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Immunological Biochips for Studies of Human Erythrocytes

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Abstract—Immunological microarrays (biochips) for detecting erythrocyte surface antigens, viz., blood group antigens (A, B, 0) and Rhesus system antigens (D, E, e, C, and c), are described. The biochips represent transparent plastic supports onto which 1.5-mm spots of specific immobilized antibodies (IgM) are coated in different dilutions. The volume of tested blood samples is rather small (1–2 μl). Binding of erythrocytes to antibodies immobilized on the biochips is specific and allows further morphological analysis of bound cells. Analysis of the dynamics of cell detachment from biochip spots using a microfluidic chamber at different flow rates of the washing solution showed that combination of a biochip with a microfluidic chamber is a promising approach to concentration of cells of various immunotypes even if their content in the mixture is very low.

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At present, protein microarrays (biochips) are widely used in basic and applied research. The main advantage of microarray technology is the possibility to perform simultaneous analysis of similar reactions using small amounts of the experimental material [1].

A protein biochip represents a solid surface (support) onto which immobilized proteins are coated in strictly set areas (spots); these proteins bind specifically the tested molecules present in the sample [2]. Nitrocellulose and chemically modified glass [1, 3, 4], less frequently gold [5, 6], polystyrene [7] and Teflon [8] are used as substrates for antibody microarrays. Among the most popular types of protein microarrays there are biochips with immobilized antibodies. These biochips can be used for simultaneous identification of a wide range of specific antigens [9].

For the first time, the ability of cells to bind to surface antigen-specific antibodies immobilized on biochips was reported by Chang [10]. In the past decade, antibody microarrays for cell phenotyping were developed independently by several groups of investigators [5, 11–18]. Most of these studies were devoted to the design of microarrays for identification of surface antigens on leucocytes [11–17], but the possibility to use them for immunophenotyping of red blood cells was also considered. Binding of erythrocytes on biochips with immobilized antibodies against their specific antigens was first described by Quinn et al. [19]. Other developments in this field include design of microarrays for grouping blood (AB0) [18] and Rhesus system antigens [5, 18].

The majority of technologies for identification of surface red blood cell antigens are based on the ability of the latter to agglutinate in the presence of antibodies [20]. In addition to agglutination in a bulk solution, agglutination on solid surfaces [21, 22] or in gels [23] is used. The salient advantage of red blood cell immunophenotyping on biochips over other techniques is the possibility to simultaneously detect antigens of blood group (AB0) and Rh system (C, c, E, e) and to use much smaller amounts of antibodies and tested samples.

However, the use of biochips is more appropriate for research purposes (e.g., study of cell interactions with antibodies immobilized on solid surfaces) than for practical applications, such as clinical diagnostics. In this respect, red blood cells represent a convenient biological model, firstly because they are isolated from the blood more easily than other blood constituents and secondly, because the repertoire of their surface antigens is well established [24].

In previous studies, nonspecifically bound cells were removed from the biochip surface by washing with buffer solutions. However, the use of microfluidic chambers for studying the effects of flow and surface conditions (shear stress) on binding of lymphocytes to solid surfaces coated with immobilized antibodies was described in one paper only [17].

Morphological analysis of bound cells is often hindered because the majority of biochips for identification of surface antigens are made of nontransparent materials. At the same time, immunophenotyping and morphological analysis are still the most popular proce-

dures in diagnostics of blood pathologies. Therefore, design of biochips combining identification of surface antigens with antibody staining and morphological analysis is a task of paramount importance. Such a combination is especially beneficial, e.g., in the analysis of lymphoid and myeloid cells. The results of our red blood cell studies provide additional evidence in favor of high clinical utility of this approach.

The aim of the present study was to develop a microarray technology for immunophenotyping human erythrocytes on biochips utilizing surface (blood group (AB0) and Rhesus system (D, E, e, C, c) antigens with simultaneous morphological analysis of bound cells and to study the effect of the flow rate of the washing solution on the binding density of tested cells.

EXPERIMENTAL

Materials. Murine monoclonal (anti-A, anti-B, anti-D, anti-C, anti-E, anti-c, anti-e) antibodies (IgM) (Tsoliclone™ diagnostic kits, Hematolog LTD, Russia) were used without additional purification. Fat-free dry milk was from Kroger, USA; gradient centrifugation solution (Ficoll-Paque), EDTA, phosphate buffer (PBS) pH 7.4, 0.05% Tween-20, and methanol were from Sigma Aldrich, USA.

Preparation of biochips. Plastic sheets used as substrates for biochips were from Fisher Scientific, USA. Antibody solutions (0.5 μl) of different dilutions were coated onto biochips in strictly specific spots using automatic pipettes. The biochips were loaded into an incubator at 100% humidity, stored overnight at +4°C, dried in air, placed into air-tight containers with a desiccant (Silicagel) and frozen to –26°C to ensure preservation of their essential characteristics for at least 12 months.

