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TECHNIQUES

MICROTECHNIQUE FOR QUANTITATIVE EVALUATION OF *IN VITRO* LYMPHOCYTE TRANSFORMATION

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SUMMARY

A microtechnique is described to quantitate *in vitro* transformation of lymphocytes from man and marmosets following stimulation by PHA and different antigens. The ^{14}C-thymidine uptake of lymphocytes grown in cultures of 0·06–0·2 ml of whole blood was measured by liquid scintillation. The magnitude, consistency and reproducibility of the results were similar to those achieved using cultures of purified lymphocytes or cell-rich plasma but fewer technical procedures were required.

INTRODUCTION

During the last 10 years, many methods have been described for *in vitro* lymphocyte transformation using cultures of purified lymphocytes or cell-rich plasma. None of these techniques can be used for serial experiments in human infants or small experimental animals because relatively large amounts of blood are required for each test. A microtechnique was developed for studying the immune response of small South American monkeys to inoculation with tumour and hepatitis viruses and is described in this report. Results were comparable to those obtained by the established standard methods but much less blood and fewer technical procedures were necessary.

MATERIALS AND METHODS

Whole blood cultures

Blood from clinically healthy humans and from nonhuman primates (white-lipped marmosets, *Saguinus fuscicollis* and *S. nigricollis*, and cotton-top marmosets, *S.* (*Oedipomidas*) *oedipus*) was drawn into plastic syringes containing 100 units of preservative-free heparin/ml of blood (Connaught Laboratories, Toronto, Canada). The heparinized blood was dispensed in 0·06–0·2 ml amounts into 16 × 125 mm plastic culture tubes (Falcon Plastics,

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Los Angeles, California, U.S.A.) containing 4·0 ml of RPMI-1640 medium (Moore, Gerner & Franklin, 1967; purchased from Grand Island Biological Corp., Grand Island, New York, U.S.A.). This medium was supplemented with 20% γ-globulin-free foetal calf, human or marmoset serum, penicillin (100 units/ml), streptomycin (50 μg/ml) and 1 ml of 200 mM L-glutamine/100 ml. Phytohaemagglutinin P (PHA) or various antigens were added to the cultures, the tubes were tightly stoppered and incubated at 37°C.

Cultures were labelled with 0·15 μCi/ml of ^{14}C-thymidine (specific activity 54 mCi/mM, Amersham/Searle Corporation, Des Plaines, Illinois, U.S.A.) 24 hr before the end of incubation. At harvest the cultures were centrifuged at 600 *g* for 15 min, the supernatant was discarded and the sedimented cells were resuspended in 4 ml of cold 3% acetic acid to haemolyse the erythrocytes. The still intact leucocytes and leucocyte nuclei were sedimented by centrifugation at 600 *g* for 15 min, were washed once with 4 ml of 0·9% NaCl and then dissolved in 1 ml of 0·1N NaOH at 37°C for 10 min. Trichloracetic acid (TCA), 4·5 ml of a 6·7% solution, was then added to each tube and the acid-insoluble precipitate was allowed to form overnight at 4°C. The contents of these original culture tubes were transferred into glass centrifuge tubes, centrifuged at 600 *g* for 15 min and the supernatants were discarded. The original culture tubes were washed once with 5 ml of 5% TCA and the washings were added to the precipitates in the glass centrifuge tubes. The contents were mixed by shaking on a Vortex mixer and were again resedimented by centrifugation at 600 *g* for 15 min. The sediment was washed with 3 ml of absolute methanol and centrifuged again (600 *g* for 15 min). The supernatant was removed and the remaining methanol was evaporated in a 56°C incubator.

The dry, acid-insoluble precipitate was solubilized in 1·0 ml of NCS Solubilizer (a quaternary ammonium base, soluble in toluene; purchased from Amersham/Searle) for 15 min at 56°C, then mixed with 15 ml of scintillation fluid (toluene based 5·0 g/l PPO = 2,5-diphenyloxazole and 0·1 g/l M2 POPOP = 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene) and counted in a Packard Liquid Scintillation Counter. The counts were corrected for quenching and recorded as disintegrations per min (dpm). All cultures were set up in triplicate and each sample was counted for 10 min. In addition to labelled cultures prepared for scintillation counting, other whole blood cultures, stimulated and unstimulated, were set up for daily cell counts and viability tests. Cell counts were performed by the technique of Wilson & Thomson (1968). Viability was checked by the dye exclusion method (incubation in 0·2% trypan blue at 37°C for 15 min).

