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Flow Cytometric Assessment of Allopurinol Susceptibility in *Leishmania infantum* Promastigote

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Background: Leishmaniasis is a major tropical and subtropical parasitic disease. Sodium stibogluconate, N-methyl -D-glucamine antimoniate, amphotericin B, pentamidine, and ketoconazole are drugs used to treat this disease. Some of these drugs cause severe adverse side effects and treatment failures are common. Allopurinol, a purine analog, has been used to treat leishmaniasis, alone or combined with the previously mentioned drugs. Low cost, ease of administration (oral), and lack of toxicity make allopurinol a particularly appealing candidate.
Methods: The effect of allopurinol on *Leishmania infantum* (MCAN/ES/89/IPZ229/1/89, zymodeme MON1) wild-type promastigotes (*wt-p229*), and an altered form of these promastigotes (*allo-p229*) resulting from long term in vitro exposure to allopurinol, was determined by [^{3}H]-thymidine incorporation assays and by diverse flow cytometric approaches.
Results: Allopurinol arrested the proliferative capacity of *wt-p229* promastigotes, reduced the proportion of viable cells, and decreased their total protein content. In contrast, *allo-p229* promastigote proliferation was only slightly decelerated and the proportion of viable cells and the protein content were not affected by the allopurinol treatment.
Conclusions: The flow cytometry approach allowed us to demonstrate differences in allopurinol susceptibility of the two promastigote forms, expanding the spectrum of flow cytometry applications in studies of parasite resistance. Cytometry 40:353–360, 2000.

Key terms: *L. infantum* promastigotes; susceptibility; allopurinol; flow cytometry; proliferation; viability; protein content

Leishmaniasis is a major tropical and subtropical parasitic disease. The yearly prevalence is estimated at 12 million people worldwide and 200–350 million people are at risk. In the Mediterranean region, leishmaniasis caused by *Leishmania infantum* has emerged as one of the important opportunistic infections of human immunodeficiency virus (HIV)-positive individuals (1,2). Moreover, the prevalence of canine leishmaniasis in this region may be as high as 42%. Dogs and wild canids are important reservoirs and are mainly responsible for the persistence of the disease in this region (3,4). Pentavalent antimonial agents (Sb^V) in the form of sodium stibogluconate (Pentostam) or N-methyl-D-glucamine antimoniate (Glucantime) are still considered the drugs of choice against leishmaniasis, despite their potential cardiac and renal toxicity, difficulty of administration, and high costs. Second-line drugs, such as amphotericin B and pentamidine, do not have a therapeutic index as favorable as that of antimonials. They also require long-term therapy and often induce toxic effects. Amphotericin B in liposomes and the nucleoside analog, allopurinol, have been considered as possible alternative drugs for replacing antimonials in certain leishmania infections (5).

Allopurinol, a purine analog, has been used for treatment of visceral leishmaniasis in humans, either alone or combined with the previously mentioned drugs with varying results. Low cost, ease of administration (oral), and lack of toxicity make allopurinol a particularly appealing candidate for the treatment of leishmaniasis (6). In leishmania, allopurinol is aminated to adenine nucleotide analogs that inhibit purine biosynthesis. Incorporation into RNA results in a net breakdown of mRNA and hence an inhibition in protein synthesis (7–9).

The therapeutic potential of allopurinol for the treatment of canine leishmaniasis alone or in combination with antimonials has been reported. However, despite clinical cure, relapses have been observed, and parasite clearance was not achieved, in dogs treated solely with allopurinol (10,11).

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In this study, the effect of allopurinol on wild-type promastigotes (*wt-p229*) and on promastigotes of the same isolate that had been cultivated in vitro in the presence of up to 800 μg/ml allopurinol for a period of 1 year (*allo-p229*) was monitored by [^{3}H]-thymidine incorporation assays and by diverse flow cytometric approaches.

The stable intracytoplasmic dye, 5-, 6-carboxyfluorescein diacetate succinimidyl ester (CFSE), was used as a quantitative method to measure cell division. It allows both identification of cell progeny and analysis of the division history of individual cells that have undergone multiple rounds of division seen as discrete peaks with a progressive reduction of CFSE fluorescence intensity (12). To determine the viability of promastigotes, the membrane-permeable nuclear stain, SYBR-14, was used in combination with propidium iodide (PI), a nucleic acid dye unable to cross intact plasma membranes of living cells (13). Fluorescein isothiocyanate (FITC), which binds covalently to positively charged groups of proteins, was used to determine total protein content in fixed promastigotes (14).

