

INHIBITION OF ^{14}C UPTAKE BY *CANDIDA ALBICANS*: A NEW METHOD FOR INVESTIGATION OF INTRACELLULAR KILLING BY PHAGOCYTES

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ABSTRACT

A radio assay employing ^{14}C -uridine to measure the intracellular killing of *Candida albicans* by phagocytes is presented.

The principle of this new method is that after killing of *Candida albicans* by phagocytes RNA synthesis stops and therefore uridine uptake is diminished whilst viable candida in cultures would take up uridine and as a result, high CPM counts can be obtained within 135 min. of incubation in test cultures. Leukocytes from 30 normal subjects showed reduced C.P.M. following killing of *Candida albicans*.

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INTRODUCTION

One of the most important aspects of phagocytosis is the intracellular killing of ingested organisms, for if this process does not occur the organism has the opportunity of growing inside the phagocyte. An example of this defect is chronic granulomatous disease (CGD) of childhood. There are several methods for assessing the microbicidal capacity of phagocytes which require the removal of extracellular organisms followed by colony counting^{1,2} or microscopic estimation of the percentage of organisms killed. One technique that is widely used depends upon microscopic evaluation of stained smears prepared from neutrophil-candida suspension.³⁻⁵ These methods are time-consuming and estimation of the percentage of organisms killed is subjective, varying from one observer to the other.

A radiometric assay of intracellular killing employing radiochromium has also been reported by Yamamura et al. and Martin, et al.,⁶⁻⁷ but the disadvantage of this method is the short half-life of chromium in comparison with ^{14}C so that CPM may differ one day from the next. In addition, greater exposure of laboratory personnel when ^{51}Cr is employed, should not be overlooked. This study was undertaken to develop a quantitative method capable of measuring the candida-

cidal activity of human leukocytes and serum. This new method describes intracellular killing of leukocytes. Also it shows that the live candida takes up ^{14}C uridine, that is compared with the sample that only has candida.

MATERIALS AND METHODS

Difco TC Medium 199 with antibiotics, pH: 7.3-7.4 was used throughout the culture.

Scintillation fluid was made by mixing 6g of PPO (2.5g diphenyloxazole) and 0.2g dimethyl POPOP 1,4 bis (2-4 methyl 5-phenyloxazole) benzene purchased from Packard. The volume was adjusted to one litre by adding toluene (Merck).

^{14}C uridine specific activity was obtained from Radio Chemical Center, Amersham.

All glassware used for this experiment were silicized and sterilized. Experiments were set up in round-bottomed plastic Cryotubes (N-1076-IA) in sterile conditions.

Preparation of *candida albicans*

Candida albicans (strain UC. 820) was cultured in 1% perion and 2% glucose at room temperature for

