***Trypanosoma (Schizotrypanum) dionisii*:
Influence of Mouse Peritoneal Macrophages and Calf
Sera on Extracellular Growth *in vitro* at 37 °C**

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Macrophages and certain batches of sera were essential for extracellular multiplication of *Trypanosoma dionisii* *in vitro* in medium 199 with 20 % (v/v) calf serum at 37 °C. In mixtures of 'good' and 'bad' batches of sera, multiplication increased as the proportion of the former was increased. Mixtures of 'bad' and 'intermediate' sera permitted virtually no growth. Phagocytosis of parasites by macrophages was unaffected by different batches of sera, though reduced in medium 199 alone. Replacement of supernatant medium by medium containing 'bad' serum reduced the macrophage infection rate no more than did transfer to medium containing 'good' serum. Daily addition of medium conditioned by prior contact with macrophages *in vitro* to cultures of trypanosomes without macrophages resulted in growth at least as good as that in the presence of macrophages. Extracellular replication in medium 199 at 37 °C apparently required at least two factors: 'M factor' provided by macrophages, but not required at 28 °C; and 'S+ factor' present in some batches of calf serum, essential also at 28 °C but not required by intracellular parasites. Some sera appeared to contain an inhibitory 'S– factor'.

INTRODUCTION

Trypanosoma (Schizotrypanum) dionisii Bettencourt & França, 1905 is a parasite of European Microchiroptera (insectivorous bats) which can be readily cultivated *in vitro* at 28 °C and which enters and multiplies within mouse peritoneal macrophages in culture at 37 °C (Baker *et al.*, 1972). Under certain conditions, reported here, extracellular multiplication also occurs at 37 °C.

METHODS

Isolation and maintenance (in medium L4NHS) of stock p3 of *Trypanosoma dionisii* and the preparation from it of a clone (no. 3) have been documented by Baker, Liston & Selden (1976). ('Stock' denotes a population derived by serial passage from a primary isolation without implying homogeneity or characterization.) This clone was used throughout the present work.

All experimental cultures consisted of 1 ml medium 199 (Wellcome) with 20 mM-HEPES buffer (Hopkin & Williams) at pH 7.2 (Baker & Liston, 1978) plus, where indicated, heat-inactivated (56 °C, 30 min) serum, in miniature Carrel flasks (Baker *et al.*, 1972). When required, peritoneal macrophages from unstimulated male Parke's (Surgically Derived) mice [strain P(SD), Animal Virus Research Institute, Pirbright, Surrey; see Baker *et al.*, 1972] were added and flasks were inoculated 1 d later with 5×10^5 washed flagellates (mainly epimastigotes, washed once in phosphate-buffered saline, 650 g, 10 min) from 4 to 7 d cultures of *T. dionisii* in L4NHS medium at 28 °C.

The bovine sera used are shown in Table 1; serum concentrations are expressed as percentage by volume.

In the experiment to determine the extent of phagocytosis, macrophage cultures were set up in medium containing no serum, 20 % serum C or 20 % serum A. One day later 5×10^5 trypanosomes, either live or

Table 1. *Origin of batches of sera**

Serum	Batch no.	Designation	Supplier	Catalogue no.
A	Z0507 }	Calf serum no. 1	Wellcome Reagents, Beckenham, BR3 3BS	CS 07
B	Z0766 }			
C	K745603	Calf serum	Gibco Bio-Cult, Paisley, PA3 4EP	BCL-002c†
D	446027 }	New-born calf serum	Flow Laboratories, Irvine, KA12 8NB	29-122‡
E	455056 }			

* A, B and D were supplied heat-inactivated (56 °C, 30 min); C and E were heat-inactivated similarly in our laboratory.

† In 1975 catalogue; number and designation subsequently changed. Collected from calves less than 10-d-old.

‡ With suffix depending on volume.

killed by immersion for 4 min in a water bath at 50 °C, were added to pairs of flasks containing one of the three media, and the cultures were fixed after 1 h incubation at 28 or 37 °C [see Baker & Liston (1978) for details of handling, fixing, staining and enumeration]. Living extracellular trypanosomes were counted with the aid of a haemocytometer. Differences between means were considered significant if their approximate 95 % confidence limits [twice the standard error of the mean (S.E.M.)] did not overlap.

Conditioned medium containing 20 % serum B was removed from cultures of macrophages (without trypanosomes) after 1, 2, 3 or 4 d at 37 °C. On day 1, this medium was used to suspend trypanosome inocula (5×10^5 ml⁻¹) for each of two flasks without macrophages. One to 3 d later the trypanosomes were harvested by centrifugation (650 g, 10 min) without washing, resuspended in conditioned medium and replaced in fresh flasks without macrophages. Control cultures containing either macrophages and trypanosomes, or trypanosomes alone, in similar but unconditioned medium were also set up on day 1. Daily trypanosome counts were made on all supernatant media. On day 3 or 4 the cultures containing macrophages and trypanosomes were fixed and the macrophage infection rates were determined.

