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Analysis and sorting of live cells according to secreted molecules, relocated to a cell-surface affinity matrix

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ABSTRACT **We have developed a technology for analysis and sorting of live cells according to secreted molecules. An artificial affinity matrix, specific for the secreted product of interest, is created on the cell surface, and the cells are allowed to secrete for a defined time period. The secreted molecules bind to the affinity matrix on the secreting cell and are subsequently labeled with specific fluorescent or magnetic staining reagents for cytometric analysis and cell sorting. Crossfeeding of the secreted products to other cells is prevented by decreasing the permeability of the incubation medium. This approach will have a wide range of applications in biotechnology and biomedical research. Here, we describe analysis and sorting of hybridoma cells, according to secreted antibodies, and of activated T lymphocytes, according to secreted cytokines.**

Proteins and other cellular products can be expressed either intracellularly, on the cell surface, or by secretion into the extracellular space. Despite its biological significance, only a few methods are presently available for the analysis of secreted products on the single cell level. This situation is mainly because secreted cellular products are hard to assign to the secreting cell, especially in a quantitative way. In the currently available protocols, the plaque assay (1), the ELISPOT assay (2), and the microdroplet technology (3), secreting cells are fixed in or on support matrices, which trap the secreted products for analysis. These approaches impose severe limitations because the secreted molecules still dissociate from the cell, and trapping matrix and cells must form a unit for further analysis and sorting of the cells. Here we describe another concept for cytometry and sorting of live cells based on secreted products. Basically, the secreted product is retained on the cell surface of the secreting cell, making it accessible to the powerful technologies for detection of surface markers. As shown schematically in Fig. 1, an affinity matrix for the secreted product is generated by attaching a specific antibody to the cell surface. Subsequently, the cells are allowed to secrete their products under defined conditions into a medium of low permeability for the secreted product. After removal of the cells from the incubation medium, they are stained for the secreted product, which is now bound to the cell-surface affinity matrix, with specific fluorochrome-, hapten-, or particle-labeled "detection" antibodies or other staining reagents.

MATERIAL AND METHODS

Biotinylation of Cells. Between 10^7 and 10^8 cells (hybridomas or spleen cells) were suspended in a solution of 200 μl of sulfosuccinimidyl-6-(biotinamido)hexanoate (1 mg/ml, Pierce). After 10 min at room temperature the cells were washed twice with 50 ml of phosphate-buffered saline (PBS)/0.5% bovine serum albumin (PBS/BSA). This protocol is based on ref. 4.

Preparation of Medium of Low Permeability. Sixty grams of gelatin (from bovine skin, 75 bloom, Sigma) was dissolved in 100 ml of warm PBS and dialyzed extensively, first against PBS and then against RPMI 1640 medium (GIBCO). For use, this stock solution was diluted (with RPMI medium/5% fetal calf serum) to a final concentration of 25% or 40% gelatin.

Embedding of Cells in Gelatin. A cell suspension of 10^6–10^7 cells in 100 ml of PBS/BSA was mixed with 1 ml of gelatinous medium (25 or 40% gelatin) prewarmed to 37°C. After incubation for various times at 37°C the cells were removed from this medium by mixing them with a 10-fold excess of PBS (prewarmed to 37°C). The cells were spun down, the medium was removed, and the cells were resuspended in PBS/BSA.

Synthesis of Antibody–Avidin Conjugates. Three microliters of 1 M dithiothreitol (Pierce) was added to 1 mg of antibody in 0.5 ml of PBS/10 mM EDTA (PBS/EDTA). After 1 hr at 4°C, excess dithriothreitol was removed by gel chromatography (Sephadex G25, Pharmacia). In parallel, 50 μg of succinimidyl-4-(*N*-maleimidomethyl)-1-carboxylate (Pierce) in 20 μl of dimethyl sulfoxide was added to 0.5 mg of avidin in 200 μl of PBS, and the mixture was incubated for 30 min at room temperature. The activated avidin was purified on Sephadex G25 and eluted in PBS/EDTA. Reduced antibody and activated avidin were mixed in a molar ratio of 2:1. After 1 hr at 20°C the reaction was stopped by adding *N*-ethylmaleimide at 10 μg/ml (Sigma).