In flow-cell studies, biochips were prepared by spotting solutions of homologous antibodies in series at dilution from 1/1 to 1/32. Each biochip contained six such spots.

If flow cells were not used in the experiments, biochips were prepared by spotting antibody solutions (anti-A, anti-B, anti-D (1/4–1/256), anti-c, anti-C, anti-e and anti-E (1/1 –1/256)) onto the same substrate (in series). Each biochip contained 57 spots.

Binding of erythrocytes on biochips. Biochips were fixed in Petri dishes, washed once with 1% defatted dry milk in PBS and thrice with 0.05% Tween-20. Then a fresh portion of 1% fat-free dry milk in PBS was added to the dishes and the mixture was incubated for 1 h at ambient temperature upon occasional shaking. After threefold washing of the solution with 0.05% Tween-20, the biochips were washed with PBS to remove the detergent and incubated with erythrocyte suspensions in PBS at ambient temperature. Erythrocyte concentration and suspension volume were selected so as to ensure complete precipitation of the cells to a monolayer. The optimum concentration of cell suspensions (2.5 ml) in 35-mm Petri dishes was 5.5×10^6 cells/ml. Blood sample volume was 1–2 μl.

In some experiments, nonspecifically bound cells were removed by several-fold washing of biochips with PBS. The purity of cell preparations was controlled by inverted-stage microscopy. The areas outside biochip spots should contain no bound cells. The biochips were dried in air.

In some experiments, incubation and washing of biochips were performed in a flow cell.

Both whole blood and red blood cells isolated from heparinized peripheral blood were used. Preliminary experiments did not establish any difference between the blood samples.

Morphological analysis of bound erythrocytes. Erythrocytes bound to immobilized antibodies were fixed with methanol for 8 min, stained according to Romanowsky–Giemsa, washed with water and dried. Prior to morphological analysis, the biochips were removed from Petri dishes and mounted on glass slides with Scotch tape.

Estimation of binding density in biochip spots. Each spot was photographed with a digital camera Olympus SP-350 installed on an OPTON-028 microscope (OPTON, Germany). To determine binding densities of red blood cells in biochip spots, at least three zones (100 × 100 μm) were selected on each micrograph and the number of bound erythrocytes in them was determined in the middle and at the edge of each spot as well as between the spots. The binding density values thus obtained were averaged.

Flow cell. The effect of binding density of red blood cells on biochips on the flow rate of the washing fluid was studied in a flow cell with a capillary cross-section of 8 mm^2 (Fig. 1). The flow cell was linked to a syringe pump Model 11 Plus (Harvard Apparatus, Inc., USA); the flow rate was 40000 μl/min. The flow cell was mounted onto the sample stage of an optical microscope in such a way that the biochip forming one of the channel walls occupied the bottom position. The flow cell was filled with a 1% degassed solution of bovine serum albumin or fat-free dry milk in PBS and incubated for 1 h after which PBS was passed through the chamber for 5 min at a flow rate of 200 μl/min. Then, a 0.3-ml aliquot of heparinized blood in PBS (erythrocyte content 1.5×10^7 cells/ml) was injected additionally into the chamber and the mixture was incubated for 10–20 min in the absence of the flow. After removal of nonspecifically bound cells by washing with PBS, the spots were photographed. In binding studies, the flow rate was increased stepwise. Imaging of each spot was performed at least 1 min after the change of the flow rate through the flow cell. The number of cells per unit of spot square was determined as described above.

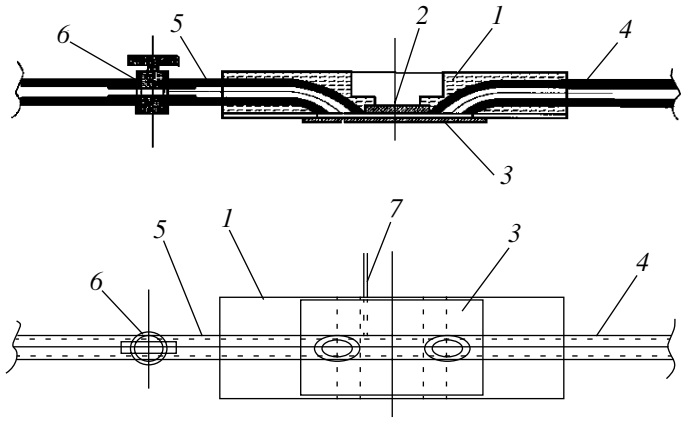

Fig. 1. A schematic representation of a flow cell. *1*—Flow cell framework; *2*—glass; *3*—biochip; *4*—inlet pipe; *5*—outlet pipe; *6*—valve; *7*—additional capillary tube. Total channel length is 33 mm, width, 6 mm, and depth, 1.3 mm. Internal inlet and outlet pipe diameter is 3 mm. The distance between the hole edges is 21 mm.

