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Tricyclic Antidepressant-Induced Lipidosis in Human Peripheral Monocytes *In Vitro*, as Well as in a Monocyte-Derived Cell Line, as Monitored by Spectrofluorimetry and Flow Cytometry after Staining with Nile Red

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ABSTRACT. Human mono- and lymphocytes from peripheral blood and the monoblastoid cell line U-937 were used in this *in vitro* study of drug-induced lipidosis. Mono- and lymphocytes were exposed for 4 days to three different tricyclic antidepressants (TCAs), imipramine (25 μM), clomipramine (10 μM) and citalopram (80 μM). The lipophilic fluorophore Nile red, which stains intracellular lipid structures selectively, was used as a lipid probe. Fluorescence microscopy, spectrofluorimetry and flow cytometry were used to detect cellular lipidosis, as verified by electron microscopy. Our results demonstrate that imipramine, clomipramine and citalopram induce lipidosis in monocytes and U-937 cells, but not in lymphocytes. An accurate quantitation of induced intracellular lipidosis can be achieved by spectrofluorimetric and flow cytometric analysis. BIOCHEM PHARMACOL 53;10:1521–1532, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. tricyclic antidepressants; monocytes; lipidosis; fluorescent probe; spectrofluorimetry; flow cytometry

Tricyclic antidepressants (TCAs) are widely used for treating psychiatric disorders and many patients undergo chronic treatment with these drugs. TCAs are cationic amphiphilic drugs (CADs) which share common structural features, i.e., a hydrophobic ring structure and a hydrophilic side-chain containing a charged cationic amino group. TCAs have been demonstrated to induce the formation of abnormal lamellated and crystalloid inclusions within cells in a variety of animal tissues, including the lung, liver, kidney [1, 2], lymph nodes [3], choroid plexus epithelium [4], dorsal root ganglion cells [5], retinal pigment epithelium [6] and vaginal and uterine epithelia [7], as well as in cultured cells, e.g., cultured human skin fibroblasts [8].

Electron microscopic examination is the most suitable and sensitive method for detecting cellular lipidosis. However, this procedure is time-consuming and, under many circumstances, difficult to quantitate. In 1985 Greenspan and coworkers [9, 10] reported that Nile red can be used as a vital stain for the detection of intracellular lipid droplets. Since then, Nile red has been extensively used in the analysis of lipids, e.g., in normal and acid lipase-deficient fibroblasts [11], the porcine renal epithelial cell line LLC-PK_1 [12], salivary gland cells of the dipteran fly *Megaselia scalaris* [13], microalgal cells [14], ciliated protozoans [15], benthic copepods [16], and mouse eggs and preimplanted embryos [17].

Nile red is a strong lipophilic fluorophore that has been shown to selectively stain intracellular lipid droplets [9, 10, 12, 14, 15]. The resulting fluorescence can be detected not only by fluorescence microscopy, but also by spectrofluorimetry [9, 10, 14, 15] and flow cytometry [9, 10, 14, 18].

In the toxico- and pharmacological field, a reliable and rapid method for quantifying drug-induced lipidosis in isolated cells is desirable. This would greatly facilitate the screening of and investigations into this drug side-effect. Since flow cytometric analysis has gained entrance into clinical laboratories, it will be simple to undertake measurements directly on clinical blood samples.

The present study was designed to investigate quantitatively the ability of the three TCAs, clomipramine, imipramine, and citalopram, to induce lipidosis in cultured human monocytes and lymphocytes, as well as in the human monocyte-derived U-937 cell line, using Nile red as a lipid probe. Cellular lipidosis was detected by fluorescence microscopy, spectrofluorimetry and flow cytometry.

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Abbreviations: CADs, cationic amphiphilic drugs; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; PBMC, peripheral blood mononuclear cells; TCAs, tricyclic antidepressants.

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MATERIALS AND METHODS

Reagents and Media

The medium employed was RPMI-1640 with L-glutamine, containing 10% fetal calf serum (FCS), 50 IU penicillin/mL and 50 μg streptomycin/mL (GIBCO, Grand Island, NY, U.S.A.). Lymphoprep was purchased from Nycomed (Oslo, Norway). Dynabeads M-450 coated with anti-CD2 monoclonal antibodies (Dynabeads M-450 Pan T) or with anti-CD19 monoclonal antibodies (Dynabeads M-450 Pan B) were obtained from Dynal (Oslo, Norway). Imipramine and clomipramine were a kind gift from Ciba-Geigy AB (Basel, Switzerland) and citalopram from H. Lundbeck A/S (Copenhagen, Denmark). Nile red (9-diethylamino-5H benzo (alpha) phenoxazine-5-one) and paraformaldehyde were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and glutaraldehyde (EM grade) was from Agar Scientific LTD (Essex, UK). All other chemicals were of analytical grade and obtained from common commercial sources.

Isolation of Mono- and Lymphocytes from Normal Human Peripheral Blood

Buffy coats from healthy blood donors were obtained from the Blood Unit, Sabbatsberg Hospital, Stockholm. Peripheral blood mononuclear cells (PBMC) were isolated from these buffy coats by gradient centrifugation on lymphoprep in accordance with the procedure described by Bøyum [19], and washed twice in RPMI-1640 medium. PBMC were then incubated for 1–2 hr at 37°C in a humidified CO_2 (5%) incubator to allow the monocytes to attach to the plastic Petri dishes. After five washes with prewarmed PBS buffer (37°C), the monocyte-enriched population was detached by further incubation at 4°C in RPMI-1640 medium containing 10 mM ethylenediamine tetraacetate (EDTA) and 10% FCS for approximately 20 minutes. Cells were recovered by gentle pipetting, washed twice and finally resuspended in the culture medium described above. These cell preparations were found to be *ca.* 70% CD14-positive by flow cytometric analysis. Viability, as reflected by trypan blue exclusion, was always greater than 90%.

Pure T and B cells were isolated using M-450 particles according to Morgan and Burke [20] and Funderud *et al.* [21]. Briefly, the PBMC suspension was cooled on ice and Dynabeads M-450 Pan T and Pan B were added to give target-to-bead ratios of 1:3 and 1:10, respectively. These mixtures were incubated at 4°C for 30 min with gentle tilting and rotation and rosetted cells were subsequently captured using a magnet. To obtain pure T and B cell populations, the cell rosettes were washed five times with PBS containing 2% FCS. Thereafter, cells still bound to Dynabeads were exposed to fresh media (controls) or to fresh media containing the TCAs.

