Lymphocyte transformation in cancer patients: variation in results according to technique

GILLIAN M. L. GYTE AND SYLVIA M. WATKINS

*From the Department of Pathology and Medicine, Lister Hospital, Stevenage, Hertfordshire SG1 4AB, UK*

**SUMMARY** Lymphocyte transformation to phytohaemagglutinin (PHA) was measured simultaneously by two methods (heparin and methyl cellulose) in 16 patients with non-lymphoid cancer and 21 normal subjects. Twelve cancer patients showed transformation levels below the normal heparin range, but only two patients showed levels below the normal methyl cellulose range. These findings suggest that in interpreting lymphocyte transformation studies close attention should be given to the methods employed.

The *in-vitro* response to PHA of lymphocytes from patients with non-lymphoid malignant disease is often impaired (Garrioch *et al.*, 1970; Scheurlen and Pappas, 1971; Thomas *et al.*, 1971; Whittaker and Clark, 1971; Watkins, 1973; Knight and Davidson, 1975). On the other hand, some workers have found normal PHA responsiveness in all patients with non-lymphoid cancers (Robinson and Hurvitz, 1966; Sutherland *et al.*, 1971; Edwards *et al.*, 1973; Roberts and Jones-Williams, 1974). Possibly the wide variety of techniques for estimating PHA transformation is the reason for these discrepancies. We have therefore studied cancer patients simultaneously by two methods to see whether technical variations can produce different results.

**Subjects and methods**

Lymphocytes from 16 patients (aged 18-73 years) with various forms of non-lymphoid cancer and 21 normal persons (aged 18-62 years) were tested by methods A and B.

Fifty millilitres of venous blood was taken from each subject and the two methods were set up simultaneously with all procedures being carried out under sterile conditions.

**METHOD A: HEPARIN**

Twenty millilitres of blood was mixed with 375 units of heparin and allowed to sediment in a syringe for 30 to 60 minutes. The leucocyte rich plasma (LRP) was decanted off, the remaining blood centrifuged for 10 minutes at 2800 g, and the plasma layer removed. The lymphocytes and total white cells in the LRP were counted and a master mix set up containing $1 \times 10^6$ lymphocytes per 3 ml, 20% autologous plasma and 80% TC199 (Wellcome: containing penicillin 200 units/ml and streptomycin 100 μg/ml). PHA-P (Difco) was added to a concentration of 1 μl of reconstituted material per ml of master mix and the master mix divided into quadruplicate cultures (whenever possible) of 3 ml in tightly stoppered tissue culture tubes (Nunc: 100 mm × 14 mm).

**METHOD B: METHYL CELLULOSE**

Thirty millilitres of blood was defibrinated (using glass beads in a universal bottle) and taken back into the syringe. The volume of serum present was calculated from the packed cell volume, and methyl cellulose (BDH, 1% in TC199) was added to give 33% methyl cellulose solution and 67% serum in the sample. The blood-methyl cellulose was mixed for 30 minutes, then allowed to sediment for 30 minutes, and the leucocyte-rich serum-methyl cellulose was decanted off and spun on the bench centrifuge for 10 minutes at 500 g. The serum-methyl cellulose was removed, the cells were washed three times in TC199 and then resuspended in 3 ml of TC199, and the lymphocytes and total white cells counted. A master mix was set up containing $1 \times 10^6$ lymphocytes per 3 ml, 30% autologous serum-methyl cellulose (hence 20% autologous serum) and 70% TC199. PHA-P (1 μl/ml of master mix) was added and quadruplicate cultures of 3 ml were set up as in method A.

The cultures set up by both methods were incubated for three days at 37°C and tritiated thymidine (Radiochemical Centre: 30 μCi, specific activity 0-1 Ci/mmol) was added to each culture four hours before harvesting. The incubation was stopped by the addition of ice-cold saline, the samples spun for

Received for publication 14 July 1977.
10 minutes at 2800 g, and the supernatant decanted. The samples were washed again with ice-cold saline, then twice with ice-cold trichloroacetic acid (5%) and once with absolute alcohol. Soluene 350 0.5 ml (Packard) was added to each, and after at least two hours the samples were transferred to liquid scintillation vials using 10 ml NE233 scintillation fluid (Nuclear Enterprises). After two to three days the radioactivity of the samples was measured on a Nuclear Enterprises NE 8312 Spectrophotometer and the efficiency of counting determined either by channels ratio or by internal standards.