Creation and Detection of a Cellular Affinity Matrix. Biotinylated cells were incubated 5 min at 4°C with antibody–avidin conjugate at 20 μg/ml in PBS. The matrix was detected by staining the cells with goat anti-rat IgG–fluorescein conjugate at 10 μg/ml (Southern Biotechnology Associates) in PBS/BSA.

ELISA. The measurement of secreted IgM is described in detail in ref. 5. Briefly, plates were coated with monoclonal antibody (mAb) R33.24.12 (5); as a standard and as detection antibody B1-8 (6) and biotinylated antibody R33-60 (5) were used, respectively. The ELISA was developed with streptavidin-conjugated alkaline phosphatase from Boehringer Mannheim and 4-nitrophenylphosphate disodium salt (Merck).

Antibodies. Antibodies were as follows: LS 136, anti-murine I (7); AN 18.17.24, anti-murine interferon γ (IFN-γ) (8); R46 A2 anti-murine IFN-γ (9); S4B6, anti-murine interleukin 2 (10); R33.18.10.1, anti-murine κ light chains (7); AS 79, as murine IgG.κ standard (7). Fluorescein-isothiocyanate (FITC)-labeled goat anti-mouse IgG from Southern Biotechnology Associates.

RESULTS

Strategy. For the specific formation of cell aggregates (rosetting; ref. 11), for negative selection of hybridoma vari-

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Abbreviations: mAb, monoclonal antibody; BSA, bovine serum albumin; IFN-γ, interferon γ.

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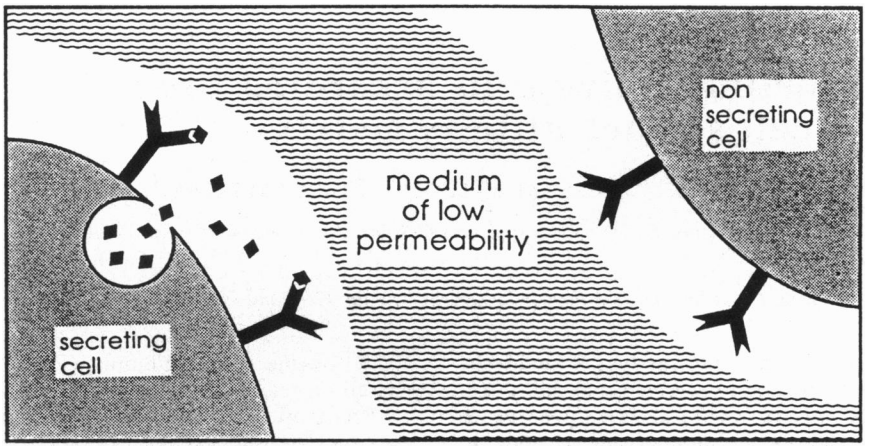

FIG. 1. Specific labeling of live cells for secreted products. Cells are artificially coated with antibodies that are directed against the secreted product, and the cells are allowed to secrete into a medium of low permeability for the secreted product. The secreted product binds to the antibodies on the cell surface and can be used like a surface marker for analysis and sorting of the live cells.

ants (12), and for "artificial" antigen presentation (13), specific ligands have been conjugated to the surface of cells before, by a variety of methods. For the quantitative analysis of secreted products, the cell-surface affinity matrix should be of ample capacity, without being harmful for the cell. We have used two approaches to attach specific "catching" antibodies to the surface of living cells without damaging them, both based on the avidin/biotin technology. In all experiments shown here we used direct biotinylation of the cell-surface proteins with biotin-hydroxysuccinimide ester. A palmitylic acid–dextran–biotin conjugate was also used (data not shown). Other methods such as the use of bispecific antibodies may be as feasible.

