

## A RAPID, RELIABLE METHOD OF EVALUATING GROWTH AND VIABILITY OF INTRAERYTHROCYTIC PROTOZOAN HEMOPARASITES USING FLUORESCENCE FLOW CYTOMETRY

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*Fluorescence flow cytometry was employed to assess the potential of a vital dye, hydroethidine, for use in the detection and monitoring of the viability of hemoparasites in infected erythrocytes, using Babesia bovis as a model parasite. The studies demonstrated that hydroethidine is taken up by B. bovis and metabolically converted to the DNA binding fluorochrome, ethidium. Following uptake of the dye, erythrocytes containing viable parasites were readily distinguished and quantitated. Timed studies with the parasitocidal drug, Ganaseg, showed that it is possible to use the fluorochrome assay to monitor the effects of the drug on the rate of replication and viability of B. bovis in culture. The assay provides a rapid method for evaluation of the in vitro effect of drugs on hemoparasites and for analysis of the effect of various components of the immune response, such as lymphokines, monocyte products, antibodies, and effector cells (T, NK, LAK, ADCC) on the growth and viability of intraerythrocytic parasites.*

Key words: protozoan hemoparasites - in vitro viability assay - fluorescent vital dye - flow cytometry

Although exceptional advances have been made in the understanding of the antigenic composition of protozoan hemoparasites affecting humans and food animals, it has remained difficult to discern which antigen(s) are the best candidates for the development of efficacious subunit vaccines. This is in part attributable to the limitations of the methods available for analysis of the immune responses elicited by the candidate vaccine antigens. Although it has been possible to localize determinants recognized by T and B cell receptors and demonstrate their capacity to elicit a proliferative response, it has been difficult to adequately assess effector activity on viable parasites under controlled conditions. Investigators have had to rely on empirical evaluation of slide preparations of organisms (Ockenhouse et al., 1984; Jones, et al., 1989; Lunel & Druilhe, 1989) or measurement of the incorporation of nucleotides (Carlin & Jensen, 1986; Goff & Yunker, 1986; Waters, et al., 1987). To address this problem we have explored the potential of using a vital fluorescent dye, hydroethidine (Gallop

et al., 1984), in conjunction with flow cytometry to detect hemoparasites in cultured erythrocytes and a parasitocidal drug, diminazene aceturate (Ganaseg; Squibb), to model effects on parasite viability that might be mediated by effector cells, cytokines, or antibodies. As shown here, our initial studies have demonstrated viable *Babesia bovis* can be readily detected and quantitated in microaerophilus stationary phase (MASP) cultures. The findings indicate the technique can be used to analyse humoral and cellular immune responses to hemoparasite antigens and their consequent effects on parasites.

### MATERIALS AND METHODS

*Parasites* – The majority of experiments were conducted with one culture-type used in our laboratory, MOBo-V4 (Goff & Yunker, 1986). Confirmatory studies were performed with two additional culture-types, M4Bo-C3 and T2Bo-S1 (Wyatt et al., 1991). The cultures were maintained in 25 cm<sup>2</sup> flasks as previously described (Goff & Yunker, 1988).

*Hydroethidine incorporation* – The potential of hydroethidine (HE) (Polysciences, Inc., Warrington, PA) for determining the percent

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of erythrocytes containing viable parasites was evaluated as previously described (Wyatt et al., 1991). Briefly, 100  $\mu$ l samples from flasks of cultured *B. bovis*-infected erythrocytes were taken for microscopic evaluation of Giemsa-stained smears. The remaining cells were pelleted by centrifugation for 5 min at 400 X g. An aliquot of stock hydroethidine was diluted into phosphate buffered saline (PBS) (Becton Dickinson Microbiological Systems, Cockeysville, MD) to a final concentration of 50  $\mu$ g/ml, filtered through a 0.45  $\mu$  filter and then mixed with the pelleted cells (1 ml HE solution for each 100  $\mu$ l packed erythrocytes). Suspensions were incubated in the dark at 37°C for 20 min, diluted with PBS and centrifuged for 5 min at 400 X g. Pellets were then resuspended in PBS and analyzed by flow cytometry (FC). Infected erythrocytes treated with diluted DMSO solution without HE (mock-treated), cultured uninfected erythrocytes treated with HE, and mock-treated cultured uninfected erythrocytes were used as controls.

