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Detection and Separation of Lymphocytes with Specific Surface Receptors, by using Microparticles

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Horse anti-(human lymphocyte) globulin was immobilized together with fluorescein-labelled dextran in spherical microparticles of polyacrylamide (AHLG-particles). The particles had a diameter of 1–5 μm and a density of 1.12 g/cm^3, with globulin exposed on the surface. Human lymphocytes bearing the antigen (thymus-derived lymphocytes) bound the particles, which were easily detected by fluorescence microscopy. In this way, about 58% of circulating human lymphocytes were able to bind AHLG-particles at 23°C. Non-specific binding was low, only 3% when human serum albumin was present in the buffer, and only 4% when non-specific horse globulins were incorporated in the microparticles. The cell–particle complexes could be separated from cells that had not reacted by density-gradient centrifugation in Ficoll/metrizoate. The viability was not changed after the separation procedure. The number of cells binding AHLG-particles corresponded well to the relative amount of T-cells. When the cells binding AHLG-particles were separated from the lymphocytes, the number of T-cells decreased remarkably, indicating that the antibodies bind preferably to the T-cell population. Concanavalin A immobilized in microparticles was sufficiently exposed to initiate the agglutination of the lymphocytes. The agglutination was completely inhibited by preincubating the microparticles with α-methyl mannoside.

The identification and detection of specific membrane structures are common problems in cell research. Different direct or indirect techniques, such as rosette or sandwich techniques, have been used for these purposes, but are in some cases laborious, time-consuming and non-specific. In addition, a further separation of the cell population is often of great interest. For this purpose proteins covalently immobilized to different solid phases, such as column beads or fibres, have been used (Edelman *et al.*, 1971; Wigzell & Andersson, 1969). Bacterial cells or sheep erythrocytes have also been utilized as a support for the protein (Ghetie *et al.*, 1974, 1975), which makes it possible to isolate the cell–cell complexes formed by gradient centrifugation.

Macromolecules can be immobilized in microparticles of polyacrylamide during polymerization in such a way that they are exposed on the surface (Ekman & Sjöholm, 1975; Ekman *et al.*, 1976). This finding opens up wide possibilities for the utilization of such microparticles to detect specific cell structures. A subsequent separation of the cells binding the microparticles is possible by gradient centrifugation. A wide variety of proteins can be immobilized, no covalent coupling to the polyacrylamide gel is needed, and to facilitate the detection of the microparticles a separate carrier can be used in the particles, e.g. fluorescein-labelled dextran or labelled inactive proteins. The density of the microparticles depends on the degree of cross-linking of the polyacrylamide network forming the particles (Ekman *et al.*, 1976) and can thus be used to increase the density of certain cells. Independently, Molday *et al.* (1975) and Quash *et al.* (1976) have prepared small latex spheres with covalently bound antibodies as visual markers for the detection and localization of cell-surface antigens by scanning electron microscopy.

The present paper describes how microparticles with non-covalently immobilized anti-(human lymphocyte) globulin (hereafter termed 'AHLG-particles') can be used to detect specific cells in a lymphocyte preparation from human blood, and to isolate the particle-binding cells by gradient centrifugation in Ficoll/sodium metrizoate. Interaction between the lymphocytes and microparticles with concanavalin A (concanavalin A-particles) has also been studied.

Materials and Methods

Materials

Horse anti-(human lymphocyte) globulin was given by Hoechst A.G., Frankfurt, Germany, and concanavalin A and fluorescein isothiocyanate-labelled

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dextran were purchased from Pharmacia, Uppsala, Sweden. Horse globulins were isolated from normal horse serum by precipitation with $(NH_4)_2SO_4$ (36%, w/v). Lymphoprep (a mixture of Ficoll and sodium metrizoate) was obtained from Nyegaard and Co., Oslo, Norway, and human serum albumin from Kabi A.B., Stockholm, Sweden.