Cultures of purified lymphocytes

Heparinized venous blood (100 units of heparin/ml) was settled for 2 hr at 37°C. The cell-rich plasma was filtered through a cotton column and the filtrate, consisting of 92–98% mononuclear cells, was diluted to a final concentration of 1×10^6 viable cells/ml with the same medium used for whole blood cultures. Two ml of this cell suspension were dispensed into 16 × 125 mm culture tubes, incubated at 37°C and labelled as described. The incorporation of label was stopped by washing the cells twice with 4 ml of cold 0·9% NaCl; the cells were dissolved and the proteins precipitated by the procedures described above for whole blood cultures. The precipitates were collected under vacuum upon filter membranes (BA6, Schleicher and Schuell, Keene, New Jersey, U.S.A.), were washed with 5 ml of 5% TCA, 2 ml of ether ethanol (1:1) and finally 1 ml of ether. The filter membranes were placed into a scintillation vial, 0·5 ml of NCS Solubilizer was added and the vials were gently shaken until

the filter membrane became transparent. Scintillation fluid (15 ml) was added and the samples were counted as described.

Stimulants

The cultures were stimulated with the following preparations (amounts given per ml of culture): 0·05 μl to 50 μl PHA (Lot no. 3110-56; Difco Laboratories, Detroit, Michigan, U.S.A.); 0·01 ml of Bacto-Streptolysin-O (SLO, Difco); 3·2 hemagglutination units of heat inactivated (56°C, 30 min), attenuated mumps virus (American Type Culture Collection VR365) (Deinhardt & Shramek, 1969); 0·025 ml of heat inactivated dried smallpox vaccine ($10^8 TCID_{50}$/ml; Dryvax, Wyeth Laboratories, Inc., Marietta, Pennsylvania, U.S.A.) diluted 1:100 in Hanks' balanced salt solution; 2 μg of tuberculin-purified protein derivative (PPD), second strength.

RESULTS

Development of the technique

PHA-responses were usually measured in cultures containing 0·06 ml of whole blood giving a final concentration of 0·5–1 × 10^5 leucocytes/ml of culture, whereas maximal stimulation

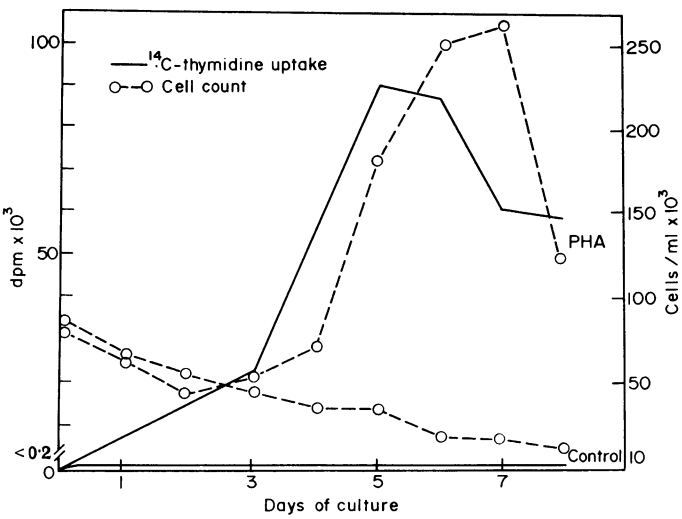

FIG. 1. Cell survival and ^{14}C-thymidine uptake of PHA-stimulated and unstimulated human lymphocytes in whole blood microcultures.

by antigens required 0·1–0·2 ml blood/culture. To check whether treatment with acetic acid, necessary for eliminating excessive quenching in the whole blood microcultures, damaged the nuclei of the leucocytes and caused loss of labelled DNA, cultures of purified PHA-stimulated lymphocytes were treated with 3% acetic acid and their ^{14}C-thymidine uptake was compared to identical cultures which were washed with 0·9% NaCl only. The mean uptake of six NaCl-washed cultures was 65,381 dpm; in six cultures washed with 3% acetic acid it was 65,103 dpm.

Cell survival and ^{14}C-thymidine uptake in PHA-stimulated whole blood microcultures

Cell survival and ^{14}C-thymidine uptake in microcultures of human blood was measured after different times of incubation with PHA (Fig. 1). Daily cell counts in twenty-two microcultures decreased during the first 2 days, due mainly to death of granulocytes. The cell concentrations in unstimulated cultures continued to drop steadily whereas the leucocyte concentration of 7-day-old PHA-stimulated cultures was three times greater than the initial concentration at day 0. ^{14}C-thymidine uptake was measured daily from 3 to 8 days in five different sets of these cultures. Optimal DNA labelling occurred at days 5–6 (Fig. 1).

