Characterization of the B Lymphocyte Response to Pokeweed Mitogen

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ABSTRACT

Human B lymphocytes activated by pokeweed mitogen (PWM) undergo a proliferative response, differentiate into immunoglobulin producing cells (IPC), and release immunoglobulin into the supernatant fluid of the lymphocyte cultures. Since the number of IPC and amount of immunoglobulin produced may be determined in part by the proportions of T and non-T lymphocytes in the culture, studies were undertaken on the kinetics of the PWM induced response in cultures containing 1:1 proportions of T and non-T cells from nine healthy adults. Blood samples were obtained from each individual on two occasions and the results compared. The proliferative response, number of cells containing intracytoplasmic immunoglobulin (IClG) and the concentration of supernatant immunoglobulin (SIg) were serially determined. The mean peak proliferative response and the mean peak number of IClG positive cells were observed on the fifth day of culture. The mean peak concentration of SIg was noted on the ninth to eleventh day of culture. The results of the initial specimens were not significantly different from those of the repeat samples. It is concluded that a 1:1 mixture of T and non-T cells respond reproducibly to PWM and that the level of response varies with the length of incubation of the cultures. It is believed these assays might be helpful in the investigation of various lymphoproliferative and immunodeficiency disorders.

Introduction

Human B lymphocytes, when appropriately activated by antigen, differentiate in vivo into immunoglobulin producing cells (IPC). Some antigens require T cell help while other antigens appear to activate B lymphocytes directly.

There are several in vitro models for this reaction, one of the most common being the pokeweed mitogen (PWM) stimulation of lymphocytes. In this system T helper lymphocytes, monocytes and PWM are needed to transform resting B lymphocytes into IPC. Several assays have been developed to measure lymphocyte...
transformation in the PWM system. These include measurement of the proliferative response of B lymphocytes by the determination of tritiated thymidine incorporation, enumeration of B cells containing intracytoplasmic immunoglobulin (ICIg), enumeration of plaque forming cells and quantitation of immunoglobulins in culture supernatants.\(^2,5,8,17\)

Pokeweed mitogen stimulation of lymphocytes involves complex cellular and humoral interactions. Consequently, assay systems measuring this stimulation have been subject to wide variation. Most previous studies have been of unfractionated peripheral blood mononuclear cells and have ignored the proportions of B and T lymphocytes present in culture. The purpose of this study is to describe the kinetics and reproducibility of a PWM driven culture system employing defined proportions of B and T lymphocytes. The proliferative response, production of intracytoplasmic immunoglobulin and concentration of immunoglobulin in culture supernatants were determined.

Materials and Methods

**SOURCE OF MONONUCLEAR CELLS**

Sixty to 100 ml of heparinized venous blood were obtained from nine normal volunteers. The blood was collected on two separate occasions with an average interval of four months between samples. Volunteers ranged in age from 25 to 58 years with a mean age of 34 years. All volunteers were in good health at the time of the study.

**CELL FRACTIONATION**

Heparinized blood was diluted with an equal volume of Hanks balanced salt solution (HBSS) and the mononuclear cell (MC) fraction separated by centrifugation over Lymphocyte Separation Medium (LSM)* at 20 to 22°C for 30 min at 400 \(\times\) g. Following three washes with HBSS, isolated MC were further fractionated into sheep red blood cell rosette (E-rosette) forming and non rosette forming (Non-T cell) populations according to a modification of the procedure of Madsen et al.\(^{10}\) Briefly, sheep red blood cells (SRBC) were treated with 0.14 M 2-amino-ethylisothiouronium bromide† (AET, pH 9.0) washed, and diluted to a one percent suspension in HBSS with 40 percent fetal bovine serum (AET-SRBC). Twenty million MC in 15 ml HBSS were combined with 15 ml of AET-SRBC and the mixture centrifuged at 20 to 22°C for five min at 200 \(\times\) g. The cell pellet was gently resuspended and centrifuged on LSM as previously described. Non-T cells were harvested from the interface, washed twice with HBSS and resuspended in lymphocyte culture medium (LCM:RPMI 1640 containing penicillin, streptomycin and L-glutamine,† with 10 percent fetal bovine serum and 0.03 M Hepes buffer).

