Photochemistry and Photobiology, 2000, 71(3): 327–332

Characterization of the Autofluorescence of Polymorphonuclear Leukocytes, Mononuclear Leukocytes and Cervical Epithelial Cancer Cells for Improved Spectroscopic Discrimination of Inflammation from Dysplasia

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Received 16 April 1999; accepted 15 December 1999

ABSTRACT

Fluorescence spectroscopy has the potential to improve the *in vivo* detection of intraepithelial neoplasias; however, the presence of inflammation can sometimes result in misclassifications. Inflammation is a common and important pathologic condition of epithelial tissues that can exist alone or in combination with neoplasia. It has not only been associated with the presence of cancer but also with the initiation of cancer by damage induced due to the oxidative activity of inflammatory cells. Microscopic examination of cervical biopsies has shown increased numbers of polymorphonuclear and mononuclear leukocytes in inflamed tissues mostly confined to the stroma. The purpose of this study was to characterize the fluorescence properties of human polymorpho- and mononuclear leukocytes and compare their fluorescence to that of cervical cancer cells. Human neutrophils were purified from peripheral blood and their fluorescence characterized over an excitation range of 250–550 nm. There are four notable excitation emission maxima: the tryptophan peak at 290 nm excitation, 330 nm emission; the NAD(P)H peak at 350 nm excitation, 450 nm emission, the FAD peak at 450 nm excitation, 530 nm emission and an unidentified peak at 500 nm excitation, 530 nm emission. Treatment of these peripheral blood neutrophils with 40 n*M* phorbol myristate acetate or with the chemotactic peptide formyl-Met-Leu Phe (1 *M*) demonstrated a significant increase in NAD(P)H fluorescence. Isolated mononuclear cells have similar emission peaks for tryptophan and NAD(P)H and a small broad peak at 450 nm excitation, 530 nm emission suggestive of FAD. Comparison of the fluorescence from leukocytes to epithelial cancer cell fluorescence has demonstrated the presence of these fluorophores in different quantities per cell. The most notable difference is the high level of tryptophan in cervical epithelial cancer cells, thus offering the potential for discrimination of inflammation.

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INTRODUCTION

Fluorescence spectroscopy has been shown to have diagnostic capability in the detection of precancerous lesions in the cervix (1), the oral cavity (2) and the colon (3). Although promising results have been obtained *in vivo,* in some cases, the presence of inflammation can lead to misclassification, reducing the accuracy of this technique (1). Inflammation is a common and important pathologic condition of epithelial tissues. It is not only associated with the presence of cancer but also with the initiation of cancer by damage induced due to the oxidative activity of inflammatory cells (4). Typically, the excitation wavelengths used in studies to develop diagnostic algorithms based on fluorescence spectroscopy have been selected on the basis of *in vitro* measurements of excised tissue specimens (2,5,6). These *in vitro* fluorescence measurements differ from *in vivo* measurements due to the lack of perfusion (5) and oxidation (7). The lack of perfusion removes the effect of hemoglobin absorption and thus affects the line shape and intensity of the measured fluorescence spectra. The oxidation state affects the fluorescence seen from FAD and NAD(P)H. The reduced form, NAD(P)H, is strongly fluorescent and its oxidized form, NAD(P) is not. The converse is true for FAD, in which the oxidized form, FAD, is fluorescent and $FADH_2$ is not (8). Oxidation of cellular fluorophores, especially NAD(P)H, can occur rapidly following excision, altering both fluorescence intensity and line shape. Thus, excitation wavelengths used in studies reported to date may not be optimal for discrimination of normal tissue, inflammation and precancer. This *in vitro* study examines viable cells to eliminate the loss of information due to oxidation of cellular fluorophores.

Tissue fluorescence spectra contain information about both cellular and extracellular components (*e.g.* collagen, elastin) of tissue. Because biochemical changes are thought to precede architectural changes during the progression of the dysplasia–carcinoma sequence or during the onset of the inflammatory response, measurements of viable cell suspensions are thought to provide insight into spectral differences important for the detection of these pathologies at their earliest stages. Therefore, this study was designed to examine the autofluorescence of living inflammatory cells and compare them to that of living epithelial cancer cells to aid in

the selection of appropriate wavelengths for differentiating inflammation from both normal tissue and precancerous tissue.

