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Immunological clearance of ^{75}Se-labelled *Trypanosoma brucei* in mice

I. ASPECTS OF THE RADIOLABELLING TECHNIQUE

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Summary. A reliable and simple technique for the *in vivo* labelling of *Trypanosoma brucei* with [^{75}Se]-methionine was developed. Between 97 and 99% of the radioactivity was protein bound in the trypanosomes and spontaneous elution *in vitro* was $<10\%$ over 4 h. The fate of the labelled trypanosomes after i.v. injection into normal and immune mice was studied. Whilst the vast majority of parasites remained in the circulation of normal animals they rapidly disappeared from the blood of immune animals. In the latter the liver was found to be the principal site of phagocytosis removing over 50% of the radiolabelled parasites within 15 min of injection.

INTRODUCTION

The immune response to pathogenic trypanosomes has been the subject of much investigation, but is still poorly understood. Considerable attention has been paid to the antibody response to infection and the apparent failure of this response to control infections because of the parasite's notable ability to undergo antigenic variation. There is evidence that macrophages and cell-mediated responses also play an active role in combating the infection (see reviews by Clarkson, 1976; Terry, 1976; Murray & Urquhart, 1977).

Quantitative evaluation of these responses *in vivo* would be aided by the availability of a suitable method for measuring the removal of circulating trypanosomes.

Several attempts to label trypanosomes with radioisotopes have been reported but so far these methods, using *in vitro* techniques, have been only partially successful. Tritiated thymidine has been used to label *Trypanosoma evansi* and *T. gambiense* (Inoki & Tadasuke, 1969) and *T. brucei* (Balber, 1971) although apparently it is not incorporated by *T. vivax* (Isoun & Isoun, 1974). *T. cruzi* has been labelled with [^{51}Cr]-sodium chromate (Kuhn, Vaughn & Iannuzzi, 1974) or [^{99}Tc]-pertechnetate (Mkwananzi, Franks & Baker, 1976). Unfortunately, the trypanosomes labelled with these various isotopes cannot readily be used for *in vivo* studies, either because radioactivity measurement is difficult or because there are adverse effects on infectivity and subsequent replication (Dahlin, Hungerer & Zwisher, 1976).

This paper describes a method of labelling *T. brucei* with [^{75}Se]-methionine, which overcomes many of the problems associated with reported techniques and which is particulary suitable for *in vivo* studies of immunological clearance.

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MATERIALS AND METHODS

Parasite

A strain of *T. brucei* derived from a stabilate of TREU 226 was used. Rats and mice which had been sub-lethally irradiated (Lumsden, Herbert & McNeillage, 1973) were infected with 1×10^5 and 1×10^4 organisms respectively by intraperitoneal (i.p.) injection. This trypanosome causes an acute and fatal infection of 5–7 days duration in rats and mice.

Laboratory animals

Female Hooded Lister rats weighing between 130 and 180 g and female CFLP mice weighing 20–30 g were used.

Radiolabelling technique

Rats and mice with fulminating parasitaemias, usually 5–7 days after infection were injected intravenously (i.v.) with 50 μCi and 10 μCi of [^{75}Se]-methionine respectively. After 20 h the animals were anaesthetized and blood removed from the heart into heparin (Lumsden *et al.*, 1973). Trypanosomes were separated from the infected blood by passing it through a DEAE-cellulose (Whatman Chromedia DE52) column according to the method of Lanham & Godfrey (1970). The isolated trypanosomes were washed three times with ice-cold phosphate glucose buffered saline (PGBS), enumerated with an Improved Neubauer haemocytometer (Lumsden, *et al.*, 1973) and their radioactivity determined. All radioactivity determinations were carried out in a Packard automatic gamma scintillation counter.

Injection of labelled trypanosomes and sampling

The suspension of washed ^{75}Se-labelled trypanosomes was diluted with PGBS to give a final concentration of 5×10^8 organisms/ml (approximate radioactivity 20,000 c.p.m./ml). Each mouse received 0·2 ml (10^8 organisms) by injection into a tail vein after sedation with 0·03 ml i.p. Hypnorm (Jansen Pharmaceuticals).