The human cell line U-937, a monoblastoid line [22], was also used in this study. These cells were grown in the same media and under the same conditions as described above.

Drug Incubations

Imipramine and clomipramine were dissolved in sterile, distilled water and citalopram was dissolved in sterile phosphate-buffered saline (PBS) prior to addition to the culture media. The final concentrations of imipramine, clomipramine and citalopram were 25 μM, 10 μM and 80 μM, respectively, for monocytes and lymphocytes. For the U-937 cell line, 65 μM imipramine, 25 μM clomipramine and 120 μM citalopram were employed. The initial number of monocytes, lymphocytes and U-937 cells added to the dishes was 1×10^6/mL. In all cases, old medium was replaced by fresh medium every second day. At the end of the incubation, cells were fixed in 1% paraformaldehyde in PBS.

Viability

Cell viability was examined after each incubation. The viability exceeded 70% after 96 hr of incubation under all conditions, as determined by trypan blue exclusion.

Staining with Nile Red

Nile red was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 mg/mL as a stock solution and maintained at room temperature protected from light. In pilot experiments, we compared the staining of fixed and unfixed cells. Both fixed and unfixed cells were washed twice in PBS, subsequently stained with Nile red (1 μg/mL final concentration) for 5–7 min at room temperature, thereafter washed a further three times with PBS and finally resuspended in the same buffer.

Fluorescence and Electron Microscopy

Fluorescence microscopy was performed using a Nikon Optiphot (Nikon, Tokyo, Japan) with a 410–485 nm excitation filter and a 515 nm barrier filter. For electron microscopic examination, cells were washed three times in 0.15 M cacodylate buffer, pH 7.3, and fixed for two days with 1.5% glutaraldehyde in cacodylate buffer, pH 7.3 (4°C). The cells were then washed three times with cacodylate buffer and maintained at 4°C until the electron microscopic studies were performed according to standard techniques.

Spectrofluorimetry

Excitation and emission fluorescence spectra were taken at room temperature using a Hitachi F-2000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). Three different sets of wavelengths for excitation and emission were used: excitation at 488 nm and emission at 540 nm;

excitation at 540 nm and emission at 650 nm; and excitation at 488 nm and emission at 650 nm. Bandpasses were set at 20 nm for both the excitation and emission wavelengths. These measurements were performed with 5×10^5 monocytes per mL and 1×10^6 U-937 cells per mL.

In order to examine if any quenching occurred, monocytes and lymphocytes were mixed at a ratio of 1:1 and the fluorescence emitted was recorded and compared to fluorescence originated from the same number of treated monocytes only.

Flow Cytometry

The same samples were also analyzed on a FACS calibur cytometer (Becton Dickinson, San Jose, CA, U.S.A.). The stained cells were excited at 488 nm and the emission recorded at both 530 ± 30 nm and ≥ 650 nm. We analyzed at least 1×10^4 cells in each experiment.

Double-Staining Analysis Using Fluorescein Isothiocyanate (FITC)-Conjugated Antibodies and Nile Red

For this technique, PBMC (1.5×10^6 cell/mL) were incubated directly with TCAs using the same concentrations as with isolated monocytes, i.e., 25 μM imipramine, 10 μM clomipramine or 80 μM citalopram. After four days of incubation, the cells were harvested by gentle scraping with a rubber policeman. These cell preparations were first stained with mouse antihuman monocyte monoclonal antibodies CD14 (Dakopatts A/S, Glostrup, Denmark) conjugated with FITC. Negative controls were performed by incubating cells with an irrelevant antibody of the same isotype (Dakopatts). The stained cells were then fixed in PBS containing 1% paraformadehyde, incubated with Nile red (0.1 μg/mL final concentration) and thereafter analyzed on the flow cytometer, in which a single 488 nm argon laser was used to excite FITC (530 ± 30 nm) and Nile red (≥ 650 nm).

Phospholipid Analysis

Two separate experiments were initiated in order to estimate the increase in total phospholipids. Monocytes were obtained and cultured as described above. In both experiments, buffy coats were obtained from five different donors. Incubation was carried out with 10 μM clomipramine for 4 days. Control cells were run in parallel. Monocytes from each incubation were pooled and stored at −20°C until analysis.

The cells (1 mg protein) were extracted by stirring with chloroform/methanol/water (1:1:0.3) at 37°C for 60 min. The protein was sedimented by centrifugation and reextracted in the same manner. The pooled extracts were adjusted to a chloroform/methanol/water ratio of 3:2:1 to obtain complete phase partition. The lower phase was evaporated under N_2, dissolved in 1 mL chloroform and applied to silica gel (230–400 mesh) columns (0.8 × 2.0 cm). The columns were washed with 6 mL chloroform and the phospholipids eluted with 6 mL methanol. The solvent was evaporated, the phospholipids redissolved in 0.5 mL chloroform and aliquots (100–200 μL) hydrolyzed in the presence of 0.3 mL 70% perchloric acid at 180°C for 60 min. After supplementation with 0.4 mL of 1.25% ammonium molybdate, 0.4 mL 5% ascorbic acid and water to give a final volume of 1.8 mL, the samples were heated in a boiling water bath for 5 min; 30 min later, the absorbance at 797 nm determined.

Statistical Analysis

The results are expressed as mean ± standard deviations (SD). Student's *t*-test was used for statistical analysis of the data.

RESULTS

Fluorescence and Electron Microscopy

Nile red staining of viable and fixed cells revealed no differences in the distribution or intensity of fluorescence, in agreement with reports in the literature [10, 15]. We therefore chose to use fixed cells in our studies, since stained, fixed cells are more stable and can be stored in a refrigerator for several days without any change in fluorescence intensity (data not shown).

When untreated monocytes were stained and observed in the fluorescence microscopy, numerous small discrete fluorescent bodies distributed throughout the cytoplasm were observed. Untreated lymphocytes were seen to display a weaker fluorescence background, with a few small discrete fluorescent bodies in single cells. In untreated U-937 cells, several small discrete fluorescent bodies were scattered throughout the cytoplasm in all cells.

In contrast, larger cytoplasmic fluorescent structures were irregularly distributed throughout the cytoplasm of the majority of TCA-treated monocytes. Treated lymphocytes, on the other hand, had the same appearance as untreated lymphocytes. An increased fluorescence was seen in treated U-937 cells, but the difference compared to untreated cells was not as pronounced as that seen in the case of monocytes.

Electron microscopy revealed abnormal cytoplasmic inclusions in TCA-treated monocytes (Fig. 1). Such abnormal alterations were not observed in untreated monocytes, or in treated or untreated lymphocytes.