The inflammatory response is a complex cascade involving many different cell types and chemical messengers. It is a nonspecific immune response that acts as a first line of defense to tissue injury or invasion in an immune-competent host. In general, white blood cells are recruited from the blood stream to the site of injury. White blood cells are typically placed into two categories based upon their nuclear appearance and the presence of granules. These categories are mononuclear leukocytes (agranulocytes) and polymorphonuclear leukocytes (granulocytes). Mononuclear leukocytes consist of monocytes and lymphocytes. Polymorphonuclear leukocytes (PMN)† consist of neutrophils, basophils and eosinophils (9).

Neutrophils, which comprise approximately two-thirds of the circulating white blood cells, are the first recruited cells to a site of injury and are ultimately present in the greatest numbers in acute inflammation and to a lesser extent in chronic inflammation. Histologically, inflammation is characterized by the infiltration of inflammatory cells, edema and increased vascularity. The infiltrate contains primarily white blood cells from the peripheral circulation. Examination of cervical biopsies (ectocervix) with inflammation in this study demonstrated cell distributions of approximately 30–45% mononuclear leukocytes and 25–40% neutrophils.

Leukocyte autofluorescence has been partially characterized by Monici *et al.* (10). They measured the emission spectra from suspensions of granulocytes and agranulocytes at excitation wavelengths from 250 to 370 nm. They also measured excitation spectra from 230 to 320 nm at 340 nm emission and excitation spectra at 330–400 nm at 440 nm emission. They reported peaks consistent with tryptophan, nicotinic coenzymes (*e.g.* NADH) and flavins (*e.g.* FAD) in both suspensions. Comparison of the total emitted fluorescence indicated that agranulocytes emitted 30–50% less fluorescence at 340 nm emission than granulocytes (10).

Eosinophil autofluorescence has been previously described (11,12). Barnes *et al.* reported peaks suggestive of tryptophan (290 nm excitation, 340 nm emission) and NADH (360 nm excitation, 440 nm emission). In addition, another peak of unknown origin at 500 nm excitation and 555 nm emission was seen (11). Mayeno *et al.* demonstrated that the granule-associated autofluorescence from eosinophils at 370 and 450 nm excitation and 520 nm emission was due to FAD and to a lesser degree flavin mononucleotide and riboflavin through the use of HPLC (12).

Other than Monici *et al.*, characterization of neutrophils has focused on the changes in NADH fluorescence with activation and environmental influences. The cytotoxic action of neutrophils is derived from its production of reactive oxygen metabolites (*e.g.* singlet oxygen, hydroxyl radicals, hypochlorous acid). Oxygen is consumed by cyanide-insensitive NADPH oxidase (13). During resting conditions, the concentration of cytosolic NADPH has been found to be 4.43 times higher than NADH levels. During activation, the level of NADPH falls, and NADH level remains constant (14). Liang *et al.* have imaged NAD(P)H levels in resting neutrophils and neutrophils activated with NAF, tumor necrosis factor, phorbol myristate acetate (PMA) and *N*-formyl-methionyl-phenylalanine (fMLP). They observed an initial increase in fluorescence intensity followed by an approximate decrease of 50% from starting levels over the course of an hour (15).

However, the autofluorescence of neutrophils and mononuclear leukocytes due to other fluorophores has not been completely characterized. In this study, we report fluorescence excitation–emission matrices (EEMs) of viable neutrophils and mononuclear leukocytes over the entire UV–visible spectral range. We compare these EEMs to that of epithelial cancer cells and suggest excitation wavelengths for future *in vivo* studies based on these results.

MATERIALS AND METHODS

Materials. *N*-formyl-methionyl-phenylalanine (fMLP), PMA, dimethylsulfoxide (DMSO), phosphate-buffered saline (PBS) tablets and Hypaque 1.077 density gradient were obtained from Sigma Chemical Co. (St. Louis, MO). MonoPoly resolving medium (MPRM) was obtained from ICN Biomedical (Costa Mesa, CA). Human blood was obtained from a consenting, normal volunteer. Approximately 8 mL of blood anticoagulated with sodium heparin was collected at the University of Texas Student Health Center in Austin.

