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# SIMPLIFIED WHOLE BLOOD METHOD FOR EVALUATING *IN VITRO* LYMPHOCYTE REACTIVITY OF LABORATORY ANIMALS

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## SUMMARY

A whole blood culture technique has been employed to quantitatively evaluate lymphocyte reactivity of small laboratory animals including mice, rats, guinea-pigs and rabbits. This simplified method has been used to study the blastogenic response to phytohaemagglutinin, concanavalin A, pokeweed mitogen and staphylococcal filtrate. Tuberculin-induced lymphocyte transformation responses have been studied in serial experiments using sensitized animals. The results of this preliminary study demonstrate that the whole blood method has numerous advantages over the conventional techniques employing leucocyte concentrates. In almost all instances the degree of lymphocyte reactivity as measured by $^{3}$H-thymidine incorporation closely approximated or exceeded previously reported results using the standard culture method.

## INTRODUCTION

During the past decade it has been the interest of many investigators to develop a suitable culture technique for evaluating lymphocyte reactivity of various laboratory animals especially those which are commonly employed for studies of delayed hypersensitivity, tumour immunity, and histocompatibility. Conventional techniques require the separation of leucocytes from whole blood and the preparation of lymphocyte-rich concentrates which are suspended in plasma or serum-supplemented media to give a final cell density of a million or more leucocytes per culture (Ling, 1968; Schellekens & Eijsvoogel, 1968; Hughes & Caspary, 1970). The methods for isolating and purifying the leucocytes are not only cumbersome, but also technically difficult. Due to these technical difficulties, the limited number of cultures which can be established, and the poor reproducibility encountered in serial experiments the scope of many of these assays has been greatly confined.

Previous studies (Pauly & Sokal, 1971) conducted in our laboratory have led to the development of a simple, expedient and efficient whole blood culture technique for evaluat-

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ing human lymphocyte reactivity *in vitro*. Similar microtechniques have been described by others for evaluating lymphocyte reactivity of humans and non-human primates (Junge *et al.*, 1970; Mumford *et al.*, 1971).

## MATERIALS AND METHODS

### *Animals*

Animals employed were healthy adult $C_3H$ and $B_{10}$ mice, Sprague-Dawley rats, Hartley guinea-pigs and New Zealand rabbits.

### *Sensitization and skin tests*

The rabbit employed in the serial studies was sensitized by means of a single intradermal injection of 0·06 mg bacille Calmette-Guerin (BCG, Research Foundation, Chicago, Illinois). Guinea-pigs used in similar studies received a 0·1 ml injection of Freund's complete adjuvant H37Ra (CFA, Difco Laboratories, Detroit, Michigan) into each hind footpad.

Skin tests were performed with 5 μg PPD (250 TU) in 0·1 ml and read at 48 hr. With exception of the serial studies, in no instance were any of the animals employed in more than one experiment.

### *Lymphocyte stimulation*

Blood, collected in preservative-free heparin (10 U/ml), was obtained aseptically from mice, rats and guinea-pigs by means of retro-orbital punctures using capillary pipettes, and from the marginal ear vein of rabbits. The blood was diluted with either 30 or 40 volumes of RPMI 1640 culture medium containing 100 U penicillin and 50 μg streptomycin per ml and was supplemented with 1% purified bovine serum (Cohn Fraction V) (Nutritional Biochemicals, Cleveland, Ohio). Three millilitre aliquots of the cell suspension were distributed to 16 × 125 mm culture tubes (Falcon Plastics, Division of B-D Laboratories, Los Angeles, California). Each of the duplicate or triplicate cultures received one of the following: 10 μg purified phytohaemagglutinin (PHA, Burroughs-Wellcome, Tockahoe, New York); 10 μg concanavalin A (Con A, Calbiochem, Los Angeles, California), 0·1 ml reconstituted pokeweed mitogen (PWM, Grand Island Biological Company, Grand Island, New York), 0·5 ml staphylococcal filtrate (SF) prepared as described by Ling, 1965, or 7·5 μg tuberculin (PPD, Parke-Davis & Company, Detroit, Michigan). Control cultures received no stimulants.

The tightly stoppered cultures were placed in an upright position in a 37°C incubator. Mitogen and antigen-stimulated cultures were harvested on the 5th and 7th day of incubation, respectively. Twenty-four hours prior to harvesting, each culture received 1 μCi $^3$H-thymidine (specific activity, 2·0 Ci/mmole). At harvest, the cells were transferred to glass centrifuge tubes and washed twice with freshly prepared cold 3% acetic acid by centrifugation at 450 *g* (1500 rev/min) for 8 min. After removing the final supernatant by aspiration, the cell pellet was bleached by adding approximately 0·03 ml of 30% hydrogen peroxide and heated for 15 min at 85°C. The cells were then dissolved in 0·5 ml of NCS Solublizer (Amersham/Searle Corporation, Des Plaines, Illinois) and 10 ml of scintillation fluid added. Samples were counted after cold and dark adaption for 10 min in a liquid scintillation counter (Mark 1, Nuclear-Chicago Corporation, Chicago, Illinois) and the counting efficiency was determined using the channels ratio method. The results were

expressed as disintegrations per minute (DPM). The blastogenic index was defined as the ratio of the result of the test culture to the corresponding control.

