virus surface antigen developed typical vesicular lesions on the tongue at the 10^2 PFU and 10^3 PFU VSV injection sites within 2 days (Table 2). In contrast, only two of six cows vaccinated with v50 developed vesicular lesions at the lower challenge dose of VSV. The remaining four cows that were vaccinated with v50 had no lesions at the 10^2 PFU VSV injection sites although they all developed lesions at the 10^3 PFU sites. In one cow (28Y) there was partial protection even against the higher VSV challenge dose since the lesion at the 10^3 PFU site remained localized. In all other cows the lesion spread to the entire surface of the tongue.

A good correlation was observed between antibody titers and protection against 10^2 PFU of VSV. All animals that were protected had neutralizing antibody titers of 1280 or greater whereas those that were sensitive had titers of 640 or lower. The degree of protection obtained is particularly noteworthy in view of the severity of the experimental challenge.

Previous experience with mass production, storage, and administration of smallpox vaccine suggests that vaccinia virus or related poxvirus recombinants would be economically feasible as veterinary vaccines. Moreover, the ability of recombinant vaccinia virus to stimulate a cytotoxic T-cell response specific for a cloned surface glycoprotein (*21*) may provide a significant advantage over subunit or inactivated whole vaccines, which in general do not prime effectively for cell mediated immunity. The large capacity of vaccinia virus for foreign DNA (*22*) also raises the possibility of multivalent vaccines for different serotypes of the same virus or even against entirely different pathogenic agents.

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16. Construction of pMM34. PGS20 (*3*) was cleaved with Xho I and partially digested with Ava I and a DNA fragment of approximately 5 kilobases (kb) was isolated. The ends of the latter were ligated together and used to transform *Escherichia coli* HB101. A resulting plasmid lacking an Xho I site was cleaved with Sma I and ligated to a phosphorylated Xho I linker. This plasmid was then cleaved with Hind III and partially digested with Pvu II. The 5′ overhanging end of the Hind III site was filled in with deoxyribonucleotides by means of the large fragment of DNA polymerase I. A 4.2-kb DNA segment was isolated. The ends of the DNA were ligated together and the resulting plasmid was designated pMM34.
17. Construction of pNJGE-2. The entire sequence coding for the VSV_{NJ} G protein was excised from pNJG6 (*13*) by partial digestion with Pst I, purified by polyacrylamide gel electrophoresis and trimmed with nuclease Bal 31 to remove the GC (G, guanine; C, cytosine) tails. Synthetic DNA linkers containing the Xho I site (CCTCGAGG) (C, cytosine; T, thymine; G, guanine; A, adenine) were then ligated to the trimmed DNA. This DNA was then cloned into the single Xho I site of plasmid JC119 (*15*). One plasmid obtained by this procedure (designated pNJGE-2) was found to have the sequence GGTATG . . . at the 5′ end following the Xho I linker. The ATG is the initiation codon for the VSV_{NJ} G protein.
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Fluorogenic Substrate Detection of Viable Intracellular and Extracellular Pathogenic Protozoa

Abstract. *Viable* Leishmania *promastigotes and amastigotes were detected by epifluorescence microscopy with fluorescein diacetate being used to mark living parasites and the nucleic acid–binding compound ethidium bromide to stain dead cells. This procedure is superior to other assays because it is faster and detects viable intracellular as well as extracellular* Leishmania. *Furthermore, destruction of intracellular pathogens by macrophages is more accurately determined with fluorescein diacetate than with other stains. The procedure may have applications in programs to develop drugs and vaccines against protozoa responsible for human and animal disease.*

Leishmaniasis causes more medical problems than any other protozoal disease except malaria. Phlebotomine sandfly vectors bite and infect man with promastigote stages of 13 *Leishmania* species. Within mononuclear phagocytes, motile promastigotes transform into small, nonmotile, oval amastigotes that induce species-dependent visceral or cutaneous disease. There are no safe, reliable vaccines, and chemotherapy is dangerous and is often ineffective (*1–4*).