One hour after the injection of labelled parasites the mice were given an overdose of tricholorethylene anaesthetic (Trilene, ICI). Blood was taken by cardiac puncture and various organs removed. The organs were washed in PGBS, weighed and placed in counting vials for radioactivity determination. The total blood radioactivity was estimated by multiplying the radioactivity of a known volume (200 or 500 μl) of cardiac blood taken at the time of killing by a previously determined factor (0·067 ml/g body weight) obtained from ^{51}Cr-red cell studies (Holmes, unpublished results). The radioactivity of each organ and the blood were expressed as a percentage of the injected activity.

Immunization procedure

Groups of mice were immunized against *T. brucei* TREU 226 either by infection and drug cure 5 days later by diminazine aceturate (40 mg/kg Berenil, Farbwerke, Hoechst) or by vaccination with supernatant, obtained by incubating trypanosomes in PGBS for 2 h at 37° (Chi, Webb, Lambert & Miescher, 1977), emulsified in Freund's incomplete adjuvant for the first inoculation and followed 2 weeks later by a second inoculation of supernatant alone. The immunized mice when subsequently challenged 2–4 weeks later with 1×10^4 organisms of the same stabilate were found to be totally resistant, in that trypanosomes were not found on any occasion despite frequent examination of wet blood films for 4 weeks.

Homogenization and protein separation

A 5 ml sample of ^{75}Se-labelled trypanosomes containing approximately 5×10^8 organisms/ml PGBS was disintegrated by alternate freezing and thawing four times. The resultant homogenate was then preserved at −20° until use.

Samples of homogenate were fractionated on a DEAE-cellulose column and the protein contents (Lanham & Taylor, 1972) and radioactivity values quantified.

Maintenance of ^{75}Se-labelled trypanosomes in vitro

Freshly separated radiolabelled trypanosomes were maintained for several hours at 37° in commercial culture medium i.e. 100 ml RPM1 1640 with 25 mM HEPES and L-glutamine (Gibco Biocult) to which was added 20 ml foetal calf serum, 1 mM HEPES, 10,000 units penicillin and 10,000 μg streptomycin, at a concentration of 10^8 organisms/5 ml medium.

Statistics

Variation around the mean is expressed as the standard error.

RESULTS

In vivo incorporation of [^{75}Se]-methionine by trypanosomes

The injection of [^{75}Se]-methionine into trypanosome-infected rats and mice produced, after 20 h, populations of viable radiolabelled organisms which were subsequently separated from the blood. The radioactivity incorporated by the trypanosomes, although representing a small percentage (0·004%) of the injected dose, gave specific activity values of approximately 4000 c.p.m./10^8 trypanosomes. The protein bound activity was 97–99% and the radioactivity was closely associated with several protein rich fractions of homogenised ^{75}Se-labelled trypanosomes (Fig. 1).

Spontaneous release of ^{75}Se from radiolabelled trypanosomes

The leakage of ^{75}Se from radiolabelled trypanosomes maintained *in vitro* was measured at hourly intervals for 4 h incubation. Aliquots of trypanosome suspensions were centrifuged at 1000 *g* for 20 min and the radioactivity of the supernatant and deposit determined. The elution of ^{75}Se was found to be $<10\%$ over 4 h.

An attempt was also made to measure the elution of ^{75}Se from labelled trypanosomes *in vivo*. The specific activity (c.p.m./10^9) of trypanosomes recovered by passage through a DEAE-cellulose column from the blood of normal rats 3 h after the injection of labelled parasites was measured and compared with specific activity of the injected trypanosome suspension. The results showed that over a 3 h period there was a fall of 26·7% in the specific activity. This reduction was not, however, due solely to leakage as seen *in vitro* but was partly attributable to replication of the labelled organisms. Such a loss of specific activity would correspond to a replication time of approximately 6 h.