Cell separation. The PMN and mononuclear leukocytes were separated with the procedure described by Kalmar *et al.* (16). Briefly, 2.5 mL of whole blood was layered upon a density-gradient solution of 1 mL Hypaque 1.077 layered on 3 mL MPRM. This solution was centrifuged at 500 ***g*** for 30 min. The two cloudy layers were aspirated and placed into separate centrifuge tubes. The top layer, mononuclear leukocytes, and bottom layer, PMN, were concentrated by centrifuging each separately for an additional 10 min at 300 ***g***. The PMN were washed with chilled deionized water for 20 s followed by the addition of an equal volume of hyperosmotic PBS (780 mOsm) to remove erythrocyte contamination from the suspension. Cellular yields and viability were determined using a hemocytometer and trypan blue exclusion assay.

Activation. The PMN were activated with PMA or the chemotactic peptide, fMLP. Activation with PMA involved the addition of 181 L of 150 n*M* PMA solution to approximately 0.6 mL of PMN suspension in PBS. The sample was incubated at 37°C for 15 min (17). The EEMs were then measured. A cell viability assay was done at the completion of the measurement. Similarly, activation with fMLP was accomplished by incubating PMN at 37°C with 10 L of 710 *M* fMLP in DMSO for 30 min (17,18).

Activation assay. The induction of the oxidative burst of the PMN was confirmed with the OxyBURST Green H_2HFF BSA assay (Molecular Probes, Inc., Eugene, OR). This reagent consists of dichlorodihydrofluorescein (H_2DCF) covalently linked to bovine serum albumin (BSA). During the oxidative burst, nonfluorescent H_2DCF is oxidized to fluorescent DCF that excites at 488 nm and emits at 530 nm. Time-based fluorometer measurements were made at these wavelengths.

Fluorometer measurements. Excitation–emission matrices were measured using a SPEX Fluorolog 2 spectrofluorometer (Instruments SA, Edison, NJ). The fluorometer consists of a 450 W xenon lamp, two double monochromators and a photomultiplier tube under computer control. Measurements were made in 10 nm increments over the range of excitation wavelengths from 250 nm to 550 nm and emission intensities measured every 5 nm up to 700 nm. Each EEM measurement required approximately 30 min. Slits were set to 1 mm, corresponding to a spectral resolution of 1.7 nm. Samples were stirred slowly with a magnetic stirrer to prevent settling in the cu-

†*Abbreviations*: BSA, bovine serum albumin; DMSO, dimethylsulfoxide; EEM, excitation–emission matrix; fMLP, formyl-Met-Leu-Phe; H_2DCF, dichlorodihydrofluorescein; MPRM, MonoPoly resolving medium; PBS, phosphate-buffered saline; PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocytes.

vette over the course of the measurement time. The sample was measured in the front focus geometry in a 5 mm pagelength reduced volume quartz cuvette (catalog number, 3-Q-5; Starna Cells, Ataseadero, CA). Measurements taken to asses the effect of activation consisted of emission spectra at 290, 350 and 450 nm excitation. All fluorescence measurements were corrected for variations in the excitation intensity using a rhodamine B quantum counter and for variations in the wavelength-dependent throughput of the instrument using correction factors supplied by the manufacturer. The resulting measurements were divided by the water Raman peak emission intensity of a water standard at 350 nm excitation.

Cervical cancer cell measurements. HeLa cervical cancer cells (ATCC CCL-2, Rockville, MD) were obtained from Dr. R. Lotan's laboratory at M.D. Anderson Cancer Center on five separate occasions. The cells were grown in a growth medium consisting of 2 m*M* L-glutamine (Fisher Scientific, Pittsburgh, PA), 0.02 mg/mL gentamycin sulfate (Bio Whittaker, Inc., Walkersville, MD), nonessential amino acids (Fisher Scientific, Pittsburgh, PA) and 10% fetal bovine serum (Sigma Chemical, St. Louis, MO). Cells were washed three times to remove the fluorescent growth medium. Each wash consisted of centrifuging the cells for 10 min at 300 ***g***, collecting the supernatant and resuspending the pellet in 30 mL of PBS. One final centrifugation was done and 5 mL of the supernatant collected and saved for use as the solvent background. All of the supernatant with exception of 3 mL was removed. The cells were resuspended and measured in the fluorometer in a stirred 1 cm pathlength quartz cuvette. Viability and cell count were measured before and after the fluorometer measurements.

