CHAPTER 5

CELL PROLIFERATION STUDIES
Cell Proliferation Studies

One of the most spectacular events in cell biology is the interaction of the mitogenic phytolectins with the human lymphocytes. The discovery of mitogenic PHA from Phaseolus vulgaris by Peter Nowell in 1960 laid the foundation of the modern human cytogenetics and gene mapping.

Many lectins differ markedly in their ability to stimulate lymphocytes of different species (Taussing et al, 1984; Roy and Das, 1990; Mannori et al, 1991; Ganguly and Das, 1994). Maximum stimulation usually occurs at a narrow lectin concentration interval. Generally, the maximum response occurs at a higher dose corresponding to the binding of only a few of the cell’s surface lectin receptors (5-20%) whereas 100% receptor occupancy give no or much lower stimulation (Ling and Kay, 1975). Some lectins are mitogenic only after various modifications of either the cell or the lectin (Sharon and Lis, 1975). Lectins such as Con. A, PHA and lentil lectin selectively activate T-lymphocytes (Janassy and Greaves, 1972), whereas the lectins from crab, Homarus americanus, chicken tissue and from the slime mold Dictyostelium purpureum stimulate B-cells only (Lipsick, 1980; Campbell et al, 1982). On the other hand PWM stimulates both type of cells (Janassy and Greaves, 1972). WGA was previously regarded as a non-mitogenic lectin, later Miller (1983) has shown that under suitable conditions it stimulates markedly both human B- and T-lymphocytes. In recent years, a surprisingly large number of other plant lectins have been shown to be mitogenic (Sandhu et al, 1985; Barralnetts et al, 1992;
Hashim et al., 1992; Oda et al., 1992; Stojanovic et al., 1994). During the last decade, the number of mitogenic lectins has markedly increased.

Most mitogenic plant lectins stimulate only the thymus-dependent (T-cells) set of lymphocytes and are inactive or inhibitory for mitosis of the other class of lymphocytes, the thymus-independent cells (B cells). An exception is the pokeweed mitogen, which stimulates both T and B cells (Van Damme et al., 1997).

Mitogenic stimulation is routinely determined by measuring the increase in the rate of incorporation of labelled thymidine into DNA after 48 to 72 h incubation of lymphocytes with varying concentrations of the mitogen tested. This method gives an average value for the whole cell population but does not provide information about the number of cells stimulated that obtained by the observation under microscope or by autoradiography of cells pulsed with radioactive thymidine.

Licastro et al. (1982) have reported the activity of lectins, which do not affect the $^3$H-TdR incorporation of human T-lymphocytes, although used a wide range of lectin concentrations, i.e. from 0.01-100 μg/ml. At the same time some lectins showed mitogenic effect on human T-lymphocytes when $^3$H-TdR used even at small concentrations of lectins.

The mitogenic power of many lectins such as Crotalaria juncea, Hura crepitans, Vicia sativa. Ononis spinosa, Phaseolus coccineus etc. is quite
strong. The mitogenic activity of other lectins such as *Glycine max* and *Datura stramonium* is weak, while other lectins such as *Erythrina indica*, *Phaseolus lunatus* etc. have an intermediate mitogenic power (Table 5.1).

**Table 5.1** Lectins with mitogenic effect on human T-lymphocytes

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Lectin concentrations (µg/ml)</th>
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<tr>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td><em>Crotalaria juncea</em></td>
<td>0.55</td>
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<tr>
<td><em>Datura stramonium</em></td>
<td>0.77</td>
</tr>
<tr>
<td><em>Erythrina indica</em></td>
<td>0.78</td>
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<tr>
<td><em>Glycine max</em></td>
<td>0.85</td>
</tr>
<tr>
<td><em>Hura crepitans</em></td>
<td>2.84</td>
</tr>
<tr>
<td><em>Ononis spinosa</em></td>
<td>1.26</td>
</tr>
<tr>
<td><em>Phaseolus coccineus</em></td>
<td>1.58</td>
</tr>
<tr>
<td><em>Phaseolus lunatus</em></td>
<td>0.56</td>
</tr>
<tr>
<td><em>Robinia pseudoacacia</em></td>
<td>2.97</td>
</tr>
<tr>
<td><em>Rutilus rutilus</em></td>
<td>1.59</td>
</tr>
<tr>
<td><em>Vicia sativa</em></td>
<td>0.69</td>
</tr>
</tbody>
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Alteration of lymphocyte surfaces by glycosidases affects the response of cells to mitogenic lectin. Neuraminidase treatment of cells enhances the mitogenic activity of lectin to certain extent. And the chemical modification of lectins also changes their mitogenic properties (Naisbett and Woodley, 1995).
There is good reason to believe that many more lectins will prove to be mitogenic if tested in a suitable system.

The results of the study of the mitogenic activity of lectin from Butea monosperma is reported below.

**Materials and Methods**

**Human Lymphocytes**

10 ml of venous blood was collected from healthy volunteers and heparinised. The peripheral lymphocyte-rich blood was separated by gradual settlement of RBCs or by density gradient centrifugation.

**Medium**

Different media have been developed for specific cell lines in order to obtain optimal growth. Mc Coy’s medium 5A has been used as a standard medium for cloning cells. It is based on BME aminoacids and vitamins from medium 199. This was modified further to form RPMI medium (Roswell Park Memorial Institute Medium). RPMI medium was supplied by Hi-Media.