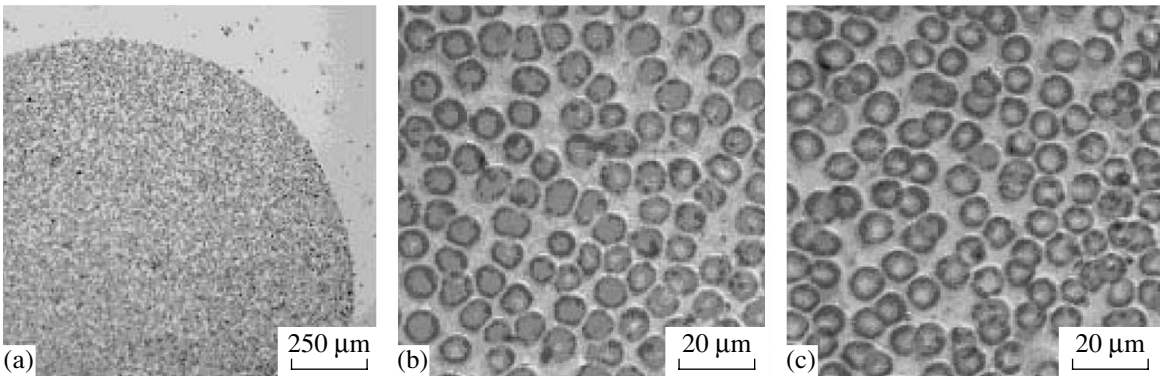

Fig. 2. Binding of healthy donor erythrocytes (blood group A(II)Rh+) to anti-D antibodies in biochip spots and in smears. Romanowsky–Giemsa staining. (a, b) Micrographs of biochip spots containing bound erythrocytes. Magnification, ×37.5 and ×600. (c) Micrograph of erythrocytes from the same donor in smear (on glass). Magnification, ×600.

RESULTS

Morphological analysis of erythrocytes bound on biochips. Biochips with immobilized anti-A, anti-B and anti-D antibodies (IgM) were incubated with Rh-positive (Rh(D)+) and Rh-negative (Rh(D)–) erythrocyte suspensions (blood groups O(I), A(II), B(III) AB(IV). After removal of nonspecifically bound cells, erythrocytes were fixed with methanol and stained according to Romanowsky–Giemsa (Fig. 2). The spots with immobilized anti-A antibodies contained only bound erythrocytes, viz., A(II) and AB(IV), while B(III) and AB(IV) erythrocytes were present in spots with anti-B antibodies. The spots with anti-D antibodies contained exclusively Rh-positive erythrocytes. These findings point to the lack of cross-reactivity of the tested antibodies.

Figure 2 shows the results of a microassay of healthy donor erythrocytes (A(II)Rh+) in smears (Fig. 2c) and on biochips with immobilized anti-D antibodies (Figs. 2a, 2b). As can be seen, the staining intensities in smear and on biochips are identical. The micrographs of patients' blood erythrocytes immobilized on biochips (Fig. 3) displayed typical morphological signs of poikilocytosis and anisocytosis (a patient with chronic renal failure kept on long-term hemodialysis) (Fig. 3a), oval (a patient with hereditary ovalocytosis) (Fig. 3b) or target (a patient with thalassemia) shape (Fig. 3c).

These findings point to the feasibility of parallel immunophenotyping and morphological analysis of red blood cells on immunological biochips with transparent support.

Effect of flow rate of the washing fluid on the density of the bound cells. Flow-cell studies made possible to select optimal conditions for washing biochips after their preincubation with erythrocytes, so that only nonspecifically bound cells were removed from the chip surface, while antibody-bound cells remained in the spots. To meet this goal, we studied the correlation between the number of erythrocytes bound on biochips and the flow rate of the washing fluid.