Spectrofluorimetry

Viable and fixed monocytes and U-937 cells were stained with Nile red as described in Materials and Methods. No apparent difference in their fluorescence intensities, as measured with a spectrofluorimetry, was observed when measurements were performed immediately after Nile red staining (data not shown). However, fixed cells proved to

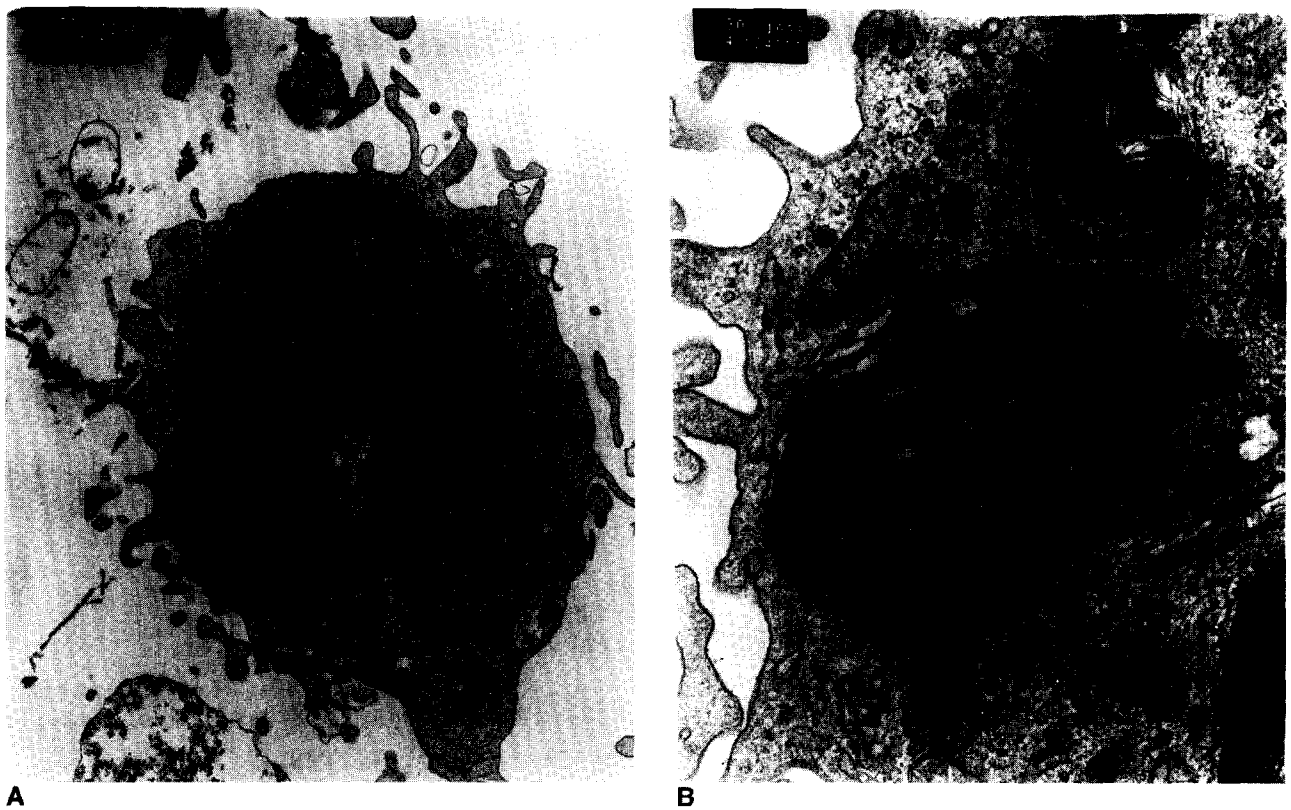

FIG. 1. Electron micrograph of a monocyte exposed to clomipramine (20 μM) for 72 hr. The cytoplasm contains abnormal lamellated inclusion bodies. (A) ×8000; (B) ×24000.

be quite convenient for use in spectrofluorimetric measurements, since it was possible to store these cells for as long as a week with no change in fluorescence intensity. Fixed cells are also less prone to undergo disruption than are viable cells.

As seen in Fig. 2, the fluorescence intensities increased almost linearly with increasing numbers of TCA-treated and untreated monocytes, as well as U-937 cells. Here only the values for yellow-gold fluorescence (excitation, 488 nm; emission, 540 nm) are presented, but similar results were obtained with the other two wavelengths. Furthermore, it can be seen that the fluorescence intensity of treated monocytes was at least two-fold greater than that of the same number of U-937 cells.

It has been reported that the yellow-gold (excitation, 450–500 nm; emission, > 528 nm) and red (excitation, 549 nm; emission, 628 nm) Nile red fluorescence reflect interaction of this fluorophore with neutral and polar lipids, respectively [9]. Thus, we selected three different wavelengths for both excitation and emission in these experiments (see also Materials and Methods): yellow-gold fluorescence (excitation, 488 nm; emission, 540 nm); red fluorescence (excitation, 540 nm; emission, 650 nm); and the same wavelengths as set in the flow cytometer (excitation, 488 nm; emission, 650 nm).

The fluorescence intensity of TCA-treated monocytes, as well as of U-937 cells, was increased significantly when all three combinations of excitation/emission wavelengths were used (Figs. 3A–C, 4A–C). As seen in Fig. 3, the intensity of the yellow-gold fluorescence of treated monocytes was higher than that of the red fluorescence. However, the intensities of yellow-gold and red fluorescence originating from treated U-937 cells were fairly equal (Fig. 4A–B). The fluorescence intensity for both monocytes and U-937 cells was much lower when measured at excitation/emission 488 and 650 nm, respectively.

We were interested in knowing whether contamination with lymphocytes interfered with monocyte fluorescence or not. As demonstrated in Fig. 5, addition of the same amount of lymphocytes and monocytes into the measuring cuvette did not reduce the fluorescence intensity originated from monocytes.

Flow Cytometry

Single-color flow cytofluorescence analysis of Nile red-stained monocytes is illustrated in Fig. 6A–B. The cells were analyzed for fluorescence at either 530 ± 30 nm (yellow-gold) or ≥ 650 nm (red or far-red). It is obvious that the treated cells demonstrated a higher fluorescence intensity than did control cells, both with respect to yellow-gold and red fluorescence (Fig. 6). Thus, cellular lipidosis could be clearly detected and quantitated. In accordance with previous findings, this effect was more pronounced in treated monocytes than in treated U-937 cells (Fig. 7).