Assays of cell viability are frequently required in research with pathogenic protozoa. *Leishmania* viability has been determined by [^{3}H]thymidine uptake (*5*), by parasite transformation or replication in vitro (*6, 7*), and by microscopic procedures with vital stains or dyes (*8–11*). However, radioisotopic procedures are not simple, some isolates grow poorly in vitro (*12*), vital staining may be difficult to detect (*9*), and no procedure rapidly identifies viable intracellular *Leishmania* by their metabolic activity.

We report the use of fluorescein diacetate (FDA) and ethidium bromide (EB) to detect viable intra- and extracellular *Leishmania* in a two-color epifluorescence microscope procedure (*13–16*). Esterases in living cells convert highly permeable, nonfluorescent FDA to fluorescein. Accumulated fluorescein imparts a striking yellow-green cellular fluorescence, termed fluorochromasia, to cells irradiated with light at 419 nm (*13*). Dead or damaged cells do not exhibit fluorochromasia and, unlike living cells, are rapidly penetrated by EB. Complexes of EB and nucleic acids cause dead cells illuminated at 546 nm to fluoresce red (*17*).

Living and dead *Leishmania donovani* promastigotes and intracellular amastigotes were incubated with EB and FDA and photographed on glass slides or in a

hemocytometer by epifluorescence illumination (Fig. 1). No fluorescence was detected in cells not treated with EB and FDA. Staining of living (yellow-green) and dead (red) promastigotes was evaluated by counting cell suspensions containing 0 to 100 percent living parasites. The medium-thickening agents Protoslo and Polyox (*15*) were used to reduce promastigote motility, permitting accurate counts of these normally active cells. Counts of viable *Leishmania braziliensis panamensis* promastigotes were not significantly different from expected values (Fig. 2). Similar results were obtained with *L. donovani*.

Table 1. Correlation between viability of *L. donovani* amastigotes determined by EB and FDA staining and transformation into promastigotes in vitro. Hydroxyurea prevented promastigote replication after transformation. Viable (FDA-stained) amastigotes transformed into promastigotes after 3 days of cultivation but Formalin-fixed (EB-stained) amastigotes did not.

Day	Hydroxyurea	Cell type	Number of cells per milliliter	Cells stained by FDA (%)	Cells stained by EB (%)
1	Yes	Amastigotes	10^6*	100	0
3	Yes	Promastigotes	10^6*	100	0
1	Yes	Fixed amastigotes	10^6*	0	100
3	Yes	Fixed amastigotes	10^6*	0	100
1	No	Amastigotes	10^6*	100	0
3	No	Promastigotes	2.4×10^7†	100	0
1	No	Fixed amastigotes	10^6*	0	100
3	No	Fixed amastigotes	10^6*	0	100

*Range, 9×10^5 to 1.1×10^6 ($n = 3$). †Range, 2.2×10^7 to 2.6×10^7 ($n = 3$).