RESULTS

Effect of macrophages

In two experiments, in the absence of macrophages 20 % serum A permitted limited multiplication (maximum sixfold after 5 d) at 28 °C whereas at 37 °C with this serum or serum B (10 or 20 %), there was (in four experiments) less multiplication (maximum fourfold) or none at all. In the presence of macrophages, multiplication in supernatant medium containing 20 % serum A or 10 to 20 % serum B at 37 °C occurred in four experiments (after a lag period of 1 to 2 d in serum A) to give a maximum parasite population of 2×10^6 to 8×10^6 ml⁻¹ after 3 to 6 d, with subsequent decline (Figs 1 and 2). With serum B, the maximum was lower (1.6×10^6 to 5×10^6 ml⁻¹) in medium containing 10 % serum than it was when 20 % serum was present (two experiments).

Effect of sera

Sera C and E did not support growth at 28 or 37 °C, whether or not macrophages were present (Fig. 3). Parasite numbers declined after an initial 2 to 5 d plateau at 28 °C; they declined more rapidly at 37 °C, especially in medium overlying a macrophage monolayer. Three experiments at 37 °C comparing media containing serum E at 10 or 20 % showed little difference with, if anything, slightly more rapid decline in the higher concentration when macrophages were present. In two of these experiments, limited multiplication occurred during the first 24 h.

Serum D allowed four- to sixfold multiplication at 37 °C during the first 4 to 5 d, in two experiments, in the presence or absence of macrophages (Fig. 4). There was no significant difference between the results for media containing 10 and 20 % of this serum.

Figs 1 to 7. Effect of different cultural conditions on numbers of extracellular *Trypanosoma dionisii* (stock p3, clone 3) in medium 199 (see Methods), plotted semilogarithmically against time. Each point is the mean of two cultures (three in Fig. 1); vertical lines indicate $\pm$ twice the standard errors of the means (where these are greater than the diameter of the symbol). Only representative experiments are illustrated.

Fig. 1. Serum A (20%): $\circ$, 37 °C, without macrophages; $\bullet$, 28 °C, without macrophages; $\triangle$, 37 °C, with macrophages.

Fig. 2. Serum B (10%), 37 °C: $\circ$, without macrophages; $\bullet$, with macrophages.

Fig. 3. Serum E (20%): symbols as in Fig. 1.

Fig. 4. Serum D (20%), 37 °C; symbols as in Fig. 2.

Figs 5 and 6. Serum E plus serum D (Fig. 5) or serum B (Fig. 6), with macrophages, 37 °C: $\bullet$, serum E 20%, serum B or D 0%; $\circ$, E 15%, B or D 5%; $\triangle$, E 10%, B or D 10%; ∇ , E 5%, B or D 15%; $\blacksquare$, E 0%, B or D 20%.

Fig. 7. Serum B (20%), 37 °C: $\bullet$, with macrophages; $\circ$, without macrophages, unconditioned medium; $\blacksquare$, without macrophages, medium conditioned by prior exposure to macrophages.

Table 2. *Proportions of mouse peritoneal macrophages containing live or heat-killed (50 °C, 4 min) Trypanosoma dionisii after 1 h exposure at 37 °C in vitro in various media*

Serum (20 %, v/v)	Inoculum	Parasitized macrophages* (% ± S.E.M.)
None	Live	14.3 ± 3.2
	Killed	32.7 ± 10.2
A	Live	52.8 ± 5.9
	Killed	52.5 ± 11.8
C	Live	55.7 ± 7.8
	Killed	51.4 ± 4.8

* Means of two cultures.

Mixtures of sera

Four experiments were performed using mixtures of sera B, D and E which had been found to support good, limited and no growth, respectively, of *T. dionisii* at 37 °C in the presence of macrophages. Serum B also allowed some multiplication at 37 °C without macrophages.

In the presence of macrophages, all mixtures of sera D and E, and 20 % serum E alone, supported virtually no growth; 20 % serum D alone, however, allowed four- to sixfold multiplication (Fig. 5). In contrast, mixtures of sera B and E supported progressively more growth as the proportion of the former (B) was increased from 0 to 20 %; least growth occurred in 20 % serum E alone (Fig. 6).

In the absence of macrophages, less growth occurred in all mixtures and no reproducible, statistically significant difference was detectable between the different mixtures.

