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NOTES

Microscopic Observation of Progressive Immobilization of Leishmania Promastigotes in Acridine Orange Stain

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To rapidly isolate *Leishmania donovani* promastigotes in samples from Novy-MacNeal-Nicolle (NNN) cultures, a method of staining with acridine orange was developed. Such vital staining combines the advantages of direct microscopic examination (e.g., observation of motility) with more accurate cytological and structural imaging of the stained parasites (usually obtained by Giemsa staining). Progressive immobilization of *Leishmania* promastigotes associated with a change in fluorescence color was also studied. Our findings may be useful for the early confirmation of a positive culture inoculated with a clinical sample.

Leishmanias are protozoan parasites which cause visceral, cutaneous, or mucocutaneous infections, which are widespread and may complicate AIDS (1, 6).

Direct diagnosis of leishmaniasis is currently done with bone marrow and spleen aspirates. Such samples are used for direct examination by microscope and for culturing. Even when a culture is started, an accurate and rapid method for isolation of the parasite in culture samples is certainly worthwhile.

Here we describe a technique which allows observation of the parasite in the liquid phase of Novy-MacNeal-Nicolle (NNN), the most commonly used leishmania culture medium.

Leishmania donovani promastigotes were maintained in a diphasic modified NNN medium with human blood substitution, and subcultures were incubated at 22 to 24°C in the dark in accordance with the technical recommendations of the World Health Organization (5). In our laboratory, this technique gave optimal results, unlike different temperatures and storage conditions, and moreover it was selected because of the worldwide distribution and strong impact of World Health Organization publications.

From 2-, 3-, and 4-day old subcultures, 20 μl of the liquid phase of the culture medium was transferred to a slide and then 10 μl of acridine orange solution (Montplet & Esteban S.A., Barcelona, Spain), at a final concentration of 1.44 mg/ml, was added. The acridine orange solution had a pH of 4.0.

Standard Giemsa staining was also applied to the same liquid phase samples used for acridine orange staining. Magnifications of ×400 and ×1,000 were used, and slides were observed 0, 15, 30, and 60 min after the samples and dye solutions were mixed. At least 20 microscopic fields were observed at each time by means of a fluorescence microscope (AXIOSCOP; Zeiss), and more than 200 parasites were differentially counted based on the colors of the nucleus, kinetoplast, and cytoplasm.

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In Fig. 1A, which was taken immediately after the dye was mixed with the leishmania suspension, the typical morphology of living promastigotesis visible due to acridine orange staining. The high contrast of the green nucleus and kinetoplast on the dark background is evident.

Figure 1B was obtained after 30 min of contact between the parasites and the dye. At the right upper corner and at the bottom, there are two dead parasites, which were immobile and showed a red cytoplasm and a pale-orange-stained nucleus and kinetoplast. However, in other fields of slides prepared at the same time, we found both living protozoa with the nucleus, kinetoplast, and cytoplasm all stained green and dead ones which appeared very similar to those in Fig. 1B.

Progressive immobilization of leishmania promastigotes associated with a change in fluorescence color was one of the main findings of our study. The kinetics of this change was monitored at different times, and the percentages of living (all green and mobile) cells decreased from 95.7% of the promastigotes observed at time zero to 59.1% at 30 min and 16.9% at 60 min. However, the kinetoplast and nucleus still appeared green within an orange-stained cytoplasm in 30.7% of the protozoa at 30 min and in 54.2% at 60 min after staining. The apparently dead protozoa were 4.3, 10.2, and 28.9% of all of the cells observed 0, 30, and 60 min, respectively, after the cell suspension and the stain solution were mixed.

Progressive immobilization of the parasites may be caused by the acidity (about pH 4.0) of the dye solution (4) or even by the dye itself. It has been reported that a number of acridine derivatives possess strong antileishmanial activity (3).

The acridine orange method required less time and was more sensitive under lower magnification than the Giemsa staining method. Even a very small number of promastigotes can be revealed easily. In 20 fields observed in each of several wet mounts obtained from 10 different 4-day-old cultures, we found a mean (± the standard deviation) of 418 ± 42 promastigotes after acridine orange staining versus 181 ± 19 after Giemsa staining. Also as reported above, the acridine orange stain allows observation of parasite morphological details very well contrasted against a dark background, which often is impossible in Giemsa-stained slides. Thus, after 2 or 3 days of

