COMPARISON OF SEPARATED-LYMPHOCYTE AND WHOLE-BLOOD LYMPHOCYTE PROLIFERATION ASSAYS FOR EVALUATING RABIES-INDUCED CELLULAR IMMUNITY

S Kasempimolporn, N Narakorn, A Saikasem and V Sitprija

Queen Saovabha Memorial Institute (WHO Collaborating Center for Research in Rabies Pathogenesis), Thai Red Cross Society, Bangkok, Thailand

Abstract. A whole-blood lymphocyte proliferation assay was compared to a standard method requiring the isolation of peripheral blood mononuclear cells (PBMC). Both methods were used to measure the cell-mediated immune responses to rabies in rabies-vaccine recipients. Whole-blood cells gave moderately higher lymphoproliferative responses in terms of stimulation indices than did separated-PBMC. The results obtained from these two methods can be considered equivalent for the purpose of quantitating cellular reactivity to rabies. The use of whole blood has advantages over the standard isolated-PBMC method.

INTRODUCTION

Both humoral and cellular mechcanisms are important in successful clearance of rabies virus. This hypothesis is supported by several studies of T or B cell depletion and adoptive transfer of immune cells in animals infected with rabies (Miller *et al*, 1978; Mifune *et al*, 1981; Prabhakar *et al*, 1981). The humoral and cellular immune responses of an individual to rabies can be measured *in vitro*. This is valuable for the evaluation of vaccines or to monitor changes in the immune responses of patients over time and following treatment. Humoral immunity against rabies is routinely assayed in many diagnostic laboratories, however, there is as yet no assay of cellular immunity that has been standardized for routine diagnostic use. One reason for this is technical. This is unfortunate, since cell-mediated immunity may be as important as humoral immunity for protection against rabies. The cellular immune response can be measured by incubation of T cells in culture with rabies antigens (Kasempimolporn *et al*, 1995). The standard method of measuring T cell responses in this way is the lymphocyte proliferation assay (LPA) which uses cultures of peripheral blood mononuclear cells (PBMC) that are separated from a venous blood sample by differential centifugation on Ficoll-Hypaque. T cell responses to the stimulating antigen in terms of DNA synthesis are measured by the amount of radio-labeled nucleotide incorporation into the cells in cultures (Hughes and Caspary, 1970). This method requires a relatively large blood sample, which makes the test unsuitable for use in some patients such as children who represent 30% of dog-bite victims in Thailand (Wilde *et al*, 1991), who are an important group for the study of immune responses. In addition, small laboratory animals including mice, rats, guinea pigs and rabbits are difficult to study using the standard LPA. The blood separation procedure itself is time consuming and complex, making the test inappropriate for use in large series.

Recently, lymphocyte proliferation studies using whole (non-separated) blood rather than lymphocyte-enriched preparations for evaluating lymphocyte reactivity of humans and laboratory animals to various antigens have been reported (Han and Pauly, 1972; Pauly *et al*, 1973; Leroux *et al*, 1985; Frankenburg, 1988; Fiavey and Frankenburg, 1992; Weir *et al*, 1994). However, a whole-blood assay has not yet been tested for rabies. Such a rapid and simple test would be useful since rabies is largely a disease confined to developing countries where laboratory facilities are often limited.

The aim of this study was to determine whether this whole-blood LPA could be substituted for the

Correspondence: S Kasempimolporn, Queen Saovabha Memorial Institute (WHO Collaborating Center for Research in Rabies Pathogenesis), Thai Red Cross Society, Rama IV Road, Bangkok 10330 Thailand.

conventional LPA for study of lymphocyte reactivity to rabies. Responses to rabies antigens are compared between both techniques using blood from rabies-vaccine recipients.

MATERIALS AND METHODS

Blood specimens

Blood samples were obtained from rabies-vaccine recipients attending the animal bite clinic of the Queen Saovbha Memorial Institute. All patients had received purified Vero cell rabies vaccine (PVRV: Institute Merieux, France) 30 days previously. Nonvaccinated subjects were used as controls. Heparin was added to each blood samples at a concentration of 10 IU/ml.

Lymphocyte proliferation assay (LPA)

The standard LPA using separated PBMC was carried out as previously described (Kasempimolporn *et al*, 1995). For the whole-blood assay, each blood sample was diluted 1:10 in growth medium without additional serum and cultured for 5 days in round-bottomed microtiter plate in the presence and absence of rabies antigens. PVRV, Lot No. K 0404, potency 11.8 IU/ml at a dilution of 1:5 was used as the stimulating antigen in this study. The lymphocyte proliferative response was assessed by the uptake of tritiated thymidine (^{3}H-thymidine) by the rabies-stimulated lymphocytes. The lymphocyte reactivity was expressed as a stimulation index (SI: mean counts per minute with antigen/mean counts per minute without antigen). SI higher than 2 was considered as evidence of proliferation. The response to phytohemagglutinin (PHA-L, Seromed) at a concentration of 2.4 µg/ml was used as a positive control for the assay. It was found to be positive in every case.