Distribution of ^{75}Se-trypanosomes in normal and immunized mice at various times after injection

Having established that the spontaneous release of ^{75}Se from labelled trypanosomes was minimal over 3–4 h, we examined the fate of radiolabelled organisms in normal and immunized mice. The distribution of ^{75}Se-trypanosomes was determined at 2, 15, 30, 60, 90 and 120 min after i.v. injection. In particular the radioactivity of the spleen, liver, kidneys, heart, lungs and blood was measured. The results are shown in Fig. 2. The most striking feature was that in the normal animals the majority of labelled trypanosomes remain in the blood, whereas in the immune animals they rapidly disappeared from the circulation. This latter effect was largely a

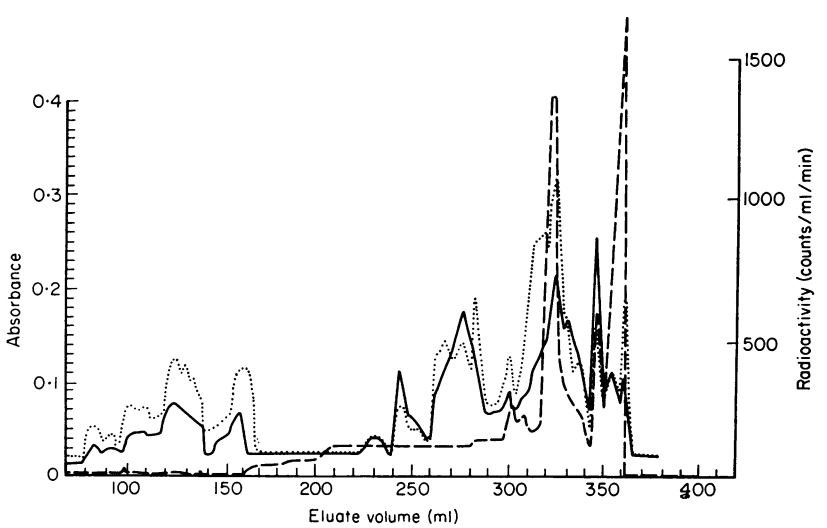

Figure 1. Chromatographic separation on DEAE-cellulose of a homogenate of [^{75}Se]-methionine labelled trypanosomes. (dashed line) ^{75}Se radioactivity; (dotted line) absorbance at 260 nm; (solid line) absorbance at 280 nm.

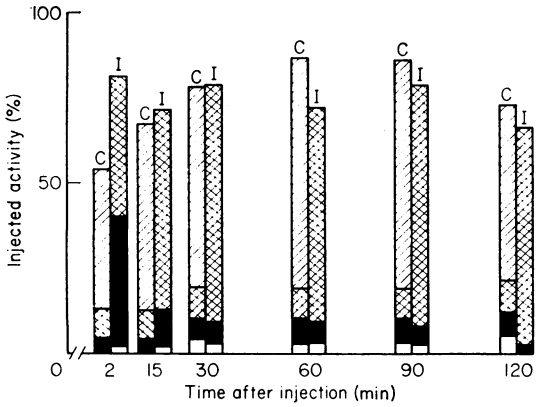

Figure 2. The distribution of [^{75}Se]-methionine labelled trypanosomes at intervals after intravenous injection into immunized (I) and control (C) mice. Hatched column, blood; cross-hatched column, liver; filled column, lungs; open column, spleen.

result of liver uptake (50–60%). In contrast the radioactivity of the liver in normal mice accounted for approximately 8% of total label. Since the liver is known to contain approximately 12% of the total blood volume (Guyton, 1971) it is likely that the majority of this radioactivity is due to the presence of circulating trypanosomes in the hepatic vasculature although some non-specific uptake of trypanosomes by hepatic macrophages cannot be excluded.

The increased uptake by the liver of immune mice was not a simple reflection of hepatomegaly since in these animals the liver weight expressed as a percentage of total body weight was only $7{\cdot}1 \pm 0{\cdot}2$ compared with $5{\cdot}8 \pm 0{\cdot}7$ for normal mice.

Distribution of ^{75}Se following the injection of [^{75}Se]-methionine into normal mice and mice immunized against *T. brucei*

In order to confirm that the radoiactivity values obtained in the previous experiment were not the result of increased uptake of free [^{75}Se]-methionine in immune mice the following experiment was undertaken. [^{75}Se]-methionine was injected i.v. into five normal and five immunized mice and the liver of each removed 1 h later. The radioactivities, when expressed as a percentage of the injected dose, were $15{\cdot}75 \pm 1{\cdot}1$ and $16{\cdot}5 \pm 1{\cdot}6$ respectively. Thus, a higher amount of radioactivity appears in the liver of normal mice following the injection of [^{75}Se]-methionine than appears after the injection of intact ^{75}Se-labelled trypanosomes. This provides further supportive evidence that the disappearance of ^{75}Se-labelled trypanosomes from the circulation is a valid clearance technique.

