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Density Gradient Separation of Lymphoid Cells Adhering to Protein-A-Containing Staphylococci

(surface-bound IgG/sodium metrizoate/lysostaphin)

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ABSTRACT **A cell separation technique was designed based on the interaction between cell-surface-bound IgG and protein A of *Staphylococcus aureus*. The density of lymphoid cells coated with IgG antibodies against one of the surface markers was increased by adherence of staphylococci. Cells with adhering bacteria were separated from cells without bacteria by density gradient centrifugation in 11.5% sodium metrizoate. Bacteria were removed from the lymphoid cells by lysostaphin digestion.**

The purity of separated cells was approximately 95% even when the proportion of a specific cell population was below 10% in the initial mixture. The viability and the ability of cells to multiply *in vitro* were not significantly impaired by the fractionating procedure.

The technique can generally be applied for cell separation, provided antibodies of the IgG class against specific surface markers are available.

It was previously shown (1) that cells of *Staphylococcus aureus* (strain Cowan 1) containing protein A (SpA) adhere to the surface of lymphoid cells pretreated with antibodies of the IgG class. Each cell binds approximately 15 to 25 bacteria. The binding is due to the reaction of SpA and the Fc region of the IgG antibodies (Fcγ) (2). The density of cells was increased by the adhering bacteria, which suggested that such cells could be separated from nonadhering cells by density gradient centrifugation.

The applicability of such a technique has now been proven by use of established human lymphoid cell lines. As a model, two cell lines were mixed in known proportions and treated with an antibody directed against an antigenic marker present only on one of the cell lines. After addition of bacteria the cell lines were separated by density gradient centrifugation. Bacteria were removed from adhering cells by enzymatic digestion under conditions that did not impair the viability and growth capacity of the cells. The cells were recovered in high purity and in a good yield.

The results suggest that the reaction of cell surface bound IgG and bacteria containing SpA can form the basis for a general technique for separation of cells.

Abbreviations: SpA, protein A of *Staphylococcus aureus*; adhering cells, lymphoid cells coated with specific antibodies that bind to staphylococci; $\beta_{2\mu}$, β_2-microglobulin; Fcγ, Fc region of IgG; aIgM, rabbit antiserum to human μ chain; arIgG, swine antiserum to rabbit IgG; a$\beta_{2\mu}$, rabbit antiserum to human $\beta_{2\mu}$; FITC-, material labeled with fluorescein isothiocyanate; PBS, phosphate-buffered saline, pH 7.2.

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MATERIALS AND METHODS

Cell Lines. The following human lymphoid cell lines of hematopoietic origin were used.

1. The Daudi cell line is a Burkitt lymphoma derived line. One of its surface markers is IgM (3). It is devoid of β_2-microglobulin ($\beta_{2\mu}$) marker. (K. T. Welsh, G. Dorval, H. Wigzell, and K. Nilsson, manuscript in preparation).
2. The 266 Bl myeloma cell line secretes IgE (4).
3. The Raji cell line is a Burkitt-lymphoma-derived line. One of its surface markers is $\beta_{2\mu}$ (Welsh *et al.*, in preparation).

All cell lines were maintained as non-stirred suspension cultures at 37°. The routine medium used was Ham's F-10 (Gibco, New York) supplemented with 10% post natal calf serum and antibiotics (100 IU penicillin per ml, 50 μg of streptomycin/ml and 1.25 μg of amphotericin B per ml). The medium was changed twice a week.

Radiolabeling of Daudi Cells. The cells were radioiodinated as described by Vitetta *et al.* (5) by adding 0.1 mCi of ^{125}I (The Radiochemical Center, Amersham) and 0.05 mg of lactoperoxidase (Sigma, St. Louis) per 2×10^7 cells. The cells were then washed six times in phosphate-buffered saline (PBS).

Antisera. Rabbit antiserum to human μ chain (aIgM) labeled with fluorescein isothiocyanate (FITC-aIgM), nonlabeled rabbit aIgM, and FITC-labeled swine antiserum to rabbit IgG (FITC-arIgG) were purchased from Dakopatts, Copenhagen. The rabbit antiserum to human $\beta_{2\mu}$ (a$\beta_{2\mu}$) was a gift from Dr. L. Rask, Uppsala. It was extensively absorbed with Daudi cells before use.