Statistical analysis

Student's *t*-test was used to calculate the significance of the difference between the two tests.

RESULTS AND DISCUSSION

Comparisons were made between the whole blood and the conventional separated-lymphocyte assays in 46 rabies vaccine recipients and 25 non-vaccinated controls. Results are summarized in Fig 1. Rabies antigens induced no response to samples from the control group in both tests. Rabies antigens induced different degrees of

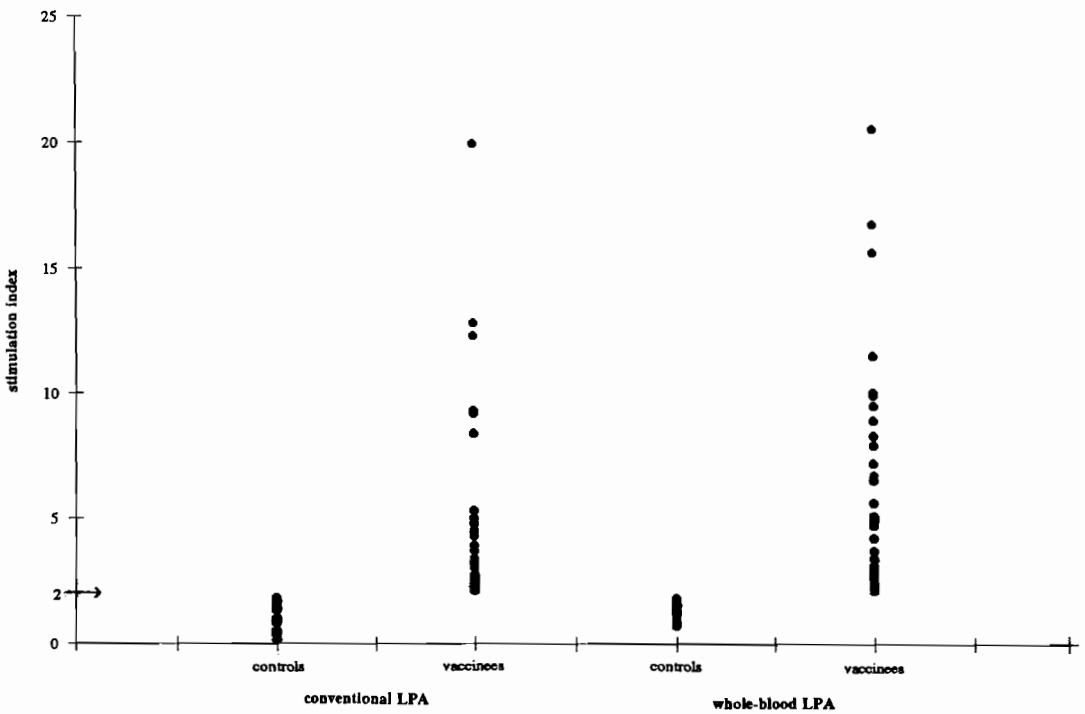

Fig 1–Comparison of rabies-induced lymphocyte stimulation by conventional and whole-blood methods.

lymphoproliferative responses in rabies vaccinees as measured in terms of SI (SI>2). Both groups of vaccinees demonstrated a positive response but results were not identical on a scattergram. Lymphocytes of individuals responsed vigorously to PHA-L in both techniques (data not shown).

The results obtained by the whole-blood method were found to be significantly higher ($p<0.001$) than by the conventional assay. This could be due to the fact that responding T-cells in whole blood are maintained in an environment similar to that found *in vivo*. By eliminating many of the steps involved in preparing cells for culture, we eliminate many possible sources of technical error which may lead to non-specific activation/suppression of the cells and selective depletion of leukocyte subpopulations. The use of non-separated blood may thus retain mononuclear cells, especially monocytes in their natural proportions, allowing maximum enhancement of the immune response through cell cooperation.

Lymphocyte proliferation *in vitro* for assessing cell-mediated immunity is usually performed by ^{3}H-thymidine incorporation of antigen-stimulated lymphocytes. However, the conventional method has long suffered from technical difficulties. Because of the need to separate PBMC, the technique has been too cumbersome for routine use. The cellular immune response to rabies antigen is thought to play a significant defensive role but has not yet been completely investigated. Only a few human vaccine immunogenicity trials have studied the cellular immune response in rabies (Ratanawongsiri *et al*, 1985; Phanuphak *et al*, 1987). One reason for this is technical. There are many attempts to simplify the usual technique. We have previously developed a colorimetric method as a proliferation assay for determining the cell-mediated immune response in rabies (Kasempimolporn *et al*, 1995). It showed no significant advantage over the conventional approach in terms of time saving. Technical aspects of measuring lymphocyte proliferation were recently simplified by adapting a well-established separated lymphocyte method to use with whole blood. Previous groups have developed whole-blood assays for measuring responses to mitogens and specific antigens for investigating a variety of infectious diseases in which T cell-mediated immunity plays an important role (Leroux *et al*, 1985; Frankenburg, 1988; Fiavey and Frankenburg, 1992; Weir *et al*, 1994) and also for assessing and following the changes in immune function which occur in asymtomatic HIV-infected subjects (Bocchieri *et al*, 1995).