MATERIALS AND METHODS

Parasites and Drug

Promastigote forms (wild-type promastigotes) of *L. infantum* (MCAN/ES/89/IPZ229/I/89, zymodeme MON 1: *wt-p229*) were maintained at 27°C in 25-cm^2 tissue culture flasks (Corning) in 5 ml of a liquid medium (pH 7.4) supplemented with 10% heat-inactivated calf serum (15). Promastigotes of the same isolate (*allo-p229*) were maintained simultaneously in the same medium to which increasing concentrations of allopurinol were added (final allopurinol concentrations: 100 μg/ml for 3 weeks, 200 μg/ml for 3 weeks, 400 μg/ml for 2 weeks, and 800 μg/ml thereafter for a total period of 12 months). Allopurinol (Sigma) was added from a 10 mg/ml stock solution prepared in 0.1 N NaOH.

[^{3}H]-Thymidine Incorporation Assay

Three-day-old promastigotes (late logarithmic growth phase) were washed three times in phosphate buffered saline (PBS, 1200*g*, 10 min at 4°C) and resuspended in fresh, serum-free medium at a density of 20–30 × 10^6 cells/ml. Drugs were serially diluted in 0.1 N NaOH. Twenty microliters of each drug dilution was added to 180 μl parasite suspension, in triplicate, in 96-well round-bottomed microtiter plates. After 24 h at 27°C, 10 μl per well [^{3}H]-thymidine (New England Nuclear, Boston, MA; final concentration of 5 μCi/ml) was added and plates were incubated for another 24 h. The cells were harvested on glass fiber filters using an automatic harvester (LKB), washed extensively with distilled water, and counts per minute (cpm) were read in a Beta plate Liquid Scintillation Counter (Wallac) without a previous TCA washing step. An assay was considered valid if the standard deviations of the triplicate counts was <15% for all dilutions and control counts were above 3,000 cpm. Data analysis was based on at least three independent valid assays.

Fluorescent Probes

All fluorescent stains were purchased from Molecular Probes Europe BV (Leiden, The Netherlands). A 2.8-mg/ml stock solution of CFSE was prepared in dimethylsulfoxide (DMSO) and stored at −20°C. Propidium iodide (PI) was supplied as a 1 mg/ml solution in water. SYBR-14 was diluted in DMSO (1 mg/ml) and fluorescein isothiocyanate (FITC) was dissolved in PBS to give a solution of 1 mg/ml.

Staining with CFSE. Promastigotes (logarithmic growth phase) were washed three times and resuspended in 2 ml PBS (6 × 10^7 cells/ml) and 2.8 μg/ml CFSE was added. Cells were incubated at 37°C for 10 min during which they were carefully mixed three to four times. Several volumes of ice-cold medium supplemented with 10% inactivated calf serum were added. After centrifugation at 1,200*g* for 10 min (4°C), stained cells were resuspended in fresh medium (density adjusted to 5 × 10^6 cells/ml) and further cultivated in 25-cm^2 tissue culture flasks at 27°C in the presence of 0, 400, and 800 μg/ml allopurinol, respectively. The CFSE mean fluorescence was determined immediately after staining and after 24, 48, 72, and 96 h.

Staining with PI and SYBR-14. Promastigote cultures were initiated at a cell density of 5–10 × 10^6 cells/ml medium with and without 400 μg/ml allopurinol. After an incubation period of 48 h at 27°C, cells were washed three times in PBS. Approximately 4 × 10^6 promastigotes were resuspended in 2 ml PBS, and 10 μg/ml PI and 0.1 mg/ml of SYBR-14 were added. The promastigotes were protected from direct light and incubated at 37°C for 30 min before the flow cytometry analysis.

Staining with FITC. Promastigotes were cultivated and washed as stated above. 4 × 10^6 promastigotes/ml were incubated in 2 ml PBS containing 4 μg/ml DNase (Type I, Sigma, St. Louis, MO) at 37°C for 10 min. After the DNase treatment, cells were fixed in 70% methanol for 6 h. The fixed cells were washed, resuspended in PBS (4 × 10^6 cells/ml), and stained with FITC (final concentration of 0.1 μg/ml) at 37°C for 30 min.

Flow Cytometric Analysis

The green fluorescence of CFSE, SYBR-14, and FITC and the red fluorescence of PI were excited at 488 nm (FACSCalibur, Becton Dickinson, Heidelberg, Germany). The cells were acquired in log forward and log side scatter and a gate was set up on the cell population. The fluorescence intensities of stained and unstained *wt-p299* and *allo-p229*-promastigotes, treated and untreated with allopurinol, were determined and compared. At least 10,000 cells were analyzed per run and each staining experiment was repeated four times. Data analysis was performed on fluorescence intensities that excluded cell autofluorescence and cell debris. CELLQuest analysis software was used to determine the fluorescence and perform most of the data analysis, whereas WinMDI was used to generate the three-dimensional histogram overlays.

