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Immunological Biochips for Parallel Detection of Surface Antigens and Morphological Analysis of Cells

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Abstract—A biochip for detecting 26 cluster differentiation (CD), HLA-DR and IgM antigens on lymphocyte surface is described. The biochip, which represents a microarray of antibodies (IgG) against a panel of selected antigens immobilized on transparent plastic surfaces in 1.5-mm spots, was used for the study of normal and neoplastic lymphocytes and can also be used for determining percent of cells expressing definite surface antigens in lymphocyte suspensions. The results are consistent with data obtained by flow cytometry. The novel biochip technology entails a combination of conventional staining of cells immobilized on biochips and morphological analysis.

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The past decades have been marked by rapid progress in microarray technology on biochips, i.e., new-generation analytical and diagnostic systems operating on the molecular recognition principle and allowing parallel multianalyte immunoassays in small volumes of the experimental material [1].

Under the term "biochip" is understood a solid surface (support) onto which molecules able to interact specifically with tested substances are coated in strictly specific spots [2]. At present, biochips are gaining increasing popularity as valuable tools for immunophenotyping large repertoires of antigens [3]. Biochips are prepared from different materials; immobilization of antibodies is achieved at the expense of covalent binding on solid surfaces [1, 4–7] or adsorption [8, 9].

For the first time, binding of cells on a biochip coated with immobilized surface-specific antibodies was described by Chang [10]. More recently, antibody microarrays for detecting surface antigens were developed by several independent groups of investigators [5, 11–18]. The majority of these studies were devoted to the design of immobilized antibody-based biochips for detecting surface antigens of leukemias [11–17].

Identification of cluster differentiation (CD) antigens expressed on the cell surface is a critical step in diagnostics of lymphomas and leukemias [19]. In modern studies, flow cytometry is used as a method of choice for immunophenotyping leucocytes. However, this procedure has one serious disadvantage, that is, it enables the detection of only a limited number of clinically important antigens. In contrast, the use of the biochip technology allows parallel detection of large repertoires of surface antigens, the only difference between different microarray models being in the number of antibody-containing spots on the biochip surface.

Yet another crucial factor is percentage of cells expressing definite antigens. Therefore, biochips must not only provide selective binding of cells expressing such antigens, but also allow determination of their content.

Antibody-based microarrays (biochips) differ in the repertoires of immobilized antibodies [6, 11–13, 20]. The biochips developed by Kato et al. [6] contain 22 specific antibodies against CD antigens and were used for blood typing in patients with T-cell acute lymphocytic leukaemia (T-ALL), Burkitt's lymphomas and acute promyelocytic leukemias. A range of CD antigens expressed on the surface of tumor cells were identified, but quantitative determination of the percent of cells expressing each of selected surface antigens was not performed. In the study of Belov et al. [11], a microarray of 48 anti-CD- specific antibodies was used for immunophenotyping of surface antigens in lymphocytes of healthy donors and patients with B-cell chronic lymphocytic leukemias (B-CLL), hairy cell leukemias, mantle cell lymphoma and T-ALL [11]. The percent of CD-expressing cells was established in lymphocytes of healthy donors and B-CLL patients. More recent stud-

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ies by these authors [12, 13] were devoted to identification of different types of tumor cells, e.g., B-cell acute lymphocytic leukaemias (T-ALL), T-cell prolymphocytic leukaemias and promyelocytic leukaemias T-ALL, etc. The antibody microarrays used in these studies enabled immunophenotyping of up to 82 CD antigens.

In addition to immunophenotyping, a crucial role in diagnostics of blood tumors is ascribed to morphological analysis. Existing diagnostic techniques do not allow parallel implementation of these two methods on the same cells as a result of which the diagnosis may be contradictory. Therefore, morphological analysis of cells immobilized on biochips seems to be a reasonable alternative provided that biochips are made of transparent materials.

The aim of the present study was to develop microarrays combining identification of cells expressing various surface antigens with morphological analysis.