Bacteria. Preparation of *S. aureus* was previously given (1). A stock suspension of living bacteria was prepared in PBS (5×10^9 bacteria per ml). This suspension could be stored at −70° for months without aggregation. After repeated freezing and thawing the bacteria became, however, somewhat more resistant to lysostaphin digestion. It is, therefore, recommended to store the bacteria in aliquots and submit them to only one freezing and thawing. Alternatively the bacteria can be lyophilized in 5% Ficoll (Pharmacia, Uppsala) in water and kept frozen. Living bacteria were labeled with FITC as previously described (1) except that the labeling time was 20 min. Formaldehyde-treated or autoclaved bacteria can not be used since such bacteria are resistant to lysostaphin digestion.

Medium for Gradient Centrifugation. A solution of 11.5% sodium metrizoate (Nyegaard & Co., Oslo) in distilled water

TABLE 1. *Outline of the cell separation technique*

Step 1.	Coating cells with specific antibody (IgG class) against a cell surface marker.
Step 2.	Density gradient centrifugation in 11.5% sodium metrizoate for removal of dead cells.
Step 3.	Adherence of *S. aureus* to antibody-coated cells.
Step 4.	Density gradient centrifugation in 11.5% sodium metrizoate for separation of adhering and nonadhering cells.
Step 5.	Lysostaphin digestion of adhering cells in the bottom fraction for removal of bacteria.

was prepared. The density of the solution was determined by sedimentation of microdroplets by gravity to a linear organic density gradient (6). The density of the sodium metrizoate was 1.065 g/ml and the osmolality, determined by an osmometer (Advance Instruments, Newton, Highlands, Mass.), was 321 mosM.

Cell Counting and Viability Tests. The number of fluorescent cells in each operational step was determined by counting approximately 200 cells in visible and ultraviolet light in a Leitz Orthoplan microscope. Since only Daudi cells and not 266 Bl cells are stained with FITC-aIgM, the number of fluorescent cells gives the percentage of Daudi cells in the cell mixture. The number of Raji cells, which react with rabbit a$\beta_2\mu$ antibody, was determined indirectly after treating the cells with FITC-arIgG.

The number of adhering cells was counted in phase contrast. In some experiments fluorescent labeled bacteria in combination with nonfluorescent antibody were used for a more rapid and accurate determination.

The viability of the cells was determined by the trypan blue exclusion method. Isolated cells were reincubated in vitro under standard culture condition to establish whether any irreversible cell damage occurred. Cells were counted in a Celloscope (Linson Instruments, Stockholm) and growth rate was determined and compared to that of optimally growing non-fractionated cells. DNA synthesis was recorded as follows: 10^6 cells in 2 ml of medium were labeled by a 1-hr pulse of 2 μCi of tritiated thymidine ([^{3}H]dThd, specific activity 2 mCi/mmol, Radiochemical Centre, Amersham). Cells were then harvested on filters in a Millipore Manifold Chamber (Millipore AB, Göteborg), washed with 15 ml of PBS and then twice with 5 ml of trichloroacetic acid (10% in distilled water). After an additional wash with 10 ml of PBS the filters were placed in Instagel (Packard Instr. Comp., Warrenville, Ill.) and the radioactivity was measured in a liquid scintillation counter.

RESULTS

The applicability of the separation technique was shown by using known mixtures of cells coated with antibodies of the IgG class. In one set of experiments Daudi cells (IgM marker) were mixed with 266 Bl cells (IgE marker); in a second series of experiments Daudi cells were mixed with Raji cells ($\beta_2\mu$ marker); and in a third type of experiments Daudi cells coated with aIgM were mixed with noncoated Daudi cells. The outline of the separation technique is given in Table 1.

Separation of Daudi and 266 Bl Cells. In a *first step* Daudi cells were coated with antibodies. Cell suspensions of known proportions of Daudi and 266 Bl cells were prepared (Table 2) and the cell concentration was adjusted to approximately 10^7 cells per ml in F-10 medium without serum. After addition of FITC-aIgM (25 μl/10^7 cells) the cells were incubated on ice for 1 hr.