Effect on infection rate of macrophages

The above results were reflected in the extent to which macrophages were infected with *T. dionisii*. No parasitized macrophage was seen in 6 d cultures containing mixtures of sera D and E, or serum E alone (10 or 20 %); parasitized macrophages were seen only in medium containing serum D alone at 10 % (18.9 % cells infected, S.E.M. ± 14.3) and 20 % (5.7 % cells infected; S.E.M. ± 14.3). In 7 d cultures containing sera B and E, however, infected macrophages (70.9 % ± 4.2 to 93.4 % ± 0.5) were seen in all media containing mixtures and in medium with 10 % serum B alone (80.6 % ± 4.6); none was seen in cultures containing serum E only (10 or 20 %). (Too few macrophages survived in medium containing 20 % serum B for their infection rate to be determinable.)

There was no significant difference in the proportions of macrophages containing parasites in medium containing serum A or C with live or dead inocula, whereas fewer live (and perhaps dead) trypanosomes were ingested in the absence of serum (Table 2).

The effect of different sera on survival and replication of intracellular parasites was determined in cultures at 37 °C in medium containing 20 % serum A. After 5 d, the cultures in four flasks were fixed and their mean macrophage infection rate was determined as 16.8 % (S.E.M. ± 4.5). The medium was removed from the remaining flasks and replaced by medium containing 20 % serum A or serum E, or no serum at all (four flasks each). After a further 2 d incubation, these cultures were fixed; the mean infection rates of each group were similarly reduced to 2.6 % (S.E.M. ± 1.2), 2.4 % (S.E.M. ± 1.4) and 6.0 % (S.E.M. ± 2.4), respectively.

Conditioned medium

Extracellular multiplication of trypanosomes in conditioned medium (see Methods) without macrophages at 37 °C was at least as good as that occurring in control flasks containing macrophages; growth in flasks containing only normal (unconditioned) medium without macrophages was significantly reduced in two experiments (Fig. 7). The macrophage infection rates were 86 % in one experiment (only one flask counted) and 60 to 68 % in the other (two flasks).

DISCUSSION

As extracellular multiplication of *T. dionisii* at 28 or 37 °C occurred only in the presence of sera A or B (good sera), it was concluded that these sera contained a growth-promoting substance or substances (S+ factor). Bad sera (C and E), which permitted virtually no multiplication, possessed an inhibitory substance or substances (S- factor), while the intermediate serum D presumably lacked appreciable amounts of either factor. Support for this hypothesis was provided by the experiments with mixtures of sera. When the good serum formed only a quarter of the total serum content (5 % of the medium), more extracellular replication occurred than in its absence, and the extent of replication increased as the proportion was increased. Mixtures of bad and intermediate sera, however, permitted no more growth than did the former serum alone. As serum D supported growth equally well on its own at concentrations of 20 and 10 %, the observed inhibitory effect of proportions of serum E as low as 5 % when mixed with serum D (15 %) could not have been due solely to dilution of a restricted amount of S+ factor present in the latter serum. The results suggested rather that there was an inhibitory substance in serum E which could be overridden by the growth-promoting property of serum B.

The enhancement of replication which occurred in the good sera when macrophages were also present, suggested that the latter cells were secreting an additional growth-promoting substance or substances (M factor), effective only in the presence of S+ and essential for growth at 37 °C but not 28 °C. A similar essential growth-promoting factor is presumably produced by the 'fibroblast-like' cells in cultures of haematozoic *T. brucei* (Hirumi, Doyle & Hirumi, 1977). This interpretation was supported by the observation that extracellular trypanosomal growth in flasks without macrophages, but replenished daily with medium conditioned by prior contact with uninfected macrophages, was as good as in flasks containing macrophages.

The similarity of infection rates of macrophages in media containing different sera, and mixtures of these, indicated that intracellular parasites did not need S+ factor. Nor were they adversely affected by S- factor, as transfer from good to bad serum resulted in no greater reduction in infection rate than did transfer to fresh medium containing good serum; presumably some intracellular digestion of parasites occurred in both media. *Trypanosoma dionisii* enters cells in our system mainly, if not entirely, by phagocytosis (Baker & Liston, 1978). As the extent of ingestion of dead and live parasites was similar in media containing good or bad sera, it appeared that these qualities of a serum did not influence, nor were they mediated by, the phagocytic activity of the macrophages. The presence of serum of any kind, however, stimulated phagocytosis, as Munthe-Kaas (1976) observed with rat Kupffer cells (but not rat peritoneal macrophages) *in vitro*. The presence in culture medium of specific antiserum has a stimulatory effect on phagocytosis *in vitro* of *T. dionisii*, and on its subsequent intracellular replication and morphogenesis (Baker & Liston, 1978).

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