Development of an Internally Controlled Antibody Microarray*

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Antibody microarrays are a high throughput technology used to concurrently screen for protein expression. Most antibody arrays currently used are based on the ELISA sandwich approach that uses two antibodies to screen for the expression of a limited number of proteins. Also because antigen-antibody interactions are concentration-dependent, antibody microarrays need to normalize the amount of antibody that is used. In response to the limitations with the currently existing technology we have developed a single antibody-based microarray where the quantity of antibody spotted is used to standardize the antigen concentration. In addition, this new array utilizes an internally controlled system where one color represents the amount of antibody spotted, and the other color represents the amount of the antigen that is used to quantify the level of protein expression. When compared with median fluorescence intensity alone, normalization for antibody spot intensity decreased variability and lowered the limits of detection. This new antibody array was tested using standard cytokine proteins and also cell lysates obtained from mouse macrophages stimulated in vitro and evaluated for the expression of the cytokine proteins interleukin (IL)-1β, IL-5, IL-6, and macrophage inflammatory proteins 1α and 1β. The levels of protein expression seen with the antibody microarray was compared with that obtained with Western blot analysis, and the magnitude of protein expression observed was similar with both technologies with the antibody array actually showing a greater degree of sensitivity. In summary, we have developed a new type of antibody microarray to screen for protein expression that utilizes a single antibody and controls for the amount of antibody spotted. This type of array appears at least as sensitive as Western blot analysis, and the technology can be scaled up for high throughput screening for hundreds of proteins in complex biofluids such as blood. Molecular & Cellular Proteomics 4:1664–1672, 2005.

Western blot analysis and ELISAs are robust low throughput methods used to analyze protein expression. A high throughput method to quickly screen for the expression of several proteins in complex biofluids is needed to provide a general overview of the proteome in disease processes. Antibody microarrays are a solid phase technology that can be used to screen expression of multiple proteins concurrently (1). Several antibody-based techniques have been developed and productively used to profile protein expression (2–6). The technologies presently used are similar to either DNA microarrays (2, 6) or sandwich ELISA techniques (7). The use of two differentially labeled protein extracts is similar to how DNA microarrays are screened and allows for pairwise comparisons. The dual label system is commonly used commercially and established in the literature (2, 6). Although this system allows for rapid pairwise comparisons based on a “control” lysate it does not control for the amount of antibody bound to the slide or label incorporation and may require amplification. Unlike labeled cDNA approaches, a “normal” is difficult to obtain and susceptible to freeze-thaw cycles. Another commonly used approach is a microsandwich ELISA technique for protein using a capture and detection antibody; it can be quantitative but requires two protein-specific antibodies and the purified antigen. A shortcoming with most of the existing antibody arrays is a lack of internal controls to quantify changes in antibody spotting density. Because the kinetics of antibody-antigen interactions depend on both antibody and antigen concentration, it is necessary to control for the amount of antibody spotted (8, 9).

Given these shortcomings we wanted to develop a high throughput antibody-based protein array detection system that could be used to screen for protein expression patterns in complex biological fluids. Specifically we developed an antibody array approach that (i) uses a general detection antibody, (ii) allows for multiple comparisons, (iii) contains internal controls for hybridization normalization, and (iv) uses an antigen labeling method that can be easily quantified for labeling efficiency. The antibody array described here uses the antibody as an internal control and a two-color detection system with one color quantifying the antigen and the second quantifying the antibody.