In a *second step* living cells were separated from dead cells. The cell suspension was diluted with cold F-10 medium to 5×10^6 cells per ml and 2 ml of this suspension was layered on 4 ml of 11.5% sodium metrizoate in plastic tubes (1.5 ×

TABLE 2. *Separation of Daudi from 266 Bl cells after treatment with FITC-aIgM and bacteria*[a]

	Daudi cells in the cell mixtures[b]		
	65%	27%	8%
Before separation			
Adhering cells	68	28	8
Viability of adhering cells	86	93	98
After separation			
Bottom fraction[c]			
Recovery of cells[d]	80	70	65
Fluorescent cells	91	84	80
Adhering cells	96	88	80
Viability of adhering cells	ND	93	95
Fluorescent cells after recentrifugation	98	ND	93
Adhering cells after recentrifugation	95	96	ND
Bottom fraction after digestion with lysostaphin			
Recovery of cells[e]	88	90	ND
Adhering cells remaining	8	2	3
Fluorescent cells	95	91	ND
Decrease in viability	17	4	8
Fluorescent cells after 24-hr recultivation	ND	83	ND
Fluorescent cells after 24-hr recultivation of recentrifuged bottom fraction	ND	97	93
Top fraction[f]			
Recovery of cells[g]	90	ND	95
Nonfluorescent cells	93	96	97
Nonadhering cells	92	97	98
Viability of nonadhering cells	88	95	96
Nonfluorescent cells after 24-hr recultivation	ND	96	98

[a] The figures represent the arithmetic mean of two experiments and are expressed in percent. The difference of two experiments was less than 10%. ND = not determined.

[b] The percentage of fluorescent Daudi cells in each cell mixture recovered in step 2 (see *Results*). All Daudi cells reacted with FITC-aIgM in step 1.

[c] The bottom fraction represents the deposit of cells after density gradient centrifugation on 11.5% sodium metrizoate (step 4).

[d] Recovery (%) = (number of cells in the bottom fraction/number of Daudi cells applied for density gradient centrifugation) × 100.

[e] Recovery (%) = (number of cells after lysostaphin digestion/number of cells digested with lysostaphin) × 100.

[f] The top fraction represents the ring of cells formed at the interphase after density gradient centrifugation on 11.5% sodium metrizoate (step 4).

[g] Recovery (%) = (number of cells in the top fraction/number of 266 Bl cells applied for density gradient centrifugation) × 100.

TABLE 3. *Separation of Raji from Daudi cells after treatment with $a\beta_2\mu$ and bacteria*[a]

	Raji cells in the cell mixtures[b]	
	32%	65%
Before separation		
Adhering cells	36	65
Viability of adhering cells	90	95
After separation		
Bottom fraction		
Recovery of cells[c]	80	85
Fluorescent cells	98	ND
Adhering cells	90	95
Viability of adhering cells	90	92
Bottom fraction after digestion with lysostaphin		
Recovery of cells[d]	90	88
Fluorescent cells	95	95
Decrease in viability	5	8
Fluorescent cells after 24-hr recultivation	95	ND
Top fraction		
Nonfluorescent cells	95	ND
Nonadhering cells	90	95
Viability	92	93

[a] The figures represent the arithmetic mean of two experiments and are expressed in percent. The difference of two experiments was less than 10%. ND = not determined.

[b] Living cells recovered in step 2 (see *Results*). The percentage of Raji cells in each cell mixture was equal to the percentage of fluorescent cells after treatment with $a\beta_2\mu$ and FITC-arIgG.

[c] Recovery (%) = (number of cells in the bottom fraction/number of Raji cells applied for density gradient separation) × 100.

[d] Recovery (%) = (number of cells after lysostaphin digestion/number of cells digested with lysostaphin) × 100.

11 cm). The cells were then centrifuged for 20 min, 18° with 300 × *g* at the interphase. Dead cells sedimented to the bottom of the tube while living cells formed a ring at the interphase. Living cells were carefully pipetted off and washed twice in F-10 medium by centrifugation at 250 × *g* for 10 min. The viability of these cells was always high (95%).

In a *third step* adhering Daudi cells were formed. The cell suspension was adjusted to 10^7 cells per ml and then mixed with bacteria (20 to 30 bacteria per cell). After centrifugation for 10 min at 250 × *g* the tube was incubated on ice for 1 hr. F-10 medium was added to give a cell concentration of 5 × 10^6 cells per ml and the cells were resuspended.

In a *fourth step* adhering and nonadhering cells were separated by layering 2 ml of the cell suspension on 4 ml of 11.5% sodium metrizoate and centrifuging (18°, 300 × *g* at the interphase). The nonadhering cells were collected as a ring at the interphase (*top fraction*) and adhering cells at the bottom of the tube (*bottom fraction*). The top fraction was carefully pipetted off and the tube was rinsed twice with F-10 medium, leaving the bottom fraction intact. The F-10 medium used for rinsing was combined with the top fraction. Both top and bottom fractions were then washed twice in F-10 medium.

