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Positive Selection by «Panning» of B Lymphocytes Using Unfractionated Anti-Immunoglobulin Antiserum

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A simple method allowing a positive selection of mouse B lymphocytes by «panning» has been achieved using rabbit anti-mouse immunoglobulin antisera without further purification. Plastic petri dishes were coated with the gamma-globulin fraction of normal mouse serum and then incubated with excess rabbit anti-mouse gamma globulin antiserum. These plates were then used for the selection of B lymphocytes by incubation of spleen cells in the plates. The adherent cells were above 95 % B lymphocytes as judged by immunofluorescence staining, proliferated in response to bacterial lipopolysaccharide and were not stimulated by concanavalin A.

Key words: «Panning», B lymphocytes, Positive selection.

The functional analysis of the immune system requires the establishment of techniques for the selection or depletion of the different types of cells implicated in the immune responses. Separation techniques become specially important in the dissection of the interactions between lymphocyte subpopulations (or between lymphocytes and «accesory» cells) which take place during immune reactions. Often, the presence of a few contaminating cells are enough to change dramatically the magnitude of a particular response.

In recent years, simple and reliable techniques for the positive selection of lymphocyte subpopulartions by incubation of the cells over antibody-coated plastic surfaces have been developed (4, 7). These techniques use the spontaneous binding to polystyrene surfaces of affinity chromatography-purified anti-immunoglobulin antibodies to separate mouse lymphocytes into surface immunoglobulin-positive (Ig^+) and Ig^- fractions. Afterwards, lymphocytes from other species coated with monoclonal mouse antibodies have been also separated by «panning» over anti-mouse immunoglobulin coated plates (1). In this study, unfractionated anti-immunoglobulin serum has been used to

selected Ig+ mouse spleen cells. To do this, the plates were previously coated with normal mouse gamma-globulin. Addition of excess serum in a second step allowed the specific atachement of Ig-bearing cells (B lymphocytes) which can then be selected on these basis.

Materials and Methods

Mice. Female Balb/c or C57Bl/6 mice 8-12 weeks old were obtained from the animal facilities of our Institute.

Rabbit anti-mouse globulin antiserum. The antiserum was raised in New Zealand rabbits by immunization with the gamma globulin fraction of normal mouse serum. This fraction was obtained by consecutive precipitation of mouse serum with 45 % saturated ammonium sulfate and then with 40 % saturated ammonium sulfate (2). Mouse gamma globulin was diluted to 1 mg/ml in saline emulsified v/v in complete Freund's adjuvant and injected in the hindlegs and in the rear footpads of several rabbits (1 mg of protein per animal). One month afterwards, the injection was repeated using incomplete Freund's adjuvant. Rabbits were bled by cardiac puncture one week after the last immunization and the serum collected. The antiserum used in the experiments described in this paper had a content of specific antibodies of 2.5 mg/ml, as determined by quantitative precipitation (2).

Separation of spleen cells on polystyrene dishes. Polystyrene dishes to be used in the separation procedures were prepared as follows: Bacteriological grade plastic petri dishes (100 mm nominal diameter, Soria-Greiner, Madrid, Spain) were incubated overnight at 4° C with 5 ml of mouse gamma globulin diluted (125 μg/ml) in phosphate buffered saline without calcium and magnesium (PBS) unless otherwise stated. The dishes were washed three times with PBS and then incubated for 45 min at room temperature with diluted rabbit anti-mouse gamma globulin serum (RAMG). The dilution was made v/v in PBS or, if less than 1.5 ml of RAMG were used per plate, the volume of serum was completed with fetal calf serum (FCS). After washing the plates three times with PBS, spleen cells suspended in PBS containing 5 % FCS (PBS-5 % FCS) were poured onto the coated plates. The plates were incubated at 4° C for 1 hour and the nonadherent cells collected by decanting. After gently washing and decanting three times with 10 ml of PBS containing 1 % FCS (PBS-1 % FCS), the adherent (immunoglobulin-positive) cells were resuspended in 20 ml of PBS-1 % by vigorously pippeting the cells. In some cases, the detachment of cells required the aid of a rubber policeman. The adherent cells and the nonadherent cells were centrifuged, washed twice in RPMI-1640 and suspended in RPMI-1640 medium supplemented with 2 mM L-glutamine and antibiotics (culture medium). Other aliquots of cells were processed for immunofluorescence staining of intracytoplasmic immunoglobulin in cytocentrifuge smears. Indirect immunofluorescence was performed using rabbit anti mouse-immunoglobulin antiserum and fluorescein labeled goat anti-rabbit gamma globulin antibiodes (2).