The E-rosette forming cells were harvested by lysis of AET-SRBC with three to five ml of 0.05 M Tris—0.14 M ammonium chloride (pH 6.2) at 37°C for three to five min. Following two washes with HBSS, the E-rosette fractions were resuspended in one to two ml of HBSS containing 10 percent fetal bovine serum (HBSS-FBS). The E-rosette cell suspensions were further enriched by filtration on scrubbed nylon wool columns.† The cell suspensions on the columns were equilibrated for 30 min at 37°C prior to elution of nonadherent cells (T-cell enriched) with 20 ml of HBSS-FBS. T-cell enriched fractions were incubated with Mitomycin-C§ at a final concentration of 12.5 \(\mu\)g per ml in LCM for 30 min at 37°C in 95 percent air, five percent CO\(_2\), and then washed three times with LCM.

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**Pokeweed Mitogen Proliferative Assay**

For determination of $^3$H-thymidine incorporation, triplicate cultures of $1 \times 10^5$ non-T cells plus $1 \times 10^5$ T cells in 100 µL LCM with and without pokeweed mitogen (PWM; 5 µg per ml)$^1$ were plated in 96 well flat bottom tissue culture plates$^1$ and incubated at 37°C in an atmosphere of 95 percent air, 5 percent CO₂. Cells were pulse labeled with one µCi $^3$H-thymidine$^{**}$ (5 Ci per mmol) on days 2, 4, 6, 8, and 10 of culture. They were harvested onto glass fiber filter discs with a Multiple Automatic Sampling Harvester (MASH)$^{† †}$ on days 3, 5, 7, 9, and 11 of culture. The filter discs were dried, suspended in 6 ml of Betalfluor$^{† †}$ and counted in a beta-scintillation counter. Values were expressed as net counts per minute (mean cpm with PWM - mean cpm without PWM).

**Pokeweed Mitogen Immunoglobulin Synthesis Assays**

For the determination of IC1g containing cells and in vitro production of immunoglobulin, $5 \times 10^5$ non-T cells were combined with $5 \times 10^5$ T cells in 1.1 ml LCM with and without PWM (5 µg per ml) in 16 x 125 mm round bottom plastic screw cap tubes.$^{§ §}$ Cultures were incubated for three to 11 days at 37°C in an atmosphere of 95 percent air, five percent CO₂. At termination after 3, 5, 7, 9, and 11 days of incubation cultures were centrifuged for 10 min at 400 x g and 0.8 ml of supernatant was carefully aspirated for determination of supernatant immunoglobulin levels (SIg). Supernatants were stored at -70°C until assayed. Cell buttons were resuspended in the remaining medium and cell count and viability (by trypan blue exclusion) performed. Cytocentrifuge slides for determination of IC1g were prepared.

** Intracytoplasmic Immunoglobulin Containing Cells**

Percent IC1g containing cells were determined by direct immunochemistry using fluorescein conjugated heavy chain specific rabbit antisera to human IgG, IgM, and IgA.$^*$ Cytocentrifuge slides of lymphocyte culture cell buttons were fixed for 10 min in cold acetone, air dried and stained with one to two drops of antisera diluted 1/20 in phosphate buffered saline (PBS) pH 7.2 for 45 min at 37°C in a humidified chamber. Stained slides were rinsed twice in PBS buffer for 10 min and once for 10 min in distilled water. Slides were air dried and coverslips applied with PBS mounting fluid.† Slides were examined at 400 x magnification on a Lietz fluorescence microscope. A minimum of 200 cells was counted and results expressed as net number fluorescent cells (percent of fluorescent cells with PWM x viable cell count minus percent fluorescent cells without PWM x viable cell count).

