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Efficient Hybridoma Screening Technique Using Capture Antibody Based Microarrays

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The hybridoma screening is a key step for the successful generation of high-affinity analyte-specific monoclonal antibodies (MAbs), particularly if the target of the antibody is a low-molecular weight analyte. This work presents an advanced screening method that makes use of antibody microarrays generated by contact printing of the hybridoma cell supernatant samples on glass chips initially coated with capture antibodies. The noncompetitive immunoassay is based on the specific binding of an analyte−horseradish peroxidase conjugate and is performed in an automated fashion using a chemiluminescence readout system. Compared to the standard ELISA screening, the work load is reduced due to a higher degree of automation. The quality of the generated data is comparable to data generated by a previously optimized microplate-based immunoassay method. Among a reference set of 373 hybridoma cell supernatant samples, three out of four high-affinity MAbs were identified as true positive, whereas none of the samples were detected as a false positive.

Monoclonal antibodies (MAbs) are useful biological tools for various immunoanalytical applications, e.g., in clinical chemistry, food analysis, and environmental monitoring. Furthermore, MAbs are increasingly used as human therapeutics.[1] They can principally be directed against high-molecular weight targets (such as proteins) or low-molecular analytes (haptens). Regardless of the intended application, high-affinity antibodies must be identified among a pool of possible candidates. For the generation of therapeutic antibodies, recombinant antibody fragment libraries have been screened with automated high-throughput techniques.[2−4] In contrast, for the generation of monoclonal antibodies of animal origin (mainly mouse), the more traditional generation of selecting antibody-secreting hybridoma cell lines is still the method of choice. It requires immunization of an animal with subsequent fusion of its spleen cells with myeloma cells.[5] After about 1−3 weeks of cell cultivation, high-affinity antibody-secreting hybridomas must be identified. This critical step usually has to be accomplished as early as possible and within approximately 1 day, as the hybridoma cell clones of interest may be mixed with other clones in the same well and thus may be overgrown. Often, hundreds or even a few thousands of hybridomas have to be screened. According to our experience, the ratio of hybridomas secreting analyte-specific antibodies can be as low as 0.1%, especially in the case of hapten-specific antibodies.[6]

The hybridoma screening can be accomplished using various techniques. The de facto standard method is the microplate-based enzyme-linked immunosorbent assay (ELISA), usually performed with antigen immobilization. A few advanced methods have been developed, among them Biacore Screening[7] and flow-based immunoassay.[8] These methods require mostly sequential measurements of the hybridoma supernatants and thus a relatively long overall screening time, even though data generated by Biacore yield more detailed kinetic binding information. In this respect, highly parallelized systems, i.e., microarray-based techniques, appear more promising due to the capability of analyte (i.e., MAb) immobilization in a multiplexed fashion.[9−11] The application of the Biacore Flexchip SPR technology to rapidly identify high-affinity human Fab fragments against human tissue kallikrein 1 (1hK1) from phage display library selection outputs was described by Wassaf et al.[12] This technology can provide estimates of kinetic constants for up to 400 different protein−protein interactions in a single experiment. As a limitation, the assay will be applicable only to antigens large enough to cause a measurable angular shift of the incident light. Further, a novel antigen-coated microarray-screening assay (AMA) was reported by De Masi et al.[13] The AMA simultaneously detects antigen-specific binding and determines the isotype of the bound antibodies by application of isotype-specific secondary antibodies conjugated to different fluorochromes. It proved useful in a scaled up

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experiment, where 80 protein antigens of different size, e.g., 13.5−650 kDa, were selected for multiplexed immunizations of mice.