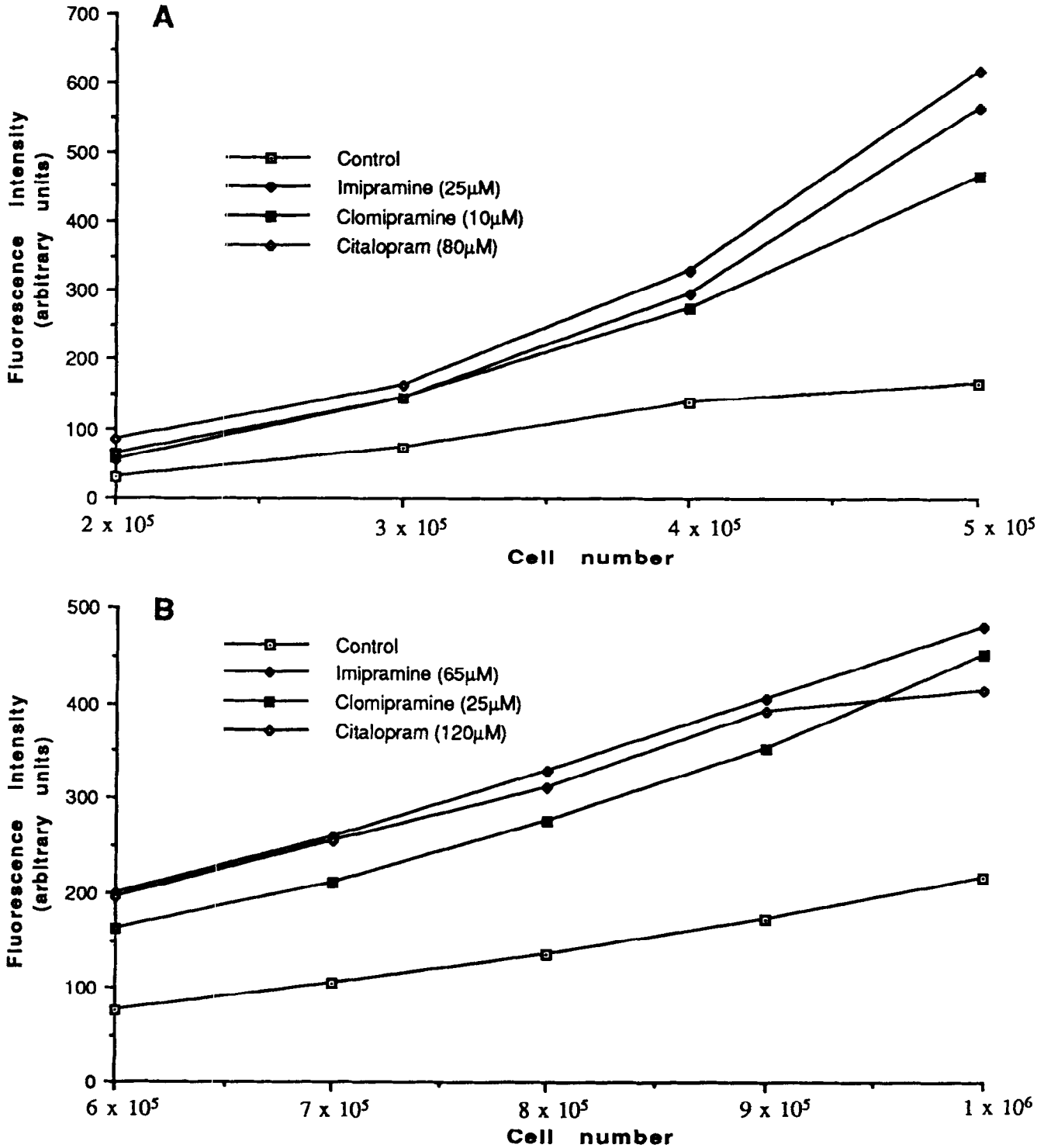

FIG. 2. Relationship between fluorescence intensity and cell number. These measurements were performed using a spectrofluorimeter with excitation at 488 nm and emission at 540 nm. (A) monocytes. (B) U-937 cells.

The results from five independent experiments on monocytes and U-937 cells are summarized in Fig. 8A–8B. These observations correspond closely to those obtained using spectrofluorimetric analysis. The fluorescent intensity ratio of treated to control monocytes was 3 ($P < 0.001$), but only 1.6 ($P < 0.05$) for U-937 cells.

Double-Staining Analysis of CD14 Expression and Nile Red Fluorescence Intensity by Flow Cytometry

To establish definitively that the increased Nile red fluorescence intensity observed originated from treated monocytes in the cell preparation, we labeled monocytes with fluorescein-conjugated monoclonal antibody towards the

