Letters to the Editor

COMMENTS ON "RESPONSE OF HUMAN LYMPHOCYTES TO PHA AND TUMOUR-ASSOCIATED ANTIGENS AS DETECTED BY FLUORESCENCE POLARIZATION". *Br. J. Cancer*, 41, 73 (1980)

SIR,—I have examined the results of Balding *et al.* (*Br. J. Cancer*, (1980) 41, 73) and find that their intracellular fluorescein polarization measurements on human lymphocytes are in disagreement with those from several other laboratories, and fraught with stray-light artifacts for the following reasons:

An asymptotic decrease of P values with increasing time of incubation in the FDA substrate (Figure, square symbols) indicates a stray-light artifact. This artifact can be eliminated by improving the resolution of the monochromators by mounting the primary blue FITC filter (Barr & Stroud Ltd., U.K.) between the excitation monochromator and polarizer as well as the GG 495 cut-off filter (Barr & Stroud) on the emission side (Cercek & Cercek, 1979). Elimination of the stray-light artifact produces a much reduced linear decrease in P values with time of incubation (Figure, circle, cross and triangle symbols) of only 0.36 and 0.88%/min in 0.65 and 2.4 μ M FDA substrate, respectively. In agreement with these artifact-free results, a linear, not asymptotic, decrease of P values by only 0.7%/min over the first 30 min of FDA hydrolysis was observed in EL4 cells by Epstein *et al.* (1977) using a calculating flow polarization-meter. As would be expected in the presence of stray-light artifacts, the results of Balding *et al.* in Fig. 2 exhibit a sharp decrease in P within the first 7 min of hydrolysis followed by a linear, much slower decrease in P of only 0.8%/min. This latter value agrees with our results in 2.6 μ M FDA substrate (0.88%/min) and also with that obtained for EL4 cells (0.7%/min) by Epstein *et al.* (1977). Therefore, to account for a 10% decrease in P at least a 10 fold increase in intracellular concentration of fluorescein over that advocated by Balding *et al.* would be required to support their suggestions. For example, the flow polarization meter results of Udkoff & Norman (1979) show that in human lymphocytes an 800% increase in intracellular fluorescein concentration is required for a 10% reduction in P. Our own results

in Fig. 1 indicate that a 10% reduction in P requires 6000%, and 400%, increase in intracellular concentrations of fluorescein in 0.65 μ M, and 2.6 μ M, FDA substrate respectively.

Evidence for stray-light artifacts in the polarization measurements of Balding *et al.* can also be inferred from their statement that more consistent results were obtained when the pipetting errors were minimized. Since the polarization values are a ratio of fluorescence intensities, changes in cell numbers should only affect polarization values if stray-light artifacts were measured (Cercek *et al.*, 1978). In addition, our own measurements on the identical fluorescence spectrophotometer to that used by Balding *et al.* in Prof. Bleehen's laboratory at Cambridge showed that both FITC and GG495 optical filters had to be installed to eliminate stray-light artifacts. Furthermore, as Dr J. A. V. Pritchard (personal communication) writes "the results presented by Balding *et al.* in his Fig. 10 were obtained in the Cardiff MPF-4 fluorescence spectrophotometer from a single blood sample, without stray-light filters, before the correct protocol had been established for successful and consistent operation of the SCM-test in Cardiff and were published without the prior knowledge of the Cardiff group". Stray-light artifacts are also evident from the asymptotic relationship between P and time of FDA hydrolysis.

Our previous report that the degree of fluorescence polarization was the same when the measurements were made 3 and 15 min after the cells were suspended in 0.7 μ M FDA (Cercek *et al.*, 1978) does not contradict the present report. For a 0.36% decrease in polarization per min, the change in polarization over 15 min is 4.3%, and could not have been distinguished from the experimental error, even less so in the SCM test when measurements are made at time constant to within ± 1 min. The decrease became apparent only when the incubation times were extended to beyond 15 min.

It follows from the above argument that,

FIGURE.—Effect of time of incubation in the FDA substrate solution on the intracellular fluorescein fluorescence polarization of human lymphocytes. In $0.65\mu\text{M}$ FDA prepared from a glacial-acetic acid FDA stock solution: emission GG495 optical filter only ($\square$); both emission GG495 and excitation FITC optical filters ($\circ$). In $2.6\mu\text{M}$ FDA prepared from an acetone FDA stock solution: emission GG495 optical filter only ($\triangle$); both emission GG495 and excitation FITC optical filters ($\times$). In all experiments cells were incubated in bulk under constant stirring in FDA solutions kept at 27°C and 3ml samples were withdrawn at various intervals. The correlation coefficient, a measure of the tightness of the linear relationship between P values and time of incubation, is 0.98.

because of stray-light artifacts, the conclusions of Balding *et al.* on the effects of time of FDA hydrolysis and of changes in the intracellular fluorescein concentration on the results of the 'SCM test' are inconsequential.

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SIR,—We are perplexed that Balding *et al.*, in their recent paper (*Br. J. Cancer*, (1980) **41**, 73) overlooked the fact that the successful accomplishment of the SCM test depends on the isolation of a specific subpopulation of lymphocytes (Cercek & Cercek, 1977, 1978a, b; Takaku *et al.*, 1977; Hashimoto *et al.*, 1978; Kreutzmann *et al.*, 1978; Pritchard and Sutherland, 1978; Pritchard *et al.*, 1978; Ørjasaeter *et al.*, 1979). In contrast to 90% pure density-specific lymphocytes (of which more than 75% are T cells) used in the SCM test, their cell preparations contained an average of only 67% lymphocytes with an undefined proportion of T cells. It is obvious that the experimental conditions for the isolation of SCM responders were not strictly followed. For example, their "cell preparations b" were isolated at 13°C instead of at 16.7°C (calculated from their data on the osmolality and density of the Ficoll-Triosil solution) resulting in the isolation of SCM-non-responding lymphocytes (Cercek & Cercek, 1978b). Other critical deviations from the SCM protocol include the use of HEPES instead of phosphate buffer in their "cell preparations a" as well as the use of acetone in the preparation of the FDA substrates. Both HEPES buffer (unpublished results) and impurities in the acetone diminish or abrogate SCM responses to PHA and cancer antigens (Cercek & Cercek, 1976, 1977). Furthermore, they claim that on PHA stimulation of lymphocytes the rate of fluorescein diacetate (FDA) hydrolysis increases and the leakage of fluorescein decreases. In contrast, a detailed study on the effects of PHA on the kinetics of FDA hydrolysis in human lymphocytes (Mullen & Campbell, 1979) and the data of Cercek & Cercek (1978a) show that the rate of FDA hydrolysis decreases on PHA stimulation by an average of 40%. It has also been reported that binding of mitogens such as PHA and Con-A to the plasma membrane of lymphocytes increases its fluidity and permeability (Greaves & Janossy, 1972; Collard *et al.*, 1977). To this we can add our own observations from hundreds of measurements on SCM-responding lymphocytes from healthy donors, cancer patients or donors with other diseases, which show that after PHA stimulation the permeation of fluorescein always increases by 5–150% according to the batch of PHA, over that of the controls. Therefore, at a constant time of FDA