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 1×10^6 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 1×10^6 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: 3.0 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/ 10^6 lymphocytes/4 h and were calculated from the following equations:

specific activity of ^3H -thymidine = 0.1 Ci/mmol

1 $\mu\text{Ci} \equiv 2.22 \times 10^6$ disintegrations/min (dpm)

hence 1 pmol of thymidine $\equiv$ 222 dpm

hence uptake of thymidine in pmol =

$$\frac{\text{counts per minute}}{\% \text{ efficiency}} \times \frac{100}{222}$$

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

Figure Lymphocyte response to PHA in normal subjects and cancer patients by two methods

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; Scheurlen and Pappas, 1971; Whittaker and Clark, 1971; Edwards *et al.*, 1973). Workers using other techniques including methyl cellulose, gelatin, dextran, Ficoll-Isopaque, Ficoll-Triosil, Ficoll-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 α_2 -globulin fraction, inhibits lymphocyte responsiveness to PHA (Riggio *et al.*, 1969; Occhino *et al.*, 1973). This peptide, immunoregulatory alphaglobulin (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 α_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 α_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

- Ablin, R. J. (1972). Cancer, immunological surveillance, and α_2 globulin (Letter). *Lancet*, **2**, 874-875.
- Barnes, E. W., Farmer, A., Penhale, W. J., Irvine, W. J., Roscoe, P., and Horne, N. W. (1975). Phytohemagglutinin-induced lymphocyte transformation in newly presenting patients with primary carcinoma of the lung. *Cancer*, **36**, 187-193.
- Blomgren, H., Wasserman, J., and Glas, U. (1975). Capacity of sera from patients with mammary carcinoma to promote PHA-stimulation of human lymphocytes. *Acta Radiologica*, **14**, 127-138.
- Catalona, W. J., Sample, W. F., and Chretien, P. B. (1973). Lymphocyte reactivity in cancer patients: correlation with tumour histology and clinical stage. *Cancer*, **31**, 65-71.
- Cooperband, S. R., Badger, A. M., Davis, R. C., Schmid, K., and Mannick, J. A. (1972). The effect of immunoregulatory α globulin (IRA) upon lymphocytes *in vitro*. *Journal of Immunology*, **109**, 154-163.
- Edwards, A. J., Rowland, G. F., and Lee, M. R. (1973). Reduction of lymphocyte transformation by a factor produced by gastrointestinal cancer. *Lancet*, **1**, 687-689.
- Garrioch, D. B., Good, R. A., and Gatti, R. A. (1970). Lymphocyte response to P.H.A. in patients with non-lymphoid tumours (Letter). *Lancet*, **1**, 618.
- Gatti, R. A. (1971). Serum inhibitors of lymphocyte responses (Letter). *Lancet*, **1**, 1351-1352.
- Glasgow, A. H., Menzoian, J. O., Nimberg, R. B., Cooperband, S. R., Schmid, K., and Mannick, J. A. (1974). An immunosuppressive peptide fraction in the serum of cancer patients. *Surgery*, **76**, 35-42.
- Kaplan, M. S., Mino, F. O., Kummerfeld, K. B., and Lundak, R. L. (1975). Phytohemagglutinin stimulated immune response: assay in colorectal carcinoma patients. *Archives of Surgery*, **110**, 1217-1220.
- Knight, L. A. and Davidson, W. M. (1975). Reduced lymphocyte transformation in early cancer of the breast. *Journal of Clinical Pathology*, **28**, 372-376.
- Kumar, S. and Taylor, G. (1975). The response to phytohaemagglutinin (PHA) of lymphocytes from cancer patients. *Journal of Clinical Pathology*, **26**, 476-479.
- McLaughlin, A. P. and Brooks, J. D. (1974). A plasma factor inhibiting lymphocyte reactivity in urologic cancer patients. *Journal of Urology*, **112**, 366-372.
- Mekori, T., Sher, S., and Robinson, E. (1974). Suppression of the mitogenic response to phytohemagglutinin in malignant neoplasia. Correlation with clinical stage and therapy. *Journal of the National Cancer Institute*, **52**, 9-12.
- Nemoto, T., Han, T., Minowada, J., Angkur, V., Chamberlain, A., and Dao, T. L. (1974). Cell mediated immune status of breast cancer patients: evaluation by skin tests, lymphocyte stimulation, and counts of rosette-forming cells. *Journal of the National Cancer Institute*, **53**, 641-646.

- Nimberg, R. B., Glasgow, A. H., Menzoian, J. O., Constantian, M. B., Cooperband, S. R., Mannick, J. A., and Schmid, K. (1975). Isolation of an immunosuppressive peptide fraction from the serum of cancer patients. *Cancer Research*, **35**, 1489-1494.
- Occhino, J. C., Glasgow, A. H., Cooperband, S. R., Mannick, J. A., and Schmid, K. (1973). Isolation of an immunosuppressive peptide fraction from human plasma. *Journal of Immunology*, **110**, 685-694.
- Riggio, R. R., Schwartz, G. H., Bull, F. G., Stenzel, K. H., and Rubin, A. L. (1969). α_2 -globulins in renal graft rejection: effects on *in vitro* lymphocyte function. *Transplantation*, **8**, 689-694.
- Roberts, M. M. and Jones-Williams, W. (1974). The delayed hypersensitivity reaction in breast cancer. *British Journal of Surgery*, **61**, 549-552.
- Robinson, E. and Hurvitz, D. (1966). *In vitro* studies of lymphocytes from cancer patients. *Israel Journal of Medical Sciences*, **2**, 80-81.
- Scheurlen, P. G. and Pappas, A. (1971). Untersuchungen über die Beeinflussung immunologischer Reaktionen durch maligne Prozesse. *Verhandlungen der Gesellschaft für innere Medizin*, **77**, 749-752.
- Silk, M. (1967). Effect of plasma from patients with carcinoma on *in vitro* lymphocyte transformation. *Cancer*, **20**, 2088-2089.
- Sutherland, R. M., Inch, W. R., and McCredie, J. A. (1971). Phytohemagglutinin (PHA)-induced transformation of lymphocytes from patients with cancer. *Cancer*, **27**, 574-578.
- Thomas, J. W., Coy, P., Lewis, H. S., and Yuen, A. (1971). Effect of therapeutic irradiation on lymphocyte transformation in lung cancer. *Cancer*, **27**, 1046-1050.
- Watkins, S. M. (1973). The effects of surgery on lymphocyte transformation in patients with cancer. *Clinical and Experimental Immunology*, **14**, 69-76.
- Whittaker, M. G. and Clark, C. G. (1971). Depressed lymphocyte function in carcinoma of the breast. *British Journal of Surgery*, **58**, 717-720.