**Preparation of Giemsa Stain**

*Stock solution*

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<table>
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<tbody>
<tr>
<td>Azure II</td>
<td>-</td>
<td>1.09 g</td>
</tr>
<tr>
<td>Azure II - Eosin</td>
<td>-</td>
<td>2.09 g</td>
</tr>
<tr>
<td>Azure B - Eosin</td>
<td>-</td>
<td>1.09 g</td>
</tr>
<tr>
<td>Azure A - Eosin</td>
<td>-</td>
<td>0.59 g</td>
</tr>
</tbody>
</table>
Preparation

1. Mix 250 ml of glycerine with 250 ml of methyl alcohol.
2. Dissolve all dyes in this solution.
3. Let stand at room temperature overnight.
4. Shake mixture well for 5-10 minutes.
5. Pour without filtering, into a dark screw cap bottle and store at room temperature.

Preparation of Giemsa working solution

A. Giemsa stock solution.
B. 0.067 M sodium, potassium phosphate buffer, pH 7.2.
   Mix 10 ml of solution A and 90 ml of solution B.

Preparation of Tissue Culture Medium (for 500 ml)

Protocol

1. Add RPMI medium with Henk's base (powder form) in 400 ml of double-distilled water in a cleaned flask and mix it gently.
2. Add streptomycin 100 mg/ml and penicillin at the rate of 100 units/ml.
3. Add about 2.6 ml of 3.5% sodium bicarbonate in the medium and adjust the pH between 7.2 to 7.4. Then make up the media to 425 ml with double-distilled water.
4. Filter the medium solution by using a Millipore filter (0.2 μ).
5. Add 75 ml of sterile foetal calf serum, i.e. 15% of the total volume of the medium.

6. Disperse the medium in 100 ml of sterile bottles and tightly close and store in the refrigerator.

**Fixative Reagents**

a) Methyl alcohol  
b) Glacial acetic acid  

Mixed the methanol and pre-cooled acetic acid in the ratio 3:1 respectively.

**Cell Proliferation Assay**

0.3 ml of lymphocyte suspension (1 x 10^6 cell/ml) was distributed in sterile screw capped culture bottles, containing 5 ml of culture medium supplemented with 15% foetal calf serum and BML. The lectin was added at the initiation of the culture period in appropriate amounts to reach final concentrations of 0.01, 0.1, 1, 10 and 100 µg/ml. Then the culture bottles were incubated in a humidified atmosphere of 5% CO₂ and 95% air for 72 h. The bottles were shaken gently to dissolve blood clot formed at intervals.

To the above suspension added 0.1 ml of colchicine (10 µg/ml) to arrest mitosis after 70 h of incubation and then incubated 2-3 h at 37°C. Then the whole culture was centrifuged at 1000 g for 10 minutes and collected the sediments and to the sediment, added 5 ml of 0.075 M pre-warmed KCl
solution and then incubated for 15-20 min. After incubation, the suspension was centrifuged at 1000 g for 10 minutes and supernatant was removed. Then to the sediment, added chilled fixative solution drop by drop with gentle shaking. After adding 1 ml fixative, the sediment is mixed so as to form an emulsion. It is then made up to 5-6 ml by adding more fixative. Then the mixture is kept in a refrigerator for 30 minutes to 12 h for fixing the cells.

The suspension was centrifuged at 1000 g for 10 minutes and the precipitate is collected. The washing is repeated several times with fixative until the sediment becomes colourless. Finally the sediment, with a small amount of fixative is smeared on a glass slide, stained with Giemsa and observed under a microscope.

Data Evaluation

The results obtained in cell cultures were expressed as stimulation index (SI). SI was calculated as the ratio between cpm of stimulated and unstimulated cultures.

\[
SI = \frac{cpm \ of \ cultures \ stimulated \ with \ lectin}{cpm \ of \ cultures \ without \ lectin}
\]

Results

The results obtained in the study showed no difference between cpm of cultures stimulated with lectin and cpm of cultures without lectin. Hence by this study, the lectin from Butea monosperma was found to be non-mitogenic.

Although extensively studied, the mechanism of mitogenic stimulation is not known. It is generally accepted that the initial step or 'first signal' is
binding of the lectin to cell surface sugars. Binding may lead to modification of membrane structure and function, resulting in the generation of a trigger or ‘second signal’. The transmission of which to the interior of the same cell initiates a series of biochemical events culminating in cell growth and proliferation (Dillner-Cenerlind et al, 1980; Skoog et al, 1980; Kimura et al, 1981; Toyoshima et al, 1982; Chilson et al, 1984; Kilpatrick, 1988).

Since the turn of the 20th century, it has been known that living organisms contain certain proteins or glycoproteins, which have various biological activities, such as cell agglutination, mitogenic activity and sometimes toxic activity for intact cells and/or cell-free systems. Lectins come under that category owing to their major biochemical properties, i.e., and their binding specificities for carbohydrates and blood groups. They are found predominantly in the seeds of plants particularly in legumes. They are also present in vertebrates, invertebrates and microorganisms. The use of lectin in biological and biochemical research is steadily increasing. In our country, even though a large number of plants have been identified, only a few attempts have been made to screen out and isolate lectins.

Since the lectin from Butea monosperma (BML) showed specificity to N-acetyl-D-galactosamine, it comes under the family N-acetyl-D-galacosamine/ D-galactose lectins. It was purified by the methods of affinity chromatography and gel permeation chromatography.