Cell preparation

Lymphocytes were obtained from fresh human or horse blood, heparinized or in citrate/glucose solution, by gradient centrifugation on Lymphoprep. Blood (4ml) diluted 1:1 (v/v) with phosphate-buffered saline (0.14M-NaCl/2.7mM-KCl/16.2mM-Na_2HPO_4/1.5mM-KH_2PO_4, pH7.4) was layered on 3ml of Lymphoprep and centrifuged at 600*g* for 30min in a Mistral 6L centrifuge. The cells were washed in buffer, and contaminating erythrocytes were lysed by a short hypo-osmotic shock treatment. Cell viability, assessed by exclusion of Trypan Blue, was always greater than 96%.

Microparticles

Microparticles were prepared and characterized as described by Ekman *et al.* (1976). A solution of acrylamide (6%, w/v) and *NN'*-methylenebisacrylamide (2%, w/v) in 0.1M-KCl/0.005M-sodium phosphate buffer, pH7.5, also containing the protein to be immobilized was homogenized in an organic phase consisting of toluene/chloroform (4:1, v/v). Then 5ml of the water phase was emulsified with an Ultra-Turrax instrument (TP 18-10) in 200ml of the organic phase with the detergent Pluronics F68, manufactured by Wyandotte Chemical Co., Wyandotte, MI, U.S.A., immediately after the catalyst system consisting of *NNN'N'*-tetramethylethylenediamine and ammonium peroxydisulphate (persulphate) had been added to the monomer solution. In the experiments described, microparticles were prepared containing concanavalin A, anti-(human lymphocyte) globulin or normal horse globulins by dissolving 100mg, 250mg or 250mg respectively, together with fluorescein isothiocyanate-labelled dextran (50 g), in 5ml of the monomer solution. The polymerization was generally complete after 5min, and the phases were allowed to separate for about 15min. The microparticles were separated and washed with buffer by centrifugation at 1100*g* for 20min. The protein incorporated amounted to about 0.02mg of concanavalin A and 0.04mg of the globulins per mg dry wt. of particles. The microparticles were stored at 4°C in 0.1M-KCl/0.005M-phosphate buffer, pH7.5, containing 0.02% NaN_3 as preservative. The density of the microparticles, as determined by density-gradient centrifugation in silica gel (Ekman *et al.*, 1976), was 1.115g/cm³ for the concanavalin A-particles and 1.120g/cm³ for the globulin particles. The diameter of the microparticles was generally 1–5μm.

Incubation of cells with microparticles

For this, 1×10^6–2×10^6 cells in 100μl of phosphate-buffered saline with albumin (1mg/ml) were mixed with the same volume of microparticles in phosphate-buffered saline corresponding to a protein concentration of 0.4–0.5mg/ml. The tube was centrifuged at 50*g* for 10min and incubated at 4°C or 23°C for 1h. The cells were then carefully suspended and the number of cells with microparticles on the surface was counted in a Leitz Dialux fluorescence microscope. At least 100 cells were counted in visible light, and those bearing three or more particles detectable in u.v. light were counted as positive.

The binding of AHLG-particles to the cells was inhibited by preincubating the cells with a solution of the anti-(human lymphocyte) globulin (5mg/ml) for 15min at 4°C. The binding of concanavalin A-particles was inhibited by preincubating the microparticles with α-methyl mannoside (0.1M, in phosphate-buffered saline) for 15min at 4°C.

Separation of cells and cell–microparticle complexes by gradient centrifugation

The mixture of cells and microparticles (diluted with 1vol. of phosphate-buffered saline) at 4°C was layered on 0.5ml of Lymphoprep ($d=1.077$g/cm³) and centrifuged at 600*g* and 4°C for 20min. The top fraction was sucked off and the Lymphoprep surface was washed once with fresh buffer. The cell pellets from the combined top fractions and from the bottom fraction were each suspended in 100μl of buffer and the cells counted.

Rosette formation

The number of thymus-derived (T-) cells in a lymphocyte population was determined from the number of cells spontaneously forming rosettes with sheep erythrocytes as described by Jondal *et al.* (1972). For this, 100μl of the lymphocyte suspension (5×10^6–1×10^7 cells/ml) was mixed with a 50-fold excess of erythrocytes previously washed and suspended in phosphate-buffered saline with albumin (1mg/ml). The mixture was centrifuged at 50*g* for 10min and was then incubated at 4°C for 1h. The pellet was then carefully suspended in buffer and the lymphocytes were counted under the microscope; those bearing more than three sheep erythrocytes were considered positive.