Microscopic examination. Five hemotoxylin and eosin-stained slides of cervical biopsies exhibiting acute or chronic inflammation were examined under light microscopy to quantitate the approximate cell type distributions present within the epithelial layer and underlying connective tissue.

RESULTS

A total of 13 samples of purified PMN were prepared for EEM measurement on 9 different days. At the time of measurement, the neutrophil suspensions were approximately 95% pure and greater than 90% viable. Typical cell concentrations were approximately 2E6 cells/mL. A typical EEM of purified circulating neutrophils is shown in Fig. 1a. It contains peaks suggestive of tryptophan at 290 nm excitation, 330 nm emission (Fig. 1b); NAD(P)H at 350 nm excitation, 450 nm emission (Fig. 1c) and FAD at 450 nm excitation, 530 nm emission (Fig. 1d). On 3 of the 8 days on which samples were taken, all of the measurements demonstrated an additional unassigned peak visible at 500 nm excitation, 530 nm emission (Fig. 1e).

Six of the purified PMN samples were activated with fMLP; six were activated with PMA. The effect of activation was assessed on paired samples. The ratio of NADH/tryptophan fluorescence after activation was compared to the ratio prior to activation for the same sample. Samples exhibiting higher tryptophan fluorescence intensities after incubation were excluded. Tryptophan is a conserved amino acid that cannot be synthesized in human cells. Therefore an increase in tryptophan concentration is likely due to cell lysis, as a result of mechanical damage, oxidative damage or disruption of the membrane from the DMSO used as a solvent for fMLP. The quantum yield and peak emission wavelength of tryptophan are dependent upon the local environment; thus, an increase in tryptophan fluorescence could also be due to an environmental change (8). However, in our data, the location of the peak tryptophan emission wavelength remained constant between 335 and 340 nm, while it is known to vary from 308 to 352 nm with changes in environment

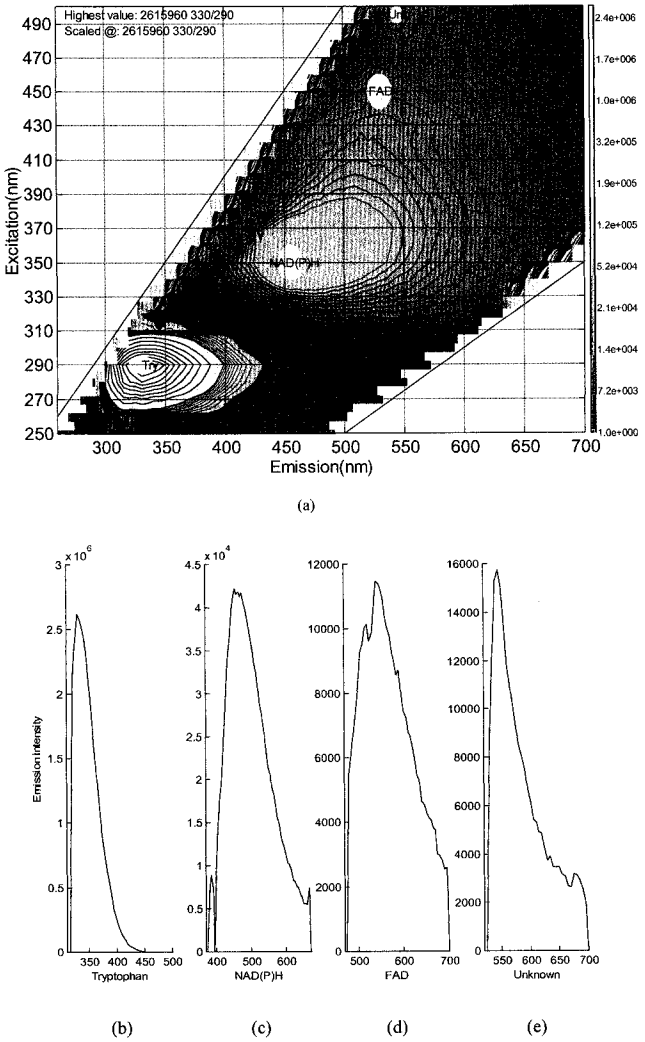

Figure 1. (a) The EEM of purified circulating neutrophils. Emission spectra at 290 nm (b), 350 nm (c), 450 nm (d) and 500 nm (e) excitation are given corresponding to tryptophan, NAD(P)H, FAD and the unknown fluorophore, respectively.