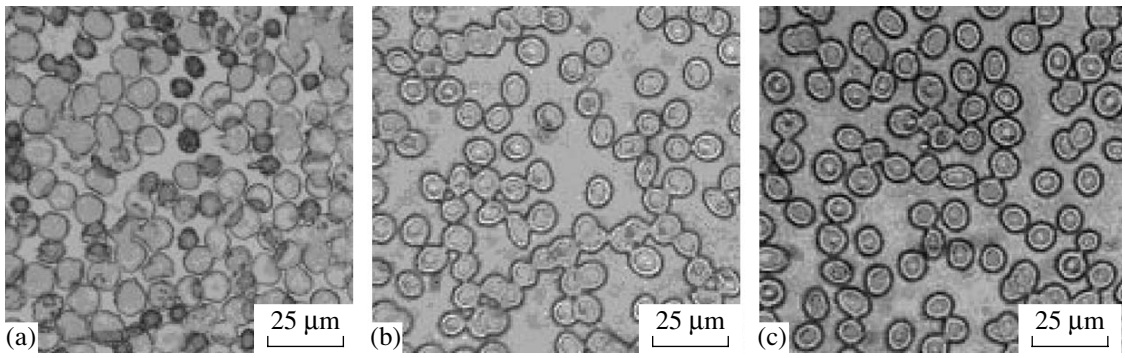

Fig. 3. Morphological changes in erythrocytes immobilized on biochips. Romanowsky–Giemsa staining. (a) Binding of erythrocytes from a patient with chronic renal failure, kept on long-term hemodialysis, to the biochip spots containing anti-A antibodies. (b) Binding of erythrocytes from a patient with hereditary ovalocytosis to the biochip spots containing anti-B antibodies (about 7% of erythrocytes have a characteristic oval shape). (c) Binding of erythrocytes from a patient with thalassemia to the biochip spots containing anti-A antibodies. Magnification, ×600.

Under the experimental conditions, nonspecifically bound cells were detached from the biochip surface at the flow rate of 4.5–6 s^{-1}, which correlated with the shear stress value (18–24 mPa). Erythrocytes devoid of the corresponding surface antigens, and, as a consequence, nonspecifically bound to the corresponding antibodies and erythrocytes bound outside biochip spots (background binding) were detached at the same rates (Fig. 4).

Figure 5 shows the effect of binding density of erythrocytes on biochips with anti-c, anti-C, anti-e and anti-E antibodies on the flow rate of the washing fluid. Each experimental curve corresponded to one antibody dilution. The measurements were performed on erythrocytes from two donors (immunophenotypes ccDEe and CCDee).

The curves reflecting the effect of the flow rate of the washing fluid on binding density of red blood cells

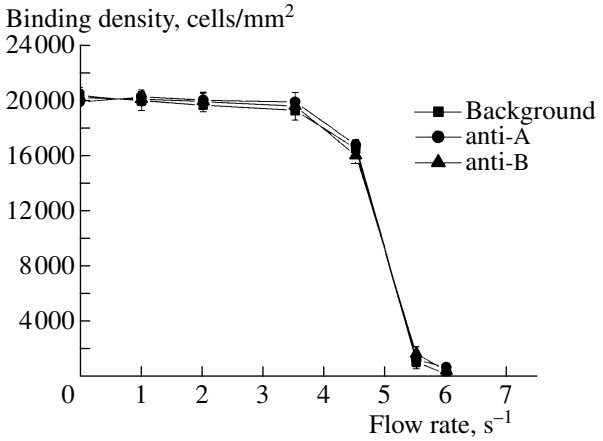

Fig. 4. Effect of the flow rate of the washing fluid on the binding density of erythrocytes (O(I)Rh+) bound nonspecifically to biochip spots containing anti-A and anti-B antibodies and outside the spots (background) (mean ± SEM from three independent measurements).

(ccDEe) on biochip spots with immobilized anti-E and anti-E antibodies are presented in Figs. 5a, 5b. In none of the cases, the maximum rate of filling the spots with erythrocytes at the minimum flow rate of the washing fluid depended on antibody dilution and differed only slightly from the maximum level (20000 ± 1000 cells/mm^2). For each antibody dilution, the density of bound cells decreased with the increase in the shearing flow rate of the washing fluid. When anti-E antibodies were used at dilution up to 1/16, binding density did not depend on the flow rate (Fig. 5a), while at dilution 1/32 it dropped down with the increase in the flow rate. At dilutions 1/8 and 1/16 of anti-E antibodies (Fig. 5b), the binding density did not change after a small initial decrease until the maximum shearing rate (450 s^{-1}) was reached. In spots with anti-E and anti-E antibodies, initial binding density was insensitive to the antibody concentration.

Figure 5 shows the effect of binding densities on the flow rate of the washing fluid in the example of CCDee erythrocytes on biochips with immobilized anti-C antibodies (Fig. 5c) and ccDEe erythrocytes on biochips with immobilized anti-C antibodies (Fig. 5d). Under these experimental conditions, the maximum binding density varied widely depending on antibody dilution, while initial binding densities on anti-C and anti-C antibodies did not change. In biochip spots with anti-C (dilution up to 1/8) and anti-C (dilution 1/4) antibodies, the binding densities did not change with the increase in the flow rate, while at higher dilutions this parameter was notably decreased with the increase in the flow rate of the washing fluid.