**Flow cytometric analysis** – For FC analysis, cell suspensions were diluted into PBS containing 0.2% sodium azide to obtain a flow rate of 300 to 600 cells/sec (approximately  $2 \times 10^7$  cells/ml). The diluted suspensions were subjected to dual parameter analysis, log side scatter (SSC) versus log fluorescence (excitation at 488 nm [range 465-607 nm]) using a Becton Dickinson FACScan and Consort 30 computer with associated software (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Each cell suspension was sampled 2-4 times at 2000-5000 cells/sampling. Data were collected in list mode for later analysis. The correlation between fluorescence and Giemsa-stain-derived PPE was determined by analysis using a SigmaPlot40 program.

**Viability assay** – To determine whether HE incorporation could be used to detect and quantitate viable *B. bovis* under experimental conditions, the assay system was modified as described (Wyatt et al., 1991). Briefly, cultures at 1 PPE were established in 24-well or 96-well plates and incubated under standard conditions. Culture supernatants were then removed and fresh medium added. One set of plates was treated with Ganaseg (final concentration of 400  $\mu$ g/ml in fresh medium) at the initiation of culture. A second set was treated with Ganaseg at 24 h. A third set of plates served as untreated controls. For each time point assayed, an infected culture and uninfected control from each

plate were collected and sampled for Giemsa stain evaluation. The remaining cells were treated with HE at a final concentration of 20  $\mu$ g/ml, with 5 min incubations. Cells were evaluated by FC in linear FI 2 data mode with amplified FI 2 signal. Each cell suspension from the 24 well plates was sampled three times. Triplicate samples were taken from the 96-well plate for each time point. Data were collected in list mode for later analysis.

The data from microscopic evaluations and from FC were plotted against time in hours after initiation of the experiment using Harvard Graphics 2.3.

## RESULTS

**Detection of parasitized erythrocytes** – To determine whether intraerythrocytic parasites take up and convert sufficient amounts of HE for detection, uninfected and infected erythrocyte cultures were incubated with HE and analyzed by flow cytometry. As shown in Fig. 1, uninfected erythrocytes exhibited low intrinsic fluorescence, as assessed by dual parameter analysis in dot plot mode. Erythrocytes containing viable parasites converted HE to ethidium and exhibited intense fluorescence.

**Comparison of percent fluorescent cells with percent parasitized cells** – To establish that detection of viable parasites labeled with HE is as accurate as evaluation of Giemsa stained

