Standardization of Lymphocyte Transformation to Antigen

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Summary. In vitro lymphocyte transformation to non-specific stimulant or specific stimulant is frequently used in the assessment of cell-mediated immunity so that a better understanding of the immune response must be gained before use. Lymphocyte transformation to bovine serum albumin (BSA) or human serum albumin (HSA) immunogen was carefully studied. The standard conditions were: (1) 100 μg of BSA or HSA immunogen; (2) 120 hours of incubation time after addition of antigen; (3) 1/30 dilution of whole blood and 0.5×10⁶ lymphocytes per culture; (4) 1 μCi of ³H-thymidine per culture.

Key words: lymphocyte transformation, cell-mediated immunity (CMI), ³H-thymidine, standardization

The peripheral blood lymphocytes of many species including animals and human beings are activated to undergo blast transformation by specific and non-specific stimulants [1-3]. This activation can be detected by the incorporation of ³H-thymidine into DNA. Lymphocyte transformation has been shown, by numerous techniques[4-8], to represent the initial lymphocyte response to antigen in vivo. In vitro lymphocyte transformation reactions have enabled researchers to investigate lymphocyte function under controlled conditions so that a better understanding of the immune response can be gained.

This study was made to determine optimal culture conditions for the in vitro measurement of cell mediated immune activity of immunised rabbits with reference to the following 4 parameters:

(1) the optimum antigen concentration in cultures;
(2) the optimum incubation time for cultures;
(3) the optimum concentration of test cells in cultures, which include whole blood and peripheral blood lymphocytes (PBLs);
(4) the optimum concentration of ³H-thymidine (³H-TdR).

MATERIALS AND METHODS

RPMI 1640 T.C medium with 20 mg per litre of gentamicin was used throughout the study. Bovine serum albumin (BSA) and human serum albumin (HSA) were obtained from Sigma Chemical Company and were diluted in phosphate buffered solution (PBS). ³H-thymidine (specific activity 5 Ci/mmol) was diluted in RPMI 1640 prior to its use in pulse labeling. Scintillation fluid was made by dissolving 6 g of Butyl-PBD in 1 litre of toluene.

Immunizations

Anti-BSA and anti-HSA animal models were produced by immunizing N.Z. white rabbits with 400 mg BSA or HSA in complete Freund's adjuvant (CFA-Difco) by subcutaneous injection into the back. Two weeks after immunization, blood was taken from the central ear artery of the rabbits. The bloods were collected in RPMI 1640 which contains heparin (20 units per ml blood).
Lymphocyte transformation assay

The whole blood macroculture system was used. The bloods were diluted 1/30 in RPMI 1640 containing heparin (20 u/ml blood). The suspensions were dispensed in 1.5 ml volumes in each culture tube, to which 50 µg—100 µg of antigen were added. All experiments were set up in triplicate. The cultures were incubated at 37°C in 5% CO₂; 95% air for a certain period of time, generally 5—7 days. On the 5th day, each culture was pulsed with ³H-thymidine for 18 hours. The cultures were harvested onto glass fibre filter pads using a millipore culture harvester. The pads were washed with tap water and methanol, placed in scintillation vials dried at 37°C overnight, and 5 ml of scintillation fluid were added to each vial before counting in a liquid scintillation counter. Results were reported as counts per minute (CPM) or the stimulation index (S.I.) which is expressed as:

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\text{S.I.} = \frac{\text{Mean CPM stimulated cells}}{\text{Mean CPM unstimulated cells}}
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The whole blood microculture assay was the same as above except the bloods were diluted 1/10 and 0.1 ml volumes were added to plastic microtitre trays (Linbro). Antigen was added in 0.05 ml per test culture, and then the trays were incubated at 37°C in 5% CO₂, 95% air for 5—7 days before they received an 18-hour pulse label of 0.5 µCi ³H-thymidine per well. Cells were harvested on a Dynatech microharvester and counted as per macroculture.

Purified blood lymphocyte cultures were prepared by density gradient centrifugation modified Boyum. Mononuclear cells were separated from whole blood on a Ficoll-Conray density gradient. The bloods were diluted 1:1 in warm RPMI. 3 ml of Ficoll-Conray were put into a sterile siliconised test tube and the diluted blood was carefully layered over the Ficoll-Conray. This was centrifuged at 400 g for 25 minutes after which the cells at the interface were removed and transferred to a sterile centrifuge tube. The cells were washed 3 times in RPMI before adjusting their concentration to 5 x 10⁶ cells per ml in RPMI 1640 containing 10% normal rabbit serum. 0.1 ml of cell suspension was dispensed in each well of the microtitre plate, and 0.05 ml of the appropriate antigen dilution was added to each well. Cells were incubated, harvested and counted as per macroculture.

Experimental protocol

1. Optimal antigen

In experiment 1 the concentration of BSA and HSA varied from 5 µg to 200 µg per cell culture. Cell cultures were incubated for a period of 5 days during which 0.5 µCi ³H-thymidine per culture was added and cells were harvested 18 hours later.

2. Optimal incubation time

In experiment 2 the time period of incubation varied from 48 to 144 hours before the addition of the ³H-thymidine using 50 µg BSA or HSA and 0.5 µCi ³H-thymidine per culture.

3. Optimal responder cell concentration

In experiment 3 the whole blood was diluted from 1/15 to 1/120 to ascertain the effect of cell concentration on the assay. The number of lymphocytes varied from 0.1 x 10⁸ to 2 x 10⁸ per well in microculture. The cultures contained 100 µg BSA or HSA and 1.0 µCi ³H-TdR. This was incubated for a period of 6 days.

4. Optimal concentration of ³H-TdR

In experiment 4 ³H-thymidine concentration was increased from 0.05 µCi to 2.0 µCi after incubating for 5 days with 50 µg of BSA or HSA.

RESULTS

Experiment 1 The BSA or HSA dose-response (fig.1).

Fig.1a indicates that immunized rabbits respond to test antigens, but normal rabbits do not. At the same time, the antigen concentration is titrable and increases response with increased concentration. The optimal response appears to be 150 µg.