The binding densities of erythrocytes in biochip spots with anti-A and anti-B antibodies (dilution up to 1/64) (Figs. 6a, 6b) did not change with the increase in the flow rate from 0 to 450 s^{-1}, i.e., maximum for this design of the flow cell. However, in the case of anti-D antibodies (dilution up to 1/32), this parameter was slightly decreased (Fig. 6c). Based on these findings, we studied the effect of antibody dilution (from 1/4 to

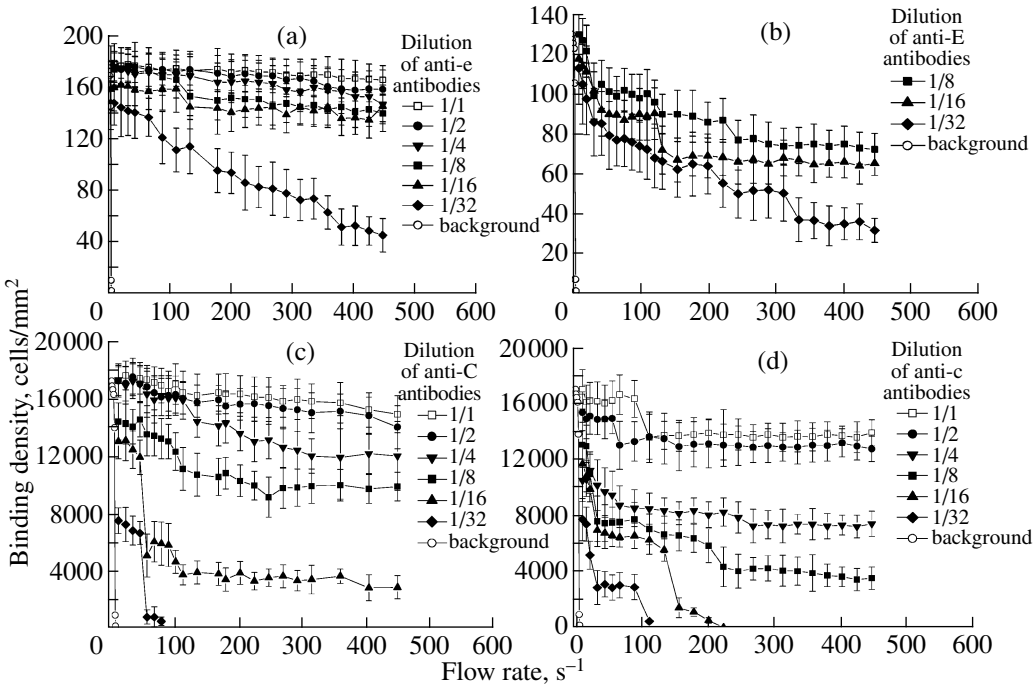

Fig. 5. Effect of the flow rate of the washing fluid on the number of erythrocytes (immunophenotype ccDEe) bound to anti-E antibodies in biochip spots (a); number of erythrocytes (immunophenotype ccDEe) bound to anti-E antibodies in biochip spots (b); number of erythrocytes (immunophenotype CCDee) bound to anti-C antibodies in biochip spots (c); number of erythrocytes (immunophenotype ccDEe) bound to anti-C antibodies in biochip spots (d) (mean ± SEM from three independent measurements).

1/256) on the binding density of erythrocytes on biochips with immobilized anti-A, anti-B and anti-D antibodies after elimination of nonspecifically bound cells by dipping into a washing solution. Erythrocytes isolated from blood samples from 12 different donors were studied, 10 of which were positive for antigen D. The results of these studies are summarized in Fig. 7.

As can be seen (Figs. 7a, 7b), the binding density of erythrocytes in biochip spots containing anti-A and anti-B antibodies did not change at dilutions up to 1/32 but decreased at higher dilutions.

The analysis of 10 blood samples from Rh(D)+-donors revealed that the binding density of erythrocytes was variable and depended on anti-D antibody dilution (Figs. 7c, 7d). This effect can be attributed to a considerable variability in the number of antigen D molecules on the erythrocyte surface [20, 24].

If biochip spots contained anti-C, anti-c, anti-E or anti-e antibodies, bound erythrocytes were completely eliminated after dipping the biochips into buffer solutions. In all probability, binding of erythrocytes to antibodies specific against surface antigens (C, c, E, and e) was not so strong as in the case of anti-A, anti-B, and anti-D antibodies; washing of biochips resulted in the detachment of all bound erythrocytes. Similar results were obtained in experiments reported in [5], where the binding density of erythrocytes on biochips containing immobilized anti-C, anti-c, anti-E and anti-e antibodies (IgG) was rather low.

Determination of relative content of erythrocytes in mixed cell suspensions. Biochips can also be used for determining the percentage of antigen-expressing cells in erythrocyte suspensions (Fig. 8). To this end, biochips with immobilized anti-D antibodies were incubated with suspensions of Rh(D)+ and Rh(D)– erythrocytes (1 : 4 and 1 : 1) from two different donors in the absence of the washing fluid and without stirring. It was found that after removal of nonspecifically bound cells, the filling of biochip spots with erythrocytes was incomplete. The ratio of binding density in biochip spots to the maximum binding density taken for 100% (20000 cells/mm^2) was 20 ± 2 and 50 ± 2%, respectively (Figs. 8a, 8b).