The hybridoma screening methods mentioned above are based on immunoassays with antigen immobilization, generally. Only very few studies were published, which describe the use of IgG-immobilized microarrays. We recently showed that the ELISA with antibody immobilization has distinct advantages over the ELISA with antigen immobilization, as it often leads to a much lower ratio of false positives.[14] The conventional ELISA with antigen immobilization allows the simultaneous binding of both paratopes of an IgG to the surface (the so-called bonus effect of multivalency, similar to the chelate effect in complex chemistry).[15] As a consequence, certain antibodies might be detected as positive, although the binding strength per paratope is relatively low. One way to compensate for this effect is to perform pairwise noncompetitive/competitive assays for each MAb, even if the number of measuements is doubled for the screening process.[16] In contrast, by application of a noncompetitive immunoassay with antibody immobilization and an analyte−horseradish peroxidase conjugate as an enzyme tracer, this bivalency effect is efficiently circumvented, especially in the case of hapten-specific antibodies.[14] For these reasons, our objective was to develop an automated hybridoma screening method using IgG antibody microarrays, in order to take advantage of the selective immunoassay format with antibody immobilization, as well as of a multiplexed assay technique that requires a minimum number of manual steps.

EXPERIMENTAL SECTION

Materials. Glass slides (26 mm × 76 mm × 1 mm) were obtained from Roth (Karlsruhe, Germany). Smaller glass slides (24 mm × 50 mm) were purchased from Marienfeld (Lauda-Königshofen, Germany). Polystyrene microplates with 96 wells (655061) were obtained from Greiner (Frickenhausen, Germany). Polypropylene microplates with 384 wells with a maximum volume of 45 μL (0030 128.508) were obtained from Eppendorf (Hamburg, Germany). All salts and reagents (purity ≥99%) were purchased from Sigma (Taufkirchen, Germany). The contents of all buffers and stock solutions used for ELISA as well as the preparation of the aflatoxin B_2−protein conjugates were described previously.[14,17] Goat antimouse IgG antibody (M5899, whole antiserum) was obtained from Sigma. The chemiluminescent substrate solutions (SuperSignal ELISA Femto Maximum Sensitivity Kit) were purchased from Pierce (Rockford, IL).

Generation of Hybridomas. The immunizations with aflatoxin B_2−bovine serum albumin conjugate and the fusion of mouse spleen cells with myeloma cells is described elsewhere.[14] For the method development, a deep-frozen (−80 °C) and previously well-characterized set of 373 hybridoma supernatants was used.

ELISA Screening. ELISAs were performed by a previously optimized method.[14] Briefly, 96-well polystyrene microplates were coated with 300 μL of goat antimouse IgG antibody (1/5000 in carbonate buffer with 0.2% sodium azide, pH 9.6) and incubated overnight at 4 °C. After washing with a 96-channel washer (ELx405 Select, BioTek, Bad Friedrichshall, Germany), the wells were filled with 100 μL of hybridoma supernatants (1/4 in phosphate buffered saline (PBS), pH 7.6), positive controls (mouse serum taken before the fusion, 1/100 in PBS), and negative controls (PBS) and incubated for 1.5 h at room temperature. After washing, plates were filled with 150 μL of aflatoxin B_2−horseradish peroxidase conjugate solution (1.0 μg/mL in a solution of 0.1% bovine serum albumin in PBS (w/v)) and incubated for 1.5 h. After the plates were washed, they were filled with 100 μL of substrate solution (3,3′,5,5′-tetramethylbenzidine/hydrogen peroxide solution in potassium dihydrogencitrate/potassium sorbate buffer, pH 3.8) and incubated for 10 min at room temperature. The color development was stopped with 100 μL of 5% sulfuric acid and the absorption determined at 450 nm with a microplate reader (Synergy HT, BioTek).