Results and Discussion

Binding of microparticles to lymphocytes

Peripheral human lymphocytes (1×10^6–2×10^6 cells in 100μl of phosphate-buffered saline) were incubated with an excess of microparticles as

Table 1. *Binding of microparticles containing horse anti-(human lymphocyte) globulin and fluorescein isothiocyanate-labelled dextran to peripheral lymphocytes*

Peripheral lymphocytes were incubated with microparticles containing anti-(human lymphocyte) globulin (AHLG) and/or fluorescein isothiocyanate-labelled (FITC-) dextran at either 4°C or 23°C. After 1 h the amount of cells binding microparticles was calculated after microscopic examination. The sample was then centrifuged on a Ficoll gradient as described in the Materials and Methods section, and the numbers of cells in both the bottom and top fraction were counted. ND, not determined.

Lymphocyte species	Microparticles	Temperature (°C)	Cells binding microparticles (%)	Cells in bottom fraction after centrifugation (%)
Human	AHLG	4	42	44
Human, preincubated with anti-(human lymphocyte) globulin	AHLG	4	10	9
Horse	AHLG	4	5	8
Human	FITC-dextran	4	2	ND
Human	AHLG	23	58	55
Human, preincubated with anti-(human lymphocyte) globulin	AHLG	23	12	34
Horse	AHLG	23	13	10
Human	FITC-dextran	23	3	3
Human	Horse globulin	23	4	ND

described in the Materials and Methods section. The cells with affinity for the protein were easily detected under the microscope by the rosette of fluorescing particles appearing round the surface. The binding increased with temperature (Table 1). About 42% of the cells were bearing AHLG-particles after 1 h incubation at 4°C. The binding was increased to about 58% if the incubation was performed at 23°C. The variability of the results amounted to ±12%.

Preincubation of the cells with excess of free anti-(human lymphocyte) globulin in phosphate-buffered saline (5 mg/ml) inhibited the binding of AHLG-particles. As shown in Table 1, only 10–12% of the cells bore microparticles after such a pre-incubation.

Non-specific binding of the microparticles to the cell surface was small, as investigated by incubating human lymphocytes with microparticles containing fluorescein isothiocyanate-labelled dextran. Only 3% of the cells bound these particles. In these experiments, the buffer contained 1 mg of albumin/ml.

The specificity of the reaction with AHLG-particles was further tested with horse lymphocytes, as anti-(human lymphocyte) globulin is prepared from immunized horses. Incubations were performed under the same conditions as with the human cells. At 4°C, 5% of the cells bound the particles, similar to the value obtained with fluorescein isothiocyanate-labelled-dextran particles. At 23°C, the cross-reaction increased to 13% (Table 1).

To exclude the possibility that anti-(human lymphocyte) globulin binds via the constant part of the immunoglobulin molecule (the Fc part) to Fc receptors on the human lymphocytes, the binding of normal horse globulin incorporated in microparticles was controlled. As shown in Table 1, an insignificant amount of cells (4%) bound these horse globulin-microparticles, corresponding to the non-specific binding of particles.

Separation of anti-(human lymphocyte) globulin-binding cells

The density of lymphocytes is about 1.07 g/cm^3 as determined by Ficoll-gradient centrifugation (Gorczynski *et al.*, 1970; Williams *et al.*, 1972). The density of the microparticles can be varied depending on the preparation procedure. Generally, microparticles with a total concentration of monomer of 8% in the water phase and a relative concentration of the cross-linking agent of 25% can be obtained with a density of above 1.08 g/cm^3 (Ekman *et al.*, 1976) and with a narrow size distribution after washing and careful centrifugation. In this case the density of the microparticles was about 1.12 g/cm^3 and the cell–particle complexes could be separated from the non-binding cells by gradient centrifugation on Lymphoprep, the density of which was 1.077 g/cm^3. Cells in both top and bottom fractions were collected, and the total number of cells and the percentage of fluorescing cells were estimated. The percentage of cells found in the bottom fraction correlated well with the number that was initially found binding microparticles, as shown in Table 1. When the cells were incubated with free anti-(human lymphocyte) globulin at 23°C, however, the yield in the bottom fraction was unexpectedly high. At this temperature, an increased tendency to cell agglutination was noticed, and aggregates were detected in the bottom fraction.