EXPERIMENTAL

Materials. The biochips used in this study represented a microarray of murine monoclonal antibodies (IgG) against a large repertoire of human antigens (CD3, CD4, CD5, CD7, CD8, CD10, CD11a, CD11b, CD16, CD19, CD20, CD21, CD22, CD27, CD29, CD31, CD36, CD38, CD44, CD45, CD45RA, CD56, CD71, CD72, CD95, CD98, HLA-DR, and IgM) (all from LLC Sorbent, Moscow). Fat-free dry milk was from Kroger (USA); gradient centrifugation solution (Ficoll-Paque), EDTA, phosphate buffer (PBS) pH 7.4, Tween-20 and methanol were from Sigma Aldrich (USA).

Preparation of cell suspensions. Lymphocytes were isolated from peripheral blood by centrifugation in the density gradient of Ficoll-Paque. The cells were resuspended in PBS containing 0.1% fat-free dry milk, 10% heat-inactivated human blood serum and 1.5 mM EDTA. The per cent content of cells in lymphocyte suspensions was determined in a Gorjaev chamber.

Preparation of biochips. Plasticized polyvinyl chloride sheets (22 × 22 mm) (Fisher Scientific, USA) were used as supports for biochips. Antibody solutions of various dilutions were spotted (0.5 μl) onto a solid surface in strictly specific spots using an automatic pipette. The biochips with immobilized antibodies were loaded into an incubator (100% humidity), stored overnight at +4°C, dried in air, placed into air-tight containers with a desiccant (Silicagel) and frozen at –26°C. Under these storage conditions, biochips fully preserved their basic properties within at least 12 months. The diameter of the spots with immobilized antibodies was 1.5 mm.

After thawing, the biochips were placed into 35-mm Petri dishes and washed once with 1% solution of fat-free dry milk in PBS and thrice with 0.05% Tween-20; then the dishes were filled with a fresh portion of 1% fat-free dry milk in PBS, incubated for 1 h at room temperature upon occasional shaking and washed thrice with 0.05% Tween-20 and a buffer to remove the detergent.

Incubation of biochips with lymphocyte suspensions. Lymphocyte suspensions were transferred to Petri dishes containing fixed biochips and incubated for 30–40 min at room temperature without stirring. Lymphocyte concentration and suspension volume were selected so as to ensure the formation of a monolayer on the bottom of Petri dishes after the sedimentation procedure was complete. The optimum concentration of lymphocyte suspensions (2.5 ml) on 35-mm Petri dishes was 5.5×10^6 cells/ml. After incubation, the biochips were washed severalfold with PBS to remove unbound antibodies. The efficiency of the washing procedure was controlled by inverted-stage microscopy; the areas outside the spots should not contain unbound cells.

Staining of bound lymphocytes with fluorescently labeled antibodies. Prior to staining, two parallel 0.2 mm-wide bands of sticky tape were pasted onto a glass slide. A mixture of 80 μl of fluorescently labeled antibodies with heat-inactivated human blood serum (10%, v/v) was added onto the biochip surface between the bands. The biochip was mounted on the top in such a way that its edges were in contact with the tape and the biochip surface coated with bound cells was directed downwards. To ensure a better adhesion, the biochip was additionally fixed with Scotch tape from above. After 30-min incubation at room temperature, the biochip was separated from the slide, washed thrice with PBS and examined under a fluorescent microscope. Each spot was photographed in normal and UV light using a Kodak DC260 digital camera installed on a Leica DM/RBE fluorescent microscope (Leica, Germany).

Staining and morphological analysis of lymphocytes. Prior to staining according to Romanowsky–Giemsa, the cells were dried, fixed with methanol (10 min), washed with water, dried and pasted onto slides. The morphological characteristics of lymphocytes bound in each spot of the biochip were analyzed under an optical microscope.

Determination of percentage of lymphocytes expressing different surface antigens. Each spot on the biochip was photographed with a digital camera Olympus SP-350 mounted on the OPTON-028 microscope (OPTON, Germany). To determine the binding density of lymphocytes, no less than three sites of predetermined size (e.g., 100 × 100 μm) were selected on all micrographs. Average binding density was determined for each spot.

