Asian Pacific Journal of Allergy and Immunology (1995) 13 : 43 - 45

# Radioactive and Non-Radioactive Lymphocyte Proliferation Assays for Measuring Rabies-Specific Cellular Immunity

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**SUMMARY** : **We compared the radioactive lymphocyte proliferation assay for measuring rabies specific cell-mediated immunity in a group of 42 rabies vaccine recipients with a new technique using rabies-stimulated lymphocytes in a colorimetric test kit (CellTiter 96™, Promega Corporation, USA). Results of both tests were good agreement (Kappa = 0.68), however, they did not show good correlation in degree (magnitude) of positivity. In addition, the conventional assay showed a higher degree of sensitivity.**

The immunogenicity and protective value of rabies vaccines is measured by determining the neutralizing antibody response in the vaccinees. The WHO Expert Committee on Rabies[1] has arbitrarily determined that an antibody response of 0.5 IU/ml is the minimum acceptable level on days 14, 30 and 90 after the first vaccine injection. The cellular immune response to rabies antigen, however, is also thought to play a significant role in protection as well as in pathogenesis of rabies but this has not been fully investigated. Only a few vaccine immunogenicity trials have also studied the cellular immune response.[2,3] Virtually no studies of cell-mediated immunity (CMI) against rabies in dogs and cats have been reported. It was, however, thought that CMI might play an important role in animal rabies. Presently, cellular immune response assays require a large quantity of blood and prompt treatment is needed. In addition, these tests are time consuming and involve expensive apparatus. Various tests are currently used to evaluate the status of CMI. These are *in vitro* tests such as radioactive lymphocyte proliferation,[3,4] a conventional technique measuring incorporation of radioactive nucleotide ([$^3$H]-thymidine) during cell proliferation, and cytotoxic T-lymphocyte assays.[5] However, these methods have a drawback in that they require radioisotopes. A colorimetric method for determining the number of viable cells as a proliferation assay has been recently developed and is being marketed. This assay is based on the conversion of the tetrazolium salt (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide) by the cellular mitochondrial enzyme succinate-dehydrogenase into a blue colored formazan which can be measured spectrophotometrically.[6-8] The aim of the present study is to determine whether this colorimetric assay could be substituted for the conventional [$^3$H]-thymidine uptake method for measuring rabies-specific CMI. The methodology and results of both techniques in the lymphocyte proliferation assay were compared in a group of 42 rabies vaccine recipients.

## MATERIALS AND METHODS

### Blood specimens

Blood specimens were obtained from 42 rabies vaccine recipients who attended the Rabies Clinic of the Queen Saovabha Memorial Institute as well as from 15 non-vaccinated control subjects. All of these vaccinated patients had purified Vero cell rabies vaccine (PVRV: Institute Merieux,

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Lyon, France) 3 to 90 days previously.

**Radioactive lymphocyte proliferation assay**

Ten millilitres of heparinised blood (10 units of heparin per 1 ml of blood) was obtained aseptically from the subjects following rabies vaccination. The peripheral blood mononuclear cells were separated on Ficoll-Hypaque gradients, and $2 \times 10^5$ mononuclear cells per well in flat-bottomed microtiter plates were cultured both in the presence and in the absence of the rabies antigen according to the method previously described.[3] One hundred microlitres of PVRV, Lot No. J 0509, potency 10.57 IU/ml, at dilution of either 1:5 or 1:10 was used as the stimulating antigen in this study. The cultures were obtained in triplicates and incubated for 5 days. The lymphocyte proliferation responses were assessed by measuring the uptake of [$^3$H]-thymidine by the rabies-stimulated lymphocyte during the last 16 hours. 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.

**Non-radioactive cell proliferation assay**

The colorimetric test kit (CellTiter 96$^{TM}$) was purchased from Promega Corporation, USA. The procedure was carried out according to the manufacturer's recommendation. The CellTiter 96$^{TM}$ assay was performed by the addition of an optimized premixed dye solution to the triplicate 5 day-incubated mononuclear cell culture wells of a 96 well plate containing either a 1: 5 or 1: 10 dilution of PVRV or culture medium as a control. During a 4 hour incubation, living cells converted the tetrazolium component of the dye solution into a formazan product. The solubilization/stop solution was then added to the culture wells to solubilize the formazan product and the absorb-