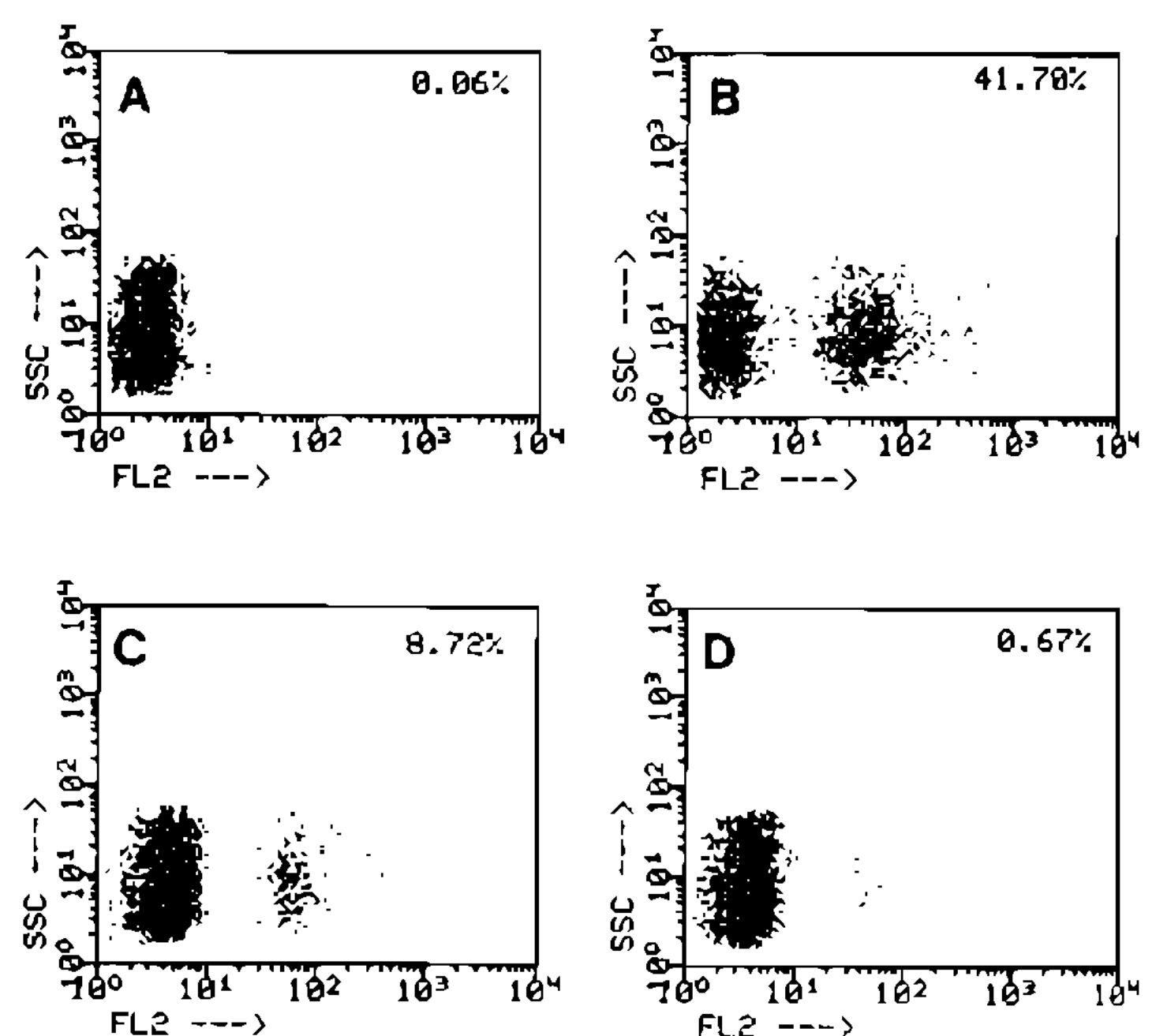


Fig. 1: representative fluorescence profiles of cultured *Babesia bovis*-infected erythrocytes. Panel A depicts uninfected erythrocytes; B, C, and D are dilutions of infected erythrocytes. The percentage of fluorescent cells is given in the upper right corner of each panel.

smears, 24 and 48 h cultures of infected erythrocytes were sampled for analysis by light microscopy and FC. A direct correlation ( $r = 0.9534$ ) was found between % infected erythrocytes as determined by microscopic examination and % fluorescent erythrocytes as determined by FC (Table). Correlation between Giemsa stain and HE for each culture-type was: MOBo-V4  $r = 0.9990$ ; M4BO-C3  $r = 0.9100$ ; T2Bo-S1  $r = 0.9707$ .

TABLE

Comparison of Giemsa stained blood film evaluation and hydroethidine conversion assay for quantitation of percentage of parasitized erythrocytes in cultures.

Culture type	Parasitized erythrocytes <sup>a</sup>	Fluorescent erythrocytes <sup>b</sup>
M4Bo-C3	12.2	10.2
	6.1	5.4
	5.5	4.5
	8.1	4.3
T2Bo-S1	2.2	2.6
	2.7	3.2
	7.2	5.6
MOBo-V4	6.51	6.0
	5.1	5.1
	5.7	3.3
Uninfected	0	0.1
	0	0.6
	0	0
	0	0

a: determined by Giemsa stain smear evaluation; expressed as percent.

b: determined by FC of HE-incubated erythrocytes; expressed as percent.

To further evaluate the sensitivity of the HE assay, dilution experiments were conducted. Doubling dilutions of infected erythrocytes with uninfected erythrocytes were prepared for analysis as above. At parasitemias lower than 0.15%, quantitation was no longer possible with either method. Thick smears were made for detection of parasitized erythrocytes by Giemsa stain evaluation. Fifty thousand erythrocytes were evaluated by FC to detect infected erythrocytes. The presence of infected cells was designated as positive or negative at these low parasitemias.