Concentration of antigen-expressing erythrocytes in biochip spots. The use of a flow cell allows concentration of erythrocytes expressing specific surface antigens. By alternating incubation in the absence (5–10 min) and in the presence of a short-term (3–5 s) flow at a rate sufficient for the detachment of nonspecifically bound cells, one can attain maximum filling of the spots with cells expressing appropriate antigens within several incubation cycles. The results of filling biochip spots with anti-D antibodies after incubation of biochips in a flow cell with Rh(D)+ and Rh(D)– erythrocyte suspensions (1 : 4) (15 incubation–washing cycles) are depicted in Fig. 8c (for comparison, the same sample after one incubation–washing cycle is shown in Fig. 8a).

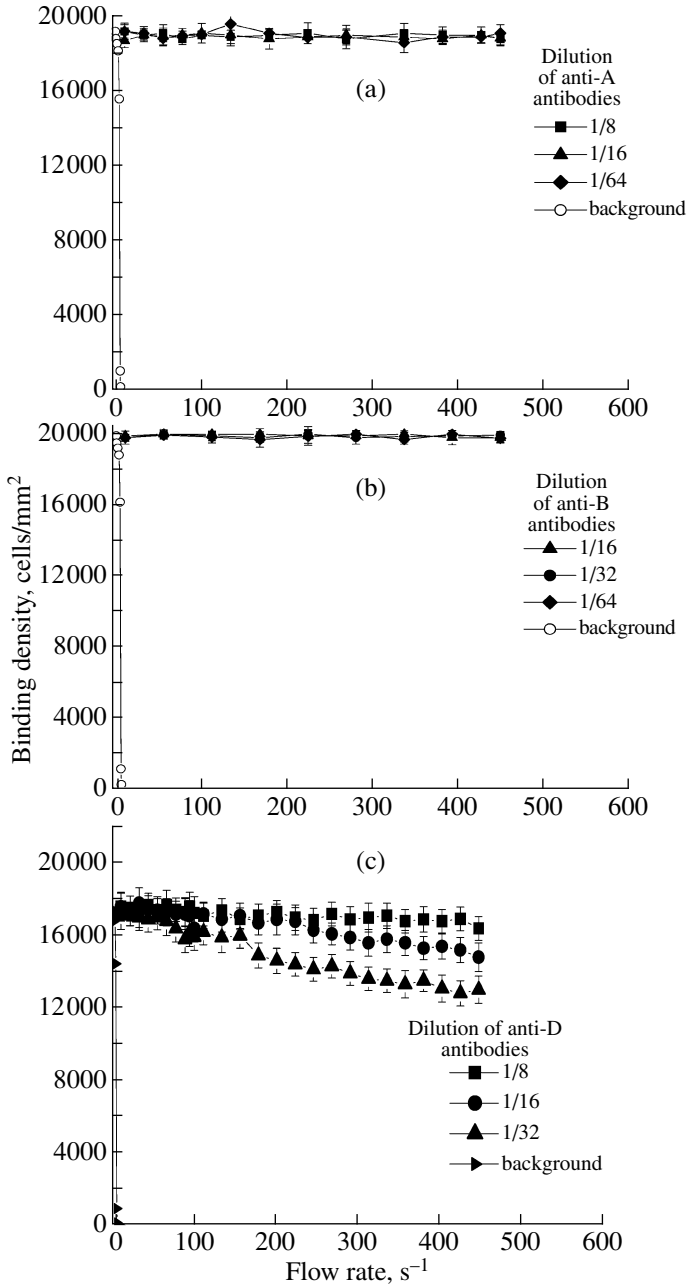

Fig. 6. Effect of the flow rate of the washing fluid on the number of erythrocytes (group A(II)) bound to anti-A antibodies in biochip spots (a); number of erythrocytes (blood group B(III)) bound to anti-B antibodies in biochip spots (b); number of erythrocytes (blood group I(0)Rh+, immunophenotype CCDee) bound to anti-D antibodies in biochip spots (c) (mean ± SEM from three independent measurements).

If a suspension contained 50% of erythrocytes expressing a specific antigen, no less than 4–5 incubation–washing cycles were required to achieve close-to-maximum filling of the spot surface (cf. 10–15 cycles for 20% erythrocyte suspensions). Cell concentration in biochip spots can also be attained by passing dilute erythrocyte suspensions (10^5 cells/ml) at the flow rate of 0.6–1.3 s^{-1} over the biochip surface. A similar approach was used for concentration of erythrocytes on a solid surface coated with immobilized antibodies [17]. In this case, the maximum binding density of cells on the spot surface was reached within 1–1.5 h.