The decrease of the green CFSE fluorescence intensity in promastigotes treated and untreated with allopurinol

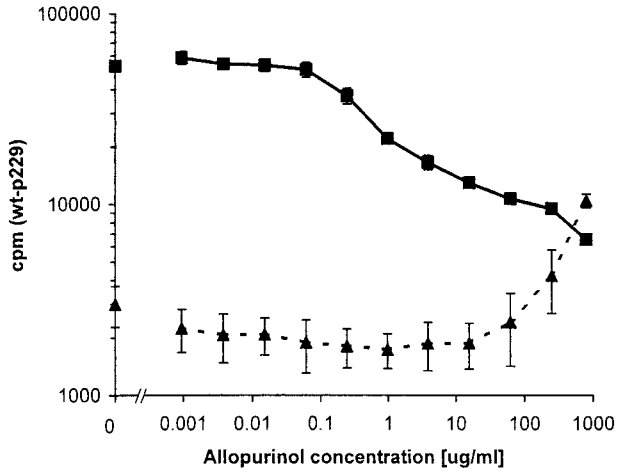

FIG. 1. [^{3}H]-thymidine incorporation of *wt-p229* (solid line) and *allo-p229* (dashed line) promastigotes in the presence of different allopurinol concentrations. Because the difference in thymidine uptake between the two populations varies by about two orders of magnitude, the X-axis is in log scale.

gave an indication of the parasite proliferation in subsequent days. The functional parameters reflecting parasite viability were quantified by generating density plots for the PI and SYBR-14–stained promastigotes. Each population appearing on the density plot was gated and quantified as a percentage of the total number of gated promastigotes. The protein content of the fixed cell was determined by the FITC fluorescence intensity of the control and allopurinol-treated promastigotes.

RESULTS

[^{3}H]-Thymidine Incorporation Assay Results

Figure 1 shows the dose-response curve of *wt-p229* and *allo-p229* promastigotes in the presence of different concentrations of allopurinol. The *wt-p229* curve is characteristically sigmoidal. There was a progressive decreased [^{3}H]-thymidine of incorporation corresponding to increased allopurinol concentrations. However, a complete inhibition of [^{3}H]-thymidine incorporation in the presence of allopurinol for these promastigotes was not observed. The maximum inhibition achieved in our system was 80%.

In contrast, the *allo-p229* promastigotes incorporated more [^{3}H]-thymidine in the presence of higher concentrations of allopurinol. Drug concentrations above 70 μg/ml led to a [^{3}H]-thymidine incorporation higher than that of the no-drug control. The incorporation of [^{3}H]-thymidine at 1,000 μg/ml was 3.5 times higher than the incorporation in the absence of the drug. However, the maximum counts per minute (cpm) observed in *allo-p229* promastigotes were about five times lower than those of the *wt-p229* promastigotes.

Flow Cytometry Results

Promastigote proliferation determined by CFSE staining. The promastigote proliferation in the presence and absence of allopurinol was determined by CFSE staining and the results from each treatment were grouped and the histogram overlaid in order to show the decrease in CFSE fluorescence intensity in subsequent days (Fig. 2). Six panels of overlaid histograms show the CFSE fluorescence for *wt-p229* (panels 1, 3, and 5) and for *allo-p229* (panels 2, 4, and 6) promastigotes. The different panels show a series of discrete peaks exhibiting a progressive serial decrease of CFSE fluorescence, at different time intervals, a feature suggestive of cell division.

Cell proliferation was further monitored by counting the cells in a Neubauer chamber. In the absence of the drug, the *wt-p229* promastigotes reached a maximum cell density of 1.1×10^8 cells/ml within 3 days. The calculated generation time of these cells was about 10 h within the first 48 h. The proliferation of *allo-p229* cells was slightly slower. These cells reached a maximum cell density of 1.0×10^8 cells/ml within 4–5 days with a generation time of about 12 h within the first 2 days. Panels 1 and 2 show histograms of the CFSE fluorescence for the untreated control *wt-p229* and *allo-p229* promastigotes in subsequent days, respectively. Loss of CFSE fluorescence was observed in both parasite forms, the loss being more pronounced on the first day.

Panels 3 and 4 show histograms of the CFSE fluorescence of the two parasite forms when incubated in the presence of 400 μg/ml allopurinol. In *wt-p229,* the fluorescence decrease was only marked on the first day; thereafter, the decrease was very slight. However, in the *allo-p229* promastigotes, a distinct continuous fluorescence decrease was observed during the subsequent 4 days. The same phenomenon was also observed when these parasites were incubated in the presence of 800 μg/ml allopurinol (panels 5 and 6).

