Technical methods

found that all the antibodies mentioned above showed equally good staining results on our smears. In normal adults the number of peripheral blood lymphocytes stained by these markers were as follows: T11: 75–90%; B1: 10–20%; I2: 10–20%; T4: 55–65%; T8: 25–35%; J5: 100% negative. These figures are in close agreement with those given by the manufacturer when using immunofluorescence.

Using this method we have successfully demonstrated various surface markers on normal lymphocytes and leukaemic cells in acute lymphoblastic leukaemia, lymphoma, and chronic lymphocytic leukaemia. We are aware that the immunofluorescence method is equally good for demonstrating cellular antigens on smeared cells, and a report describing such technique has recently been published in the *Journal of Clinical Pathology*.⁷ The advantage of the method described in the present paper over immunofluorescence is that a simultaneous study of morphology and immunocytochemistry is possible and the stained smears can be filed as a permanent record.

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Fluorimetric method for measuring chemotaxis

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Polymorphonuclear leucocyte chemotaxis is a fascinating phenomenon and its molecular, biological, and clinical aspects have been widely studied. Most investigators have used a variation of the test developed by Boyden.¹ The chief disadvantage of this test is that it requires the chemotactic response to be measured microscopically on stained filters, either by cell counting or by measuring the distance travelled into the filter. Gallin *et al*² tagged granulocytes with ⁵¹Cr and measured the response by counting the radioactivity present on collecting filters with a pore size impermeable to leucocytes. The labelling step increases the duration of the experiment and the hazard of cell damage.

We have also used a second filter to collect migrating cells but have developed a chemical method for counting them. The method assays for DNA as described by Kapuscinski and Skoczylas.³ The natural fluorescence of the dye 4', 6-diamidino-2-phenylindole (DAPI) is enhanced

when complexed to DNA. The fluorescence enhancement is linearly proportional to the concentration of DNA at appropriate DAPI:DNA ratios, even in the presence of protein and RNA. By means of a standard curve, changes in fluorescence can be read directly as cell numbers.

Material and methods

Polymorphonuclear leucocytes were purified from venous blood using the method described by Boyum.⁴ Contaminating red cells were removed by hypotonic lysis. The polymorphonuclear leucocytes were suspended at a concentration of 1×10^6 /ml in medium 199 containing 0.9% glucose and 1.75% sodium bicarbonate and applied to 8 μ m and 0.45 μ m cellulose nitrate filters (Millipore) sandwiched in Teflon Boyden chambers. The chemoattractant was 0.5% casein in medium 199. The chambers were incubated in 5% CO₂ at 37°C for 90 min. For some experiments cell concentrations and incubation times were varied. The variables were tested in duplicate. After incubation, two 0.45 μ m filters were removed from the chambers, placed in 1 ml of 5 mM Hepes buffer containing 12 mM NaCl, and sonicated with a Dawe Soniprobe for 20 s. One millilitre of DAPI at an appropriate concentration in Hepes buffer was added to each vial, mixed by inversion, and the fluorescence measured on a Perkin-Elmer 204 spectrophluorimeter. Filters from chambers containing casein but no cells were used as

fluorescence controls. Standard curves were constructed by diluting a counted sample of the original cell suspension in triplicate in DAPI and measuring fluorescence. Chemotaxis values were obtained by subtracting control fluorescence from cell fluorescence and converting to cell numbers by means of the standard curve. The remaining two filters were fixed and then stained with Harris' haematoxylin stain.⁵ Five randomly selected fields were counted and the total number of responding cells calculated.

The fluorescent dye 4', 6-diamidino-2-phenyl indole dihydrochloride was obtained from Sigma Chemicals, dissolved in 5 mM Hepes buffer containing 12 mM NaCl to make a stock solution of 4 mg/100 ml and stored at 4°C. We used 8 μ m and 0.45 μ m filters obtained from Millipore Corporation. Only the filters from a single lot were used.

Results

Fig. 1 shows the relation between fluorescence and cell numbers (log plot) for two concentrations of DAPI—4 ng/ml and 40 ng/ml. For the higher concentration of DAPI the relation is clearly linear for cell counts ranging from 1×10^4 to 1×10^6 . For the lower DAPI concentration there is a suggestion that the fluorescence is saturated at the higher cell concentrations. On the other hand, the lower DAPI concentration may still be sensitive to low numbers of cells. A DAPI concentration of 40 ng/ml was used for further experiments.