About 85% of the cells found in the bottom fraction had particles on their surfaces. The remaining fraction consisted of empty dead cells (as assessed by Trypan Blue exclusion), and contaminating erythrocytes and macrophages, which also passed through the Lymphoprep gradient. In a control experiment, when the same number of cells was centrifuged without particles in the same manner, 7% of the cells were found in the bottom fraction.

Some cells in the top fraction were found with microparticles on their surfaces. The microparticles were relatively small and the amount on each cell was low. The number of these cells always amounted to less than 10% of the cells present in the top fraction. The total recovery of cells after the gradient centrifugation with or without preincubation with microparticles was about 75%. The loss of cells may be due to adhesion to pipettes and tubes, as the volumes used were relatively small.

The viability of the cells, as tested by Trypan Blue exclusion, was high (more than 90% after the treatment with microparticles and centrifugation). Normally, the incubation with microparticles was preceded by centrifugation at low speed (50*g*). Some experiments were also done by incubating cells and microparticles overnight without pre-centrifugation. The same results were obtained as with the standard procedure and the viability was decreased only slightly.

Correlation between anti-(human lymphocyte) globulin-binding cells and T-cells

Anti-(human lymphocyte) globulin is clinically used as immunosuppressive agent in, for example, patients who have undergone renal transplantation. The treatment causes a suppression of the T-cell subpopulation, as the number of peripheral blood cells spontaneously forming rosettes with sheep erythrocytes is decreased (Bach *et al.*, 1969; Lundgren *et al.*, 1976; Quadracci *et al.*, 1976).

The number of cells reacting with AHLG-particles correlates well with the normal content of T-cells in human peripheral blood, 52–76% (Jondal *et al.*, 1972; Barker *et al.*, 1975; Lundgren *et al.*, 1976). A closer investigation showed that the amount of anti-(human lymphocyte) globulin-binding cells in a specific sample correlated well with the number of T-cells forming rosettes with sheep erythrocytes (Table 2). In addition, the number of T-cells decreased remarkably after separation of the anti-(human lymphocyte) globulin-binding cells. As shown in Table 2, only a small amount of T-cells was found among the cells that did not bind AHLG-particles and remained in the top layer after the Ficoll-gradient centrifugation. The values in Table 2 are the mean values from several experiments with lymphocytes from one person (variability ±1–2%).

Table 2. *Number of cells binding anti-(human lymphocyte) globulin in relation to the number of T-cells*

Peripheral human lymphocytes were incubated either with AHLG-particles or with sheep erythrocytes. The cells that bound the particles were separated on a Ficoll gradient and the empty cells in the top layer were incubated a second time with AHLG-particles or sheep erythrocytes.

	Cells binding (%)	
	AHLG-particles	Sheep erythrocytes
Total cell sample	46	47
AHLG-particle-depleted cells	10	8

Concanavalin A-particles

When lymphocytes and concanavalin A-particles were incubated for 1 h at 4°C, large aggregates of cells and particles were formed and very few free cells could be detected. Obviously, the concanavalin A in the microparticles is sufficiently exposed on the surface to initiate cell agglutination (Rutishauser & Sachs, 1974). The effect seems to be specific for concanavalin A-particles, as AHLG-particles do not cause the same aggregation of the cells. Binding and agglutination were drastically inhibited when the microparticles were preincubated with 0.1 M-α-methyl mannoside.

Conclusions

Microparticles of polyacrylamide with immobilized cell-reactive macromolecules are useful for detecting and separating cells. The macromolecules are exposed on the surface of the particles, they preserve their biological activity and they are not modified by covalent coupling reactions. They can also easily be combined with other substances bearing the label wanted for detecting the particles. In our study we used fluorescein isothiocyanate-labelled dextran, but obviously a range of different fluorescing agents can be used, as well as, for example, enzymes for histochemical detection or radioactively labelled substances for radioautographic detection or quantitative analysis. The diameter of the particles can be varied according to need, e.g. smaller than 1 μm for the detection of specific cell-surface structures, or larger (1–5 μm) for cell separations in density gradients. The method shows a high degree of specificity and the binding experiments are easy to perform. Further, the microparticles are stable over several months of storage.

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