In the representative experiment shown in Fig. 2, parasitemia was allowed to increase to 38%. Parasitized erythrocytes could be quantitated by both methods to a theoretical PPE of 0.1475%. Below these quantitation levels, the presence of parasitized erythrocytes could be

seen by thick Giemsa-stained smears as low as 0.0012% and by HE incorporation (counting 25-50,000 cells per sample) to 0.0184%.

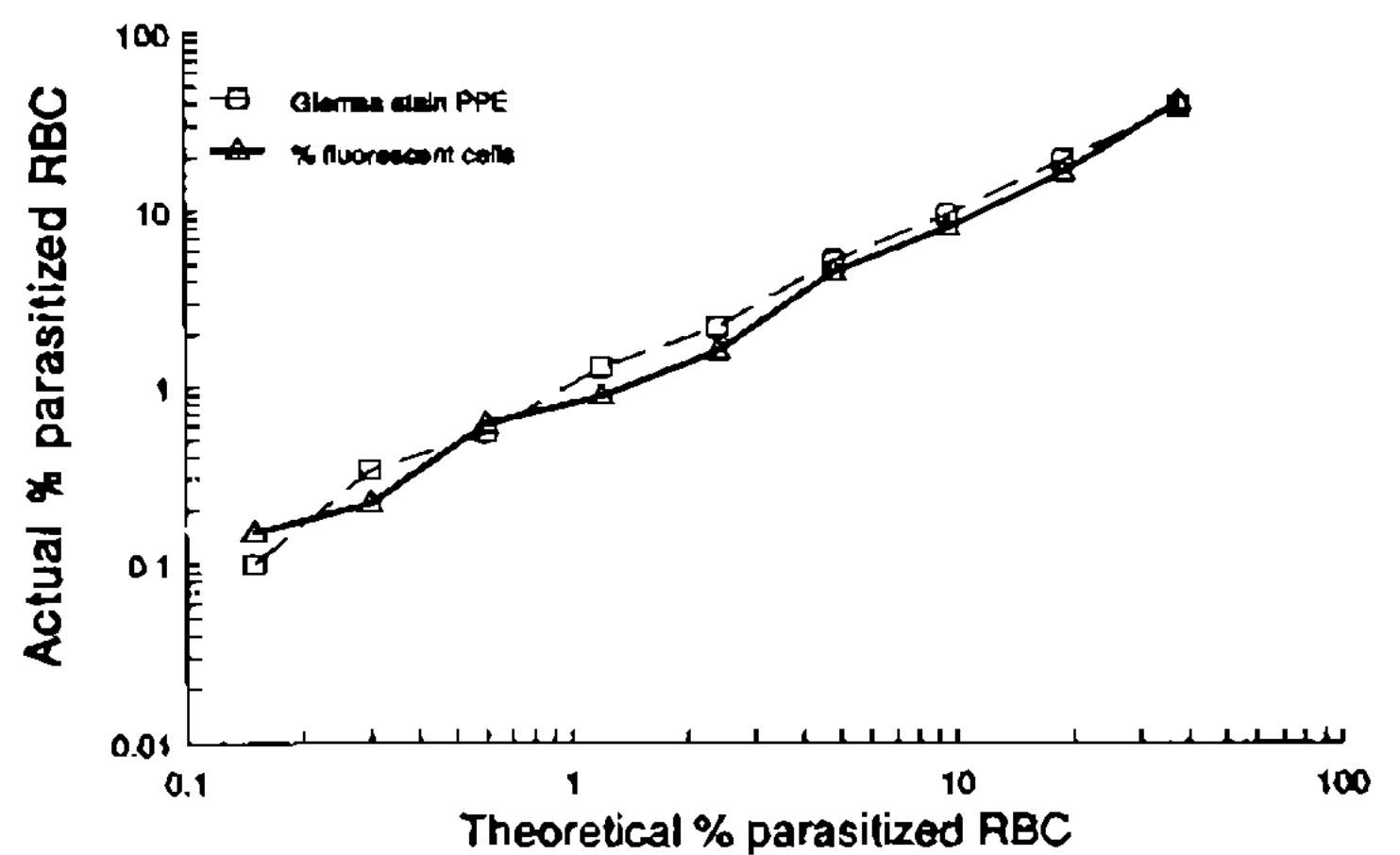


Fig. 2: comparison of PPE quantitation by Giemsa stain and by vital dye incorporation with theoretical percent parasitized erythrocytes. Infected erythrocytes were serially diluted with uninfected erythrocytes for each data point. Theoretical values were calculated by serially dividing initial Giemsa stain PPE determination by 2. The experiment was repeated with similar results.