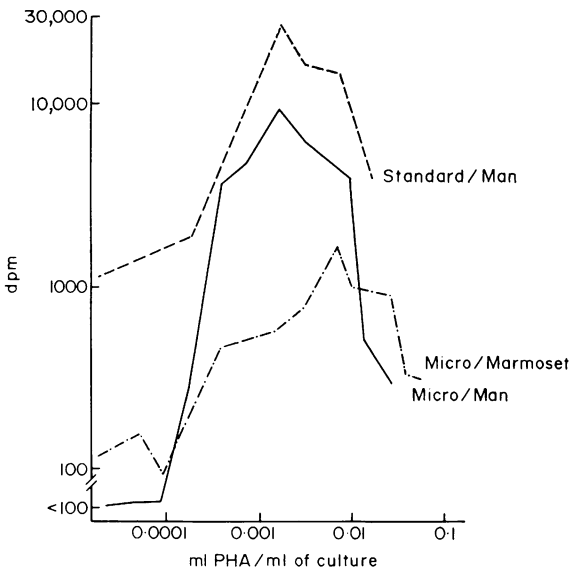

FIG. 2. Effect of different PHA concentrations on the ^{14}C-thymidine uptake of human and marmoset lymphocytes measured by the standard technique and the microtechnique after 72 hr of culture.

PHA dose–response curves for cultures of purified lymphocytes and whole blood microcultures

Four PHA dose–response curves were established in individual experiments for microcultures and for standard cultures of purified lymphocytes from the same human donor; both were incubated for 72 hr with the same batch of PHA and a representative example is given in Fig. 2. Both sets of cultures showed the best response when incubated with 1·5 μl PHA/ml of culture, and this PHA concentration was used in all further experiments with human cells. Fig. 2 also shows a typical PHA dose–response curve for marmoset lymphocytes in whole blood microcultures in which the optimal PHA-concentration was 9·4 μl/ml. This curve could not be compared with a dose–response curve for standard cultures because sufficient blood cannot be obtained from a single marmoset.

Sensitivity of the microtechnique

The average uptake of ^{14}C-thymidine by PHA-stimulated lymphocytes in whole blood microcultures from twenty human donors was 430 times higher than in unstimulated control microcultures (Table 1). These values were obtained after a 72-hr incubation with PHA

because only after these experiments were completed was it realized that an even better response may be obtained after 5 days of incubation. This PHA-response was compared to the response in standard cultures of purified lymphocytes from eleven of these twenty donors and no significant difference was seen: the average uptake in PHA-stimulated standard cultures exceeded the uptake in the control cultures by 353 times whereas in the microtechnique the control value of those eleven donors was exceeded by a factor of 341. Marmoset lymphocytes responded much less to PHA: the mean uptake of stimulated cultures from

TABLE 1. Response of human and marmoset lymphocytes to PHA and antigens as measured by the microtechnique

				Stimulated cultures / Control cultures		
Donor	Stimulant	Number studied	Number responding	dpm	Ratio	Mean ratio
Human	PHA-P	20	20	2652–12255 / 23 14	= 113·0–875·0	429·6
	SLO	5	5	350–704 / 113 55	= 3·0–13·0	6·7
	Vaccinia	4	3	12–12504 / 9 871	= 1·3–14·3	9·3
	PPD	2	2	4946–631 / 1678 85	= 3·0–7·4	
	Mumps	1	1	160 / 16		10·0
Marmoset	PHA-P	30	30	7621–8665 / 889 56	= 8·4–156·0	53·3
	Vaccinia	3	3	1897–8016 / 526 711	= 3·6–11·3	8·2
	Mumps	3*	2	1781–2642 / 1133 564	= 1·6–4·7	3·0

* One animal was not sensitized.

thirty different animals was only fifty-three times higher than in the corresponding unstimulated control cultures.

The response of human and marmoset lymphocytes to stimulation by different antigens in whole blood cultures after 5–7 days of incubation is also given in Table 1. The height of the vaccinia antigen induced response was about the same as the response obtained in parallel experiments using the standard technique: lymphocytes from one of the four human donors vaccinated 3 years prior to these experiments responded to vaccinia with a 7-fold increase in thymidine uptake in the microtechnique and an 8·3-fold increase in the standard technique. Both techniques yielded negative results (less than 2 × control) when lymphocytes were used from another of the four donors, who was vaccinated 14 years prior to these studies.

Consistency and reproducibility of results obtained by the microtechnique

The internal consistency of results obtained with the microtechnique was compared to the consistency of results obtained with the standard technique by comparison of their variation coefficients. Results from 100 stimulated or unstimulated sets of three cultures each (a total of 300 microcultures) had a variation coefficient of 18·2%; eighty similar sets (240 cultures) evaluated by the standard technique had a variation coefficient of 25·6%. The reproducibility of results obtained by the microtechnique was evaluated by checking the response of the cells from one human donor to the same batch of PHA four times at 4-week intervals; the response varied between 211 and 297 times control.