Microarray Fabrication. Glass slides (26 mm × 76 mm × 1 mm) were PEGylated and activated by reaction with *N*-hydroxysuccinimide (NHS) as described elsewhere.[18] Then, 75 μL of goat antimouse antibody solution (as obtained from the distributor) was given in the center of each chip, covered with a smaller second glass slide (24 mm × 50 mm) and incubated overnight in a humid chamber at room temperature. The antibody-coated chips were rinsed with deionized water and dried in a stream of nitrogen. Hybridoma supernatants were directly spotted on the chip using a BioOdyssey Calligrapher miniarrayer (Bio-Rad, München, Germany) and a solid pin (SNS 12, Bio-Rad) that transferred 5.1 nL of cell supernatant. A grid of 7 × 15 spots with a spot interspacing of 1.00 mm in the *x*/*y*-direction was printed. The 96 samples were spotted together with two spots for positive controls (mouse serum taken before the fusion, diluted 1/100 in PBS, top right and bottom left spots of each chip) and an additional row of seven spots for negative controls (PBS). All microarrays were generated in duplicate. Humidity in the spotting chamber was set to 50%. During the spotting process, supernatants were kept in the 96-well polystyrene plates at 15 °C. Alternatively, supernatants were transferred to the 384-well polypropylene plates before spotting. The chips were subsequently blocked by shaking in a 2% solution of casein in PBS (w/v) for 4 h at room temperature, then rinsed with water, and dried in a stream of nitrogen.

Automated Immunoassay. An automated flow-through assay with chemiluminescence readout was performed using a previously described setup.[19] The flow cell has a volume of 60 μL and consists of a transparent polycarbonate slide with inlet and outlet perforations attached to the glass slide by means of an adhesive sheet. The automated assay consisted of rinsing the flow cell with a 0.5% solution of casein in PBS (w/v) (800 μL, 350 μL/s), followed by purging the flow cell with aflatoxin B_2−horseradish peroxidase conjugate solution (2 μg/mL in a 0.1% solution of bovine serum albumin in PBS (w/v), 180 μL, 100 μL/s). Then, another 620 μL of this enzyme conjugate solution was pumped through the flow cell in intervals of 20 μL (20 μL/s). After another rinsing step with casein solution (8 mL, 100 μL/s), the chemiluminescent substrates (mixture of SuperSignal ELISA

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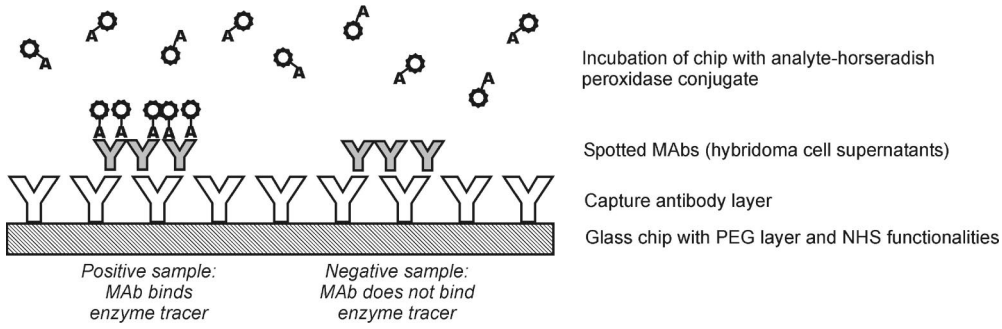

Figure 1. Chip layout (cross section) and immunoassay principle for the screening of hybridoma cell supernatants using an analyte−horseradish peroxidase conjugate as an enzyme tracer.

Femto Luminol Enhancer Solution and SuperSignal ELISA Femto Stable Peroxidase Solution) were pumped into the flow cell in a ratio of 1:1 (120 μL each, 20 μL/s), followed by the measurement of the signal intensities with the CCD camera (exposure time, 60 s). The system was finally purged with casein solution. The total assay run time was 7 min.

Data Processing. Raw data were automatically processed using the program SIP 0.4 (Karsunke Softwarebüro, Wolnzach, Germany). The software eliminates electronic artifacts of the CCD camera. Spots are identified automatically according to signal intensity and consistency with the spot grid. Spots that do not fulfill one of these criteria are identified as outliers and are not evaluated. SIP 0.4 subsequently sums up the pixel intensities within a square of a given side length around each spot center (0.6 mm corresponding to 9 pixels of the CCD camera). The size of the square can be adapted to the size of the spots.