The mean fluorescence intensities (MFI) of the untreated control, 400 μg/ml, and 800 μg/ml allopurinol-treated *wt-p229* and *allo-p229* promastigotes in subsequent days were determined (Table 1). There was a marked MFI decrease in the untreated *wt-p229* and *allo-p229* promastigotes. The overall decrease after 4 days in culture was higher in the *wt-p229* promastigotes, where it was reduced by a factor of 75.5 and by a factor of 66 in the *allo-p229.* The decrease in fluorescence intensity in the allopurinol-treated *wt-p229* promastigotes was pronounced only on the first day. Thereafter, the daily decrease was by an average factor of 1.3.

The overall decrease in MFI for the 400 μg/ml allopurinol-treated *wt-p229* promastigotes was by a factor of 5.3 and by a factor of 3.0 for 800 μg/ml allopurinol-treated *wt-p229.* In contrast, the corresponding MFI overall decreases for the *allo-p229* promastigotes were more than twice that of the *wt-p229* (11.0 and 7.9, respectively).

Promastigote viability. The viability of promastigotes was determined by SYBR-14 and PI dual staining. The results are given as two-parameter density plots in Figure 3. Panels 1 and 2 show the density plots for the untreated *wt-p229* and *allo-p229* controls, respectively. Panels 3 and 4 show the density plots of the 400 μg/ml allopurinol-treated *wt-p229* and *allo-p229* promastigotes. In panels 1 and 3, three subpopulations of *wt-p229* can be identified:

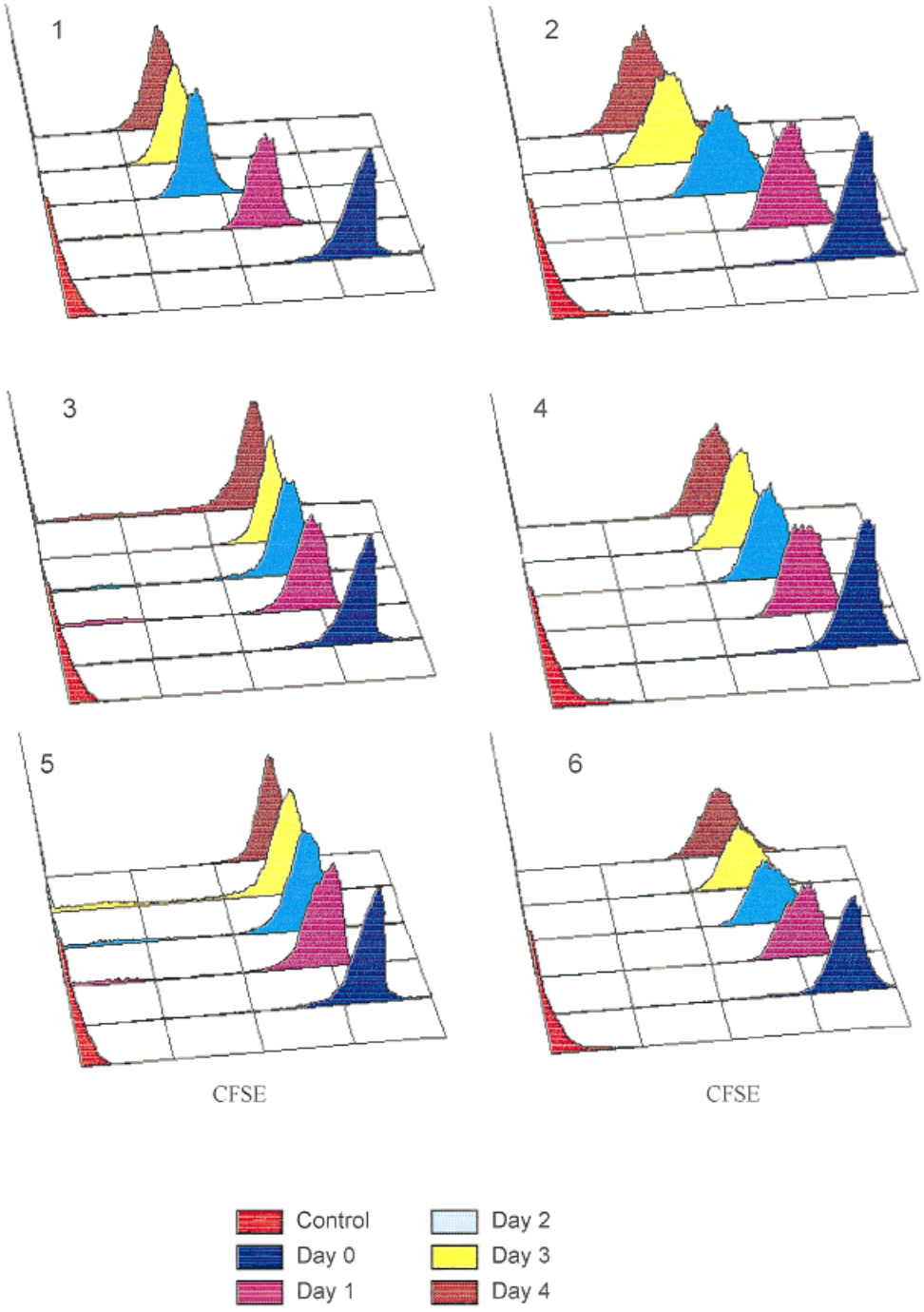

FIG. 2. Promastigote proliferation determined by CFSE staining of *wt-p229* (**panels 1,3,5**) and *allo-p229* (**panels 2,4,6**) promastigotes untreated (panels 1 and 2), exposed to 400 μg/ml allopurinol (panels 3 and 4), or to 800 μg/ml allopurinol (panels 5 and 6). Y-axis, cell counts; X-axis, CFSE fluorescence intensities (scale: 4 order logarithmic intensity).

one contains cells showing a high PI but a low SYBR-14 staining pattern (PI); another with cells stained strongly with SYBR-14 but not with PI (SYBR-14); and the third contains cells with an intermediate SYBR-14 and PI staining pattern (dual). The proportion of SYBR-14 cells was most prominent in both the control and allopurinol-treated groups. However, in the presence of 400 μg/ml allopurinol, the proportion of PI or dual-stained promastigotes was clearly increased.

In contrast, panels 2 and 4 show no distinct differences in the proportions of the PI, SYBR-14, and dual-stained subpopulations between the untreated and allopurinol-treated *allo-p229* promastigotes. Once again, most of the cells were stained with SYBR-14, whereas very few were PI or dual stained. The proportion (percentage) of *wt-p229* and *allo-p229* promastigotes stained with either PI, SYBR-14, or both (dual) is shown in Table 2. Twice as many *wt-p229* promastigotes are stained with PI in the

Table 1
CFSE MFI ± SD for wt-p229 and allo-p229 Promastigotes in the Absence and Presence of Allopurinol

	wt-p229			*allo-p229*		
Day	Untreated control	400 μg/ml	800 μg/ml	Untreated control	400 μg/ml	800 μg/ml
0	1,671.25 ± 43.2	1,671.25 ± 43.2	1,671.25 ± 43.2	2,412.07 ± 63.8	2,412.07 ± 63.8	2,412.07 ± 63.8
1	251.74 ± 34.3*(6.6)	794.04 ± 91.5*(2.1)	974.70 ± 27.0*(1.7)	624.68 ± 51.8*(3.9)	837.96 ± 93.9*(2.9)	1108.42 ± 102.1*(2.2)
2	43.47 ± 10.5*(38.4)	629.97 ± 90.3*(2.7)	748.11 ± 58.7*(2.2)	162.62 ± 20.9*(14.8)	478.55 ± 53.5*(5.0)	690.75 ± 89.1*(3.5)
3	27.31 ± 13.6*(61.2)	477.05 ± 58.3*(3.5)	654.63 ± 88.7*(2.6)	51.34 ± 16.4*(47.0)	296.73 ± 24.0*(8.1)	450.21 ± 73.3*(5.4)
4	22.15 ± 12.5*(75.5)	316.26 ± 53.3*(5.3)	562.49 ± 64.8*(3.0)	36.52 ± 15.0*(66.0)	219.98 ± 30.5*(11)	307.25 ± 59.8*(7.9)

[a]Ratio of MFI reduction between day 0 and the subsequent days (in parentheses).