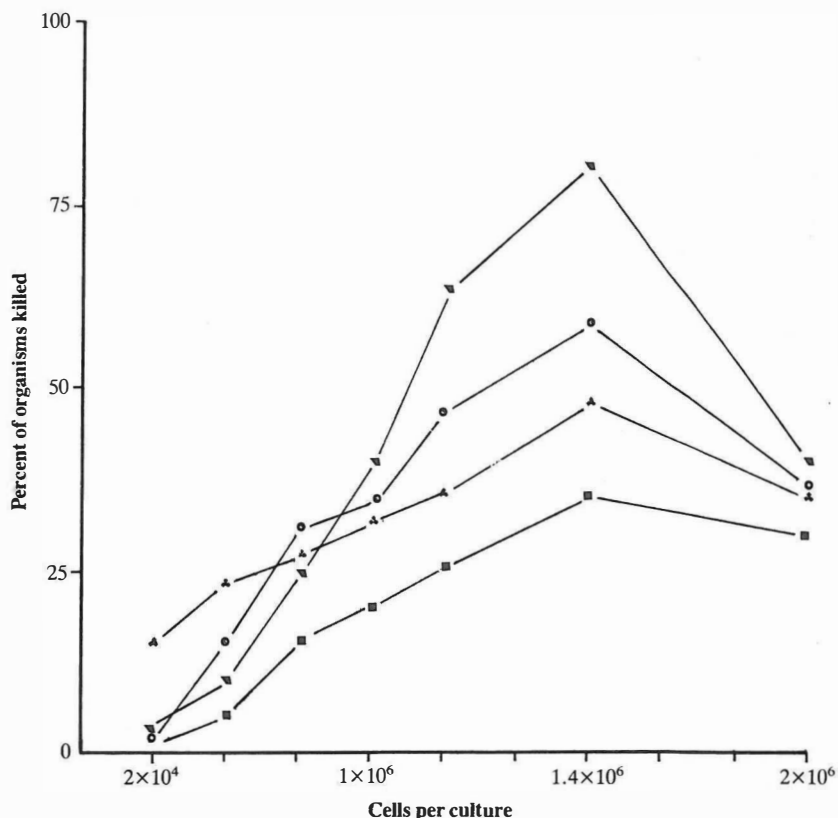


Fig 1. Influence of different numbers of phagocytes added to cultures which initially contained 1×10^6 *C. albicans*.

three days and kept at 4°C for further use. This culture can be kept at the above temperature for a period of four weeks.

Preparation of leukocytes

Leukocytes were separated from heparinized venous blood by dextran sedimentation. Separated white cells were washed three times with TC199 and finally resuspended in TC199 to obtain a WBC count of $7 \times 10^6/\text{ml}$.

Leukocyte/candida suspension

Test tubes containing a known number of phagocytes suspended in TC199 containing 1×10^6 *Candida albicans* and enriched to a known percentage of autologous plasma in final total volume of 1 ml. All experiments were set up in triplicate. The incubation time was 90 min. at 37°C . After this time 0.2 ml of 2.5% sodium deoxycholate was added in order to lyse the phagocytes and release the killed candida. Simultaneously 0.1ml of a DNase and 0.1ml of uridine with known specific activity was mixed with the culture. The mixture was incubated at 37°C for another 45 minutes. These cultures were harvested in a millipore macroharvester

through glass fiber filters. 5ml scintillation fluid was then added and counted in a Packard model 3390, and the percent of inhibition of uridine incorporation was determined by the following formula, according to Yamamura, et al. (1977).

$$100 - \frac{\text{CPM (C. albicans + PMNs)}}{\text{CPM (C. albicans)}} \times 100$$

Optimization

1. To find a suitable number of phagocytes to kill *Candida albicans* experiments were set up with 2×10^4 up to 2×10^6 phagocytes per culture enriched with 20% plasma.
2. To find a suitable plasma concentration, various concentrations of plasma ranging from 10% to 50% per culture were employed.
3. To find a suitable incubation time prior to addition of uridine, cultures were incubated from 30 min. to 120 min.
4. To find an optimum time for labelling *Candida albicans* by uridine, incubations were made ranging from 15 min. to 60 min. Each set of experiments were harvested at 15 minute intervals.

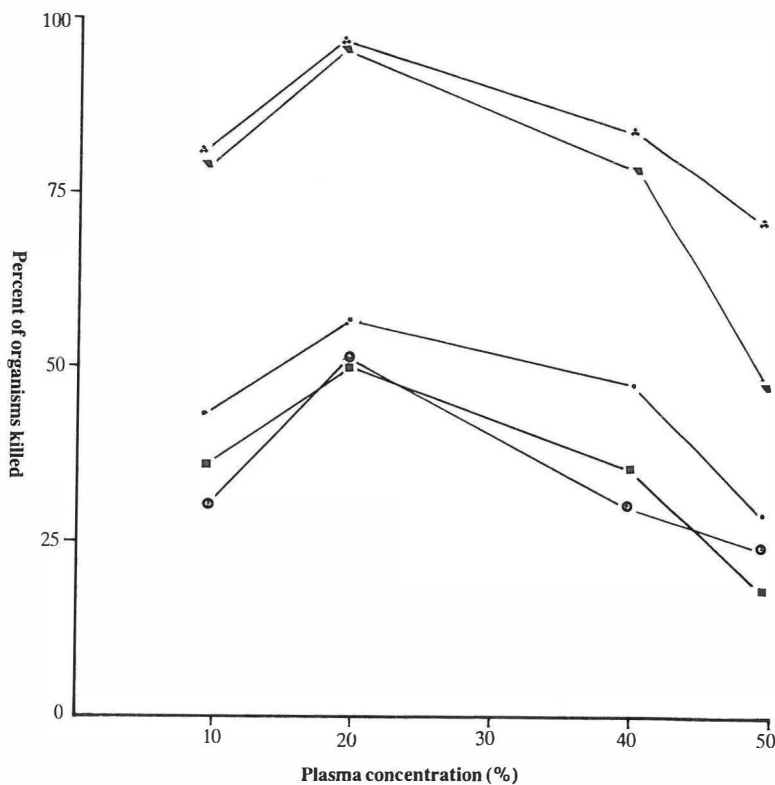


Fig. 2. Effect of plasma concentration on candida killing.