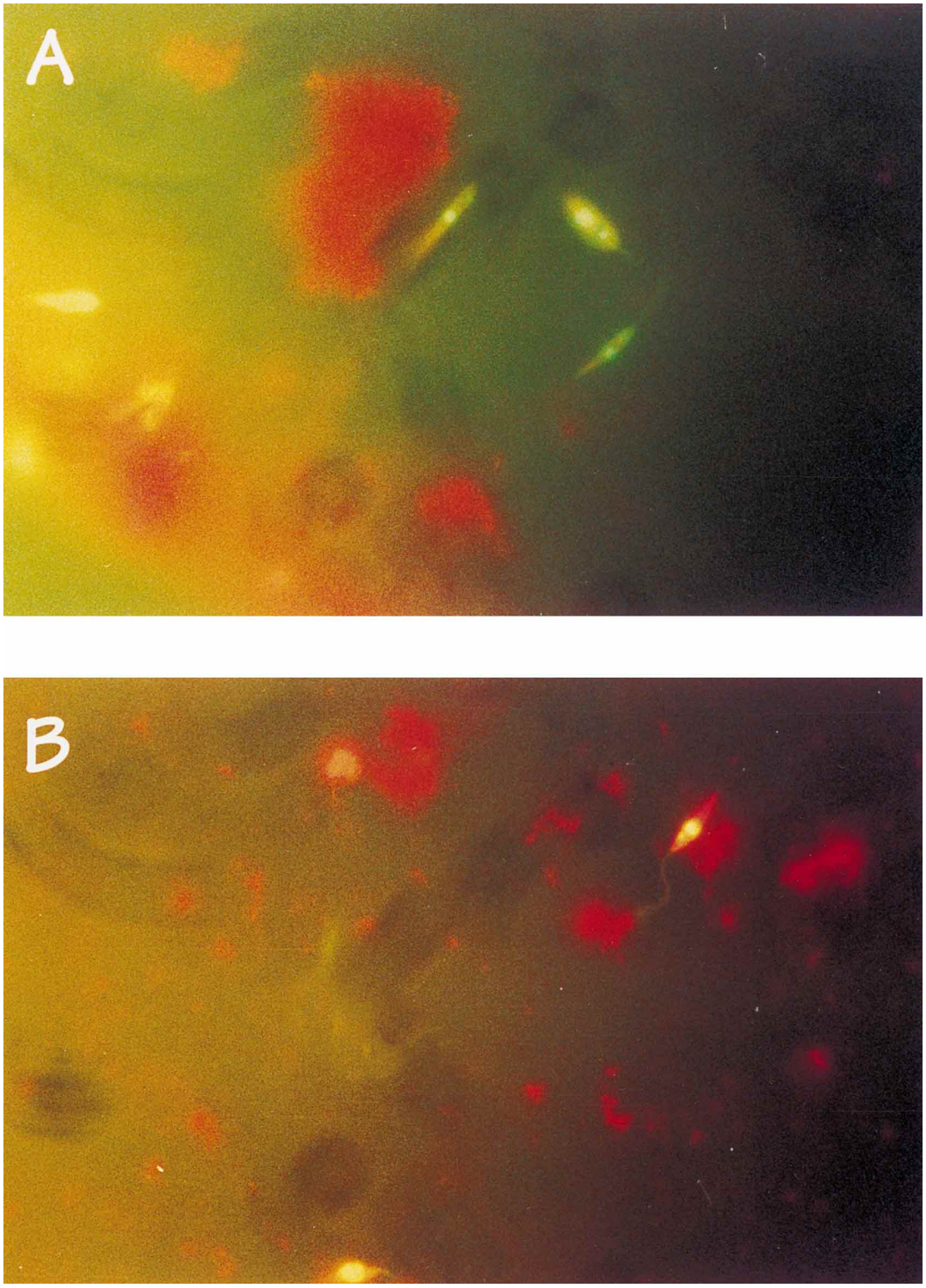

FIG. 1. *Leishmania* promastigotes taken from NNN medium, stained with acridine orange, and observed by fluorescence microscopy. Magnification, ×1,000. Immediately after staining, promastigotes appeared apple green with a highly contrasting dark green nucleus and kinetoplast (A). At the right upper corner and at the bottom, two dead parasites, with red cytoplasm and a pale-orange-stained nucleus and kinetoplast, were evident 30 min after staining with acridine orange (B).

subculturing, we found that acridine orange-stained slides were clearly positive for leishmania promastigotes while Giemsa-stained slides seldom were.

Acridine orange has also been reported to be a valuable tool for revealing malaria parasites in blood smears and to provide a good alternative to Giemsa staining (2).

The advantages of the technique reported here include both vital staining and the ability to evaluate the rate of immobili-

zation of promastigotes very accurately. The percentages of vital parasites (that is, all green-stained cells) will diminish earlier within the observation time (e.g., from 0 to 60 min) in samples obtained from patients treated with effective antileishmanial therapy than in untreated control samples, as we found in preliminary experiments (data not shown). Therefore, the present techniques may also be a useful tool for laboratory follow-up of leishmaniasis therapy.

However, in light of our present definitive findings, we recommend that acridine orange-stained slides be observed immediately after staining.

In conclusion, the staining method proposed here appeared very useful and time-saving for early and accurate checking of reticuloendothelial tissue cultures for leishmania promastigotes.

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