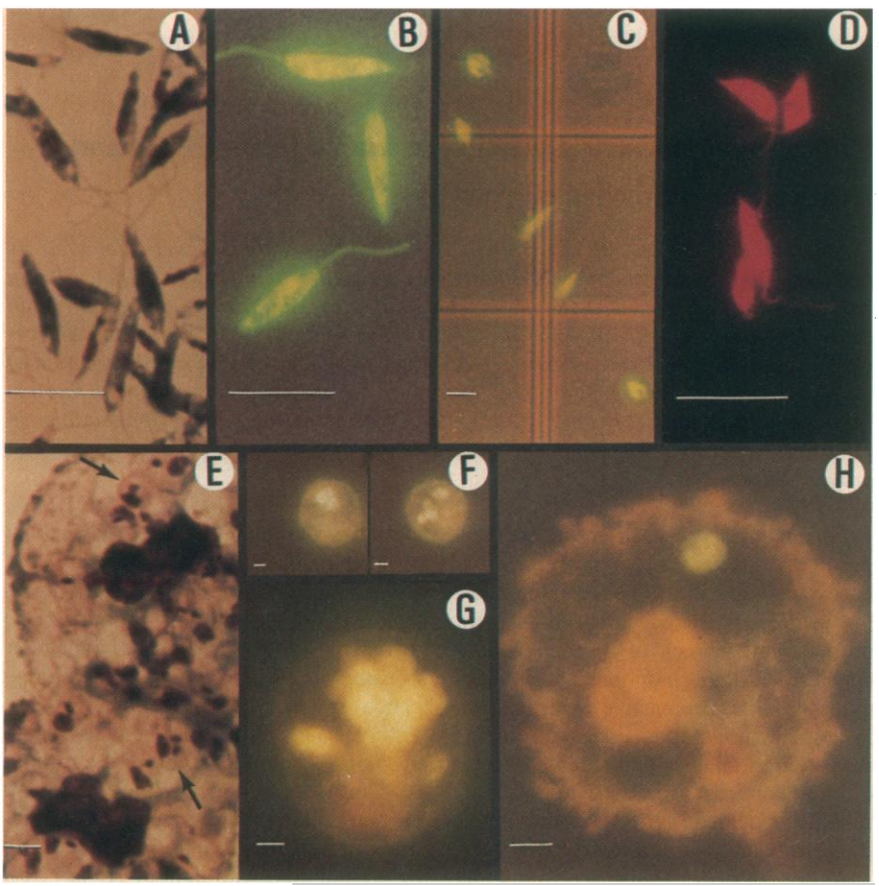


Fig. 1. Light and fluorescence micrographs of *Leishmania* promastigotes and macrophages infected with amastigotes. (A) Methanol-fixed *L. tropica* promastigotes stained with Diff Quik. (B) *Leishmania tropica* promastigotes showing fluorochromasia due to FDA metabolism. (C) Fluorochromasia-positive *L. tropica* promastigotes in a hemocytometer. (D) Formalin-fixed *L. tropica* promastigotes showing the red fluorescence of EB-nucleic acid complexes. (E) Methanol-fixed *L. donovani*–infected murine macrophages stained with Diff Quik (arrows indicate intracellular parasites). (F) Macrophages infected with *L. braziliensis panamensis*, with both parasites and host cells showing fluorochromasia. (G) Fluorochromasia of macrophages and *L. braziliensis panamensis* amastigotes. (H) Fluorochromasia of a single living *L. braziliensis panamensis* amastigote in a dead, EB-stained red macrophage. Host cell death was due to adverse conditions during photography. Scale bars: 20 μm (promastigotes) and 5 μm (amastigotes).

The EB-FDA procedure identified living and dead *L. donovani* or *L. braziliensis panamensis* promastigotes cultured in vitro (Fig. 3). Cultures initiated with 1×10^7 cells per milliliter ceased division by day 6, but most *Leishmania* (about 98 percent) were alive and positive for fluorochromasia. Cultures initiated with dead, Formalin-fixed promastigotes (1×10^7 cells per milliliter) did not increase in number.

We also used a transformation assay (*14*) to ascertain whether viable amastigotes show FDA-induced fluorochromasia. Living and Formalin-fixed extracellular amastigotes were cultured in vitro for 3 days in the presence or absence of 10 m*M* hydroxyurea, an inhibitor of promastigote replication (*14*). Amastigotes exhibiting FDA-induced fluorochromasia transformed into viable promastigotes, but EB-induced red fluorescence marked dead parasites (Table 1).

We evaluated EB-FDA and Diff Quik staining of amastigotes in macrophages previously incubated with living or dead parasites (Table 2) (*14*). Diff Quik, a protein stain, did not differentiate living from dead intracellular amastigotes; both were equally stained. Whereas living amastigotes showed strong fluorochromasia in macrophages, dead intracellular amastigotes did not fluoresce after incubation with EB and FDA. This is because dead amastigotes cannot metabolize FDA and because EB, at the low concentrations used, did not enter living macrophages in levels high enough to cause dead amastigotes to fluoresce red.

After 1 hour fewer macrophages containing living parasites were detected with EB and FDA than with Diff Quik (23.1 and 44.6 percent, respectively (Table 2). This difference could be due to a low percentage of viable amastigotes in the infecting inoculum, suboptimum intracellular concentrations of FDA, or differences in macrophage uptake of viable and nonviable amastigotes. These possibilities are unlikely, however, since (i) 95 percent of the living extracellular amastigotes were FDA-positive before and after incubation with macrophages, (ii) a twofold increase in FDA failed to stain more intracellular amastigotes, and (iii) frequency distributions of viable intracellular amastigotes were very similar to those of dead intracellular parasites (Fig. 4). It is more likely that some living amastigotes were killed during or shortly

after uptake by macrophages. This is supported by the finding that, at 48 hours (Table 2), equivalent numbers of infected macrophages were observed with EB and FDA or Diff Quik, suggesting degradation of nonviable parasites in macrophages similar to that observed at 48 hours in macrophages given dead amastigotes.