Mitogenic stimulation of lymphocytes. After fractionation, the spleen cells were adjusted to 1.5×10^6 cells/ml in culture medium. Cultures were set in flat-bottomed 96 well plates (Nunclon, 167008) containing 0.2 ml of cells plus 10 μl of mitogen dilution in PBS per well. The final concentration of mitogens in culture was 1 μg/ml (Con-

canavalin A, Calbiochem, Grade A) and 50 μg/ml (lipopolysaccharide from *E. coli* 055:B5, Difco Laboratoires). The cultures were incubated for 72 hours in a 5 % CO_2 atmosphere. Three hours before termination of cultures, they were pulsed with 1 μCi/culture of ^{3}H-thymidine (Amersham, The Radiochemical Centre, Sp. act 21 Ci/mmol). The cultures were harvested onto GF/A (Whatman) glass fiber filters using a Mash II harvester. The filters were dried and the radiactivity incorporated into the cells determined in a scintillation spectrometer.

Results and Discussion

Two different aspects of «panning» in this system were investigated: First, plates coated with an equal amount (5 ml at 0.125 mg/ml) of mouse gamma globulin were incubated with different volumes of RAMG (table I) and then 12×10^7 spleen cells were poured per plate. As shown in table I, augmenting the volume of RAMG from 1.5 to 3.75 ml did not enhance the number of cells adhered. However, higher amounts of serum made the adherent cells more difficult to detach and decreassed their viability. So, 1.5 ml of RAMG were used in the following experiments. The percentage of Ig^+ cells changed from

Table I. *Adherence of spleen cells to plates incubated with different amounts of rabbit anti-mouse gamma globulin antiserum.*

Antiserum per plate (ml)	Num. of adherent cells (x 10^{-6})	Per cent Ig^+ cells	
		Adherent	Non adherent
0 (a)	—	—	35
1.25	25.2	95	N.D.
2.5	21.6	94.3	N.D.
3.75	21.3	96	N.D.

(a) Control plates received 3.75 ml of normal rabbit serum.

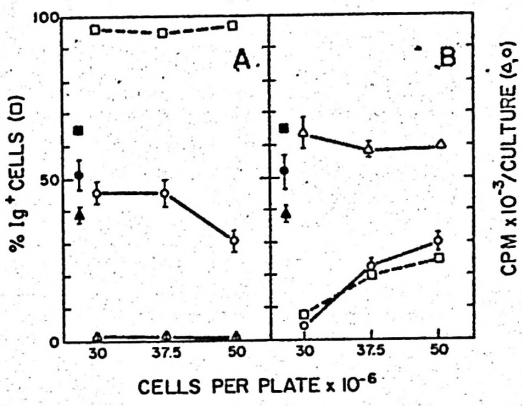

Fig. 1. *Response to Concanavalin A (△ — △) or lipopolysaccharide (○ — ○) and number of Ig-positive cells (□ - - - □) in cells adherent (A) or not (B) to plates coated with mouse gamma-globulin and rabbit anti-mouse gamma globulin antibodies.* The plates were poured with the number of cells indicated in the abcissa. Closed symbols represent the response to mitogens and the percentage of Ig^+ cells in unfractionated spleen cells.

35 % in unfractionated cells to about 95 % in the adherent cells.