**Table 1.** Comparison of rabies-specific proliferative responses measured by the conventional radiothymidine incorporation and the colorimetric CellTiter 96™ assays

| Vaccinee no. | Result of tests | | Vaccinee no. | Result of tests | |
|---|---|---|---|---|---|
| | SI[a] | O.D.[b] | | SI[a] | O.D.[b] |
| 1 | < 2 | 0.101 | 22 | < 2 | 0.133 |
| 2 | < 2 | 0.194 | 23 | < 2 | 0.150 |
| 3 | < 2 | 0.159 | 24 | 4.5* | 0.335** |
| 4 | 5.2* | 0.112 | 25 | < 2 | 0.158 |
| 5 | 21.3* | 0.749** | 26 | 5.0* | 0.164 |
| 6 | < 2 | 0.117 | 27 | 3.7* | 0.192 |
| 7 | 2.8* | 0.112 | 28 | 2.2* | 0.298** |
| 8 | 4.2* | 0.275** | 29 | < 2 | 0.237 |
| 9 | 15.7* | 0.258** | 30 | 2.1* | 0.437** |
| 10 | 3.3* | 0.773** | 31 | 2.4* | 0.336** |
| 11 | 6.4* | 0.294** | 32 | 2.1* | 0.281** |
| 12 | 3.6* | 0.426** | 33 | 2.1* | 0.178 |
| 13 | 4.9* | 0.198 | 34 | < 2 | 0.174 |
| 14 | 4.1* | 0.341** | 35 | < 2 | 0.242 |
| 15 | 3.0* | 0.460** | 36 | < 2 | 0.230 |
| 16 | 3.0* | 0.377** | 37 | < 2 | 0.243 |
| 17 | < 2 | 0.133 | 38 | < 2 | 0.236 |
| 18 | < 2 | 0.199 | 39 | 2.0* | 0.247** |
| 19 | 2.6* | 0.131 | 40 | 5.9* | 0.469** |
| 20 | < 2 | 0.143 | 41 | 3.2* | 0.312** |
| 21 | < 2 | 0.175 | 42 | < 2 | 0.166 |

[a] The lymphocyte reactivity was expressed as a stimulation index (SI),measured by the [$^3$H] thymidine incorporation assay.

[b] Expressed as an actual O.D. from the CellTiter 96™ assay.

* A stimulation index higher than 2 was considered as evidence of proliferation.

** Considered positive, if the O.D.levels were above 0.245.

**Table 2.** Comparison of the results of commercial colorimetric test with radiothymidine incorporation assays

| CellTiter 96™ | [$^3$H] thymidine incorporation | | |
|---|---|---|---|
| | + | - | Total |
| + | 17 | 0 | 17 |
| - | 7 | 18 | 25 |
| Total | 24 | 18 | 42 |

| | | |
|---|---|---|
| Kappa analysis | = | 0.68 |
| Sensitivity | = | 70.8% |
| Specificity | = | 100% |
| Positive predictive value | = | 100% |
| Negative predictive value | = | 72.0% |

ance at 570 nm was recorded using an ELISA reader and a cut-off value was established. The cut-off value was calculated by reference to the mean + 2 SD of the non-vaccinated control samples. Any specimen having an absorbance value equal to or greater than the cut-off value was considered positive. In the study this was determined to be 0.245.

**Analysis of results**

The results of both tests were analyzed. The conventional radiothymidine uptake assay and the commercial colorimetric test were compared based on sensitivity, specificity and kappa coefficients.[9,10]

## RESULTS AND DISCUSSION

Table 1 shows the results obtained by the two methods for measuring CMI in 42 rabies vaccinees. Of the 24 specimens that tested positive for [$^3$H]-thymidine uptake, 17 (70.8%) were CellTiter 96$^{TM}$ positive and 7 (29.2%) turned out to be negative. But with the 18 [$^3$H]-thymidine negative, 18 (100%) were also CellTiter 96$^{TM}$ negative and no sample that tested positive for CellTiter 96$^{TM}$ was [$^3$H]-thymidine negative. None of the control subjects showed response to rabies antigen neither in the conventional nor in the colorimetric tests (data not shown). By kappa analysis, there was good agreement between these two tests (kappa = 0.68). However, they did not show good correlation in degree (magnitude) of positivity (Table 1). The sensitivity, specificity and positive and negative predictive values of the two tests were 70.8, 100, 100 and 72.0 percent, respectively (Table 2). The discordant results might be due to a different degree of precision and/or diversity among the two test systems. The colorimetric assay measures the number and activity of liviing cells at the end of the procedure, whereas [$^3$H]-thymidine incorporation measures the number of cells synthesizing DNA during the last few hours of the assay. In addition, the lesser sensitivity of results in the colorimetric assay depends not only on the metabolic activity of the tested cells but also on the variation background due to the turbidity of the stimulating antigen which is added. As a form of lymphocyte proliferation test, the non-radioactive assay (CellTiter 96$^{TM}$) has shown no significant advantage over the radioactive approach, ie time consuming, reagent hazard risk ([$^3$H] as radioactivity risk, N,N,-dimethylformamide containing in solubilization solution is possibly carcinogen). In conclusion, if a laboratory has good facilities and budgets, use of the colorimetric test instead of the conventional radiothymidine incorporation assay is not recommended. However, for laboratories which are not equipped for performing the conventional method, the colorimetric technique may be a useful tool for the specific measurement of rabies-specific CMI.

## ACKNOWLEDGEMENTS

We thank Professor Henry Wilde for reviewing the munuscript and Professor Narongsak Chaiyabutr for helpful suggestion.

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