Viability of the biotin-labeled cells was not impaired as judged by propidium iodide uptake in flow cytometric analysis (data not shown), or function, as far as tested (see below). To create a specific immunoaffinity matrix on the biotinylated cells, antibodies specific for the secreted molecules were conjugated to avidin, and the cells were labeled with the avidin–antibody conjugates. These catch-antibodies were detectable on the cell surface by staining with fluorescence-labeled antibodies. After 48 hr in culture no antibody–avidin conjugates were detectable on B1-8 hybridoma cells (data not shown).

Sensitivity of the Affinity Matrix. The suitability of an affinity matrix for quantitative analysis of secreted products depends largely on the binding constants of the affinity receptor and on the local concentration of the secreted product in the vicinity of the cell, which, in turn, depends on the package size, frequency, and concentration of exocytosis and the speed of diffusion through the medium of low permeability. For steady-state concentrations of the secreted product, the sensitivity and dynamic range of a given cellular affinity matrix can be measured by titrating various amounts of isolated "secreted product" into the culture medium, staining the cells with the detection antibody, and quantitating the staining by flow cytometry. In the example shown in Fig. 2, the mean fluorescence of affinity-matrix-coated cells stained with externally added "secreted product" at 0.03 μg/ml is clearly distinguishable from the mean fluorescence of unstained cells and thus defines the limit of sensitivity of this particular affinity matrix. Staining increases linearly until a maximum capacity of the affinity matrix at $\approx$10 μg/ml. While the sensitivity of the matrix is a function of the affinity of the catch-antibody, the maximum capacity depends on how many catch-antibodies can be attached to the cell surface. It will usually be sufficient to control the characteristics of a particular matrix by adding a saturating amount of secreted product externally (Fig. 3) to determine the capacity of that matrix.

Kinetics of Capturing. The usefulness of a cellular affinity matrix for the analysis of secreted products depends entirely on (*i*) whether it is sensitive enough to detect the secreted products at all and (*ii*) whether it has sufficient capacity to collect secreted molecules in the linear dynamic range over an acceptable time period. This relationship is shown in Fig. 3 for an Ls 136-affinity matrix [anti-murine λ light chain (7)] and B1-8 murine hybridoma cells, secreting an IgM,λ antibody (6). The mean fluorescence of anti-IgM–fluorescein [mAb R33.24.12 (5)]-stained cells is plotted against the time of secretion—i.e., the time cells were in the incubation medium. It is obvious that the sensitivity of the affinity matrix is not