## RESULTS

The results of the mitogen-induced lymphocyte blastogenic response in thirty-five animals are presented in Fig. 1. PHA induced a significant response in all animals. The blastogenic indexes were 81, 40, 50 and 32 for rats, mice, guinea-pigs and rabbits, respectively. Of particular interest is the observation that Con A induced a significantly higher response

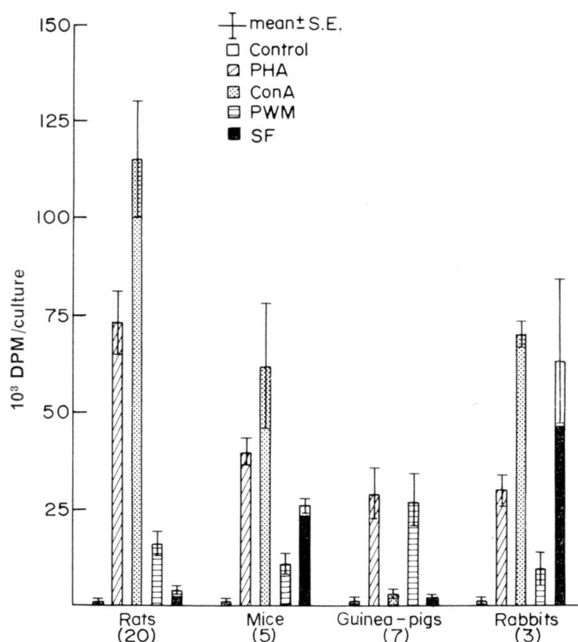


FIG. 1. Mitogen-induced blastogenic response in whole blood cultures established from various laboratory animals. Blood dilution was 1 : 40. All cultures harvested after 5 days of incubation.

than PHA in cultures from rats, mice and rabbits. In guinea-pig cultures, on the other hand, the response to Con A was consistently poor. In all instances, PWM proved to be significantly blastogenic; in the guinea-pig cultures, it induced a response closely approximating that of PHA. The lymphocyte responses to SF in rabbit and mouse cultures were unequivocal, whereas negligible or only slight responses were observed in rat and guinea-pig cultures. In all instances, control cultures showed a consistent and uniformly low response.

Fig. 2 presents the results of serial experiments employing a BCG-sensitized rabbit. Prior to sensitization, PPD did not induce an *in vitro* lymphocyte transformation response; however, a significant response was recorded for all of the mitogens. A maximal PPD-induced response was elicited 20 days after sensitization at which time it showed a greater than five-fold increase over the corresponding control. Significant, although transient,

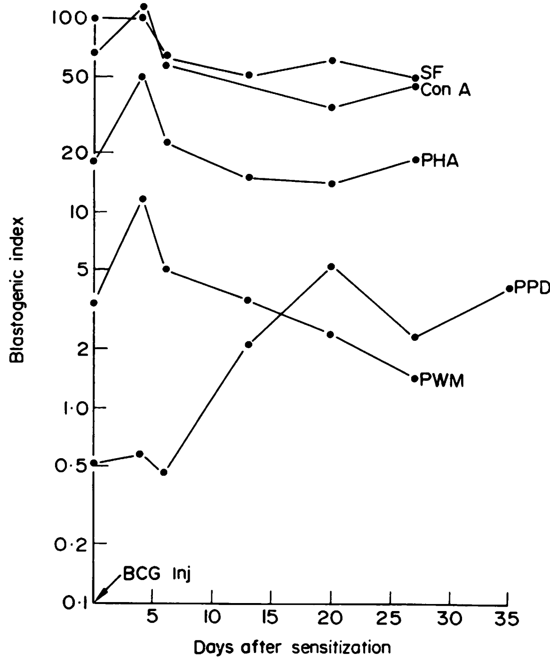


FIG. 2. Results of serial experiments demonstrating PPD- and mitogen-induced blastogenic responses of a BCG-sensitized rabbit. Blood dilution was 1 : 40. All cultures harvested after 7 days of incubation for PPD and after 5 days of incubation for mitogens.

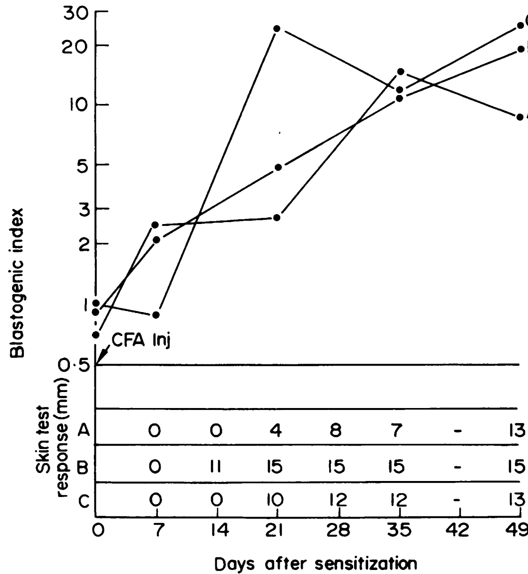


FIG. 3. Results of serial experiments demonstrating PPD-induced blastogenic and skin test responses of three CFA-sensitized guinea-pigs. Blood dilution was 1 : 30. All cultures harvested after 7 days of incubation.

increases in three of the mitogen-induced responses (PHA, PWM and SF) were recorded on the fourth day after sensitization.