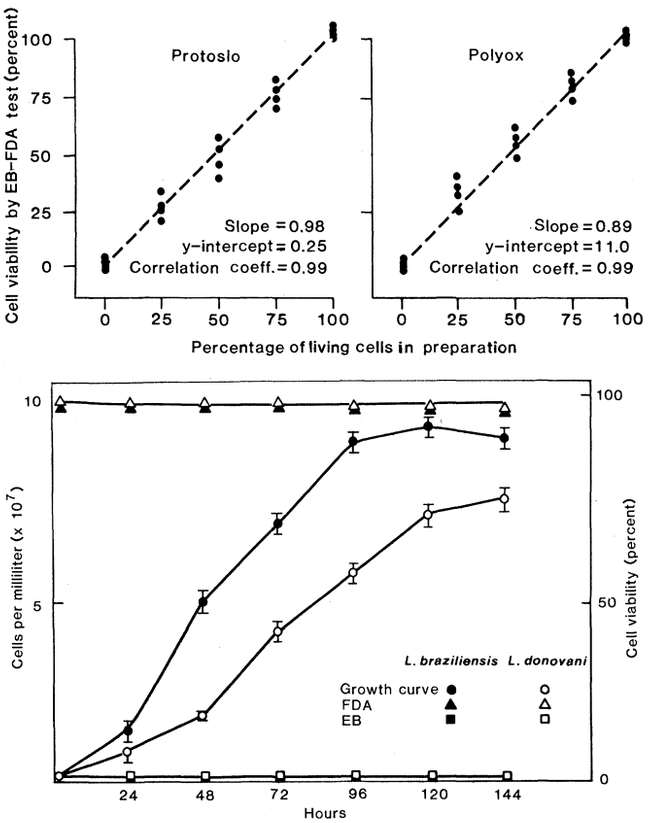
We also have used EB and FDA to detect *Leishmania* in preparations of thawed promastigote cryostabilates (*18*), heat- or fixative-killed promastigotes (*14*), and intracellular or extracellular amastigotes in homogenates of infected hamster spleen (*11*). Thus the procedure is versatile, and, since esterases are present in all *Leishmania* examined so far (*19*), FDA-induced fluorochromasia should be exhibited by any viable *Leishmania*.

Fluorochromasia-positive promastigotes eventually cease movement and die. Killing is caused by fluorescence, since illuminated, unstained promastigotes remain motile. Fluorescence has been reported to kill other protozoa stained with fluorescent compounds (*20*). Under prolonged illumination (5 to 10 minutes), promastigotes, amastigotes, and macrophages gradually lose FDA-induced fluorochromasia and fluoresce red as EB–nucleic acid complexes form. (A red, dying macrophage containing a living amastigote is shown in Fig. 1H.) Toxicity of FDA to *Leishmania* in vitro was not evaluated, but promastigotes

Table 2. Percentage of infection of murine macrophages incubated in vitro with living or dead (Formalin-fixed) *L. braziliensis panamensis* amastigotes, as determined with EB and FDA or Diff Quik. In four separate experiments living and dead amastigotes in 200 macrophages were stained by Diff Quik, but FDA-induced fluorochromasia marked only viable intracellular *Leishmania*. The percentages of infected macrophages (means ± standard error, $n = 8$) detected by these stains were significantly different at 1, 24, and 72 hours of incubation, reflecting the superior ability of FDA-induced fluorochromasia to detect viable *Leishmania*. No dead amastigotes were detected in living macrophages by EB; N.S., not significant.