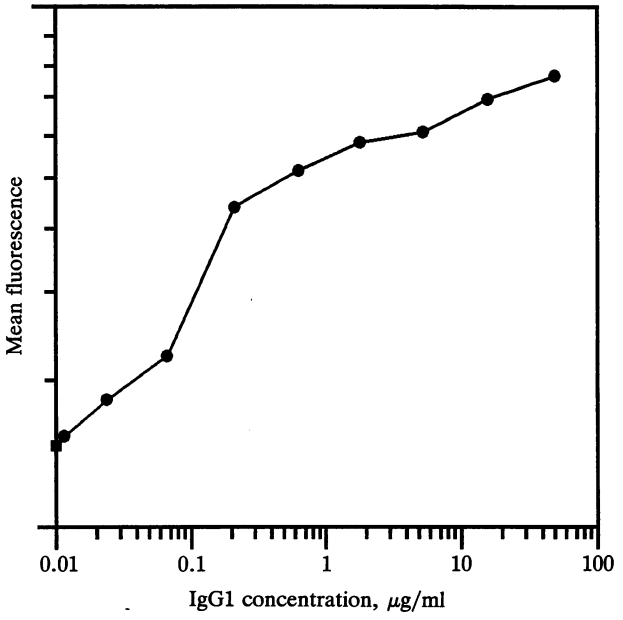

FIG. 2. Sensitivity of an affinity matrix. About 10^7 nonsecreting (X63 Ag.8. 653; ref. 14) cells were biotinylated and coated with an avidin–anti-κ-antibody (R33.18.10.1; ref. 7) conjugate. Aliquots of 10^6 of these cells were incubated for 10 min with different concentrations of As79 (IgG2b,κ; ref. 7) antibody, as indicated (●) or no As79 (■), and stained with fluorescein isothiocyanate-labeled goat anti-mouse IgG (Southern Biotechnology Associates, 10 μg/ml in PBS/BSA). The staining was analyzed by flow cytometry using a FACScan and FACScan research software (Becton Dickinson). The mean fluorescence of stained cells is plotted against the concentration of As79 used for labeling.

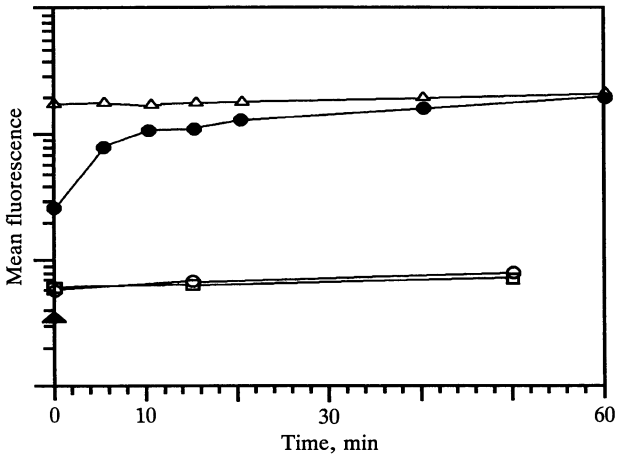

FIG. 3. Kinetics of an affinity matrix. B1-8 IgM, λ antibody-secreting hybridoma cells were coated with an anti-λ affinity matrix and allowed to secrete into RPMI 1640/40% gelatin for various time periods (●) in a humid atmosphere with 5% CO_2/95% air. The cells were then washed, stained for IgM, and analyzed by flow cytometry as described above. ▲, Unstained cells; ○, cells not coated with an affinity matrix but stained for IgM after incubation in gelatinous medium for various time periods; □, cells without affinity matrix, which had been incubated in gelatin medium and subsequently labeled with B1-8 IgM at 10 μg/ml and stained for bound IgM; and △, cells coated with an affinity matrix, first incubated in gelatinous medium, then labeled with B1-8 IgM at 10 μg/ml to saturate the affinity matrix, and then stained for IgM, were used as controls.

limiting. Secreted IgM is readily detected at the earliest time point measured. After ≈10 min, the capacity of the matrix approaches saturation. Thus, within the first 10 min, this affinity matrix can be used to detect quantitative differences in secretion on the single-cell level.

Protection Against Crossfeeding. By definition, the process of secretion separates the secreted product from the secreting cell. In normal medium the secreted product will readily diffuse away and, in the approach described here, will label all cells covered with an affinity matrix, whether they are secreting or not. Fig. 4 shows this condition for B1-8 hybridoma cells, secreting IgM,λ antibodies. We solved this problem by decreasing the permeability of the incubation medium for the secreted product, adding 5% (wt/vol) hydroxymethylcellulose or, in the experiment shown in Fig. 4, 25% (wt/vol) gelatin. Because higher concentrations of gelatin appear to minimize crossfeeding, which is particularly relevant for smaller secreted products such as INF-γ, in all later experiments we used 40% (wt/vol) gelatin. For the time of secretion, the cells labeled with the affinity matrix were incubated in this semisolid, highly viscous medium. The incubation medium was then solubilized by addition of a 10-fold excess of warm (37°C) PBS, and the cells were recovered by centrifugation. The detection antibody will then see two defined populations in a mixture of secreting and nonsecreting cells (Fig. 4).