In a *fifth step* bacteria were removed from adhering cells. The bottom fraction was suspended in PBS (5 × 10^6 cells per ml) and the bacteria were lysed by adding 5 μl of 0.2% lysostaphin/ml (Schwarz/Mann, New York) and incubation for 20 min at 37°. The suspension was diluted 2-fold with cold F-10 medium, and washed twice in F-10 medium.

The top and bottom fractions were then separately recultivated.

Table 2 summarizes three different experiments in which the Daudi cells represented 65%, 27%, and 8% of the initial number of cells. A good agreement is found between the number of fluorescent cells and the number of adhering cells in each mixture before separation, which shows that cells coated with FITC-aIgM react specifically with SpA of the bacteria. Only a minor proportion of adhering cells were nonfluorescent.

After separation, the recovery of Daudi cells in the bottom fractions varied in the three fractions from 65 to 80%. The enrichment of Daudi cells was 1.4-, 3.1-, and 10-fold, respectively. The percentage of adhering cells in each bottom fraction was similar to that of fluorescent cells.

The viability of adhering cells was determined for two of the bottom fractions and was in both instances over 90%.

Recentrifugation on sodium metrizoate of the three fractions resulted in a better separation of the cell lines. The percentage of Daudi cells (fluorescent and adhering cells) increased to approximately 95%.

After lysostaphin digestion of the bottom fractions only a minor proportion of the cells remained with bacteria attached to the cell surface. The cell recovery was almost 90%. A decrease in cell viability of 4–17% was noted.

The viability during 24 hr of recultivation usually decreased by 10%. No further cell death was recorded during prolonged cultivation. All viable cells looked normal in phase contrast. The population doubling time and the rate of [^{3}H]-thymidine incorporation was comparable to control cultures that were not subjected to fractionation.

The recovery of cells in the top fractions was approximately 90%. The purity of 266 Bl cells, as shown by the percentage of nonfluorescent and nonadhering cells, was better than 92%. The viability was very high and almost 100% after 24 hr of recultivation.

Separation of Daudi and Raji Cells. A similar type of experiment was performed with Raji and Daudi cells. A different antibody ($a\beta_2\mu$) against a different antigenic marker was used to attach bacteria to the Raji cells.

Each cell mixture was treated with rabbit $a\beta_2\mu$ antiserum (15 μl/10^7 cells) and separated as described for Daudi and 266 Bl cells. Table 3 shows that the Raji cells were recovered in the bottom fraction and the Daudi cells in the top fraction in high yields (80–90%) and with a high purity (90–95%). The viability of the cells was also high, as demonstrated by the trypan blue exclusion method, [^{3}H]thymidine incorporation, and cell growth.

Separation of Antibody-Coated from Noncoated, Radioactively Labeled Daudi Cells. Daudi cells were treated with FITC-aIgM (25 μl/10^7 cells) and washed three times in F-10 medium. The cells were then mixed with another batch of nonfluorescent, ^{125}I-labeled Daudi cells. Living cells were recovered by centrifugation in 11.5% sodium metrizoate (step 2). After addition of bacteria, fluorescent cells were separated from nonfluorescent cells (step 3 and 4). Sheep erythrocytes

were added to the top and bottom fractions before washing to avoid losses of radioactive cells. The number of fluorescent cells in the top and bottom fractions was determined. The radioactivity of each fraction was counted in an autogamma scintillation spectrometer. No recultivation of separated cells was performed.

The results are presented in Table 4. Similar to previous experiments, a good correlation between the number of fluorescent cells and adhering cells was found. The recovery of antibody-coated cells in the bottom fraction was almost 90% while the radioactivity was low (4%). In contrast, the top fraction contained a low number of fluorescent and adhering cells (6%) but most of the radioactivity (96%).

The Effect of Lysostaphin Digestion on the SpA-Antibody Complex of Adhering Cells. Lysostaphin digestion of *S. aureus* releases SpA from the bacteria (7). As shown in Tables 2 and 3, lysostaphin digestion of adhering cells does not remove the antibodies from the cell surface. That SpA is still bound to the antibodies after digestion was proved by the following experiments.

Adhering cells were isolated after treatment of Daudi cells with aIgM and nonfluorescent bacteria. After lysostaphin digestion adhering cells were exposed to a lysostaphin digest of FITC-labeled bacteria, washed, and examined in a ultraviolet microscope. No fluorescent cells were detected. In contrast, aIgM-coated Daudi cells became fluorescent after treatment with a digest of FITC-labeled bacteria.