To be certain that polymorphonuclear leucocytes were actually eluted from filters, preparations were fixed and stained after overnight elution and after sonication. Sonication alone thoroughly removed the cells.

The stability of the DNA-DAPI complex was assessed by measuring fluorescence of sonicated and unsonicated samples immediately and after 4, 18, and 42 h at 4°C. DNA fluorescence decreased by 39% within 4 h of sonication. Unsonicated filters, however, could be stored for 4 h at 4°C without any reduction in fluorescence.

After chemotaxis, polymorphonuclear leucocytes assayed by the DAPI method were compared with cells counted visually (Fig. 2). Data were generated by nine experiments in which cell concentrations and incubation times were varied. As expected the two methods were highly correlated ($p = 0.002$). There was also a consistent and significant bias between the results given by the two methods. The mean of the differences between the two methods was 0.44 ± 0.29 . Student's paired *t* test value was 9.2 ($p < 0.001$).

Fig. 1 Log plot of polymorphonuclear leucocyte DNA complexed with DAPI concentrations of 40 ng/cm³ and 4 ng/cm³ against cell numbers. Bars represent standard error of the mean of three readings. Suitability of 40 ng/cm³ of DAPI for assaying DNA from 1×10^4 to 1×10^6 cells is evident.

Fig. 2 Plot of log₁₀ visual cell count against DNA measure. Mean of differences between two methods = 0.44 ± 0.29 . Paired *t* test value = 9.2 ($p < 0.001$).

Discussion

The procedures described simplify the measurement of chemotaxis. Elimination of visual counting facilitates replication of the procedure and allows a larger number of samples to be tested in a short time.

We had anticipated that counting all the cells crossing the membrane rather than a random sample of them would reduce the variance of the test readings. This did not prove to be the case, however; most of this variance seemed to be due to differences between the filters. Cell concentrations of 1×10^6 are used routinely because they produce visually acceptable numbers of cells on the collecting filters. Counting cells by chemical means permits use of a much wider range of polymorphonuclear leucocyte concentrations, especially physiological ones. Because chemotactic responses are affected by the cell density, it is important to use physiological concentrations.⁶

The DNA method gave higher estimates of cell numbers than visual counts of the membranes. One explanation for this is that cells may drop off the collecting membranes during the process of fixation and staining. It is likely that the DNA method also measures cells that disintegrate after transverse the membrane and thus are not visible to the staining.

A portion of the polymorphonuclear leucocyte DNA producing fluorescence enhancement appears to be susceptible to cellular nucleases. It is impor-

tant to measure fluorescence soon after the collecting membranes are sonicated. Several hours delay can be tolerated, however, if the membranes are not sonicated immediately on removal from the chemotaxis chambers.

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Rapid identification of *Clostridium difficile* by direct detection of volatile organic acids from primary isolation media

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The importance of *Clostridium difficile* in pseudomembranous colitis¹⁻⁵ and its increasing role in antibiotic-associated diarrhoea⁶⁻⁹ have encouraged many laboratories to screen routinely for the organism in all samples of faeces.

Isolation of *C. difficile* has been greatly facilitated by the development of more effective selective media.¹⁰ Identification by conventional biochemical

techniques, however, is often confounded by the variable and fastidious nature of the organism. Detection by gas chromatography of characteristic volatile organic acids in broth cultures of *C. difficile* provides a more reliable method of identification, but entails a delay in reporting of 24-48 h because of the need to incubate broth cultures.

An observation that the same volatile organic acids are present in plugs of agar removed from beneath suspected colonies of *C. difficile* provides the basis for the rapid method described.

Material and methods

All samples of faeces received for bacteriological investigations were inoculated on to cycloserine-cefoxitin fructose agar (CCFA) (Oxoid Ltd). Cultures were incubated at 37°C in an atmosphere of CO₂ (5%), H₂ (5%), and N₂ (90%) for up to 48 h.

A hollow glass tube (approximate internal diameter 4 mm) was pushed through suspected colonies of *C. difficile* and into the underlying agar. A rubber