Hours of incubation	Infected macrophages (percent)		Significance*
	EB-FDA	Diff Quik	
	Living amastigotes		
1	23.1 ± 3.2	44.6 ± 4.1	$0.01 > P > 0.001$
24	74.1 ± 4.3	85.1 ± 2.6	$P < 0.001$
48	65.9 ± 3.9	71.4 ± 3.2	N.S.
72	56.8 ± 5.7	63.2 ± 4.1	$0.05 > P > 0.02$
	Dead amastigotes		
1	0 ± 0	43.5 ± 6.0	$P < 0.001$
24	0 ± 0	35.5 ± 8.7	$0.01 > P > 0.001$
48	0 ± 0	6.5 ± 1.0	$P < 0.001$
72	0 ± 0	3.3 ± 1.0	$0.01 > P > 0.001$

*Student's *t*-test.



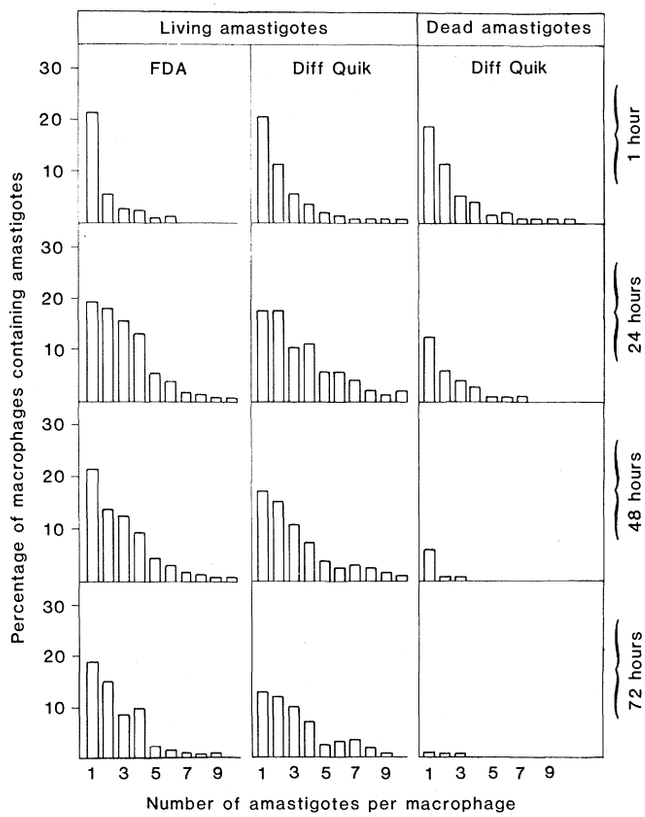


Fig. 2 (top left). Detection by EB and FDA staining of the percentage of living and dead *L. braziliensis* promastigotes in preparations (10^7 cells per milliliter) containing 0, 25, 50, 75, or 100 percent living cells. Promastigote motility was reduced with Protoslo or Polyox. Each point represents the percentage of living promastigotes in 100 counted cells; four separate preparations were examined. Dashed lines represent expected cell counts. Linear regression analysis provided the *y*-intercept and slope of the lines. Fig. 3 (bottom left). Growth and viability of EB- and FDA-stained *L. donovani* or *L. braziliensis panamensis* promastigotes during 6 days of cultivation in vitro. Cell counts are means ± standard errors for three experiments. Fig. 4 (right). Detection by EB and FDA or Diff Quik staining of *L. braziliensis panamensis* in cultivated murine macrophages infected with living or dead amastigotes. The number of amastigotes per macrophage is similar in cells incubated with living parasites and stained by either method. Dead intracellular amastigotes declined in number, as determined by Diff Quik staining. No dead intracellular *Leishmania* were stained by EB and FDA.

were motile and fluorochromasia-positive after a 3-hour incubation with EB and FDA at 5°C.

Ethidium bromide or FDA has been used in viability assays of free-living protozoa and other cells (*13, 17, 21–25*), and parasitic protozoa of the genera *Acanthamoeba, Babesia, Crithidia, Entamoeba, Giardia, Plasmodium*, and *Trypanosoma* exhibit fluorescent substrate–induced fluorochromasia (*26*). We believe, therefore, that the EB-FDA procedure may be of value in immunologic, biochemical, and pharmacological research with protozoan pathogens responsible for many human and animal diseases.