In order to compare experimental results with flow cytometry data (FACS Calibur, Becton Dickinson, USA), the binding density of lymphocytes in biochip spots was expressed on a percent basis. Average bind-

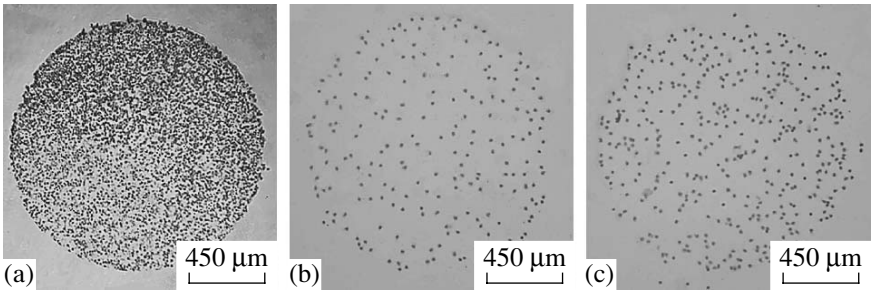

Fig. 1. Binding density of lymphocytes in biochip spots containing anti-CD45 (a), anti-CD16 (b), and anti-CD8 (c) antibodies. Magnification, ×37.5.

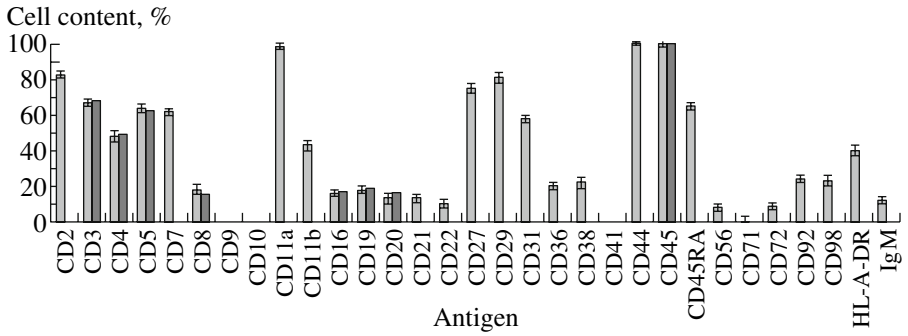

Fig. 2. Comparative analysis of healthy donor lymphocytes using biochips (light columns) and flow cytometry (dark columns).

ing density of anti-CD45 antibodies was taken for 100%.

RESULTS

Determination of percentage of CD-expressing healthy donor lymphocytes on biochips. Suspensions of lymphocytes from healthy donors were analyzed on biochips containing immobilized antibodies. After incubation of biochips with lymphocyte suspensions, washing of nonspecifically bound cells, fixation and staining, the highest binding density was found in biochip spots containing immobilized antibodies; outside the spots, bound cells were absent. The binding densities varied considerably, depending on antibody type (Fig. 1).

These findings suggest that binding densities of lymphocytes in biochip spots reflect the concentration of CD-expressing cells. In order to test this hypothesis, we determined the concentration of lymphocytes in biochip spots containing immobilized antibodies and calculated relative densities (%) of filling the spots with lymphocytes. As expected, the maximum binding density was found in spots containing anti-CD45 antibodies, since all the cells tested in this study expressed the CD45 antigen on their surface.

Relative binding densities of lymphocytes in biochip spots containing different antibodies were compared to blood serum levels of lymphocytes expressing appropriate surface antigens established by the flow cytometry method. (Noteworthy, the lymphocytes were obtained from the same donor). It is of note that the number of surface antigens determined in biochip-based microassays significantly exceeded that established in clinical flow cytometric studies, while the relative content of lymphocytes expressing CD3, CD4, CD5, CD8, CD10, CD16, CD19, CD20, and CD45 antigens determined by two different methods showed a good correlation (Fig. 2) [21].

It can thus be concluded that the percentage of lymphocytes in antibody-containing spots correlates with their content in lymphocyte suspensions expressing the corresponding antigens.