In this study, the results obtained from these two different LPA can be considered equivalent for the purpose of quantitating cellular reactivity to rabies. However, the use of whole blood is more productive and has advantages over the usual isolated-PBMC procedure. The whole-blood LPA is less cumbersome, less time consuming, less blood is used and it is cheaper to carry out than the conventional isolated-PBMC method. It would be applicable in a clinical laboratory as well as in research setting. In addition, this technique has proved particularly useful in serial animal experiments for which only limited volumes of blood are available (Han and Pauly, 1972; Fasanmade and Jusko, 1995).

ACKNOWLEDGEMENTS

We are indebted to Professor Henry Wilde for his critical review of the manuscript.

REFERENCES

Bocchieri MH, Talle MA, Maltese LM, Ragucci IR. Whole blood culture for measuring mitogen induced T cell proliferation provides superior correlations with disease state and T cell phenotype in asymtomatic HIV-infected subjects. *J Immunol Methods* 1995; 181: 233-43.

Fasanmade AA, Jusko WJ. Optimizing whole blood lymphocyte proliferation in the rat. *J Immunol Methods* 1995; 184: 163-7.

Fiavey NP, Frankenburg S. Appraisal of the total blood lymphocyte proliferation assay as a diagnostic tool in screening for tuberculosis. *J Med Microbiol* 1992; 37: 283-5.

Frankenburg S. A simplified microtechnique for measuring human lymphocyte proliferation after stimulation with mitogen and specific antigen. *J Immunol Methods* 1988; 112: 177-82.

Han T, Pauly J. Simplified whole blood method for evaluating *in vitro* lymphocyte reactivity of laboratory animals. *Clin Exp Immunol* 1972; 11: 137-42

Hughes D, Caspary EA. Lymphocyte transformation *in vitro* measured by tritiated thymidine uptake I. Lymphocyte culture techniques. *Inst Arch Allergy* 1970; 7: 506.

Kasempimolporn S, Chomchey P, Sitprija V. Radioac-

tive and nonradioactive lymphocyte proliferation assays for measuring rabies specific cellular immunity. *Asian Pac J Allergy Immunol* 1995; 13: 43-5.

Leroux M, Schindler L, Braun R, Doerr HW, Geisen HP, Kirchner H. A whole blood lymphoproliferation assay for measuring cellular immunity against herpes viruses. *J Immunol Methods* 1985; 79: 251-62.

Mifune K, Takeuchi E, Napiorkowaki PA, Yamada A, Sakamoto K. Essential role of T cells in the post exposure prophylaxis of rabies in mice. *Microbiol Immunol* 1981; 25: 895-904.

Miller A, Morse HC, Winkelstein J, Nathanson N. The role of antibody in recovery from experimental rabies. *J Immunol* 1978; 121: 321-6.

Pauly JL, Sokol JE, Han T. Whole-blood culture technique for functional studies of lymphocyte reactivity to mitogens, antigens and homologous lymphocytes. *J Lab Clin Med* 1973; 82: 500-12.

Phanuphak P, Khawplod P, Sirivichayakul S, Siriprasomsub W, Ubol S, Thaweepathomwat M. Humoral and cell-mediated immune responses to various economical regimens of purified Vero cell rabies vaccine. *Asian Pac J Allergy Immunol* 1987; 5: 33-7.

Prabhakar BS, Fishchman HR, Nathanson N. Recovery from experimental rabies by adoptive transfer of immune cells. *J Gen Virol* 1981; 56: 25.

Ratanavongsiri J, Sriwanthana B, Ubol S, Phanuphak P. Cell-mediated immune response following intracutaneous immunisation with human diploid cell rabies vaccine. *Asian P J Allergy Immunol* 1985; 3: 187-90.

Weir RE, Morgan AR, Britton WJ, Butlin CR, Dockrell HM. Development of a whole blood assay to measure T cell responses to leprosy: a new tool for immuno-epidemiological field studies of leprosy immunity. *J Immunol Methods*1994; 176: 93-101.

Wilde H, Chutivongs S, Tepsumethanon W, Choomkasien P, Polsuwan C, Lumlertdacha B. Rabies in Thailand. *Rev Infect Dis* 1991; 13: 644-52.