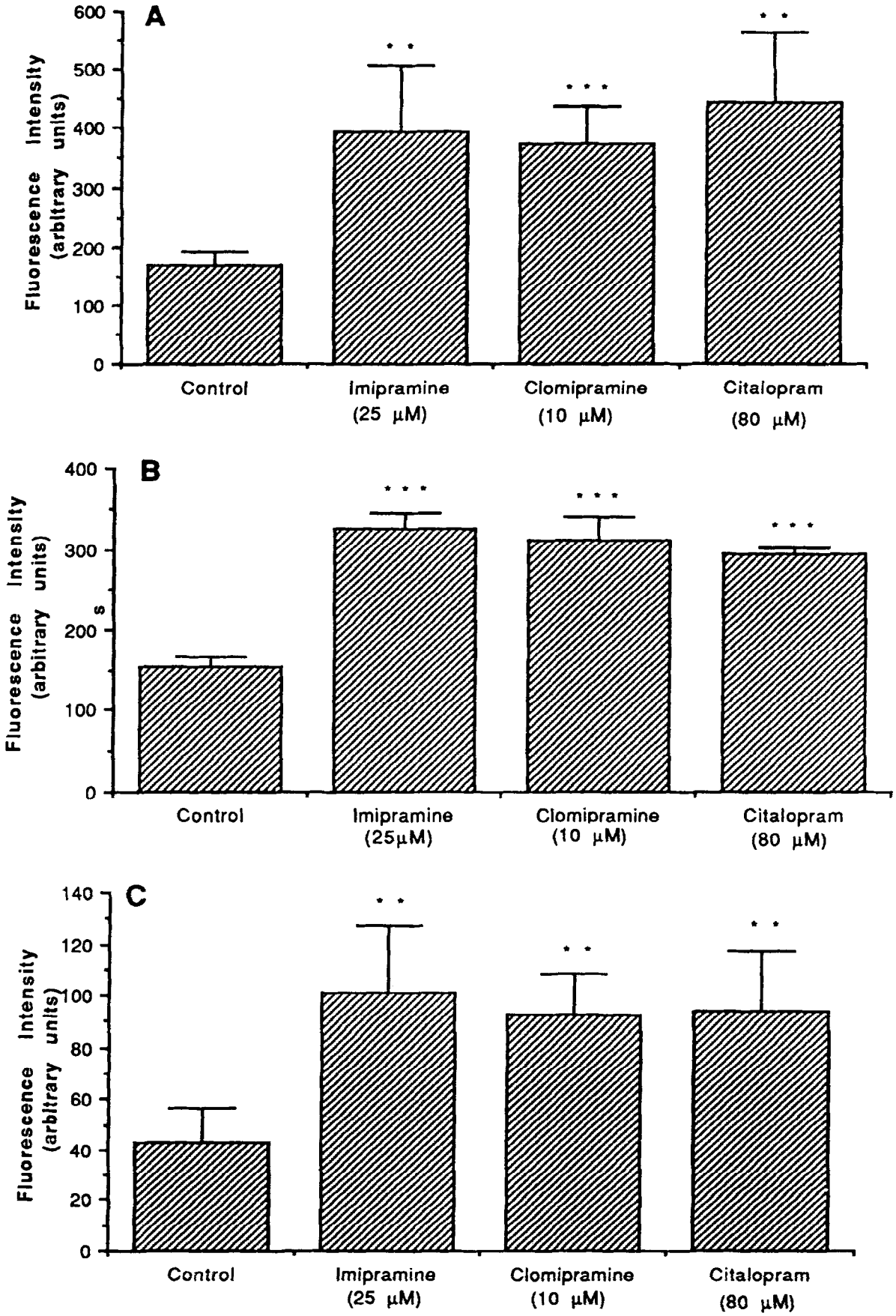

FIG. 3. Quantitation of the Nile red fluorescene of TCA-treated and untreated monocytes using different excitation and emission wavelengths. A. excitation 488 nm/emission 540 nm. B. excitation 540 nm/emission 650 nm. C. excitation 488 nm/emission 650 nm. These results are the means ± SD of five independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

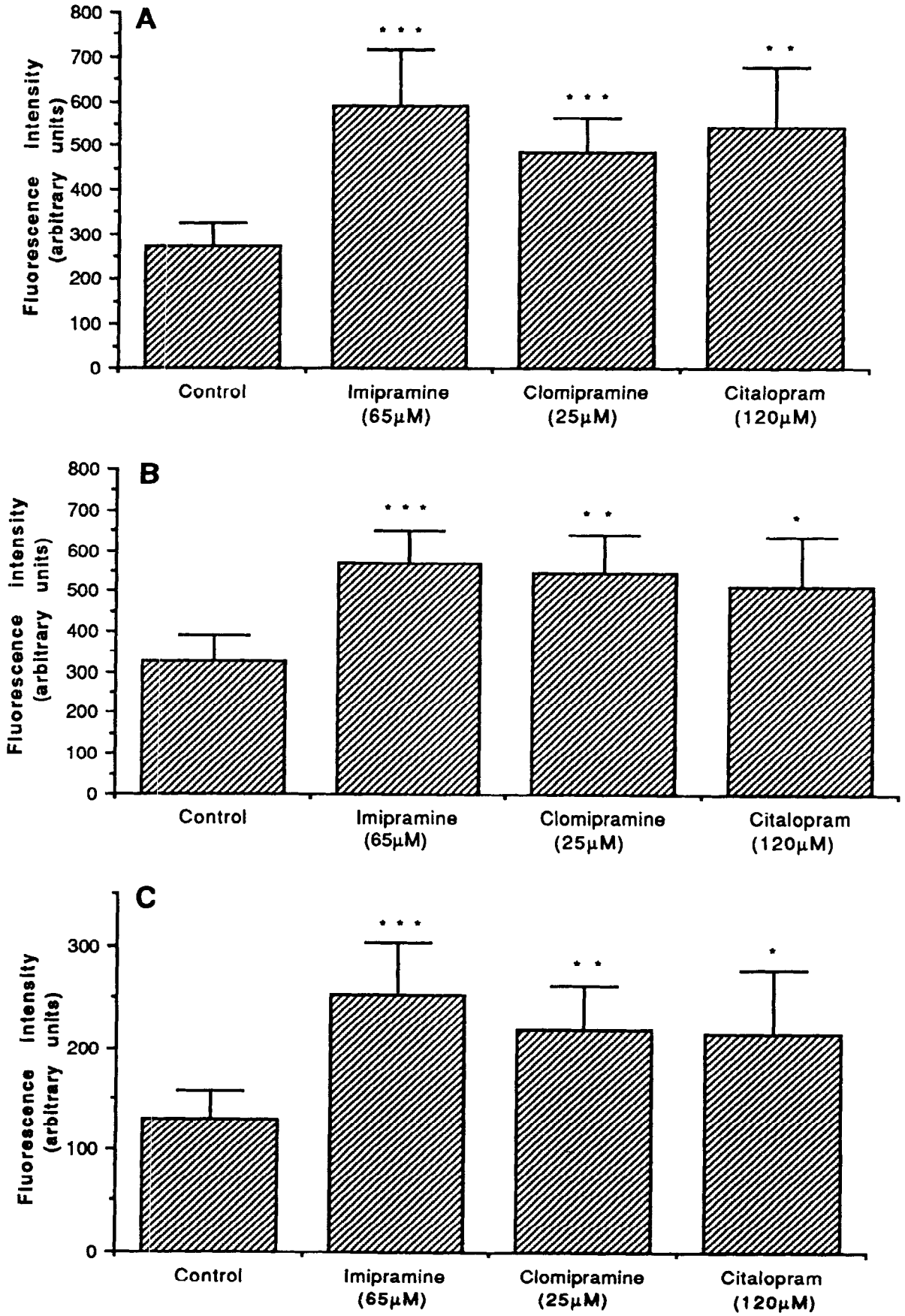

FIG. 4. Quantitation of the Nile red fluorescence of TCA-treated and untreated U-937 cells. A. excitation 488 nm/emission 540 nm. B. excitation 540 nm/emission 650 nm. C. excitation 488 nm/emission 650 nm. These results are the means ± SD of five independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