Determination of percentage of lymphocytes expressing different CD antigens in patients with lymphoproliferative diseases. A change in the normal ratio of CD-expressing cells is one of the main markers of leukemia. In this study, we used biochips for the analysis of peripheral blood lymphocytes from three patients with B-CLL. The results obtained were compared with flow cytometry data (Fig. 3) and showed a good correlation. Moreover, morphological analysis on biochips enabled immunophenotyping of much greater repertoires of surface antigens.

Staining and morphological analysis of lymphocytes immobilized on biochips. The use of transparent supports as a base for biochips allows a combination of standard staining procedures with morphological analysis, which, in its turn, enables immunophenotyping of lymphocytes expressing different antigens, especially if

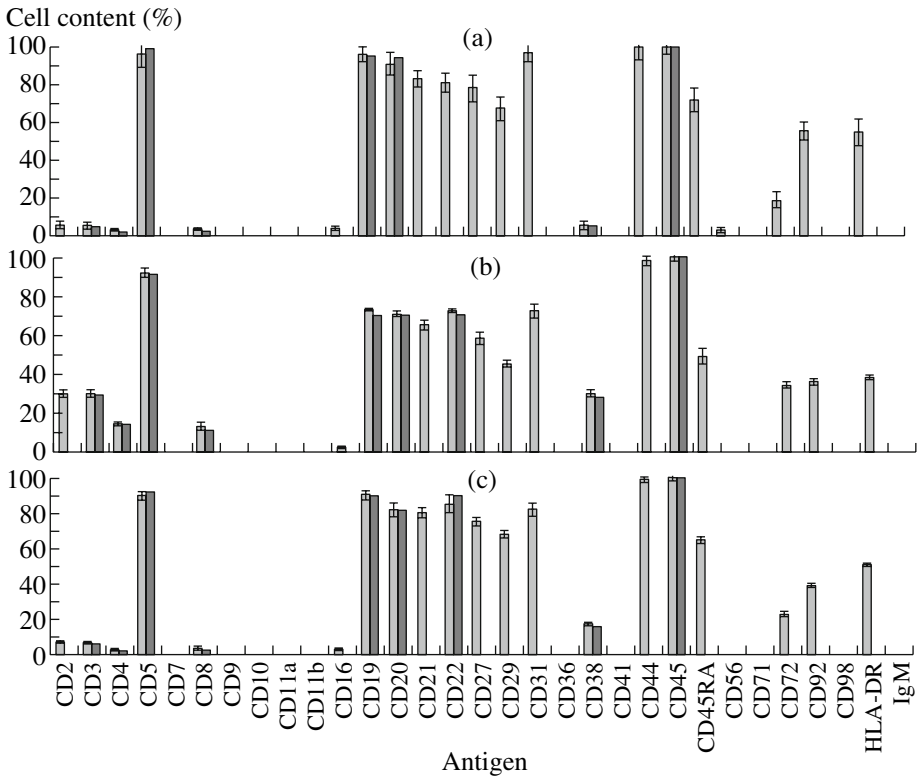

Fig. 3. Comparative analysis of lymphocytes from three patients with chronic B-cell chronic lymphocytic leukemias (B-CLL) using biochips (light columns) and flow cytofluorimetry (dark columns). (a) patient A.; (b) patient K.; (c) patient S.

the same antigen is expressed by cells of various types. The micrographs of healthy donor lymphocytes bound to anti-CD45 antibodies and stained according to Romanowsky–Giemsa displayed the presence of well-shaped large and small lymphocytes (Fig. 4a).

Binding of B-CLL lymphocytes to anti-CD5 and anti-CD19 antibodies on biochips is shown in Fig. 5. A high percentage (up to 90%) of lymphocytes expressing simultaneously CD5 and CD19 antigens was characteristic of B-CLL (Fig. 5). Yet another salient feature of CLL was that all the lymphocytes bound to anti-CD5 and anti-CD19 antibodies represented mature cells.