DISCUSSION

The microtechnique is now used routinely in our laboratory, even when larger amounts of blood are available, because fewer technical procedures are involved and results are comparable in magnitude, consistency and reproducibility to those obtained by the standard technique. The mean variation coefficient for the microtechnique lies within the same range reported for the standard technique (Hartog, Cline & Grodsky, 1967; Schellekens & Eijsvoogel, 1968). More reproducible results could probably be obtained if the whole blood microcultures were initiated with a constant lymphocyte concentration (calculated from the total white cell count and the percentage of mononuclear cells in the donor's peripheral smear).

The main difference between the technique reported here and those described in the literature is the presence of large amounts of erythrocytes and granulocytes in the culture. Transformation of lymphocytes grown in whole blood cultures after stimulation by PHA and antigens was reported by Heitmann (1967) who only evaluated the response morphologically and did not measure DNA-metabolism. The addition of granulocytes to purified, PHA-stimulated lymphocytes does not significantly alter their ^{14}C-thymidine uptake (Schellekens & Eijsvoogel, 1968) but responses to antigens are usually higher in lymphocyte cultures containing other white cell elements than in cultures of lymphocytes purified by cotton wool or glass bead column filtration (McFarland, 1969). Knight & Ling (1969) have shown that the addition of erythrocytes does not alter the response of purified lymphocytes to staphylococcal filtrate but we had to eliminate erythrocytes from the microcultures prior to processing for liquid scintillation counting to avoid inconsistencies due to quenching. Treatment of the cells with 3% acetic acid resulted in complete lysis of red cells without damage to the lymphocyte nuclei or change in their thymidine content.

There seems to be a difference in the division and the survival of cells grown in whole blood and purified lymphocyte cultures after PHA stimulation: in comparison to most of the reports on purified lymphocytes (Hartog *et al.*, 1967; Schellekens & Eijsvoogel, 1968; Yam *et al.*, 1967) we found a generally better and prolonged lymphocyte growth rate and a delay of 48 hr for maximal DNA-metabolism and maximal cell count in the whole blood cultures.

Further experiments are necessary to clarify the role of the serum in the microcultures. All the experiments with human cells and most of the PHA experiments with marmoset cells described in this report were performed with 20% γ-globulin-free foetal calf serum. However, ten times higher responses were found in human cells stimulated by SLO and vaccinia when autologous instead of foetal calf serum was used and marmoset lymphocytes responded

to antigens only when cultured in medium supplemented with either human, homologous or autologous serum.

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REFERENCES

DEINHARDT, F. & SHRAMEK, G.J. (1969) Immunization against mumps. *Progr. med. Virol.* **11,** 126.

HARTOG, M., CLINE, M.J. & GRODSKY, G.M. (1967) The response of human leucocyte cultures to stimulation by tuberculin and phytohaemagglutinin measured by the uptake of radioactive thymidine. *Clin. exp. Immunol.* **2,** 217.

HEITMANN, H.J. (1967) Vereinfachte Methodik der Lymphocytenkultur als Allergietest. *Hautarzt*, **18,** 459.

KNIGHT, S. & LING, N.R. (1969) A comparison of the responses of lymphocytes from blood, spleen, thymus and appendix of rabbits of different ages to stimulation '*in vitro*' with staphylococcal filtrate. *Clin. exp. Immunol.* **4,** 667.

MCFARLAND, W. (1969) Factors affecting the immunological reactivity of human lymphocytes '*in vitro*'. II. 'Pure' lymphocytes versus total leucocytes. *Proc. 3rd ann. Leucocyte Culture Conference*, p. 77. Appleton-Century-Crofts, New York.

MOORE, G.E., GERNER, R.E. & FRANKLIN, H.A. (1967) Culture of normal human leukocytes. *J. Amer. med. Ass.* **199,** 519.

SCHELLEKENS, P.TH.A. & EIJSVOOGEL, V.P. (1968) Lymphocyte transformation '*in vitro*'. I. Tissue culture conditions and quantitative measurements. *Clin. exp. Immunol.* **3,** 517.

WILSON, J.D. & THOMSON, A.E.R. (1968) Death and division of lymphocytes. *Lancet*, **ii,** 1120.

YAM, L.T., CASTOLDI, G.L., MITUS, W.J. & LOMANNO, V. (1967) Quantitative evaluation of PHA-stimulated lymphocyte cultures. *J. Lab. clin. Med.* **70,** 669.