A similarly conducted serial study employing CFA-sensitized guinea-pigs is shown in Fig. 3. Prior to sensitization there was neither an *in vivo* or *in vitro* response to PPD. As early as seven days after sensitization, two of the three animals showed a modest but significant *in vitro* response despite the fact that they exhibited negative skin tests. Twenty-one days after sensitization all animals showed positive skin test responses to PPD as well as significant *in vitro* responses. The range of peak responses was from 14 to 25 times the corresponding control cultures.

## DISCUSSION

The whole blood culture method is being employed routinely in our laboratory for evaluating human lymphocyte reactivity. The parameters used in this study such as cell density, harvest day and concentration of the various stimulants may not represent the optimal conditions for eliciting a maximal blastogenic response. The results of this preliminary study, however, clearly indicate that this method has numerous advantages over the conventional method. Perusal of the literature showed that the results presented here closely approximate and in some instances exceed those previously reported using the conventional techniques in which the cultures contain ten or more times as many lymphocytes than the whole blood cultures (Knight *et al.*, 1965; Festenstein, 1968; Oppenheim, Wolstencroft & Gell, 1967; Metcalf, 1965).

This method enables one to prepare a large number of cultures and to evaluate the lymphocyte response to a relatively large battery of stimulants. Our results indicate that there is no need to remove the erythrocytes, polymorphonuclear leucocytes, platelets or residual autologous plasma to obtain a significant mitogen or antigen-induced lymphocyte transformation response. By eliminating many of the steps previously preparing cells for culture, it eliminates many possible sources of technical error which give rise to considerable variability of the results. Due to the fact that there are numerous modifications of the conventional technique, it is virtually impossible to compare the results of studies conducted in different laboratories. In addition, it should seem reasonable to assume that an *in vitro* assay should be a true representation of the *in vivo* system under evaluation.

By using this whole blood culture technique we have demonstrated (1) that *E. coli* L-asparaginase completely inhibits the blastogenic response of mouse peripheral blood lymphocytes to PHA, Con A and PWM and (2) that mouse lymphoid cells responsive to PHA are thymus-dependent lymphocytes while cells responsive to PWM are bursa-equivalent-dependent lymphocytes (unpublished observation).

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## REFERENCES

Festenstein, H. (1968) Mouse peripheral-blood lymphocytes in culture. *Lancet*, **i**, 182.

Hughes, D. & Caspary, E.A. (1970) Lymphocyte transformation *in vitro* measured by tritiated thymidine uptake. I. Lymphocyte culture techniques. *Int. Arch. Allergy*, **37**, 506.

Junge, U., Hoekstra, J., Wolfe, L. & Deinhardt, F. (1970) Microtechnique for quantitative evaluation of *in vitro* lymphocyte transformation. *Clin. exp. Immunol.* **7**, 431.

Knight, S., Ling, N.R., Sell, S. & Oxnarc, C.E. (1965) The transformation *in vitro* of peripheral lymphocytes of some laboratory animals. *Immunology*, **9**, 965.

Ling, N.R. (1968) *Lymphocyte Stimulation.* North-Holland Publishing Company, Amsterdam.

Metcalf, W.K. (1965) Some experiments on the phytohemagglutinin cultures of leukocytes from rats and other mammals. *Exp. Cell Res.* **40**, 490.

Mumford, D.M., Barsales, P.B., Ball, K.D. & Gordon, H.L. (1971) Microlymphocyte transformation studies with seminal antigen. 1. Technique and patterns of responsiveness to autologous and allogeneic semen from normal and infertile male subjects. *J. Urology*, **105**, 858.

Oppenheim, J.J., Wolstencroft, R.A. & Gell, P.G.H. (1967) Delayed hypersensitivity in the guinea-pig to a protein-hapten conjugate and its relationship to *in vitro* transformation of lymph node, spleen, thymus and peripheral lymphocytes. *Immunology*, **12**, 89.

Pauly, J.L. & Sokal, J.E. (1971) A simplified technique for *in vitro* studies of lymphocyte reactivity. *Proc. Soc. exp. Biol.* (*N.Y.*), (In press).

Schellekens, P. Th. A. & Eijsvoogel, V.P. (1968) Lymphocyte transformation *in vitro*. 1. Tissue culture conditions and quantitative measurements. *Clin. exp. Immunol.* **3**, 571.