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15. Fluorescein diacetate (5 mg/ml in acetone) was kept at −20°C until use. For each experiment FDA (30 μg/ml) was prepared in PBS. Ethidium bromide (20 μg/ml in PBS) was kept at −20°C before use. Promastigotes were stained with EB and FDA by mixing 50 μl of the cell suspension in PBS with 25 μl of FDA and 25 μl of EB. After 2 minutes at 25°C, 50 μl of this was mixed with 50 μl of Protoslo or 1 percent (weight to volume) Polyox in PBS [D. M. Spoon, C. O. Feise, R. S. Youn, *J. Protozool.* **24**, 471 (1977)]. When extracellular amastigotes or infected macrophages were stained with EB and FDA, PBS was substituted for Protoslo or Polyox.
16. Yellow-green fluorescence due to FDA metabolism and red fluorescence due to EB–nucleic acid complexes were detected by epifluorescence microscopy of cells on glass slides under cover glass or in a phase hemocytometer. An American Optical Fluorostar microscope with a mercury-vapor lamp was used with various filter combinations. No cells showed autofluorescence. Viable promastigotes, macrophages, and intracellular or extracellular amastigotes showed FDA-induced fluorochromasia. Dead promastigotes, macrophages, and extracellular amastigotes exhibited red fluorescence. Slow penetration of EB into living cells at the concentrations used prevented detection of dead amastigotes in living macrophages.
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Ascorbic Acid and the Behavioral Response to Haloperidol: Implications for the Action of Antipsychotic Drugs

Abstract. *Haloperidol, a widely used antipsychotic drug, was tested for its ability to block the behavioral response to amphetamine and to elicit catalepsy in rats treated with saline or ascorbic acid (1000 milligrams per kilogram of body weight). By itself, ascorbic acid failed to exert significant behavioral effects, but it enhanced the antiamphetamine and cataleptogenic effects of haloperidol (0.1 or 0.5 milligrams per kilogram). These results, combined with a growing body of biochemical evidence, suggest that ascorbic acid plays an important role in modulating the behavioral effects of haloperidol and related antipsychotic drugs.*

Haloperidol is one of the most widely prescribed antipsychotic drugs in the United States today. It is often the drug of choice in the treatment of schizophrenia and the paranoid psychosis produced by chronic amphetamine abuse (*1*). Apart from its therapeutic benefits, haloperidol also causes muscular rigidity and other extrapyramidal side effects (*2*). An overwhelming body of evidence suggests that both the antipsychotic and the motoric effects of haloperidol are mediated, in large part, by a blockade of forebrain dopamine receptors. In animal tests of antipsychotic efficacy, for example, haloperidol blocks the open-field behavior produced by amphetamine and other dopamine agonists in rats (*3*). The clinical potency of haloperidol is closely related to its ability to bind and presumably block dopamine receptors in vitro (*4*). Moreover, when administered alone, haloperidol elicits catalepsy, an animal analog of muscular rigidity that is believed to reflect a blockade of dopamine receptors in the neostriatum (*5*).

The dopamine agonists that have been tested with haloperidol either release ascorbic acid (AA) from brain tissue (*6, 7*) or are prepared with AA before injection (*8*). Furthermore, AA appears to be an essential ingredient of assays in vitro that have demonstrated a high affinity of haloperidol for the dopamine receptor (*9*). When combined with evidence that both AA and haloperidol accelerate the firing rate of neostriatal neurons (*6, 10*), these data suggest that an AA-induced interaction with the dopamine receptor modulates at least some components of the behavioral response to haloperidol. We now report results consistent with this hypothesis that, in standard animal tests, AA enhances the antipsychotic and extrapyramidal actions of haloperidol.

The behavioral response to *d*-amphetamine (1.0 mg per kilogram of body weight) was recorded from male Sprague-Dawley rats (approximately 300 g) housed individually under standard laboratory conditions (*11*). Each rat had been treated 10 minutes earlier with an intraperitoneal injection of haloperidol (0.1 mg/kg), AA (1000 mg/kg), haloperidol (0.1 mg/kg) mixed with AA (1000 mg/kg), or a comparable volume of saline (*12*). The *d*-amphetamine was adminis-