Correlation with Intracellular Staining. To prove that the labeled cells are indeed the secreting cells, the mixture of cells, stained on the surface for secreted IgM with a phycoerythrin-conjugated anti-IgM, were fixed in formaldehyde, permeabilized with saponin (15), and counterstained for cytoplasmic IgM with a fluorescein-conjugated anti-IgM, mAb R33.24.12 (Fig. 5). Compared with an unpermeabilized control, the permeabilized cells show complete correlation between staining in the cytoplasm and staining via the surface affinity matrix—i.e., staining of secreted IgM.

Cell Sorting According to Secretion. With fluorescence-activated cell sorting and high-gradient magnetic cell sorting (16) available as state-of-the-art technologies, staining of cells for secreted products with fluorescent and/or magnetic anti-

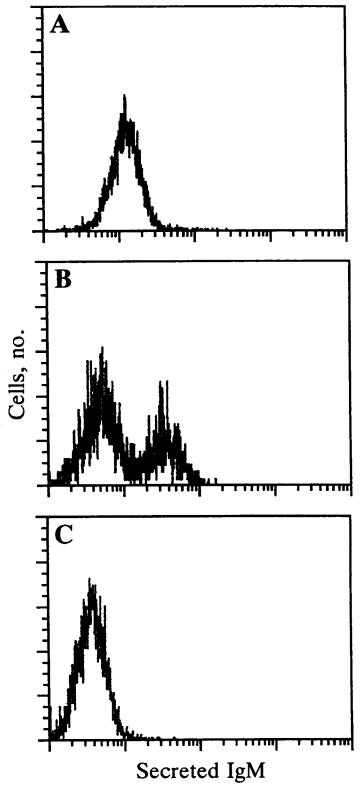

FIG. 4. Effect of medium permeability. B1-8 hybridoma cells were coated with an anti-λ affinity matrix and incubated in RPMI 1640 medium/5% fetal calf serum (Biother, Kelkheim, Germany) (*A*) or RPMI 1640 medium/5% fetal calf serum/25% gelatin (*B*). After incubation for 45 min at 37°C, the cells were washed, stained for IgM, and analyzed by flow cytometry as described above. Fluorescence profiles of 5000 cells are shown, gated for live cells according to exclusion of propidium iodide. Control cells were coated with an affinity matrix but directly stained for IgM (*C*). It should be noted that the positive cells from medium with low permeability are stained brighter than the cells from normal medium, reflecting the effect of the local concentration of secreted product at the cell surface on labeling efficiency.

bodies implies that such cells can be sorted according to their secreted products. Here we use cell sorting to further confirm the correlation between cell-surface-affinity-matrix-mediated staining and secretion and to demonstrate that labeling and cell sorting do not affect the cells functionally.

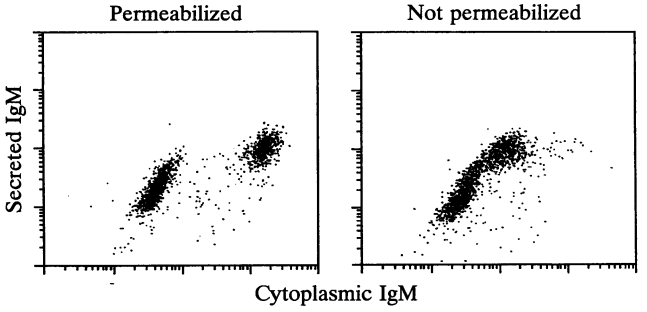

FIG. 5. Specificity of an affinity matrix. Anti-λ affinity matrix-coated B1-8 cells were allowed to secrete IgM for 45 min in gelatin/medium, washed, and stained with an anti-IgM–phycoerythrin conjugate (mAb R33.24.12). (*Left*) The cells were fixed in 2% formaldehyde/PBS, washed, and stained for intracellular IgM with anti-IgM-fluorescein isothiocyanate, using saponin to permeabilize the cell membranes. (*Right*) Control cells were stained with anti-IgM–fluorescein in the absence of saponin—i.e., under conditions disabling intracellular staining. Dot plots displaying red (phycoerythrin) versus green (fluorescein) fluorescence are shown for ≈10^4 cells each.