5. Determination of the normal range for this 1.4×10^6 phagocytes of all 40 healthy volunteers were tested in test tubes in a one ml volume, containing 20% fresh plasma and 1×10^6 *Candida albicans* incubated at room temperature for 90 min. and labelled with uridine for 45 min. thereafter.

RESULTS

Using cells from healthy individuals, the candidacidal activity of PMN leukocytes was assessed over a wide range of *C. albicans*/leukocyte ratios. Results from four such experiments are shown in Fig. 1. In the presence of 20% plasma there was a progressive percentage of candida killing with increasing PMN leukocytes/*C. albicans* ratio, reaching the maximum at 1:1.4 ratio. Since this ratio repeatedly ensured maximum-candidacidal activity it was used in subsequent assays.

Next, the effect of varying concentrations of plasma (10-50%) on the inhibition of ^{14}C -uridine uptake into *C. albicans* was measured using the 1:1.4 ration PMN/*C. albicans*. Maximum intracellular killing produced by PMN leukocytes from individuals consistently occurred at plasma concentrations of 20% (Fig. 2). It should be noted that in these assays, plasma concentrations of 30% or more did not induce a statistically significant inhibition.

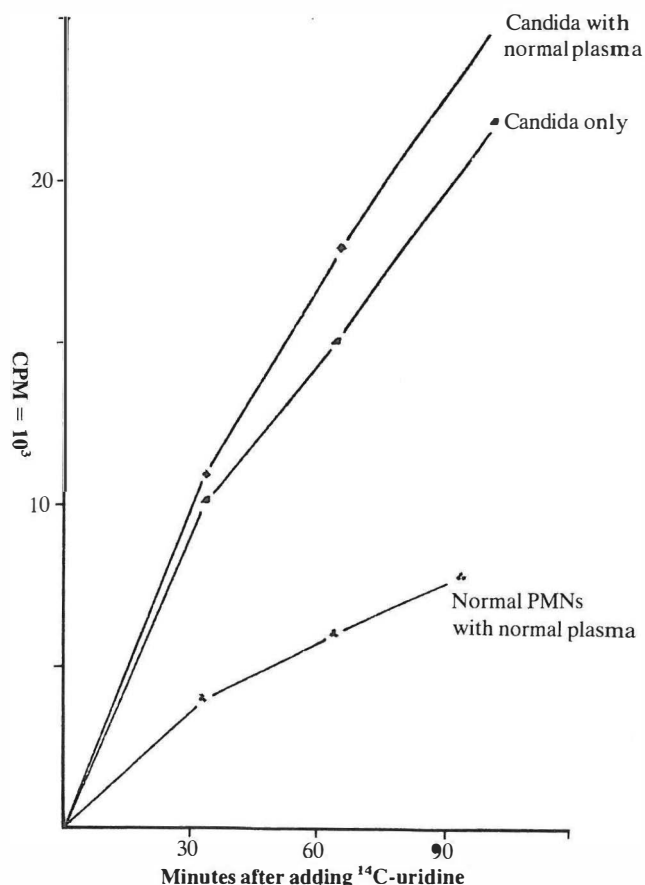


Fig. 3. ^{14}C -uridine uptake against time, in relation to whether the *Candida albicans* were incubated alone, with normal plasma, and with normal cells plus normal plasma.