**ELISA Assay for Supernatant Immunoglobulin (SIg)**

Supernatant IgG, IgM and IgA levels were determined by double antibody sandwich enzyme labeled immunosorbant assay (ELISA).$^{13}$ Optimal dilutions of horseradish peroxidase conjugated and un-conjugated heavy chain specific rabbit antisera to human IgG, IgM and IgA$^*$

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were determined by checkerboard titration. Standard curves ranging from 10 to 90 ng per ml were constructed from fractions of human IgG, IgM and IgA.‡ Gilford PR 50 microcuvets§ were coated for five to nine days at 4°C with 200 μl aliquots of antisera diluted in 0.015 M NaH₂CO₃ - 0.034 M NaHCO₃ buffer (pH 9.6). Prior to use the coated cuvets were washed three times with phosphate buffered saline, pH 7.2, containing 0.05 percent Tween 20 (PBS-T). Two hundred μl of PBS-T diluted standards and lymphocyte culture supernatants were then incubated in the cuvets for two hours at room temperature (RT), followed by three washes with PBS-T. Two hundred μl of peroxidase conjugated antisera diluted in PBS-T were then added to each cuvet and incubated for two hours at RT, followed by three washes with PBS-T. Two hundred μl of 0.005 percent H₂O₂ in 0.0052 M 5-aminosalicylic acid¹ were then added and allowed to react for 30 min at RT. The enzymatic reaction was stopped by the addition of 100 μl of 0.3 N NaOH and the absorbance read at 450 nm on a Gilford EIA 50 instrument.§ All determinations were run in duplicate and the values averaged. Levels of SIg are expressed as net ng (ng SIg with PWM - ng SIg without PWM).

### TABLE I

Pokeweed Mitogen Assay Proliferative Response

<table>
<thead>
<tr>
<th>Days of Culture</th>
<th>Tritiated Thymidine Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Net Counts per Minute ± 1 SE</td>
</tr>
<tr>
<td></td>
<td>First Sample</td>
</tr>
<tr>
<td></td>
<td>Second Sample</td>
</tr>
<tr>
<td>3</td>
<td>16,230 ± 2,031</td>
</tr>
<tr>
<td>5</td>
<td>37,469 ± 3,893</td>
</tr>
<tr>
<td>7</td>
<td>24,415 ± 4,745</td>
</tr>
<tr>
<td>9</td>
<td>12,293 ± 6,233</td>
</tr>
<tr>
<td>11</td>
<td>2,829 ± 2,016</td>
</tr>
</tbody>
</table>

N.D. = Not determined

#### Statistical Analysis

When appropriate these data were tested for significance using an analysis of variance, randomized complete-block design, linear regression analysis and a Student’s unpaired t-test.¹⁶

#### Results

**Pokeweed Mitogen Proliferative Assay**

The kinetics of the proliferative response following incubation with PWM of lymphocytes from nine normal volunteers are summarized in table I. A significant variation in the results of the proliferative responses was observed among the 11 culture days (p < 0.005). In both sets of samples, peak³ H-thymidine incorporation was observed on day 5. The mean responses of the first sample were not significantly different from those of the second sample except on day 7.

**Pokeweed Mitogen Induced Intracytoplasmic Immunoglobulin Production**

The kinetics of ICIg production are shown in figure 1. A significant variation in the number of cells containing ICIg was observed among the 11 culture days (p < 0.05). The greatest number of ICIg positive cells for each class of immunoglobulin usually occurred on the fifth day of culture with the greatest increase in ICIg positive cells occurring between the third and fifth culture day. For all nine individuals, the net number of cells positive for IgM was usually greater than or equal to the number of cells positive for IgG. The net number of cells positive for IgA was always the least of the three classes of immunoglobulins. No statistically significant differences between the first and second sets of samples were noted with the exception of the net number of IgM containing cells present on day 7.
POKeweED MITOGEN INDUCED SUPERNATANT IMMUNOGLOBULIN

The kinetics of immunoglobulin accumulation in culture supernatants are presented in figure 2. A significant variation in net SIg was observed among the 11 culture days (p < 0.005). As summarized in figure 2, the mean net SIg for each class of immunoglobulin rises sharply between days 5 and 9 and then remains relatively constant. The concentration of IgM is greater than IgG. In addition, the concentration of IgA is less than either IgM or IgG. Although the SIg concentrations from the first sample tend to be higher than those of the second sample, the differences are not statistically significant.

CORRELATION BETWEEN VIABLE CELLS POSITIVE FOR ICIg AND SIg

When the total number of viable cells positive for ICIg on the peak day is compared to the concentration of SIg on day 9, an excellent correlation is observed (r = 0.89; figure 3).

