodies to this parasite (Smith and Louria, 1972). Similar inhibitory effect against the trypanolytic and neutralizing activity of human serum could not be demonstrated with hyperimmune antisera (Le Ray *et al.,* 1972) directed against common trypanosome antigens or against variant specific antigens.

Concentration of trypanosomes — Incubation time — Reversibility

Enormous concentrations of *T. brucei* may be lysed and neutralized by undiluted adult serum, as well *in vitro* as *in vivo.* In *in vitro* experiments, using column-isolated trypanosomes, it was found that a pure trypanosome concentrate may be completely neutralized by its fivefold volume of serum. Similarly, mice with heavy parasitaemia often are completely cleared from trypanosomes by injecting as less as a few tenths of a ml of serum.

Non incubated suspensions, containing 10^6 of trypanosomes per ml were seldom infective to mice, similar suspensions never were infective after 1 or 2 hours of incubation. Trypanosomes, not or shortly incubated, still have a quite normal aspect and their potential infectivity could easily be demonstrated by washing them free from human serum. Consequently, the inability of the analogous unwashed trypanosomes to infect must be explained by the after-effects of the serum in the mouse. After 3 hours of incubation of suspensions, containing 10^6 organisms, the infectivity of all trypanosomes was practically always irreversibly lost.

For neutralization of suspensions, containing many millions of organisms per ml, a prolonged incubation time was needed. Briefly, the minimal incubation time needed for complete neutralization of an unwashed suspension was found to be proportional to the original concentration of trypanosomes. Similarly, the higher this concentration, the longer the neutralization procedure was reversible by washing, whether or not for a minority of organisms.

Incubation temperature

Comparative analysis of the cinetics of the lytic reaction at various temperatures indicated that its rate is directly proportional to the temperature. Incubation of *T. brucei* in undiluted serum at temperatures between 0 ° and 5 °C for 5 hours and even much longer, had not the slightest effect upon the viability nor upon the microscopic aspect of the trypanosomes. Such suspensions however were seldom infective to mice; this result probably being of the same nature as that obtained with non incubated suspensions. Incubation at 37 °C, subsequent to incubation at low temperature did not reveal a particular high trypanocidal reaction rate. Moreover, trypanosome suspensions, incubated at low temperature, regained their infectivity after washing with guinea-pig serum.

Absorption experiments

Some of the above mentioned results suggest that the trypanocidal factor is not firmly bound to its substrate, independantly of temperature. This assumption was further examined by absorption tests with trypanosome concentrates, incubated with their fivefold volume of serum at 0° — 5 °C and at 37 °C; for 1 and 4 hours. Serum samples were then separated and titrated on the remaining activity. No significant decrease of activity could be detected in any instance.

Immunofluorescence

Several attempts were made for demonstrating IgM on incubated trypanosomes by indirect immunofluorescence, using monospecific conjugate, but never succeeded.

Discussion

The trypanolytic factor in human serum, probably being identical with the neutralizing one, was found to be not complement-dependant in classical terms and not absorbable. Both of these characteristics are unusual for a conventional lytic antibody. The characteristics of the trypanocidal reaction seem to resemble those of an enzymatic process.

Further investigations should be undertaken to find out whether a particular IgM may fulfill these criteria. Until further notice, the possibility might be faced that the trypanocidal factor is a substance of high molecular weight which is not detected by a common precipitating antiserum, the latter however possibly containing inhibitory antibodies against it.

Because of its unabsorbability, precise identification of the factor may be particularly difficult. Even more complicated may be the identification of the corresponding trypanosome substrate, although electron microscopic observations might be helpful.

For the present time, important information can be gained from an empirical incubation infectivity test, the validity of which would be increased by the introduction of better standardized methods. The use of high titered human serum, whether or not lyophilized, would like to be more convenient than the use of plasma or whole blood. For most purposes, mice instead of rats seem to be usable. The concentration of incubated trypanosomes should not be exaggerated, especially in case of a relative short incubation time. For avoiding all needless complications, a concentration of one million of trypanosomes per ml of serum, easy to express in a number of organisms per microscopic field, and an incubation at 37 °C for 5 hours, seem to be suitable. In such test conditions, lysis of susceptible trypanosomes is nearly completed and their neutralization irreversibly established, thus permitting injection of any desired volume of suspension with minimal risk of inducing irrelevant parasitaemia.

Still greater safety might be obtained by retesting trypanosomes of any parasitaemia appearing in the inoculated animals, probably the best after previous passage in order to avoid possible interference of host antibodies. The latter method would reveal the real nature of trypanosomes from weak transient parasitaemias in test animals and from those appearing after uncommonly long prepatent periods, as exceptionally observed by Rickman and Robson (1970).

Résumé — **Nouvelles recherches sur le phénomène de lyse et de neutralisation de *T. brucei* dans du sérum humain.**

L'influence de plusieurs facteurs sur le phénomène de lyse et de neutralisation de *T. brucei* dans du sérum humain a été étudiée. Certains des résultats peuvent contribuer à une méthode plus efficace pour tester l'infectivité de trypanosomes après incubation préalable dans du sérum humain.

L'activité trypanocide du sérum se manifestait également en absence du système intact du complément et elle n'était pas éliminable par absorption.

Une faible activité a été trouvée dans certains sérums de nouveau-nés.

Une résistance de *T. brucei* envers l'action du sérum humain n'a pu être induite.

Samenvatting — **Verdere studie nopens het proces van lyse en neutralisatie van *T. brucei* in mensenserum.**

De invloed van verschillende faktoren op het proces van lyse en neutralisatie van *T. brucei* in mensenserum werd nagegaan. Sommige resultaten kunnen bijdragen tot een meer doeltreffende methode voor serum inkubatie infektiviteitstesten.

De trypanocide aktiviteit van menselijk serum bleek niet afhankelijk van het intakt complementsysteem en kon niet verwijderd worden door absorptie.

Een lage aktiviteit werd aangetroffen in sommige sera van pasgeborenen.

Weerstandigheid van *T. brucei* tegenover de werking van mensenserum kon niet opgewekt worden.

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