Intracellular Killing Of Candida Albicans

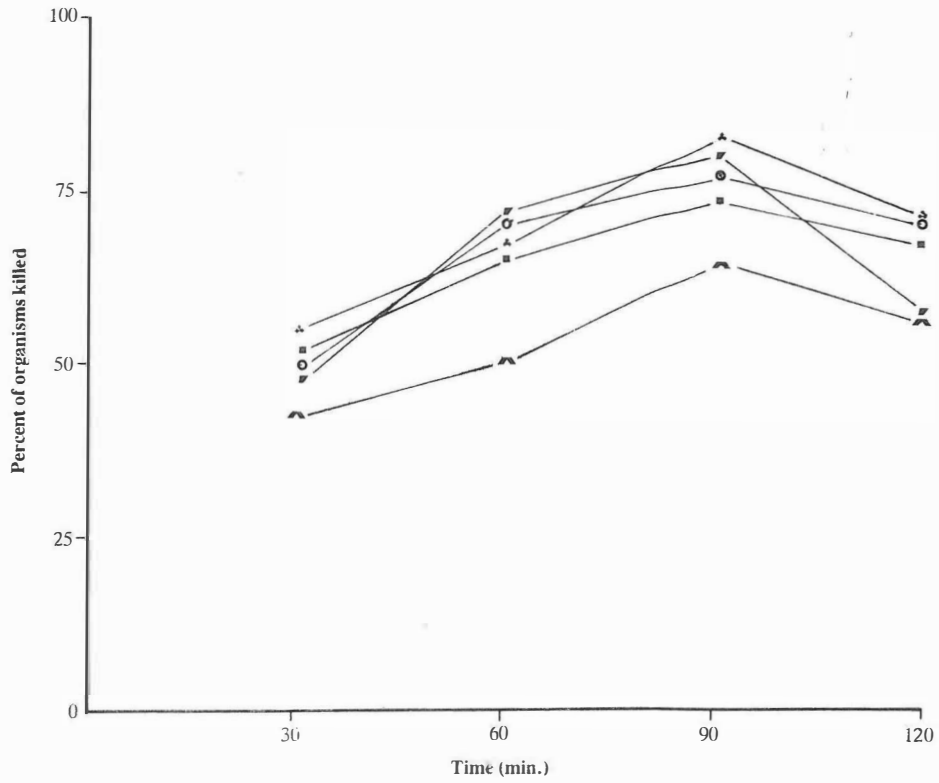


Fig. 4. Time required for intracellular killing of candida by 1.4×10^6 PMNs plus 20% of plasma.

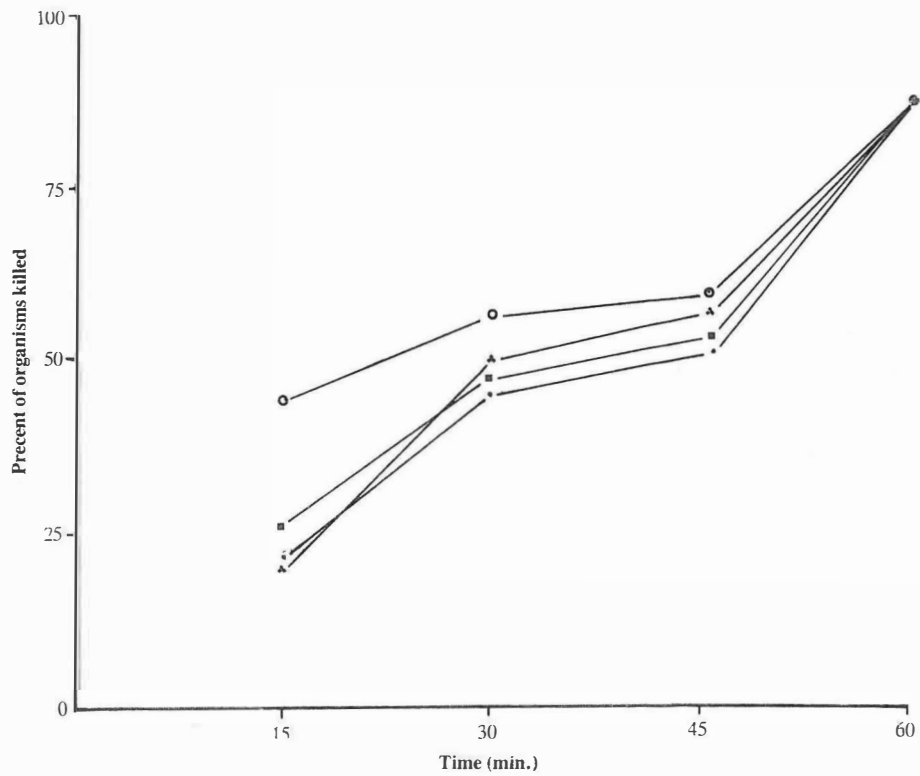


Fig. 5. ^{14}C -uridine uptake of *C. albicans* after phagocytosis and release of killed candida.