(8). An increase in tryptophan fluorescence intensity following activation was seen most often in PMA-activated cells (three of six samples) and in two of six samples activated with fMLP. A Student's *t*-test showed that the activation of neutrophils with fMLP ($n = 4$) resulted in a significant elevation ($\alpha = 0.05$) in the fluorescence intensity corresponding to the NAD(P)H level. The average increase in NAD(P)H fluorescence was by a factor of 2.1 ± 0.5. Activation with PMA ($n = 3$) exhibited an increase that was not statistically significant due to the large variance. The procedures for activation with both fMLP and PMA were validated with the OxyBURST Green fluorescent label.

Mononuclear leukocytes were purified on five different occasions. At the time of measurement, the mononuclear leukocyte suspensions were approximately 95% pure and greater than 90% viable. Typical cell concentrations were approximately 2E6 cells/mL. A typical fluorescence EEM of mononuclear leukocytes is shown in Fig. 2. The fluorescence EEM exhibits peaks suggestive of tryptophan, NADH and FAD.

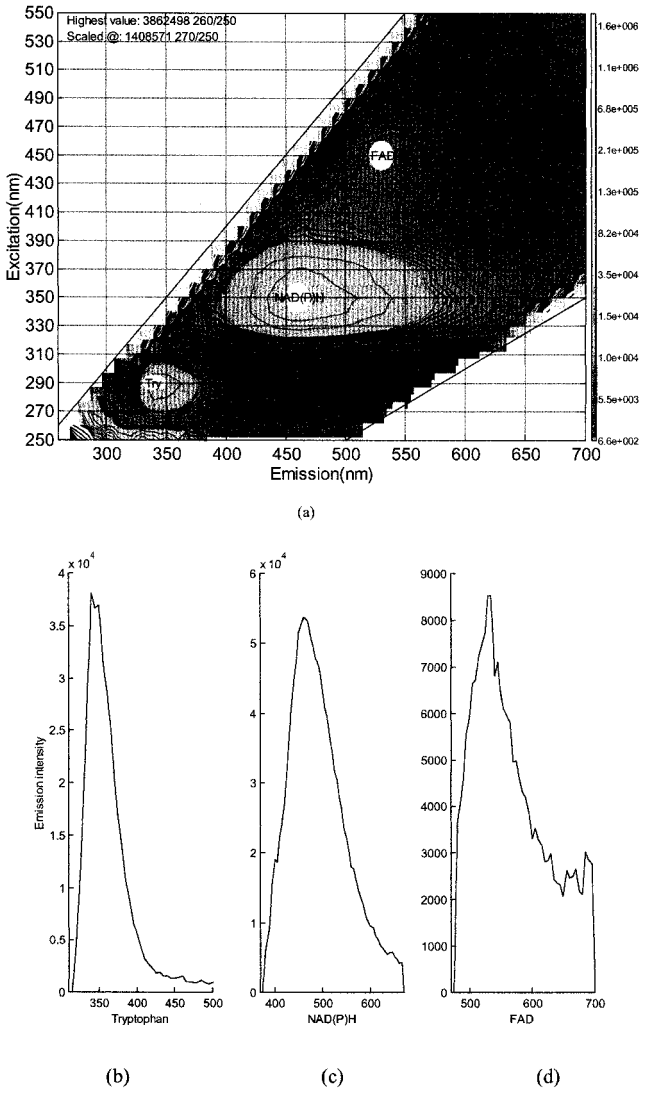

Figure 2. (a) The EEM of purified circulating mononuclear leukocytes. Emission spectra at 290 nm (b), 350 nm (c) and 450 nm (d) excitation are given corresponding to tryptophan, NAD(P)H and FAD, respectively.