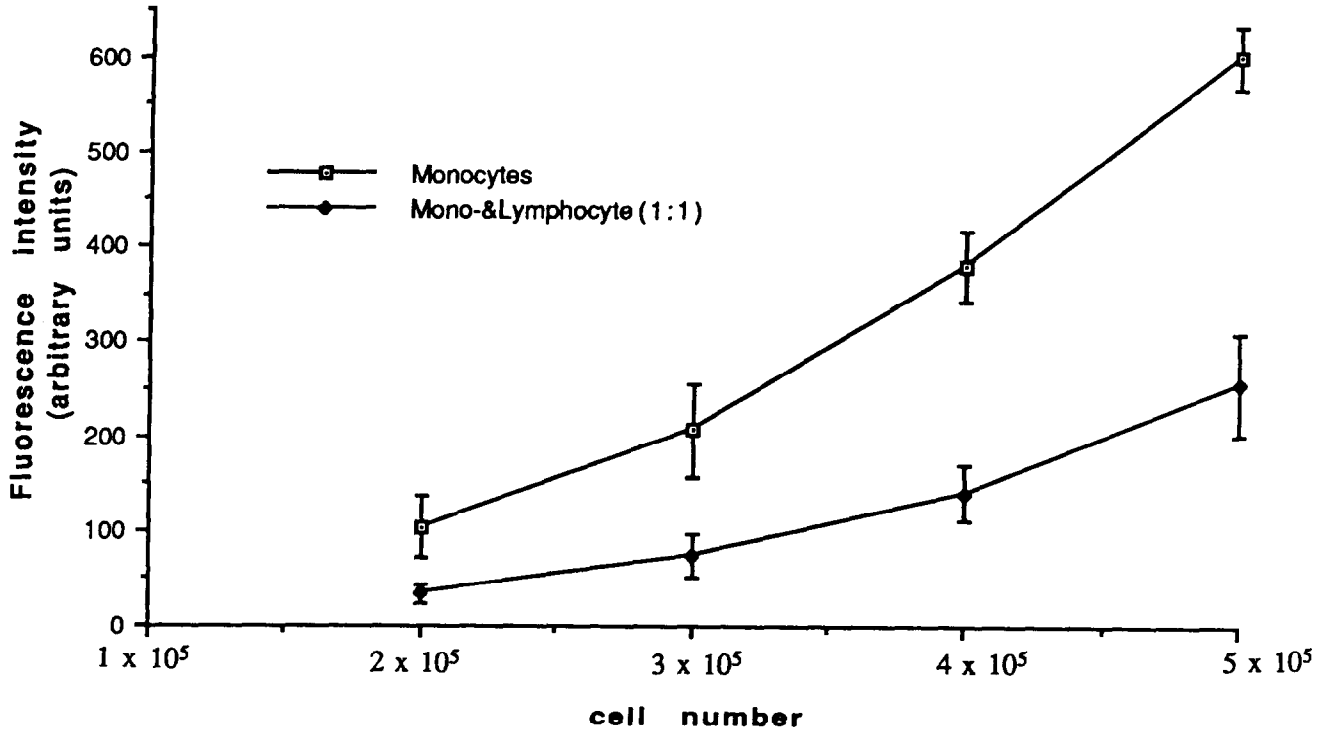

FIG. 5. Effects of an equal number of treated lymphocytes on the fluorescence of treated monocytes. Measurements were performed as described in Materials and Methods.

CD14 surface antigen and examined Nile red fluorescence intensity in this $CD14^+$ population by flow cytometry (Fig. 9A). Simultaneously, we measured the fluorescence intensity in the $CD14^-$ population (T + B lymphocytes) (Fig. 9B). No significant increase in fluorescence intensity was observed in these latter cells (Fig. 9B) and their basal fluorescence intensity was much lower. These results correspond well with our observations from fluorescence and electron microscopic examination.

Phospholipid Determination

The total amount of phospholipids expressed as the ratio phospholipids/protein in the treated populations was 0.35 and 0.36 compared to 0.20 and 0.15, respectively in the untreated monocytes.

DISCUSSION

Lipidosis induced by drugs is normally demonstrated by light or electron microscopic examination. Phospholipid deposition is seen as the formation of membrane-bound lamellated or crystalloid cytoplasmic inclusions of a lysosomal nature [5, 23, 24]. However, quantitation of lipidosis by these methods is extremely time-consuming and somewhat uncertain. In the present study, we report that TCA-induced lipidosis observed qualitatively using fluorescence and electron microscopy can be quantitated with spectrofluorimetry and flow cytometry using Nile red as a probe.

Nile red is a benzophenoxazone dye with a very high partition coefficient from water to hydrophobic solvents. The fluorescence of this dye is strongly dependent on the polarity of its environment [25]. This fluorescence is quenched in aqueous solution, making Nile red a suitable stain for cellular lipids [15].

Greenspan *et al.* [9] reported that cellular neutral or polar lipids can be measured fluorescently at selected excitation and emission wavelengths. Using this approach, Cole [15] carried out semi-quantitative estimation of cellular neutral lipid content by spectrofluorimetry, using excitation and emission wavelengths of 488 nm and 540 nm, respectively. Polar lipids could be measured with an excitation wavelength of 535 nm and emission at 680 nm. Cooksey *et al.* [14] found that the fluorescence generated at excitation and emission wavelengths of 525 nm and 580 nm, respectively, by intracellular lipids in *Navicula sp.* was linear.

In the present experiments we selected three different wavelength combinations for the examination of intracellular lipids. We found that the fluorescence intensity of TCA-treated monocytes and U-937 cells was higher than that of the corresponding untreated cells, both for yellow-gold and red fluorescence. This indicates that TCAs induce accumulation of both neutral and polar lipids in these cells. Drug treatment may lead to an increase in the cellular content of many classes of lipids, with accumulation of different classes of lipids in specific organelles [26]. The nature of the lipids accumulating in human peripheral monocytes treated with imipramine, clomipramine and citalopram can be determined definitively by isolation and characterization of the lamellated inclusion bodies, as has been performed for these types of organelles in the kidney proximal tubule cells of gentamicin-treated rats [27]. In the