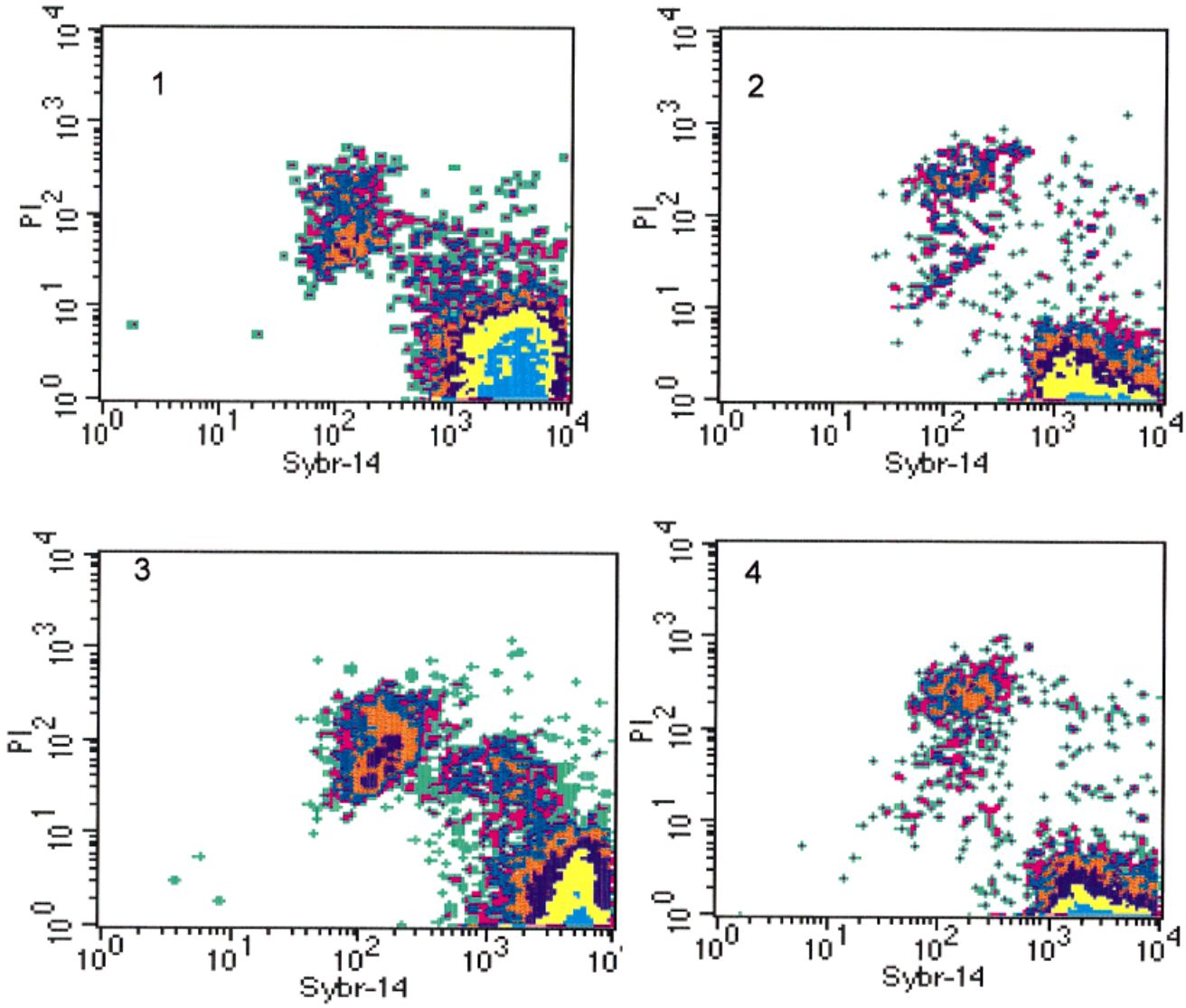

FIG. 3. Promastigote viability. SYBR-14 and PI staining of *wt-p229* (**panels 1 and 3**) and *allo-p229* (**panels 2 and 4**) promastigotes exposed to 400 μg/ml allopurinol (panels 3 and 4). The corresponding controls without allopurinol exposure are shown in panels 1 and 2. Y-axis, PI fluorescence intensities; X-axis, SYBR-14 fluorescence intensities.

allopurinol-treated group when compared with the control. This finding is also observed for the dual-stained promastigotes. Consequently, allopurinol treatment led to a clearly decreased proportion of SYBR-14–stained *wt-p229* promastigotes.

There was no significant difference in the proportion of PI, SYBR-14, or dual-stained *allo-p229* promastigotes between the untreated control and the allopurinol-treated cells.

Protein content determined by FITC. The total cellular protein content of fixed promastigotes was deter-

Table 2
Proportion (%) ± SD of wt-p229 and allo-p229 Promastigote Stained With PI, SYBR-14, or With Both Stains in the Absence and Presence of 400 μg/ml Allopurinol

	wt-p229		*allo-p229*	
Stain	Untreated control	400 μg/ml	Untreated control	400 μg/ml
PI	4.20 ± 1.69	8.75 ± 1.27	3.44 ± 0.65	4.24 ± 0.82
PI + SYBR-14	3.24 ± 0.41	5.31 ± 2.04	3.30 ± 0.60	3.96 ± 2.10
SYBR-14	92.45 ± 1.39	85.78 ± 2.18	92.31 ± 1.51	90.82 ± 2.22