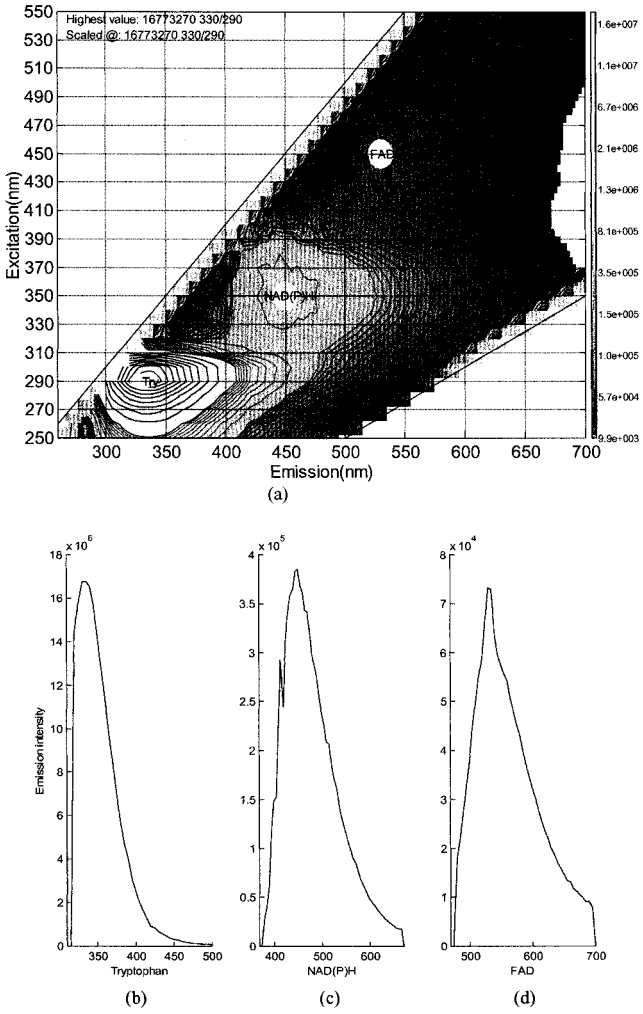

Figure 3. (a) The EEM of HeLa cervical cancer cells. Emission spectra at 290 nm (b), 350 nm (c) and 450 nm (d) excitation are given corresponding to tryptophan, NAD(P)H and FAD, respectively.

To better assess the role of neutrophils and mononuclear leukocytes in the fluorescence measured from cervical tissue, epithelial cells from a cervical cancer cell line were measured. An EEM of cervical cancer cells from an immortalized cell line, HeLa, was measured to compare with the EEMs from the leukocytes (Fig. 3). At the time of the measurement, cells were approximately 65% viable and concentrations were approximately 6E6 cells/mL. The lower viability was the result of the washing procedure used to remove fluorescent components contained within the growth medium. Again, the typical cellular fluorophores are visible: tryptophan, NAD(P)H and FAD. However, the ratio of the tissue fluorophores differed from the leukocytes characterized in this study (Fig. 4). Figure 4 depicts the average peak fluorescence intensity for a given fluorophore normalized to the cell count for mononuclear leukocytes, resting neutrophils (iNeutrophils), activated neutrophils (aNeutrophils) and HeLa cervical epithelial cells.

DISCUSSION AND CONCLUSIONS

The EEMs of cell suspensions of resting neutrophils and mononuclear leukocytes show contributions from tryptophan, NAD(P)H and FAD; in addition, contributions from an unidentified fluorophore (500 nm excitation 530, nm emission) were present in approximately a third of the neutrophil suspensions. To address concerns that the unidentified fluorophore was produced by photobleaching, fluorometer measurements were taken from 450 to 550 nm excitation before measurements in the UV were taken. However, the presence of this peak did not correlate with measurement order. It is interesting to note the presence of fluorophores at 500 nm excitation in both the neutrophils measured here and eosinophils measured by Barnes *et al.* (11).

The effect of activation of neutrophils was consistent with the observations of Liang and Petty (15). However, PMA did not induce a significant increase in NAD(P)H fluorescence as seen with fMLP. Both were found to initiate activation with the OxyBURST Green assay, but the extent of activation, the rate of release of oxidative species, was not measured.