RESULTS AND DISCUSSION

This work describes an improved and automated hybridoma screening technique which is potentially useful for the generation of MAbs against low-molecular weight analytes. As a model analyte, aflatoxin B_2 was chosen, a common and highly toxic food contaminant of fungal origin. A set of 373 hybridoma cell supernatants was used. The samples were examined previously by standard ELISA methods in a detailed study[14] and thus appeared ideal as a set of reference samples for the method development. Among the 373 samples, four MAbs were previously identified as high-affinity aflatoxin-specific MAbs (minimal IC_{50} (aflatoxin, nanograms per liter) for MAb 62, 195, and 287: 2.7 ± 0.2, 30 ± 7, and 10 ± 5).

The automated hybridoma screening technique described here makes use of glass slides with a PEG layer and electrophilic succinimidylester functionalities.[18] The center area of each chip (24 mm × 50 mm) was coated with a capture antibody (goat antimouse IgG). Volumes of about 5 nL of all 96 cell supernatants generated in one standard 96-well microplate were then spotted directly on the chip without pretreatment or dilution. The use of antimouse capture antibodies yielded significantly better results than spotting the samples directly on the activated glass surface. Also, coating the whole center area of the chip with capture antibody could be carried out in a more reproducible way compared to spotting the capture antibody solution with subsequent overspotting of the hybridoma supernatant samples. With the use of this coating technique, a volume of only 75 μL of commercially available goat antimouse IgG solution was required.

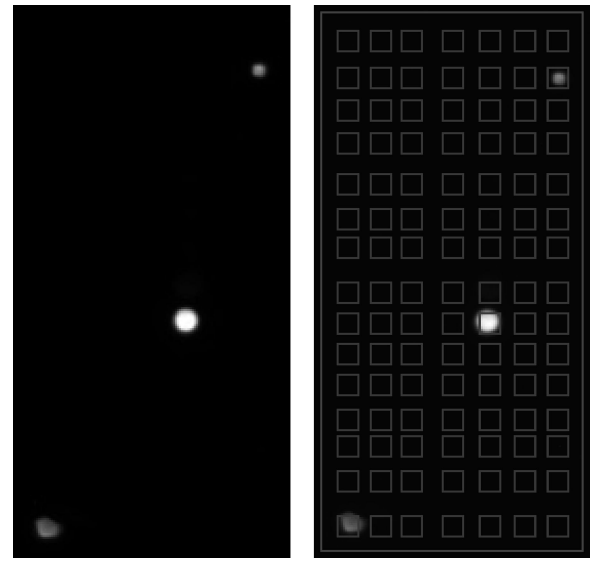

Figure 2. Picture of a chip before (left) and after automatic spot identification (right) including one positive sample and two positive control spots.

The flow-through immunoassay was performed in an automated fashion making use of a setup described earlier.[19] The measurement is based on the detection of chemiluminescent light by a CCD camera placed in a dark chamber underneath the flow cell and makes use of the horseradish peroxidase-catalyzed oxidation of luminol.[20] The noncompetitive immunoassay includes incubation with an analyte−horseradish peroxidase conjugate (in this case an aflatoxin B_2−enzyme conjugate) that binds specifically to high-affinity analyte-specific MAbs. For the synthesis of such low-molecular weight analyte−enzyme conjugates, the standard NHS coupling chemistry is recommended. This method is also conventionally applied for the synthesis of immunogens and coating conjugates for ELISA within such an antibody generation. Figure 1 summarizes the layout of the chip and the immunoassay principle.