Table 3
FITC MFI ± SD for wt-p229 and allo-p229 Promastigotes in the Absence and Presence of Allopurinol

wt-p229		*allo-p229*	
Untreated control	400 μg/ml	Untreated control	400 μg/ml
5,104.86 ± 152.31	3,874.72 ± 224.18	3,006.59 ± 456.0	2,793.31 ± 337.75

mined by staining with FITC, an acidic dye that binds covalently to the positively charged groups of proteins. Figure 4 shows the histograms of FITC fluorescence of *wt-p229* and *allo-p229* promastigotes incubated in the absence and presence of 400 μg/ml allopurinol for a period of 24 h. Allopurinol treatment led to a clear decrease in the MFI in *wt-p229* promastigotes only, whereas no distinct effect could be detected in *allo-p229* promastigotes (Table 3).

DISCUSSION

The effect of allopurinol on *L. infantum* (MCAN/ES/89/IPZ229/1/89, zymodeme MON1) wild-type promastigotes (*wt-p229*) and promastigotes cultivated in vitro in the presence of up to 800 μg/ml allopurinol for a period of 1 year (*allo-p229*) was determined by [^{3}H]-thymidine incorporation assays and flow cytometric approaches. [^{3}H]-thymidine incorporation clearly decreased in *wt-p229* promastigotes in the presence of increasing drug concentrations. However, absolute inhibition of [^{3}H]-thymidine incorporation was not achieved in our assay.

The [^{3}H]-thymidine incorporation results of *allo-p229* gave a completely different drug-response curve. High [^{3}H]-thymidine incorporation was only observed in the presence of high allopurinol concentrations.

Counting the promastigotes during the incorporation assay indicated that both *wt-p229* and *allo-p229* cells did proliferate best in the absence of allopurinol (proliferation factor 2). At increased drug concentrations, the proliferation was gradually inhibited in the *wt-p229* cells (no proliferation at 1,000 μg/ml allopurinol). In the *allo-p229* promastigotes, a decrease of cell proliferation rate was observed at lower drug concentrations (proliferation factor 1.5 at 1 μg/ml) followed by an increase of the proliferation rate in the presence of higher drug concentrations (proliferation factor 1.7 at 1,000 μg/ml). However, this proliferation rate remained lower than that of the no-drug control.

Differences between the two promastigote forms at the cellular level were further demonstrated by the different flow cytometry experiments. This article describes a new technique for the investigation of *Leishmania* promastigote proliferation using CFSE stain, which permits us to resolve and track population of cells that have undergone cell divisions. Until now, incorporation of [^{3}H]-thymidine has been the most common method for determining cell division. However, this approach can only give an indication of total amount of cell divisions and does not give any information on the division of individual cells. Tritiated thymidine is only incorporated by cells that undergo DNA

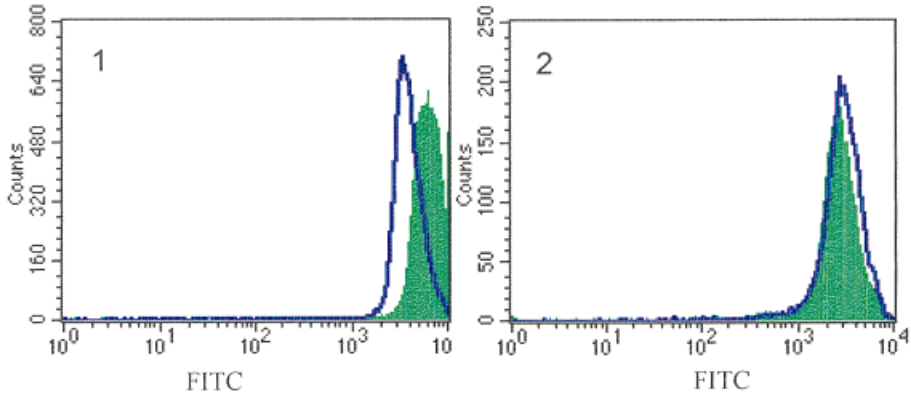

FIG. 4. Total cellular protein content. FITC staining of *wt-p229* (**panel 1**) and *allo-p229* (**panel 2**) promastigotes exposed to 400 μg/ml allopurinol (blue line) in direct comparison to untreated controls (green area). Y-axis, cell counts; X-axis, fluorescence intensity.

replication at the time the culture is pulsed, which underestimates proliferation if the majority of division occurs at an early phase of the culture (12). In our incorporation assay, [^{3}H]-thymidine was added after 24 h of allopurinol exposure. Therefore, the allopurinol effect within the first 24 h was not quantified. The use of CFSE complemented the [^{3}H]-thymidine assay, whereby the effect of allopurinol on the proliferation of these promastigotes at different time intervals was determined and the difference in susceptibility of the two parasite forms was further demonstrated. The proliferative capacity of *wt-p229* was clearly arrested by treating these cells with either 400 or 800 μg/ml allopurinol, whereas it was only slightly decelerated in the altered *allo-p229* promastigotes. This new flow cytometric application on *Leishmania* opens up potential studies in antileishmanial drug pharmacokinetic and toxicology studies.