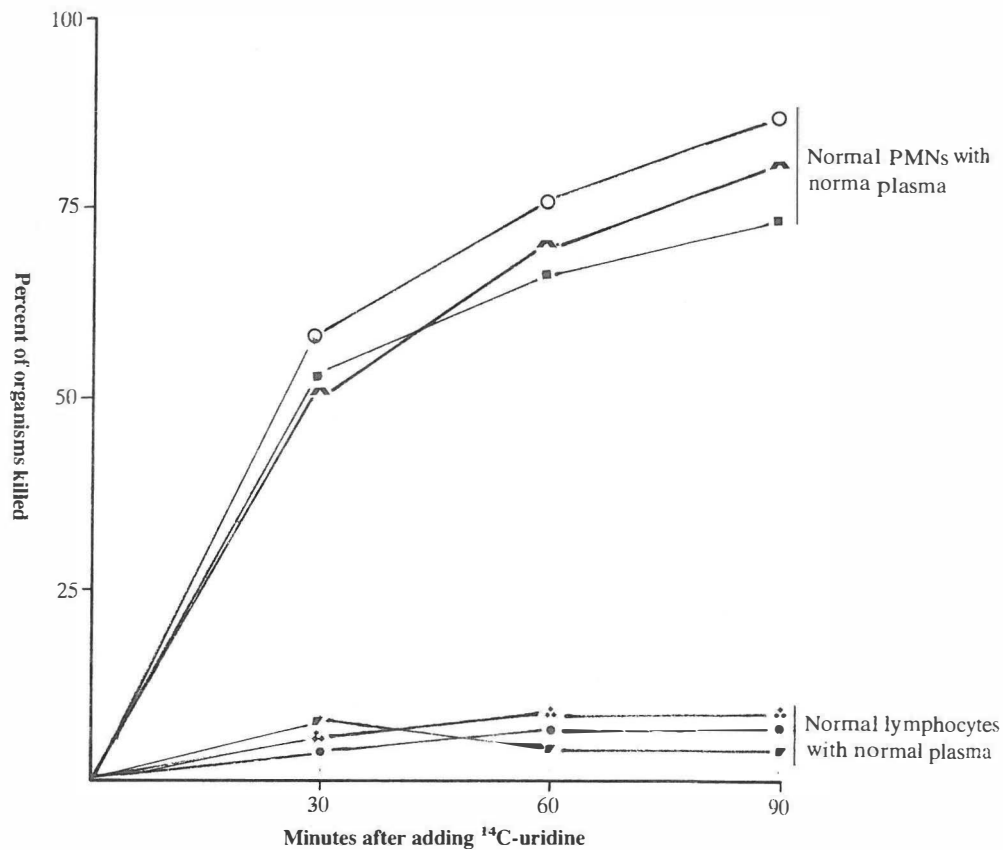


Fig. 6. Showing the percent of candida killing with time, comparison between normal PMNs plus normal plasma against normal lymphocytes and normal plasma.

In addition, in the absence of PMN leukocytes, plasma concentrations of 20% or more were unable to induce candida killing significantly. Uptake of radioisotopes by these organisms in standard-culture was comparable to that observed when *C. albicans* were incubated in plasma alone (Fig.3).

To determine the suitable incubation time, culture containing 1.4×10^6 PMN leukocytes and 1×10^6 *C. albicans* were pulsed with ¹⁴C-uridine at 30 min. intervals. Fig.4 demonstrates the results of 5 such experiments. In every case, killed candida were measured 60 min. after addition of the radioisotope. This pre-labelling incubation period was used in the subsequent assays (Fig. 5).

Fig. 6 depicts the results from three experiments in which inhibition of ¹⁴C-uridine uptake was used to compare the candidacidal activity of the two preparations-PMN leukocytes and that of the lymphocytes. As expected, there was a significant decrease in the amount of killed candida when 1.4×10^6 PMN were incubated with 1×10^6 of *C. albicans*. However, addition of similar number of lymphocytes to the cultures containing *C. albicans* did not induce significant intracellular killing by this organism.