The results were expressed as uptake of thymidine in pmol/10⁶ lymphocytes/4 h and were calculated from the following equations:

\[
\text{specific activity of } ^3\text{H-thymidine} = 0.1 \text{ Ci/mmol} \\
1 \mu\text{Ci} \equiv 2.22 \times 10^6 \text{ disintegrations/min (dpm)} \\
\text{hence 1 pmol of thymidine} = 222 \text{ dpm} \\
\text{hence uptake of thymidine in pmol} = \frac{\text{counts per minute}}{100} \times \frac{\% \text{ efficiency}}{222}
\]

**Results**

The normal range of results using the two techniques is shown in the Figure. The heparin method gave a higher range and mean value in the normal subjects compared with the methyl cellulose method. The results in four out of the 16 cancer patients were normal by both methods and will not be considered further. The results in all 12 of the remaining patients were below the normal heparin range but only two were below the normal methyl cellulose range.

**Discussion**

Most of the cancer patients had normal lymphocyte responses when assessed by the methyl cellulose method, while three-quarters of the results were below the normal range by the heparin method. By contrast the normal range was much lower using the methyl cellulose technique.

Analysis of the results of other workers shows that all those who used heparin and autologous serum in preparing the lymphocyte cultures found a proportion of cancer patients with subnormal responsiveness to PHA (Garrioch et al., 1970; Scheurlen and Pappas, 1971; Thomas et al., 1971; Whittaker and Clark, 1971; Watkins, 1973; Knight and Davidson, 1975). On the other hand, those who used washed cells and non-autologous serum found consistently normal PHA responses (Robinson and Hurvitz, 1966; Sutherland et al., 1971; Roberts and Jones-Williams, 1974). These results are easily explained since the
Lymphocytes themselves are probably normal and the impaired responsiveness is due to humoral factors, possibly produced by or near the malignant tissue (Silk, 1967; Gatti, 1971; Scheurlem and Pappas, 1971; Whittaker and Clark, 1971; Edwards et al., 1973). Workers using other techniques including methyl cellulose, gelatin, dextran, Ficol-Isopaque, Ficol-Triosil, Ficol-Hypaque, and whole blood have found variable results (Whittaker and Clark, 1971; Catalona et al., 1973; Mekori et al., 1974; Nemoto et al., 1974; Roberts and Jones-Williams, 1974; Barnes et al., 1975; Blomgren et al., 1975; Kaplan et al., 1975; Kumar and Taylor, 1975).

We have shown that the heparin and methyl cellulose techniques give very different results in the same patient. This discrepancy may be caused through chemical interference by the reagents used in the methyl cellulose method on the humoral factors which depress lymphocyte responsiveness.

A peptide, found in small amounts in normal human serum and associated with the \( \alpha_2 \)-globulin fraction, inhibits lymphocyte responsiveness to PHA (Riggio et al., 1969; Occhino et al., 1973). This peptide, immunoregulatory alphas-globulin (IRA), is believed to play a role in the feedback control of the cellular immune response (Riggio et al., 1969; Glasgow et al., 1974). Patients with various forms of non-lymphoid cancer have been shown to have raised amounts of \( \alpha_2 \)-globulin (Ablin, 1972) and of IRA and IRA-like peptides in their serum compared with normal subjects (McLaughlin and Brooks, 1974; Nimberg et al., 1975). This serum peptide may inhibit lymphocyte responsiveness to PHA (and antigen) by binding to a plasma membrane receptor which is different from the mitogenic receptor. This binding either interferes with the mitogenic binding or prevents, at an early stage, the metabolic processes which the mitogen normally stimulates, hence inhibiting lymphocyte blastogenesis (Cooperband et al., 1972).

In this methyl cellulose technique for measuring lymphocyte responsiveness to PHA possibly the methyl cellulose prevents the binding of the inhibitor at the lymphocyte surface (either competitively or non-competitively) or the methyl cellulose binds the \( \alpha_2 \)-globulin in solution, thus permitting a normal blastogenic response to PHA.

We have looked at only two methods for determining PHA responsiveness. Many other techniques have been employed by others, while some have not stated their method. In view of our findings we suggest that reports on lymphocyte responsiveness in cancer patients should be interpreted with caution and with due regard to the technique used. Clearly it would be ideal to have a standard technique in all laboratories.

We are indebted to the North-West Thames Regional Health Authority for financial support.

References


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*J Clin Pathol* 1978 31: 125-128
doi: 10.1136/jcp.31.2.125

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