The difference between the results of the [^{3}H]-thymidine incorporation assay and CFSE experiments is not fully understood. In *allo-p229* cells, [^{3}H]-thymidine incorporation seemed to be stimulated by higher allopurinol concentrations when compared to the no-drug control, whereas CFSE experiments showed a reduced proliferation rate in the presence of the drug. However, unlike *wt-p229* cells, the proliferation of the *allo-p229* cells was not completely arrested. These observations corresponded well with the actual cell counts determined during the [^{3}H]-thymidine incorporation assay. Thus, the CFSE approach seemed to give a more appropriate picture of the effects of allopurinol on these cells than the [^{3}H]-thymidine incorporation assay, which seemed to measure the overall [^{3}H]-thymidine uptake by the cells rather than the actual cell proliferation. Whether the observations made on the *allo-p229* promastigotes were indicative for resistance or higher tolerance to allopurinol, is not clear as yet.

The combination of SYBR-14 and PI has been used extensively in sperm viability studies (13). However, according to our knowledge, this is the first time that this dual staining is used to determine the viability of parasites. SYBR-14 stained the nucleus and mitochondrial DNA of the live promastigotes brilliant green and PI stained the nucleus of the dead promastigotes red. Some of the promastigotes were dual stained, with a resulting yellowish color of the nucleus. Flow cytometry was effective in quantifying the resultant fluorescent populations, SYBR-14–stained, PI-stained, and dual-stained promastigotes. Both dyes label DNA, thus avoiding the ambiguity of stains that target separate cellular organelles (13). This staining method had the advantages of being rapid (30 min) and the cells did not require extra processing prior to the staining. This study demonstrates that SYBR-14, when used in combination with PI, was effective for simultaneously visualizing the living and dead population of *Leishmania* promastigotes before and after treatment with allopurinol. The significant increase of dead (PI-stained) and dying (PI- and SYBR-14–stained) cells in the *wt-p229* promastigotes after exposure to allopurinol indicates a clear allopurinol susceptibility of these promastigotes. In contrast, the proportion of dead and dying cells in the *allo-p229* promastigotes was not significantly influenced by drug exposure. The CFSE assay and the viability assay with SYBR-14 and PI are new tools in the flow cytometric measurement of cell toxicity in parasitology. Our data on total protein content of *wt-p229* promastigotes were consistent with an earlier study (14), which indicated that allopurinol treatment leads to a decrease in the total protein content in *L. infantum* promastigotes. However, this effect on total protein was not observed in the *allo-p229* promastigotes.

The different experiments in our study clearly indicated that allopurinol had at least a partial antileishmanial effect on *wt-p229 Leishmania* promastigotes. These promastigotes seemed to have adapted in vitro to a selection pressure resulting from long-term drug exposure. The study suggests that long-term in vitro exposure of promastigotes to allopurinol might lead to the development of an allopurinol-resistant (or less sensitive) form of the parasite.

Clinical studies done by Cavaliero et al. (10) showed that allopurinol treatment of *L. infantum*-infected dogs led to clinical cure of the disease, but not to an absolute elimination of the parasites. Relapses of the disease were observed in dogs when treatment was discontinued. Based on our observations, it is tempting to speculate that long-term allopurinol treatment in theses dogs led to the development of some degree of resistance or decreased sensitivity of the parasites. However, although the clinical observations seem to correspond well to our in vitro data, more detailed studies are required to establish a true causality.

The assessment of proliferation capacity, viability, and cellular changes by flow cytometry proved to be a promising way of evaluating the susceptibility and resistance of *Leishmania* promastigotes to allopurinol. The successful application of flow cytometry to determine cellular changes in *Leishmania* cells further opens up future perspectives in determination of effects of antileishmanial compounds.

The flow cytometry approaches complemented the [^{3}H]-thymidine incorporation assay, enabling us to detect, differentiate, and quantify cellular changes in these parasites as a result of allopurinol treatment. We were able to demonstrate differences in allopurinol susceptibility of the two promastigote forms, thereby expanding the spectrum of flow cytometry applications in the field of parasitology and in studies of parasite-drug interactions as well as cellular toxicity.

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