**Viability assay** – Following the demonstration that comparable measurements are obtained by FC and light microscopy, further studies were conducted to determine if the HE assay could be used to monitor parasite viability under experimental conditions that might be used to study immune responses to parasites. Replicate sets of cultures of uninfected and infected erythrocytes were set up in 96-well plates. Two sets of cultures were treated with Ganaseg, one at the initiation of culture and the second at 24 h. A set of untreated cultures served as controls. All cultures were sampled at intervals for 48 h and evaluated as above. Cultures of untreated parasites exhibited a linear increase in organisms by both assays (Fig. 3, panel A). When Ganaseg was included in the cultures at initiation, both detection methods indicated absence of growth for 8 h followed by decreased viability, which lasted through the 48 h period (Fig. 3, panel B).

When Ganaseg was added at 24 h after initiation of culture, immediate arrest of replication could be detected and the decline of viable organisms followed (Fig. 3, panel C). Both assay procedures yielded comparable results.

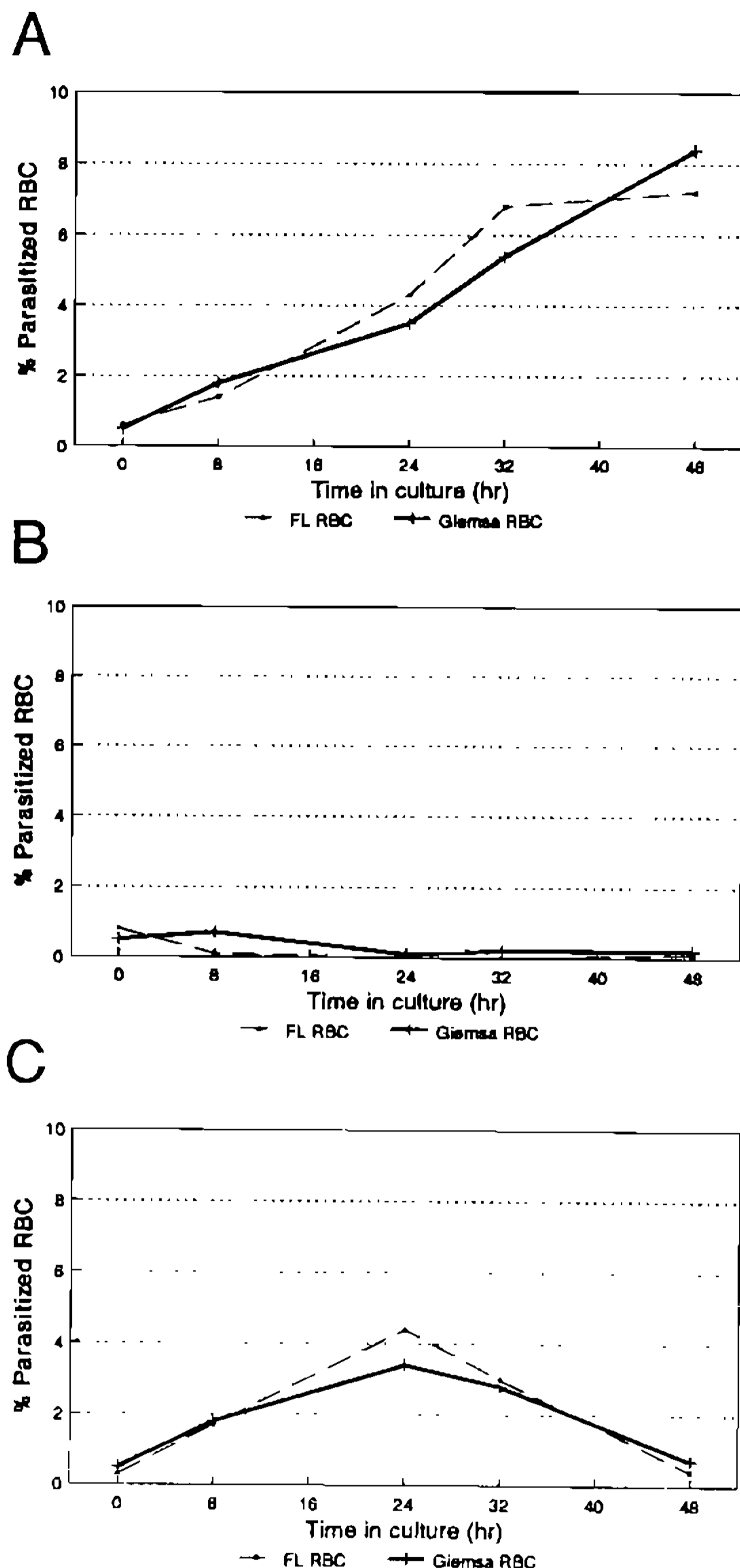


Fig. 3: comparison of viability detection by Giemsa stained blood film evaluation and by hydroethidine conversion in the presence of a parasitocidal drug. Samples were taken from a 96-well plate over a 48 h period. Panel A depicts untreated control cultures. Panel B depicts cultures which received Ganaseg at 0 h. Panel C depicts cultures which received Ganaseg at 24 h. The experiment was repeated in a 24-well plate with similar results.

## DISCUSSION

Hydroethidine is a relatively new vital dye which is currently being used in the study of nucleated cells such as tumor cells (Bucana et al., 1986) and analysis of target and killer cell interactions following conjugate formation (Cavarec et al., 1990). When taken up by living cells, HE is converted to ethidium, a fluo-

rochrome that intercalates into DNA (Gallop et al., 1984). Since ethidium is excited at 488 nm and emits at 585 nm (range 563-607), it can be used in flow cytometry in conjunction with other fluorochromes coupled to antibodies. Thus it is now possible to monitor the viability and growth characteristics of nucleated cells (including protozoan parasites *in vitro*) following exposure to drugs, immune