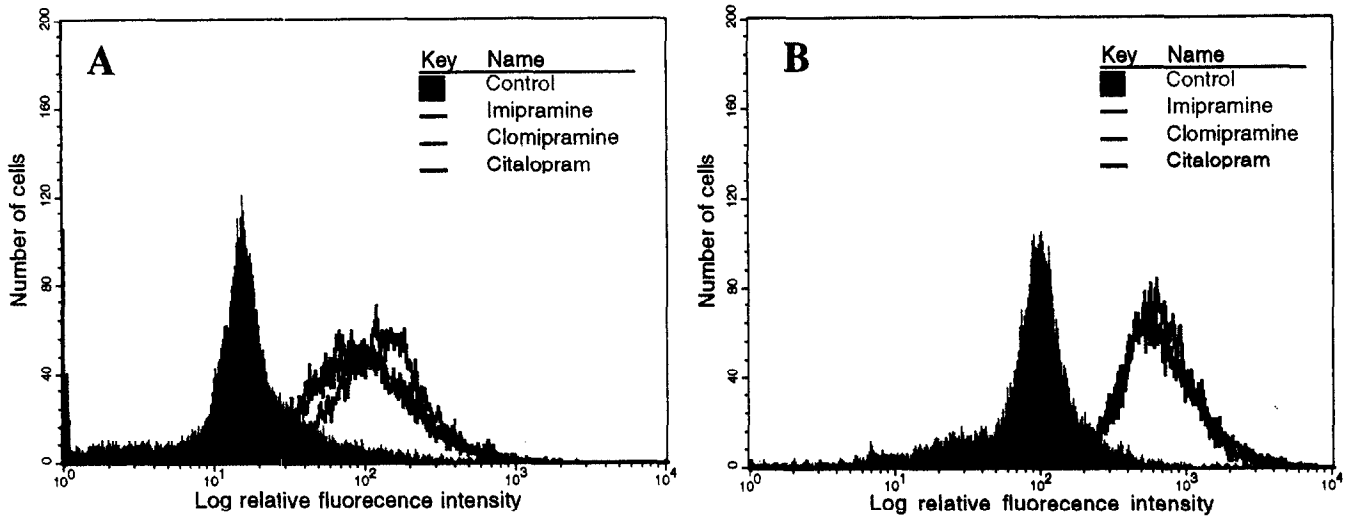

FIG. 6. Flow cytometric analysis of TCA-treated and untreated monocytes stained with Nile red. Cell fluorescence was measured using a laser excitation wavelength of 488 nm and an emission wavelength of either 530 (A) or ≥ 650 nm (B). The log of the fluorescence intensity is plotted on the x-axis and the number of cells possessing a given intensity is plotted on the y-axis.

present study, we undertook analysis of the total amount of phospholipids only, and found an approximately two-fold increase.

Flow cytometric analysis offers a unique approach to the examination of the accumulation of lipid-enriched organelles within individual cells. Greenspan *et al.* [10] found that the yellow-gold fluorescence (515–560 nM) of Nile red-stained cells is a better discriminator of cells which accumulate neutral lipids, as analyzed by flow cytometry, than is red fluorescence. However, when phospholipid-altered and control cells were stained with Nile red and subjected to flow cytometric analysis, a larger difference was obtained when the cells were examined for red fluorescence (> 630 nm) than for yellow-gold fluorescence (530–560 nm) [18]. In the present study, differences between control and treated cells were clearly observed for both red (≥ 650 nm) and yellow-gold fluorescence (530 ± 30 nm) (Figs. 6, 7). However, the intensity of the red fluorescence was higher than that of the yellow-gold.

Quantitation of TCA-induced lipidosis in monocytes by flow cytometry has great advantages over quantitation by spectrofluorimetry. The spectrofluorimeter is less sensitive. To achieve an accurate response with the spectrofluorimeter, *ca.* 0.5–1 × 10^6 cells are required. Furthermore, with the flow cytometric technique it is possible to differentiate between positive and negative cell populations in the same sample, which is not the case with the spectrofluorimeter. The cell number needed is only 1/50 of that required in the spectrofluorimeter. This is an advantage when working both with experimental and clinical samples.

In conclusion, our results demonstrate that the tricyclic antidepressant drugs imipramine, clomipramine and citalopram induce lipidosis in human peripheral monocytes, but not in lymphocytes *in vitro*. Nile red is a useful, convenient and inexpensive probe for detecting cellular lipid/phospholipidosis in connection with semi-quantitative analysis by fluorescence microscopy and accurate quantitative determination by spectrofluorimetry and/or flow cytometry. Flow

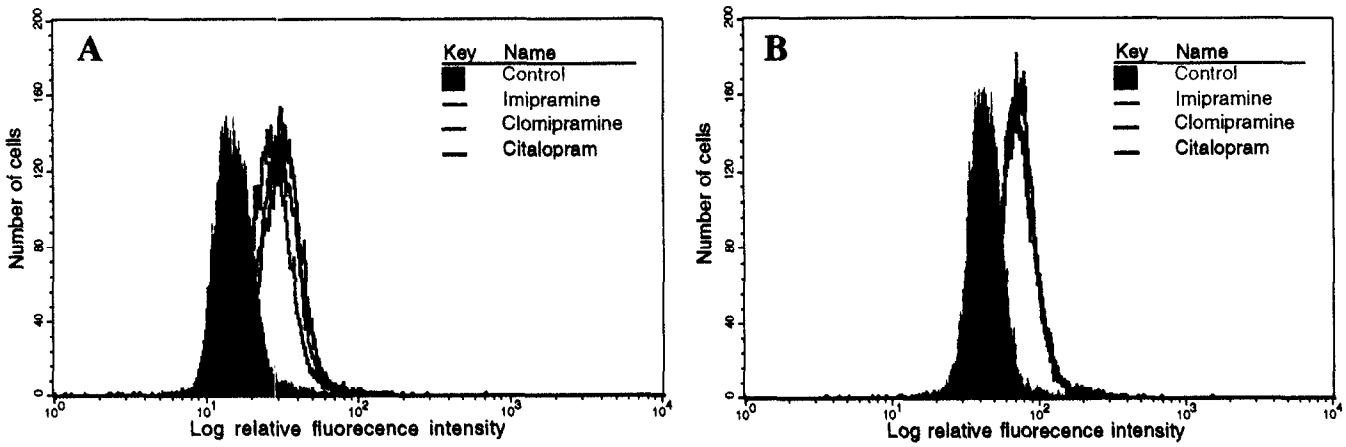

FIG. 7. Flow cytometric diagram of TCA-treated and untreated U-937 cells stained with Nile red. Cell fluorescence was measured using a laser excitation wavelength of 488 nm and an emission wavelength of either 530 (A) or ≥ 650 nm (B).