In the second set of experiments, we tested the ability of the plates to retain Ig^+ cells (fig 1). Adherent cells were highly enriched in Ig^+ cells and responded well to lipopolysaccharide (LPS) whatever the initial number of cells poured, and the response to Concanavalin A was negligible (fig. 1A). On the other hand, nonadherent cells varied in the percentage of Ig^+ cells as well as the response to LPS as increasing number of cells were poured in the plate. The capability of the plates to retain Ig^+ cells was saturated using 30-45 × 10^6 cells, depending on the initial percentage of Ig^+ cells. Higher numbers of input cells implied that some B lymphocytes (i.e. Ig^+ cells) were not retained by the plates and, consequently, an appreciable response to LPS was found in nonadherent cells. The number of B cells among nonadherent cells could be substantially reduced by a sec-

Table II. *Influence of mouse gamma-globulin concentration on binding capacity of the plates.*

Mouse gamma globulin (mg/ml)	Antiserum per plate (ml)	Input cell/plate ($\times 10^{-6}$)	Number of cells ($\times 6^{-6}$)(a) Adherent	Non adherent
0.05	0.5	12	6.2 (96.1)	4.9 (6.2)
0.125	1.25	30	17.0 (97.4)	13.3 (7.3)

(a) Figures in brackets represent the percentage of Ig^+ cells in each fraction. The percentage of Ig^+ cells in unfractionated spleen cells was 51.1 %.

ond incubation under the same conditions, as has been described for «direct» systems (4, 7, 8). The retaining capacity of the system could be easily scaled down by simphy decreasing the concentration of mouse gamma globulin used to coat the dishes and the amount of RAMG employed. In the experiment described in table II, incubation of plates with 50 μg/ml of mouse gamma globulin instead of 125 μg/ml and addition of 0.5 ml of RAMG per plates instead of 1.25 ml was enough to decrease in the same proportion (i.e. 2.5 fold) the number of bound cells without altering the ratio of bound to unbound cells.

In our experiments, the principle first described by WIGZELL *et al.* (6) to remove Ig^+ lymphocytes by chromatography on colums filled with glass or plastic beads coated with normal mouse gamma globulin reacted *in situ* with anti-mouse gammaglobulin antiserum has been applied. However, the chromatographic systems have the disadvantage of the cumbersome preparation of the columns and the need for a previous depletion of cells adhering nonspecifically to the column in order to select positively Ig^+ cells. In the system described here, the easy handling of cells typical of «panning» systems has been combined with the use of simple immunological reagents characteristic of Wigzell's method. A problem is posed by the existence of 3-5 % of adherent cells which remain unstained by anti-Ig immunofluorescence. However, binding of cells due to attachment by Fc receptors seems to be negligible in our hands (see table I for nonspecific binding) as well as in others (4, 7). In addition, nonspecific esterase staining (5) of adherent cells indicate that macrophages represent less than 0.1 % of the population, and the same figure applies for the number of polymorphonuclear leukocytes found in Wright-stained smears. On the other hand, the adherent cells have virtually no contaminating T lymphocytes as judged by treatment with monoclonal anti Thy-1.2 antibodies and complement. Nevertheless, it would be useful a previous depletion of macrophages (3) and/or T cells when very stringent separation conditions are required. So, we have developed a simple method for the effective separation of functional lymphocytes into Ig^+ and Ig^- populations using easily obtainable reagents without previous purification of anti-immunoglobulin antibodies. This fact will allow a better study of B lymphocyte function under different physiological conditions (age, nutrition, hormone or pharmacological treatments, etc.).

Resumen

Se ha desarrollado un método sencillo para la selección positiva de linfocitos B de ratón por «plateado» («panning»). Para ello se ha empleado un suero de conejo anti-inmunoglobulina de ratón no fraccionado. En una primera incubación se recubrieron placas de petri con gamma-globulina de ratón, y a continuación se añadió en exceso el suero de conejo anti-gamma globulina de ratón. Al añadir las células de bazo a estas placas, quedan adheridos los linfocitos B, poseedores de inmunoglobulinas de membrana. Así, más del 95 % de las células adherentes eran linfocitos B por inmunofluorescencia, eran estimulados a proliferar por lipopolisacáridos bacterianos (mitógenos para linfocitos B) y no por mitógenos de linfocitos T.

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