DISCUSSION

[^{75}Se]-methionine has not been used previously, to our knowledge, to label protozoan parasites, although there have been reports of its use as a radiolabel for nematodes (Maclean, 1977) and trematodes (Christensen, 1977).

Our results show that [^{75}Se]-methionine can be used effectively as a radiolabel for African trypanosomes and furthermore that such organisms can be used for *in vivo* clearance studies.

The technique we have described has several important advantages over previously reported methods. It fulfils most of the essential criteria required of a trace labelling method: (a) the labelled organisms are still viable and pathogenic; (b) there is insignificant release of ^{75}Se over several hours; (c) as a gamma-emitting radioisotope ^{75}Se is easily counted with the minimum of sample preparation; and (d) the relatively short labelling period of 20 h enables specific populations of trypanosomes to be labelled.

A possible disadvantage of the technique is that its use in clearance studies is limited to situations where relatively large numbers of organisms can be used otherwise the low specific activity of the radiolabelled trypanosomes makes their detection difficult.

The exact way in which trypanosomes become labelled with [^{75}Se]-methionine has not been determined although it seems most likely that initially they pinocytose free [^{75}Se]-methionine. Also, it is known that within 24 h of injection proteins labelled with [^{75}Se]-methionine can be found throughout the body (Awwad, Potchen, Adelstein & Dealy, 1966) and presumably pinocytosis of these labelled proteins by trypanosomes may occur (Brown, Armstrong & Valentine, 1965). A third possibility, the coating of the parasite surface by ^{75}Se-labelled globulins must also be considered, since adherence of plasma proteins to the surface of trypanosomes has been reported (Vickerman, 1972; Seed, 1974).

There are two reasons why the latter seems unlikely. First, the principal fraction of radioactive

trypanosome protein from homogenized parasites after DEAE-cellulose chromatography did not correspond to plasma protein fractions but was closely related to the protein-rich fractions of trypanosomes previously reported by Lanham & Taylor (1972), and secondy, the donor animals were irradiated and unable to produce anti-trypanosome antibodies (Luckins, 1972).

The distribution of ^{75}Se-labelled trypanosomes in normal and immune mice demonstrated the value of this technique in immunological studies. Whereas the labelled parasites remained in the circulation of normal animals, they rapidly disappeared from the blood of immune animals. In the latter the liver was shown to be the principal site of phagocytosis removing over 50% of the trypanosomes within 15 min of i.v. injection. Whether the hepatic uptake represents the uptake of intact or lysed organisms will be the subject of a subsequent paper.

^{75}Se-labelled trypanosomes clearly have several advantages compared with previously reported techniques in exploring the role of macrophages and antibodies in the immune response to trypanosomiasis. For example, while foreign red cells, carbon and various colloids can be used to measure phagocytic function in infected mice (Murray, Jennings, Murray & Urquhart, 1974; Longstaff, 1974) the results do not necessarily reflect the host's ability to remove trypanosomes from the circulation. Tests of antibody function such as agglutination, complement fixation and trypanolysis are subject to similar reservations (Seed & Gam, 1966). In contrast, the removal of labelled organisms from the circulation provides a direct index of the host's capacity to eliminate specific populations of trypanosomes. Furthermore, it enables the site of phagocytosis to be determined with certainty.

Radiolabelled trypanosomes may be of value in at least three other situations. First, to show whether innate resistance to trypanosomiasis such as is found in certain wild mammals and N'dama cattle (Chandler, 1952), is associated with enhanced clearance of trypanosomes. Second, in the improved quantification of trypanolytic antibody *in vitro* which currently depends on visual assessment of lysis (Lumsden *et al.*, 1973). Third, in the measurement of trypanosome replication rates both *in vitro* and in the host.

Recent experiments (unpublished) have shown that this method of labelling trypanosomes is equally applicable to *Trypanosoma congolense*.

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