The experiments demonstrate that lysostaphin digestion of adhering cells does not remove SpA from the cell bound antibodies.

DISCUSSION

The purpose of this investigation was to show (*1*) that cells coated with IgG-antibodies react with SpA-containing staphylococci, become denser, and, therefore, can be separated from noncoated cells by density gradient centrifugation; (*2*) that bacteria adhering to antibody-coated cells can be removed by lysostaphin; and (*3*) that the growth capacity of isolated cells was not affected by the separation procedure.

To our knowledge *S. aureus* has not been used before for cell separation. Cells with individual surface markers, can be separated from other cells by the use of *S. aureus* with wall-bound SpA as a reagent for IgG. The technique can be advanced and used as a general method for cell separation, provided specific antibodies are available and provided the reactions involved do not lead to cell damage.

To show the feasibility of the technique, established human lymphoid cell lines with defined antigenic markers were chosen. Several media were studied in which adhering cells could be separated from nonadhering cells by centrifugation. Only the sodium metrizoate (11.5%) was found suitable. In sodium metrizoate–Ficoll (8), bovine serum albumin (6), and sodium silicate-polyvinylpyrrolidone (9) a good separation of nonadhering cells was obtained at the interphase but adhering cells did not sediment satisfactorily because of extensive cell clumping. For separation of other cells than those used in the present work one may have to find another medium.

The technique gave a high purity of isolated cells (>90%) also when the starting mixture contained only 8% of antibody-coated cells. The method seems to be suitable for isolation of cells of even lower concentrations but in such cases several recentrifugations are necessary to reach a similar high purity.

TABLE 4. *Separation of Daudi cells coated with FITC-aIgM from noncoated, radioactively labeled Daudi cells*[a]

	Antibody-coated Daudi cells in the cell mixture[b] (33%)
Before separation	
Adhering cells	31
Viability of adhering cells	92
After separation	
Bottom fraction	
Recovery of cells[c]	88
Fluorescent cells	86
Adhering cells	91
Radioactivity	4
Viability of adhering cells	85
Top fraction	
Recovery of cells[d]	90
Nonfluorescent cells	94
Nonadhering cells	94
Radioactivity	96
Viability	98

[a] The figures represent the arithmetic mean of two experiments and are expressed in percent. The difference of two experiments was less than 10%.

[b] The percentage of fluorescent Daudi cells in the cell mixture recovered in step 2 (see *Results*).

[c] Recovery (%) = (number of cells in the bottom fraction/number of FITC-aIgM coated cells applied for density gradient centrifugation) × 100.

[d] Recovery (%) = (number of cells in the bottom fraction/number of nonfluorescent cells in the top fraction) × 100.

The decrease in viability during fractionation was found to be correlated to the initial viability of the cells and to the duration of the fractionation procedure. A similar degree of cell damage was observed after repeated washing of the cells in a serum-free F-10 medium at 4° for a similar length of time. Thus, fixation of antibody and bacteria *per se* to the cells does not impair the viability of the cells.

As shown, the SpA–antibody complex remains on the cell surface after lysostaphin digestion. Trypsin treatment of the cells will, however, remove the complex. Recultivation of trypsin-digested cells for 24–48 hr will permit the cells to resynthesize the cell surface markers (10).

That the SpA-antibody complex does not harm the cells was proved by recultivation. The growth rate of fractionated cells was similar to that of nonfractionated control cells. In some of our experiments fractionated Daudi and Raji cell lines have been kept in culture for over 1 month. In addition, no significant cell damage was observed when the technique was applied for separation of subpopulations of lymphocytes from mouse spleen and from human peripheral blood (unpublished results).

The method described could be generally applied for fractionation and purification of several types of cells from a heterogeneous cell population. However, some restrictions of the technique must be pointed out.

1. The antibody fixed to the cells must be of the IgG class (11). The antibody must also be derived from an animal

species whose IgG has a relatively high affinity for SpA, e.g., from human, monkey, guinea pig, rabbit, swine, and goat.

2. The majority of antibody-coated cells must react with SpA-containing bacteria. If not so, the yield of adhering cells will be low and the top fraction will be contaminated with antibody-coated cells.

3. A low but significant number of cells may react with the bacteria before coating cells with antibodies (1). Such cells should first be removed by addition of bacteria followed by density gradient centrifugation.

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