Discussion

In the current report an assay system employing PWM has been described to initiate proliferation and differentiation of B lymphocytes into immunoglobulin producing cells. These studies indicate that the mean peak proliferative response and mean peak number of ICIg positive cells occurred on the fifth day of culture. In contrast, the maximum concentration of immunoglobulin in the cell culture supernatant occurred on the ninth to eleventh day of culture. The number of ICIGM positive cells and the concentration of IgM in the supernatant fluid was equal to or slightly greater than that for IgG immunoglobulin. In addition, the number of cells positive for ICIGA and the concentration of IgA in the supernatant was
the least of the three classes of immunoglobulins. Similar findings have been observed in PWM stimulated lymphocytes from adults when only IC1g positive cells were enumerated. When lymphocytes from infants up to two months of age were tested, however, IC1gM positive cells predominated over cells containing either IgG or IgA.

Although the mean proliferative response, the total mean number of IC1g positive cells and the total mean concentration of immunoglobulins in the supernatants were less in the second than in the first samples, these differences were not statistically significant (p > 0.05). This observation suggests that the day to day variation for a group of normal individuals is within the 95 percent confidence interval.

A moderate variation existed in the results of these assays among individuals, with coefficients of variation approaching 50 percent. Therefore, it seems likely that there may be other variables in these assays.

Keightley found that the number of IC1g positive cells was proportional to the number of T cells in the initial culture. Since a constant number of T and non-T cells were used throughout this study, a variation in the number of T cells seems unlikely to be the cause of variation observed among different individuals. It is possible that the differences observed among individuals are not an artifact of the technique but rather due to variations in the proportion of various B and T cell subsets. Studies with murine and human lymphocytes have demonstrated that the responses to polyclonal B cell activating substances are characterized by the presence of a subpopulation of B cells. Therefore, the relative proportion of a given B cell subpopulation which is responsive to PWM might vary among individuals. In addition, since the PWM response of B cells is T cell dependent, and since it has been shown that subpopulations of functionally distinct inducer and cytotoxic/suppressor T cells exist, the pro-
portion and state of activation of these T cell subsets may also be important in determining the magnitude of the B cell response in vitro. The effect of T cell subpopulations in terms of selective recovery and/or alteration of function by various fractionation procedures designed to provide T-enriched populations from PB may also be a factor. Larrson et al. and Silva et al. have reported that several functions of human T cells are enhanced after rosette formation with sheep red blood cells.

Mononuclear phagocytes are a necessary accessory cell in the PWM induced maturation of B lymphocytes to IPC. Studies by de Vries indicate that the proportion of monocytes present can influence both the kinetics and the magnitude of the proliferative response of unfractionated lymphocytes to phytohemagglutinin. In addition, Gmelig-Meyling and Waldmann have reported that a high ratio of monocytes to lymphocytes can be associated with suppression of PWM-induced B cell differentiation. In the current study no attempt was made to control the proportion of monocytes present in the non-T cell fraction. Previous studies in our laboratory have indicated that the T-enriched population is markedly depleted of monocytes while the non-T enriched population contains approximately 50 to 60 percent monocytes. Therefore, sufficient monocytes were present in our cultures to provide the necessary function as required accessory cells; however, it is possible that the non-T cell fraction from different individuals contained different proportions of monocytes and that this may in part account for variation in the magnitude of response seen among normals.

George and Cohen studied the kinetics of immunoglobulin synthesis and secretion of plasma cells, resting lymphocytes and PWM transformed lymphocytes by pulse labeling with H-leucine. They found that PWM transformed cells demonstrated enhanced intracellular immunoglobulin synthesis but no significant rise in secreted immunoglobulin, a pattern similar to lymphocytes but different from plasma cells. In the current study, a significant rise in ICIg positive cells and concentration of SIg was observed following PWM stimulation. An excellent correlation (r = 0.89) was observed between the peak number of viable cells positive for ICIg and the peak supernatant concentration of immunoglobulin. While the present studies do not address the mechanism by which immunoglobulin accumulates in culture supernatants, whether by active secretion or by cell death and lysis, the levels achieved correlate well with the number of cells synthesizing immunoglobulin.
The assay system described in the present report differs from most previously reported applications of PWM to access B lymphocyte function. In the present experiments, the proportions of T and non-T cells present were controlled by recombinating enriched cell populations. This approach was undertaken to define a system which would permit reproducible results.

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References