Lastly, PMN leukocytes from 35 healthy individuals

were tested in the presence of 20% autologous plasma for their candidacidal capacity during a 90 min. incubation period. A range of 27 to 77% candida killing was observed with a mean value of $62.2 \pm 6.4\%$. This result has been compared with chronic granulomatous disease (CGD) and two immunodeficient patients (Fig.7).

DISCUSSION

Previously published methods for the assay of thymidine uptake (Foroozanfar, et al.⁸) and inhibition of uridine uptake by *Candida albicans* (Yamamura, et al.⁹) are essentially a measure of phagocytosis rather than an assessment of intracellular killing.

Our method is a simplified and sensitive assay for measuring intracellular killing of *Candida albicans* which avoids tedious microscopic counting and therefore eliminates observer error. In this approach, *C. albicans* is labelled with ¹⁴C-uridine after being incubated with phagocytes. Our study demonstrates that all live candida take up ¹⁴C whereas dead organisms can not. Further, we have employed a pure culture of candida with no phagocytes and a pure culture of

Intracellular Killing Of Candida Albicans

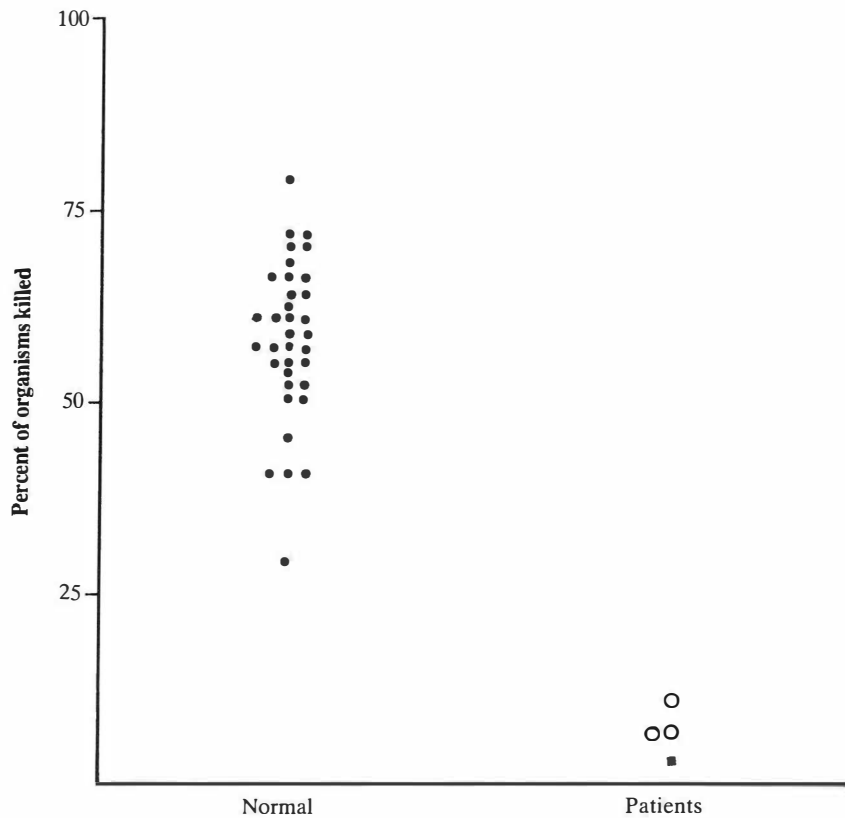


Fig. 7. Showing percentage of *C. albicans* killed in 90 minutes by normal PMNs (●) against PMNs from patients with immunodeficiency (○) and chronic granulomatous disease (●).

Candida plus normal plasma that compared with the normal PMNs and normal plasma, which allowed us to compare two systems together and to calculate results by the formula given previously in this paper. For answering this question that why killed *C. albicans* can not incorporate ^{14}C -uridine, we used patients with chronic granulomatous disease (CGD), whose PMNs are known to have normal capacity to phagocytose but are not capable of killing ingested *C. albicans* (Lehrer, 1970). It was found that intracellular killing of PMNs of one patient was reduced.¹⁰

Yamamura and Martin introduced a method for the assay of intracellular killing based upon the use of ^{51}Cr -labelled candida. This method differs from ours however in the nature of the radioisotope and labelling procedure. Our technique is clearly less hazardous to laboratory workers who deal with radioactive materials on a daily basis; it is sensitive, easy to carry out and probably more economical in that half-life of ^{14}C is longer than that of ^{51}Cr .

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