A spot pattern and identification is displayed in Figure 2. As shown as the upper right and bottom left spots, the positive control sample (diluted mouse serum taken before the fusion) was spotted. The positive control was taken as a qualitative control only and not considered for data evaluation, i.e., to confirm that the immunoassay works. In addition, seven spots with negative control solution (PBS) were also spotted (first row). For spot

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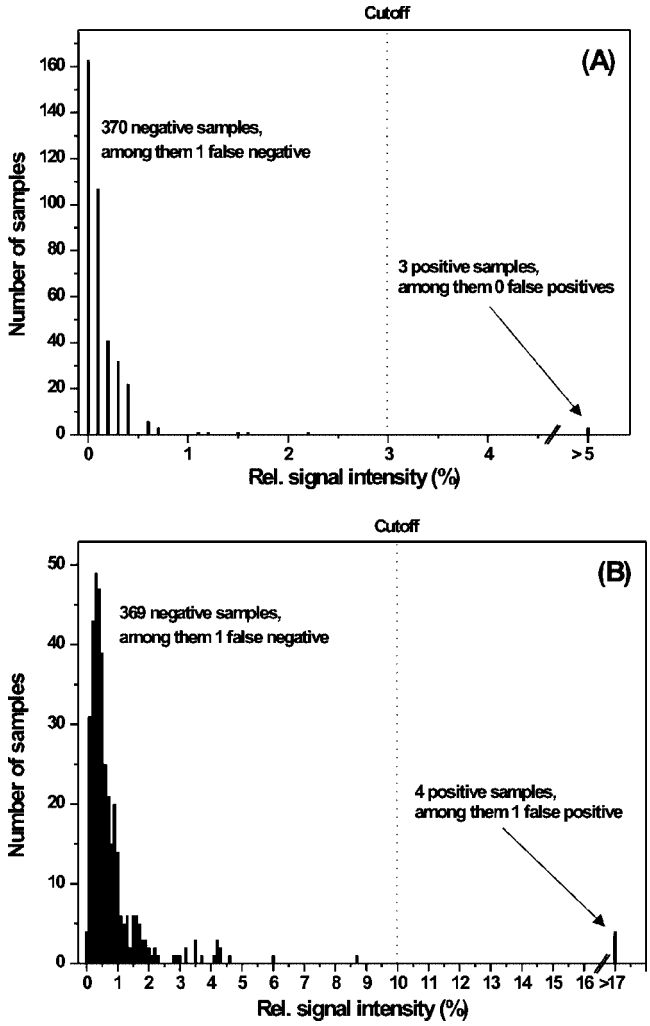

Figure 3. Screening results showing relative signal intensities for a set of 373 hybridoma cell supernatants comparing microarray-based flow-through immunoassay (A) and microplate-based ELISA (B). The cutoff value was arbitrarily set to distinguish between positive and negative samples.

identification, the software used for data evaluation identifies the spot centers automatically. Furthermore, the software is fed with the *x*/*y*-coordinates of the positive controls and the number of spots in the *x*/*y*-direction, enabling a reliable identification. The data is finally displayed as sums of pixel intensities within a square of a given side length around each spot. The resulting 373 values were then normalized to the highest value, which was set to 100% relative signal intensity (MAb 62). Hence, the signal intensities generated by using different chips can be compared to each other in Figure 3.

Figure 3 shows the screening results for the 373 hybridoma supernatant samples comparing the microarray-based flow-through immunoassay with a microplate-based ELISA. It has to be mentioned that the ELISA method was already optimized previously yielding substantial improvements of the overall selectivity compared to standard ELISA methods with antigen immobilization.[14] The value of the cutoff is arbitrary. It was set lower for the microarray-based screening, as the distribution of the intensities of the negative samples is significantly narrower compared to the microplate-based ELISA. With the use of cutoff values of 3% and 10%, respectively, both methods clearly identified 3 out of 4 high-affinity analyte-specific MAbs (relative signal intensity of MAbs 62, 195, 287: 100%, 12%, 6% (microarray) and 79%, 100%, 75% (ELISA)). Both methods could not identify one high-affinity MAb (relative signal intensity: 0.4% (microarray) and 4.2% (ELISA)). The number of false positives is very low for both methods, often a critical point for hybridoma screening.[8,14] For ELISA, the relative signal intensity of the false positive sample was 50%. The same sample had a relative intensity of 0.1% in the microarray-based screening and thus was not detected as false positive. The reason for the disparity between both assays regarding this finding remains unclear. Consistently, the relative signal intensity of this antibody was significantly lower than the three true positives in both assays. Therefore, we speculate that this antibody because of low affinity could only bind the enzyme tracer after extended incubation time, i.e., after 1.5 h as was applied in ELISA. All chips and microplates were measured in duplicate yielding similar results (relative signal intensity (microarray): 100% and 96% for MAb 62, 12% and 9% for MAb 195, 6% and 6% for MAb 287). Altogether, both methods yield data of comparable quality with very good overall selectivity.