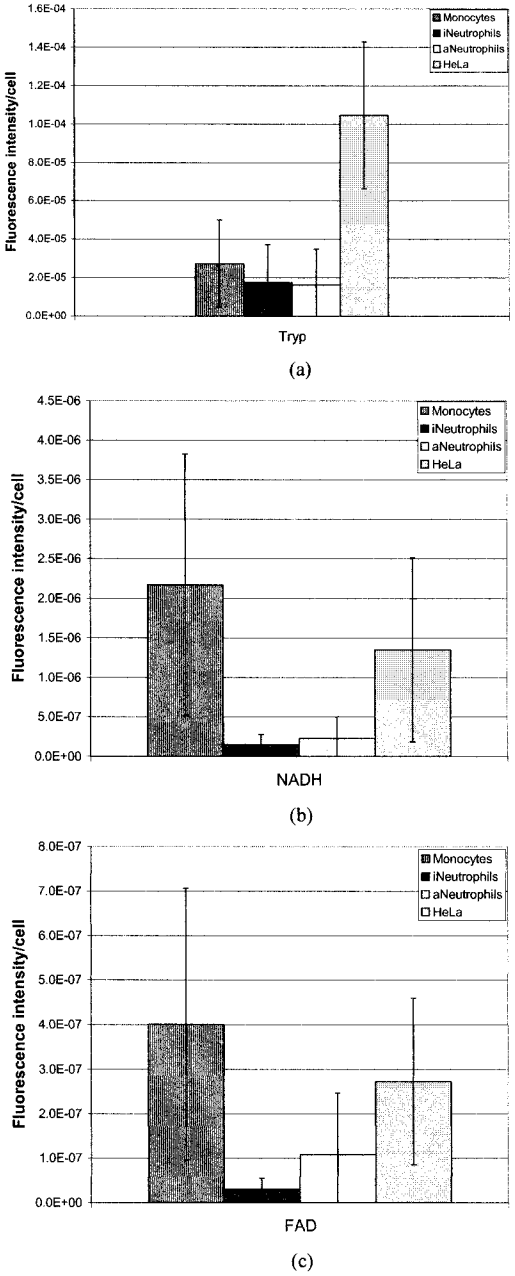

Figure 4. Relative average peak fluorescence intensity of tissue fluorophores in mononuclear leukocytes (n = 5), resting neutrophils (iNeutrophils, n = 13), neutrophils activated with fMLP (a-Neutrophils, n = 4) and HeLA cervical cancer cells (n = 5) at (a) 290 nm excitation, (b) 350 nm excitation and (c) 450 nm excitation.

A summary of the relative fluorescence intensity from each of the three assigned tissue fluorophores is shown in Fig. 4. The leukocytes demonstrate a significantly lower intensity of tryptophan fluorescence per cell than the cervical cancer cells as assessed with a Student's *t*-test ($\alpha = 0.05$). This is expected due to the low protein expression of inactive leukocytes, especially lymphocytes (18). While these differences are statistically significant, it should be noted that comparison is made between neutrophils with 90% viability and cancer cells with lower viability due to the washing procedures to remove growth medium. To determine whether differences in tryptophan fluorescence reflected differences due to cell lysis during the wash procedure, we measured the supernatant fluorescence of the final wash from the cancer cells. The tryptophan fluorescence in the supernatant was at least two orders of magnitude weaker than that associated with the cells; thus, the tryptophan measured was associated with intact cells and not with lysed cells. Furthermore, because tryptophan is an amino acid that cannot be made by these cells, it is not a strong metabolic indicator and would not be expected to be sensitive to viability.

The neutrophils demonstrate the lowest NAD(P)H and FAD fluorescence per cell, while the level in mononuclear leukocytes is similar to that of cervical cancer cells; however, none of these differences were statistically significant as assessed with a Student's *t*-test ($\alpha = 0.05$).

Figure 4 suggests that excitation near 290 nm to measure tryptophan fluorescence may be useful to discriminate dysplasia and inflammation in the cervix. Ultraviolet radiation is known to damage DNA through the induction of thymine dimers (19). As a result, clinical results reported to date have concentrated on excitation wavelengths longer than 330 nm due to these safety concerns. However, the quantum yield of tryptophan is high enough that the fluorescence can be measured at illumination levels below the skin and eye threshold limit value (3 mJ/cm^2 at 270 nm) given by the American Conference of Governmental Industrial Hygienists (ACGIH®). These guidelines allow the total effective exposure at 270 nm to be calculated based upon the spectral output of a proposed device. Therefore, the incorporation of excitation wavelengths near 290 into a spectroscopic device may offer improved discrimination of inflammation from dysplasia in the cervix.

Acknowledgments—This research was funded in part by a Cost-Reducing Health Care Technology grant from the Whitaker Foundation and by the NIH (RO1CA72650). We acknowledge Sharon Thomsen, M.D. (M.D. Anderson Cancer Center) for her contributions in identifying and categorizing the different types of leukocytes present in cervical biopsies.

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