cells, cytokines, and antibodies. At least two fluorochromes coupled with antibodies can be used with HE labeled parasites to analyze the phenotype and functional activity of effector cells placed in culture with parasites. As illustrated here, we have established HE can also be used to identify and quantitate parasites present in erythrocytes. The technique is easy to use and offers a way to obviate the difficulties encountered with other methods of assessing viability (Ockenhouse et al., 1984; Carlin & Jensen, 1986; Goff & Yunker, 1986; Waters et al., 1987; Jones et al., 1989; Lunel & Druihe, 1989). The method is rapid and sensitive, allowing the analysis of 2,000 to 100,000 erythrocytes in minutes. The ease and speed of analysis permits the monitoring of the effects of treatment throughout the period of study. The results obtained are objective and not reliant on evaluation by light microscopy or incorporation of radionucleotides. Moreover, the technique is as sensitive as previously used methods.

Efforts to standardize conditions for use of HE with *Babesia* demonstrated *Babesia* can be cultured in different sized culture vessels without reduction in viability and importantly, that uptake of the dye is uniform under all conditions tested. Studies in culture vessels including small flasks, 24-well plates and 96 well plates showed good correlations over a range of 0.06% to 70% parasitemias between the HE and Giemsa stain techniques of quantitation (Wyatt et al., 1991), indicating that viability of organisms can be accurately measured over a wide range of parasitemias.

Further studies involving the use of Ganaseg to model viability of *Babesia* under conditions to be used to study the effects of immune cells, cytokines, and antibodies demonstrated that it will be possible to detect both immediate and delayed effects on parasite viability. Treatment at the initiation of culture caused immediate cessation of growth of organisms. Treatment during the rapid phase of growth led to a gradual loss of viable parasites associated with uptake of the drug by metabolically active organisms.

The technique described here provides a useful approach to elucidate the mechanisms governing the regulation and expression of immune responses to intraerythrocytic parasites. Such information is critical to the development of improved vaccines.

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