The spotting routine was tested by immobilizing polyclonal anti-HRP antibody as described elsewhere,[18] yielding a standard deviation of 2.9% for the chemiluminescence signal intensity between spots. For testing the assay reproducibility, the cell culture supernatant of the high-affinity MAb 62 was spotted in a grid of 7 × 15 spots on 6 chips on different days. The intrachip coefficient of variation (CV) of the relative signal intensity was between 9 and 15%, whereas the interchip CV was lower (6%).

The time required for printing 96 samples on duplicate chips using one solid pin is 4 h. The microarray printing system used for this work allows simultaneous spotting of up to eight samples using eight solid pins, which reduces the spotting time down to 30 min per chip printed in duplicate. One advantage of the microarrray fabrication is that the hybridoma cell supernatants can be spotted directly on the surface without any previous dilution step. Hence, a microplate used for cell cultivation can directly be placed into the arraying system (humid chamber). Another advantage compared to most standard methods is that a volume of only a few nanoliters of cell supernatant per spot is required. The incubation of the supernatants on the chip surface and the subsequent blocking step can be carried out over a 4 h time period (each) or overnight, yielding the same results. The flow-through assay is performed using an automated fluidic and readout system and only requires 7 min per chip. Altogether, the time required for the automated screening method is comparable to the standard microplate-based ELISA for the same number of samples (here: 373). However, the work load is significantly reduced as the number of steps that have to be carried out manually is minimized. An advantage of the new method is that printing a chip duplicate instead of a single chip does not increase the work load, whereas performing the measurements in duplicate by ELISA doubles the number of pipetting steps. Additionally the maximum spot density has not been fully attained yet. By using a smaller pin (SNS 9, Bio-Rad), as well as a smaller spot distance (0.70 mm), one can make use of the whole spot area (9 mm × 26 mm) and apply a grid of 12 × 33 spots. So, all samples in a 384-well microplate plus 6 positive and 6 negative controls can be spotted on one chip. This was not demonstrated here, because at the time cell culture

supernatants were cultivated by default in 96-well microplates, which were put directly in the spotter.

CONCLUSION

The hybridoma screening method presented here describes a reliable and highly automated microarray-based technique for the identification of high-affinity monoclonal antibodies (MAbs) against a given target in hybridoma cell supernatants. The method proved especially valuable for the generation of MAbs against low-molecular weight analytes but can also be applied for high-molecular weight targets. The screening method requires a comparable amount of time compared to the standard ELISA (which is still one of the fastest hybridoma screening methods). However, there is a significant reduction of required work load. The new method might furthermore allow improvements of the initial cell cultivation step of the fused cells, which is usually carried out in 96-well plates. With the use of the hybridoma screening method described here, only nanoliter volumes of supernatant per clone are required. Consequently, the hybridoma cells can be cultivated in low-volume 384-well microplates and the hybridoma screening can be carried out earlier after the cell fusion. This is favorable because cell stability generally decreases with the time elapsed in cell culture. The chance that interesting, analyte-specific MAbs are overgrown by other clones present in the same well might be reduced when shortening this cell cultivation step.

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