Determination of coexpression of antigens by staining bound lymphocytes with fluorescently labeled antibodies. Identification of a single antigen is not a necessary prerequisite to its adequate immunophenotyping. If the experimental task entails more exact identification of cells, coexpression of two or more antigens (e.g., by additional staining of cells with fluorescently labeled antibodies) can be required.

Identification of T-, B-, and NK-lymphocytes was carried out by staining healthy donor lymphocytes immobilized on anti-CD4, anti-CD8, anti-CD19, anti-CD45, and anti-CD56 antibodies in three different bio-

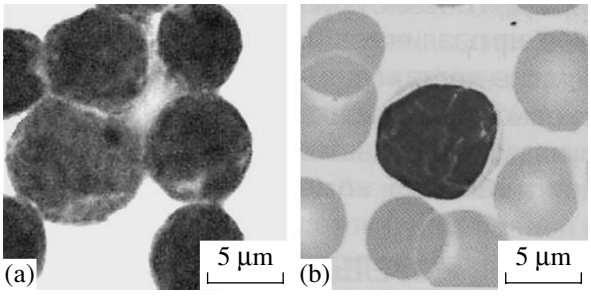

Fig. 4. Mature healthy donor lymphocytes immobilized on biochips containing anti-CD45 antibodies. (a) Staining according to Romanowsky–Giemsa. Magnification, ×1350. (b) A micrograph of a mature lymphocyte stained in smear (from the handbook [22]).

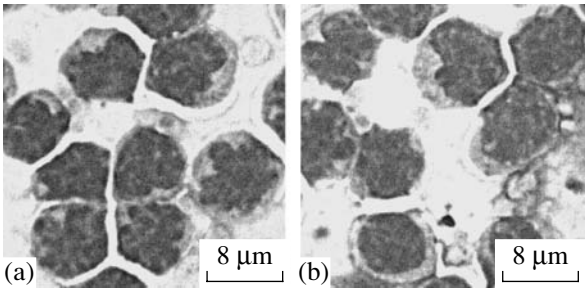

Fig. 5. Lymphocytes from a patient with B-cell chronic lymphocytic leukemias (B-CLL) immobilized on biochips containing anti-CD5 (a) and anti-CD19 (b) antibodies. Staining according to Romanowsky–Giemsa. Magnification, ×1350.

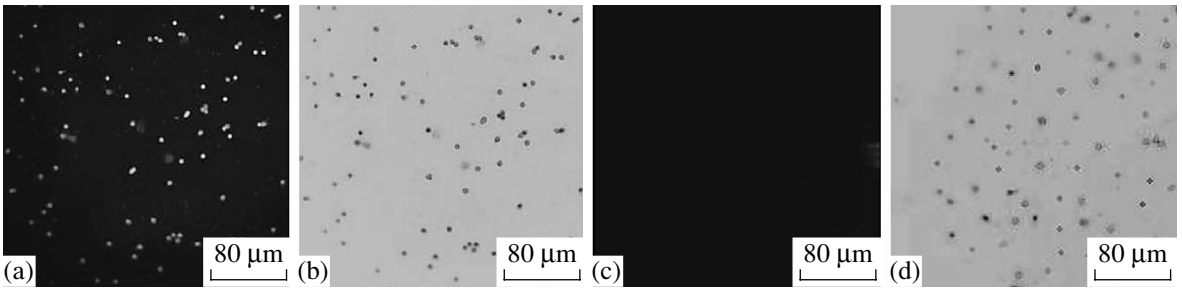

Fig. 6. Staining of healthy donor anti-CD20 lymphocytes with FITC-labeled antibodies on a biochip. Magnification, ×150. (a) Staining of lymphocytes bound in biochip spots with anti-CD19 antibodies (coexpression of CD19/CD20), illumination with UV light. (b) The same spot, brightfield view. (c) Non-stained lymphocytes bound to anti-CD4 antibodies after illumination with UV light. (d) The same spot, brightfield view.

chip spots containing FITC-labeled antibodies (anti-CD3, anti-CD16, and anti-CD20, respectively). Fluorescent microscopy established the absense of nonspecific binding (Table 1, Figs. 6c, 6d), but demonstrated normal coexpression of CD3/CD4, CD3/CD8, CD3/CD45, CD19/CD20, CD20/CD45, CD16/CD45, and CD16/CD56 antigens.