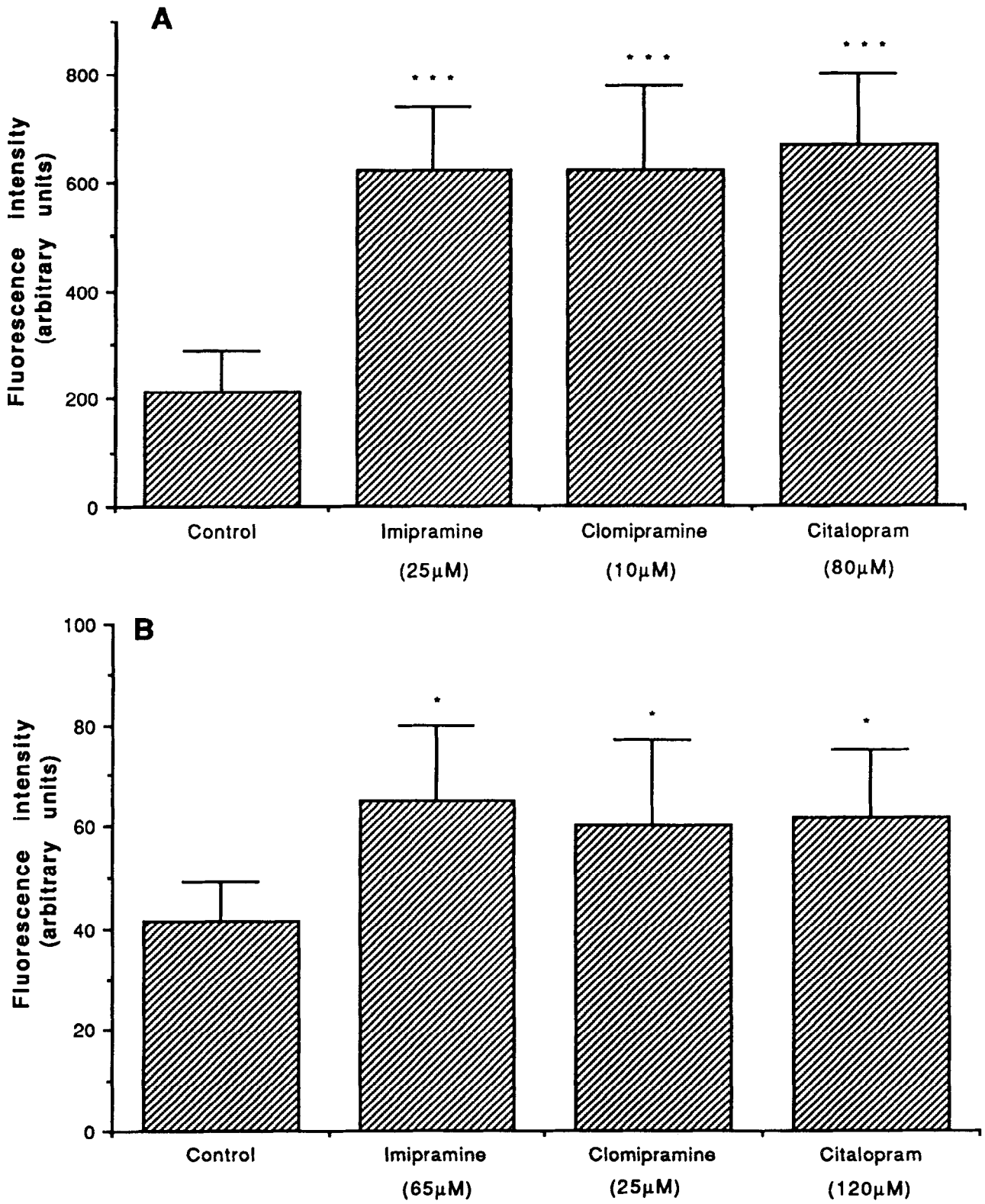

FIG. 8. Quantitative analysis of cellular Nile red fluorescence by flow cytometry with an emission wavelength of ≥ 650 nm. Monocytes (A) and U-937 cells (B) were treated and stained as described in Materials and Methods. These results are the means ± SD of five independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

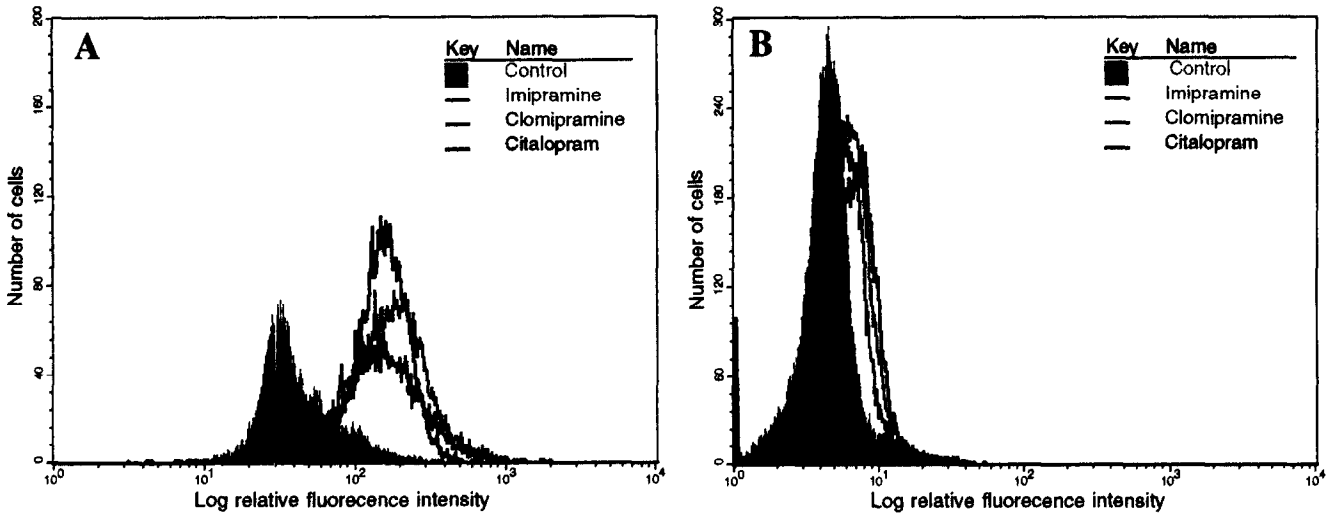

FIG. 9. Two-color analysis of CD_{14} expression and Nile red fluorescence of TCA-treated mononuclear cells by flow cytometry. A: Nile red fluorescence intensity of CD_{14}-positive cells; B: Nile red fluorescence intensity of CD_{14}-negative cells.

cytometry is the optimal method for quantitation and is less time-consuming for screening drug-induced lipidosis in experimental, as well as in clinical studies.

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