LETTERS TO THE EDITOR

Comments on the Fluorescein Excitation and Emission Polarization Spectra in Living Cells

Dear Sir:

We are very perplexed by a recent paper of Cercek et al. (1) in this journal. They report results of fluorescence polarization studies in mammalian cells that apparently resolve a disagreement between their work and that of Lindmo and Steen (2). However, their results also indicate that it should be impossible for us to make measurements that we have now been making routinely for more than three years.

In several instances, biologically significant changes in the state of a living cell have been detected by measuring changes in the polarization of fluorescence from fluorescein molecules located in the cytoplasm of the cell. However, not all investigators have been successful in demonstrating the phenomenon. Thus, Lindmo and Steen (2), in a study of cultured NHIK 3025 cells, found no difference in polarization values for cells in S and G_1 phases of the cell cycle, whereas previously Cercek et al. (3), in a study of cultured CHO cells, found a large difference. There are many possible reasons for this disagreement. To name a few: (*a*) Different cell lines were used. (*b*) There are small differences in the labeling procedures used. (*c*) The methods used for measuring polarization were very different. Thus, Cercek et al. (3) made their measurements on a bulk suspension of cells, a procedure that requires careful calibration and correction for leakage of fluorescein molecules from the cells into the supporting fluid, whereas Lindmo and Steen made their measurements on individual cells in a flow cytometer, a procedure in which calibration and background correction requirements are much less demanding.

In a recent paper in this journal (1), Cercek et al. offer an explanation different from any of the above for the disagreement. They claim that the polarization measured for fluorescein molecules in the cytoplasm of a mammalian cell is a strong function of both the wavelengths used to excite and to measure the fluorescence. In particular, they present data showing that polarization shifts are minimal unless the excitation wavelength is near 470 nm and the emission wavelength near 510 nm, and are nonexistent for an excitation wavelength of 488 nm (as used by Lindmo and Steen). They show this wavelength dependence in cell cycle studies of cultured S3 fibroblasts and also in studies of the response of human lymphocytes to phytohemagglutinin (PHA), a system which they have previously shown (4) also shows polarization shifts shortly after PHA addition.

We are making extensive studies of shifts in polarization after biological activation in several biological systems, including the response of human lymphocytes to PHA. These measurements are being made in a flow cytometer-cell sorter system basically similar to that of Lindmo and Steen, and we have used an excitation wavelength of 488 nm from an argon ion laser as described in detail elsewhere (5). Filters are used to exclude the scattered laser light and long wavelength light, also as described elsewhere (6). Fig. 1 shows the wavelength distribution of the fluroescence spectrum actually analyzed. It is centered in a broad band around 540 nm and almost entirely excludes 510 nm.

We are presently in the middle of a double-blind study to determine whether we can confirm and extend the results of Cercek and Cercek (7) that fluorescence polarization measurements can distinguish women with early recurrent breast cancer, not evident by simple clinical investigation, from normal individuals. Some of the controls for this study are shown in Fig. 2. Each point represents the polarization values (P) measured for the peripheral blood lymphocytes from a single individual within 1 h after exposure to 2% PHA. Clearly, for normal individuals, P has decreased on exposure to PHA. The values obtained for P in the absence of PHA and the average downshift obtained on adding PHA (4.8%) are in general agreement with the results of Cercek and Cercek (4). However, for patients with

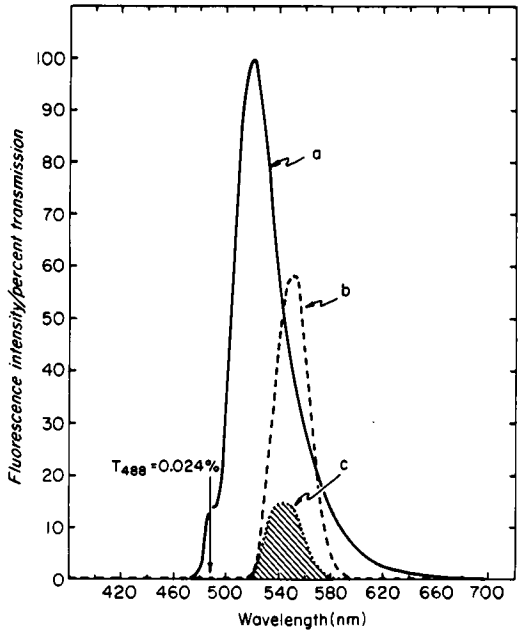

FIGURE 1 Wavelength distribution of fluorescence spectrum: (*a*) fluorescence intensity spectrum of fluorescein isothiocyanate (FITC) in aqueous solution, arbitrary scale with maximum of 100; (*b*) measured filter transmission, percent; (*c*) fluorescence transmitted through the filter stack, same scale as *a*. For details, see reference 6.

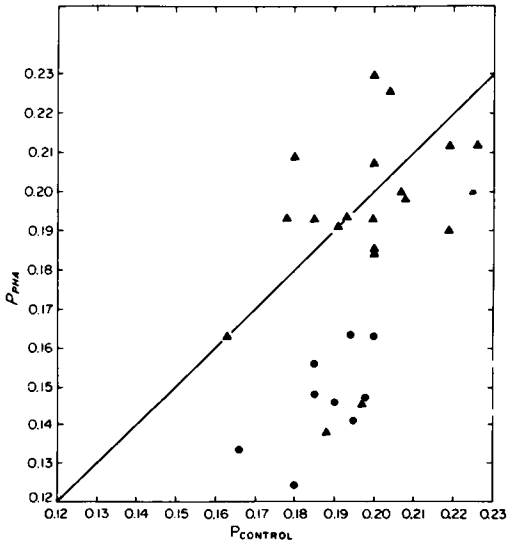

FIGURE 2 Polarization values for individual peripheral blood lymphocyte samples from either normals (●) or patients with recurrent breast cancer before treatment (▲) measured with or without prior exposure to PHA. See text.

recurrent breast cancer before any treatment, P values have changed little, if at all, in response to PHA addition. The difference between the two groups is statistically significant ($p << 0.005$) as judged by the Wilcoxon rank sum test. We have recently made a few polarization measurements using an argon ion laser line at 472.8 nm and get results qualitatively similar to those of Fig. 2 which we obtained with measurements at 488 nm.

We can find no ready explanation for our disagreement with Cercek et al. (1), i.e., that we are observing shifts in polarization similar to those they observe but at an emission and excitation wavelength for which they observe no effect. However, there are two major differences in procedure which may ultimately lead to an explanation. First, their measurements are made on a carefully selected subpopulation of lymphocytes (1), whereas our measurements are made on all lymphocytes: All cells in the peripheral blood buffy coat are run through the flow cytometer, and the scatter signal is used to distinguish lymphocytes from other cells (8). Second, their measurements must be made within minutes of introducing fluorescein inside the cells (1), whereas our measurements are made at least 20 min after fluorescein introduction (8). It is possible that we are looking at different phenemona.

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