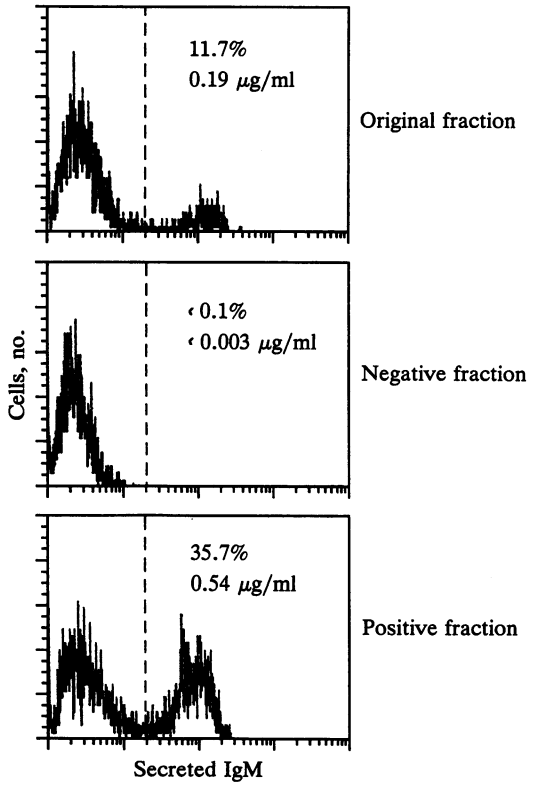

FIG. 6. Sorting of B1-8 cells according to IgM secretion. B1-8 IgM, λ antibody was captured on the surface of anti-λ affinity-matrix-coated B1-8 hybridoma cells, as described above. The cells were then stained for IgM with magnetic beads (anti-IgM beads, 1:5, diluted from the stock in PBS/BSA from Miltenyi Biotec GmbH) and fluorescent anti-IgM antibody. The cells were sorted with a magnetic cell sorter (Miltenyi Biotec) and analyzed by flow cytometry; 10^5 cells of each fraction were cultured in 3 ml of RPMI 1640 medium/5% fetal calf serum. Two days later the concentration of IgM in the culture supernatants was determined by ELISA. All cultures show normal cell growth, and no significant number of death cells could be detected by trypan blue staining. Fluorescence profiles of 5000 cells each are shown for the original cell mixture and positive and negative fractions of magnetic cell sorter-separated cells.

Again, we used B1-8 hybridoma cells with ≈10% of the cells expressing an IgM,λ antibody. The cells were labeled for secreted IgM as described above, by staining them with anti-IgM magnetic cell sorting beads and fluoresceinated anti-IgM. The cells were then sorted by high-gradient magnetic cell separation under sterile conditions, by using the fluorescent label to monitor the sort (Fig. 6). Original, positive, and negative fractions were collected and cultured overnight. After 48 hr, viability of the cells was assessed, and the amount of secreted IgM in the culture supernatant was determined by ELISA. In the experiment shown here, secreting cells were depleted below the level of detection by both staining and ELISA. In the positive fraction, a 3-fold enrichment of stained cells correlated with a 2.5-fold enrichment in secreted IgM. Viability of the cells was not impaired in any fraction.