DISCUSSION

The use of transparent supports in antibody microassays allows combined application of standard staining techniques and morphological analysis to cells immobilized on biochips. Figures 2 and 3 show that staining of erythrocytes in smears and on biochips occurs with similar intensity. The micrographs of bound erythrocytes from patients with chronic renal failure, ovalocytosis and thalassemia display pronounced morphological changes manifested as poikilocytosis, anisocytosis, ovalocytosis, target erythrocytes, etc.

Our study demonstrated the feasibility of simultaneous identification of human red blood cells on biochips and their morphological analysis. This method can find wide clinical application, e.g., in the study of lymphoid and myeloid cells.

The dynamics of changes in the number of erythrocytes in biochip spots depending on the flow rate of the washing fluid is shown in Figs. 4–6. As can be seen, at the flow rate of 7 s^{-1} virtually all nonspecifically bound cells were detached from the biochip surface (Fig. 4). In contrast, at flow rates below 10–20 s^{-1}, all specifically bound cells remained on the biochip (Fig. 5).

The number of cells remaining on biochip spots depends on three different parameters: concentration of antibodies, their affinity, and the assortment of specific antigens on the erythrocyte surface. A comparative study of biochips containing the same antibodies at different dilutions revealed that the smaller the concentration of antibodies, the lower is the flow rate sufficient for the detachment of erythrocytes from biochip spots. The data obtained provide more information about optimum dilutions for immobilization of anti-c, anti-C, anti-e, and anti-E antibodies. At dilutions <1/16 (anti-e, anti-E), 1/4 (anti-c), and 1/8 (anti-C), the use of antibodies is inexpedient, since further increases in antibody concentration do not induce any further increase in the number of erythrocytes bound on biochips (Fig. 5).

Interestingly, the use of different antibodies in identical dilutions for quantitative determination of bound erythrocytes gave different results. It was found that erythrocytes bind differently to antibodies, which can be attributed to different affinities of the respective antibodies or different levels of expression of the corresponding antigens on erythrocyte surface.

The rate of expression of Rh antigens on erythrocyte surface is lower than that of antigens A and B (810000–1170000 per erythrocyte) [20, 24]. Thus, erythrocytes (immunophenotype CCDee) express on their surface 46000–56000 antigens C, 18000–24000 antigens e

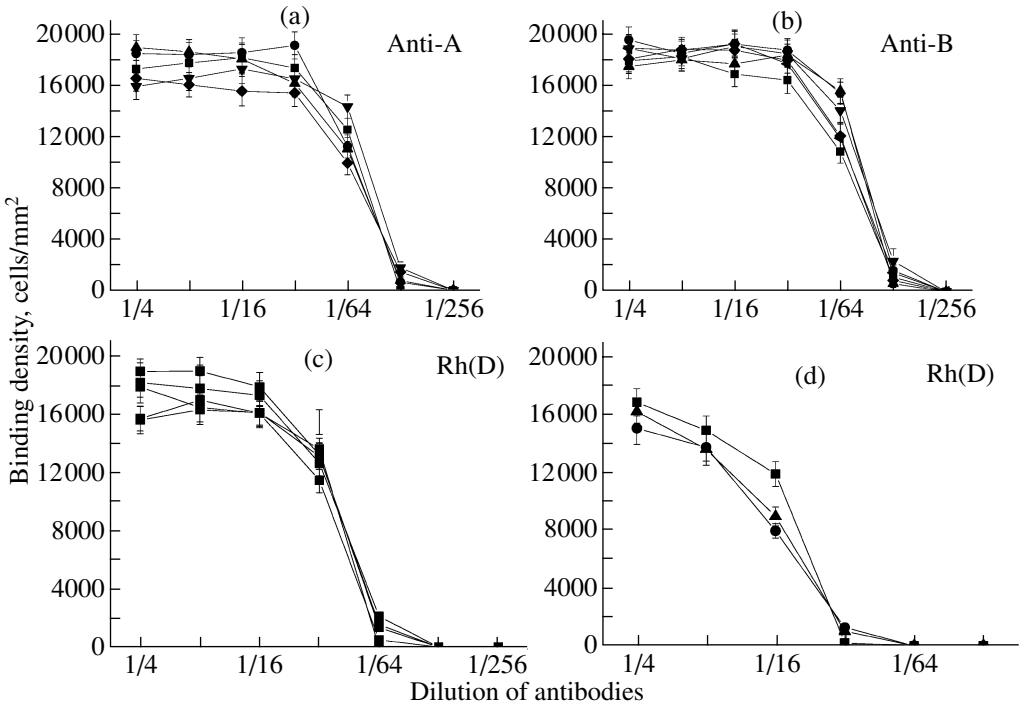

Fig. 7. Effect of antibody dilution on the binding density. (a) Binding density of erythrocytes (blood groups A(II) and AB(IV)) on anti-A antibodies. (b) Binding density of erythrocytes (blood groups B(III) and AB(IV)) on anti-B antibodies. (c, d) Binding density of Rh(D)-positive erythrocytes on anti-D antibodies (means ± SEM from three independent measurements). Each curve corresponds to erythrocytes from the same donor. The biochips were washed with a buffer solution.