The micrographs of lymphocytes stained with fluorescently labeled anti-CD20 antibodies and immobilized on anti-CD19 antibodies in biochip spots are depicted in Figs. 6a, 6b. A comparison of the images obtained under normal (Fig. 6b) and UV-illumination (Fig. 6a) showed that all the cells immobilized in biochip spots were stained with fluorescently labeled antibodies (anti-CD20). Coexpression of CD19 and CD20 was more typical of B-lymphocytes.

Lymphocytes bound to anti-CD4 antibodies (Fig. 6d) were not stained with fluorescently labeled anti-CD20 antibodies (Fig. 6c).

DISCUSSION

Determination of percentage of lymphocytes expressing different CD-antigens. Percentage of lymphocytes expressing different CD antigens determined by means of biochips or flow cytometry was similar (Figs. 2 and 3), which testifies to a high efficiency of the biochip technology. After incubation with lymphocyte suspensions and washing of the biochip, only antibody-bound lymphocytes were present on the spot surface. The binding density of lymphocytes in antibody-containing spots correlated with the percent of lymphocytes expressing the corresponding antigens.

Analysis of lymphocytes from patients with leukemias revealed that the number of cells expressing surface T-cell antigens (CD2, CD3, CD4, CD7) was sharply decreased, while the number of cells expressing surface B-cell antigens (CD19, CD20, CD21, CD22) was increased suggesting the B-cell origin of leukemias. In this group of patients, about 90% of lymphocytes expressed CD5; percentage of CD19+ cells varied from 73 to 97%. It was concluded that the majority of these lymphocytes coexpressed CD5/CD19.

The use of biochips allows simultaneous identification of much larger assortment of surface antigens than flow cytometry. Furthermore, antibody microassay on biochips does not require expensive equipment. However, the use of this technology without additional staining with fluorescently labeled antibodies allows determination of only one antigen per cell, whereas flow cytometry allows simultaneous detection of several antigens on each cell.

Figures 4 and 5 show that the biochip technology allows the use of standard fixation and staining protocols similar to those used for cell analysis in smears. The images obtained thereupon allow identification of all major cell types. It is noteworthy that the structure of chromatin observed after staining of cells in smears (Fig. 4b) and on biochips (Fig. 4a) is different, which can be attributed to a significant spreading of cells in the

Estimation of antigen coexpression by staining of healthy donor lymphocytes with fluorescently labeled antibodies immobilized on biochips

FITC-labeled antibodies for staining bound lymphocytes	Antibodies immobilized on biochips				
	anti-CD4	anti-CD8	anti-CD19	anti-CD45	anti-CD56
Anti-CD3 FITC	+	+	–	+	–
Anti-CD16 FITC	–	–	–	+	+
Anti-CD20 FITC	–	–	+	+	–

Note: (+) staining; (–) lack of staining.

course of smear preparation. In future studies, these problems will be overcome or minimized through the use of more advanced cell preparation techniques.

After binding to immobilized antibodies against some particular antigen, bound cells can repeatedly be stained with fluorescently labeled antibodies against other antigens. A comparison of the micrographs of biochip spots obtained after illumination with normal and UV light (Fig. 6) allows one to determine the number of bound cells expressing the second antigen.

Yet another application for biochips is detection of diagnostically important variants of antigen coexpression in neoplastic blood cells. Staining of blood cells with mixtures of several fluorescently labeled antibodies is a promising approach to simultaneous determination of expression of several antigens. In future studies, this technique can find wide use for determination of nuclear and cytoplasmic antigens in cells immobilized on biochips.

The biochips described herein allow the identification of surface lymphocyte-specific antigens combined with morphological analysis, which opens up fresh opportunities for large-scale immunophenotyping of lymphocytes bound in each spot of a biochip. And, finally, repeated staining of cells with fluorescently labeled antibodies is a helpful tool for studying coexpression of several antigens on each bound cell.

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