Detection of Secreted IFN-γ. IgM has a rather high molecular mass of ≈600 kDa. Many secreted molecules of biological significance have lower molecular masses. Of particular immunological interest are cytokines—i.e., regulatory molecules of the immune system and the cells that secrete them. To demonstrate the feasibility of the cellular affinity matrix for the analysis of cytokine secretion, murine splenic lymphocytes were activated *in vitro* with staphylococcal enterotoxin B, as described (17). After 43 hr the cells were collected and biotinylated. After creation of the affinity matrix consisting of an avidin-conjugated anti-IFN-γ [mAb AN 18.17.24 (8)], the cells were incubated for 30 min in medium/40% gelatin at 37°C. Then, the cells were retrieved from this medium, washed, and stained with indirectly fluoresceinated anti-IFN-γ [mAb R46A2 (9)] or, in control samples, with indirectly fluoresceinated anti-interleukin 2 [S4B6 (10)]. Fig. 7 shows that ≈23% of the gated blast cells are specifically labeled, with the matrix not yet saturated completely, as is obvious from comparison with a control sample, with exogenous IFN-γ added. By microscopic analysis, 35 of 36 cells stained in the cytoplasm also stained on the surface for secreted IFN-γ.

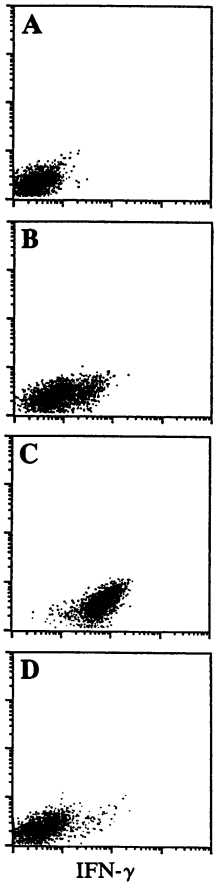

FIG. 7. Detection of secreted IFN-γ. Murine spleen cells were cultured for 43 hr with staphylococcal enterotoxin B, then biotinylated, labeled with avidin-conjugated rat monoclonal anti-murine IFN-γ AN18.17.24 (in the presence of unconjugated anti-IFN-γ mAb AN 18.17.24 and R46A2), and incubated for 30 min in secretion medium/40% gelatin. Cells were then washed and stained with digoxigenin-conjugated rat mAb to mouse IFN-γ R46A2 and fluorescein-conjugated sheep anti-digoxigenin (1 μg/ml) from Boehringer Mannheim. Stained cells were analyzed by flow cytometry and fluorescence microscopy. Dot plots show IFN-γ staining of large activated lymphocytes (blasts), gated according to forward and side scatter (*B*). As negative control, affinity matrix-coated cells not incubated at 37°C in secretion medium (*A*) and cells not labeled with the affinity matrix but incubated in secretion medium for 30 min (*D*) are shown. As high control, affinity matrix-coated cells were stained with IFN-γ-containing culture supernatant for 10 min (*C*).

DISCUSSION

Compared with previously available methods, the cellular affinity matrix technology allows analysis and sorting of large numbers of cells for secreted products on the single-cell level. Limited only by the number of labels available for detection, various secreted and/or surface and/or intracellular products can be combined at will for correlated, quantitative multiparameter analysis of individual cells. Magnetic and fluorescent

labels can be used for analysis and sorting, as well as large particles, such as antibody-conjugated beads or liposomes.

This method should have a wide range of applications in biotechnology and biomedical research, from (*i*) analysis and purification of secreting cells before or in the course of production processes, (*ii*) isolation of rare cells or genetic variants of interest—e.g., high producers, hybridomas or transfectants—to (*iii*) the analysis of the secretion process and its regulation itself—e.g., by directly analyzing the control of exocytosis or directed secretion—and (*iv*) the isolation of secreting cells for subsequent molecular and functional studies. For clinical diagnosis and treatment of dysregulation of expression of secreted products, like hormones, viruses, etc., the technology opens additional possibilities for assigning the secreted products to distinct cells in distinct environments. Here, we have described the technology in principle and established it for two different systems of general interest—the characterization and sorting of hybridoma cells and cytokine-secreting T lymphocytes.

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