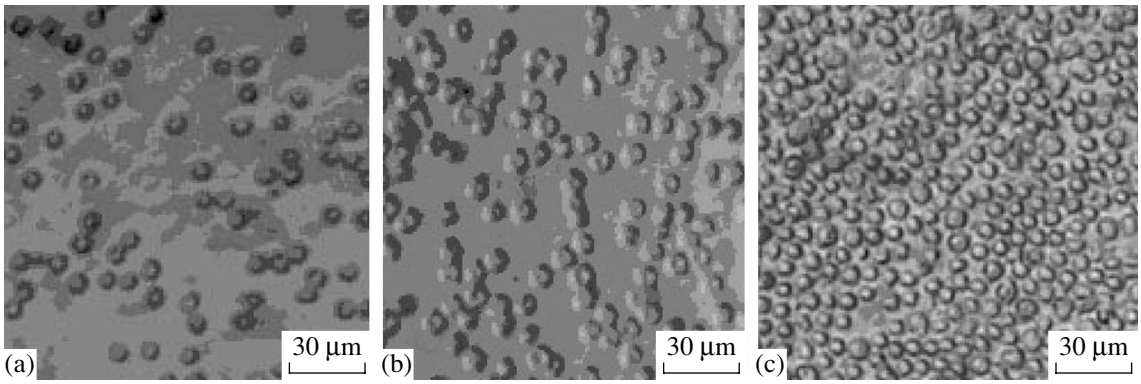

Fig. 8. Filling of spots containing anti-D antibodies with (Rh(D)+) erythrocytes during incubation of biochips with mixed cell suspensions containing Rh(D)+ and Rh(D)– erythrocytes from two different donors in a microfluidic chamber. (a) Partial filling of spots. Rh(D)+ to Rh(D)– ratio is 1 : 4, one incubation/washing cycle. (b) Partial filling of a spot after one incubation/washing cycle, Rh(D)+ to Rh(D)– ratio is 1 : 1. (c) Close-to-maximum filling of the spot with bound erythrocytes after 15 incubation/washing cycles. Rh(D)+ to Rh(D)– ratio is 1 : 4. Magnification, ×300.

and 14000–19000 antigens D (on a per cell basis), while erythrocytes (immunophenotype ccDEe) express 70000–85000 antigens C, 13000–14000 antigens e, and 14000–16000 antigens D [20, 24]. This finding sheds additional light on the fact that binding of erythrocytes to immobilized anti-A and anti-B antibodies in biochip spots does not depend on antibody dilution in the range from 1 to 1/32 (Figs. 6a, 6b), whereas the binding density of erythrocytes on biochips with anti-C, anti-c, anti-E, and anti-e antibodies decreases even at 1/4–1/16 dilutions (Fig. 5).

The number of antigens D expressed by erythrocytes (immunophenotypes CCDee and ccDEe) is commensurate with that of antigens c, C, e, and E [20, 24]. At the same time, the detachment of erythrocytes bound to anti-D antibodies on biochips (Fig. 6c) required much higher flow rates than the detachment of erythrocytes bound to anti-c, anti-C, anti-e, and anti-E antibod-

ies (Fig. 5). Moreover, erythrocytes bound to anti-D antibodies were not eliminated from the biochip surface even after washing (Figs. 7c, 7d). In this case, bound cells were absent even from spots containing non-dilute anti-c, anti-C, anti-e and anti-E antibodies. These findings testify to higher affinity of anti-D antibodies for red blood cells in comparison with anti-c, anti-C, anti-e, and anti-E antibodies.

Fig. 8 shows the results of filling the spots containing anti-D antibodies during incubation of biochips with suspensions of Rh(D)+ and Rh(D)– erythrocytes used in different ratios. As can be seen, the binding density of erythrocytes on immobilized antibodies directly correlates with the number of cells carrying specific antigens.

Incubation of biochips with polymorphous erythrocyte suspensions without stirring was accompanied by precipitation of erythrocytes on the spot surface in amounts corresponding to their percentage in the suspension. Under these conditions, only erythrocytes expressing appropriate surface antigens could bind to antibodies, while cells devoid of surface antigens remained unbound to the biochip surface, but shielded antibodies from attacks of other cells. After removal of nonspecifically bound cells, the rate of filling of biochip spots with specific antibodies correlated with their percentage in mixed cell suspensions expressing the corresponding antigen.

Analysis of erythrocyte suspensions containing cells of different immunophenotypes in flow cells enables not only the separation of cells expressing different antigens, but also their concentration (Fig. 8c). The use of biochips for concentrating of definite types of cells opens up fresh opportunities for further analyses.

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