EXPERIMENTAL PROCEDURES

In Vitro Protein Expression from Mouse Peritoneal Macrophages—Mouse macrophages were the source of cellular proteins used to test the antibody array system. Mouse peritoneal macrophages were in-

*Antibody Microarray*

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duced from pathogen-free CD-1 mice in a method described previously (10). These experiments were approved by the Unit for Laboratory Animal Medicine at the University of Michigan and were in accordance with standards described in “The Guide for the Care and Use of Laboratory Animals.” Macrophages were induced by a 1-ml intraperitoneal injection of sterile 5% thioglycollate solution (Sigma). Four days post-injection the mice were euthanized by carbon dioxide asphyxiation, and the peritoneal cavity was lavaged three times with 5 ml of ice-cold phosphate-buffered saline (Invitrogen) and diluted 1:1 in ice cold RPMI 1640 medium (Invitrogen). Cells were pelleted by centrifugation at 450 × g for 15 min at 4 °C (Beckman Coulter), and resuspended in growth medium (RPMI 1640 medium with 50 μg/ml BSA (Invitrogen)). Yield and viability were determined by hemocytometer counts in diluted trypan blue solution. Macrophages were plated at a density of 2 × 10^6 cells/well in a 6-well plate (BD Falcon, San Jose, CA) and incubated for 18–24 h under standard conditions (37 °C, 5% CO2, and 95% relative humidity). Resting cells were treated with medium (unstimulated), lipopolysaccharide (LPS) (10 μg/ml diluted in RPMI 1640 medium) (serotype 055:B5, catalogue number L2880, Sigma), LPS (10 μg/ml)/interferon-γ (IFN-γ diluted in RPMI 1640 medium) (25 units/ml) (11, 12), or BSA/anti-BSA immune complexes (13). After 24 h, medium was removed, and cells were lysed in 200 μl of lysis buffer (1 × PBS (Invitrogen), 1% Nonidet P-40 (Sigma), 0.5% sodium deoxycholate, and 0.1% SDS (Sigma) supplemented with proteinase inhibitors (mini-Complete, Roche Applied Science). Cell lysates were collected in microcentrifuge tubes, centrifuged for 30 min at 14,000 × g at 4 °C, and transferred to a new tube. Protein concentration was determined using BCA protein quantification method (Pierce).

DNP-SE Labeling of Protein—The macrophage cellular protein lysates and the recombinant proteins IL-1β, IL-5, IL-6, MIP-1α, and MIP-1β (R&D Systems, Minneapolis, MN) were labeled using 6-(2,4-dinitrophenyl) aminohexanoic acid, succinimidyl ester (DNP-SE) (Molecular Probes, Inc., Eugene, OR). Protein (100 μg) in lysis buffer was diluted by at least 2-fold with water and adjusted using 100 mM sodium bicarbonate buffer to pH 9.0. As a control for labeling efficiency and to act as positive controls, carbonic anhydrase and trypsin inhibitor (Sigma) were spiked into the extracted cellular protein at a concentration of 250 and 50 pg/ml, respectively. DNP-SE (100 μg/ml) in spotting solution on a vertically rotating platform at 25 °C. Spotted slides were washed once for 5 min in TBS and blocked for 1 h in antibody array blocking buffer (1% BSA, 1% powdered milk in TBS-t). The slides were dipped in TBS-t for 30 s, and the liquid surrounding the array grid was removed by vacuum. Labeled protein (10 μg) was applied to the array, and a clean coverslip was placed over the solution. Slides were placed in a humid chamber and were incubated on a horizontally rotating platform for 1 h at 25 °C. Coverslips and labeled protein were removed by dipping the slides in TBS-t. The slides were washed once in high salt TBS-t (TBS-t containing 500 mM NaCl) for 5 min followed by two washes for 5 min in TBS-t. Excess liquid was removed, and universal secondary antibody solution (Invitrogen) containing a 1:2500 dilution of biotin-conjugated donkey anti-goat secondary antibody (Chemicon) was applied under a coverslip. The universal secondary antibody is a mixture of different antibodies that react with the constant region of antibodies from a range of different sources. The universal antibody was incubated for 30 min at 25 °C in a humidified rotating chamber. The slides were washed three times in TBS-t. The labeled protein and universal antibodies were detected by incubating the slide with Cy5™-anti-DNP and Cy3™-streptavidin (Zymed Laboratories Inc.) diluted 1:2500 in antibody array block solution. Slides were placed into a heat-sealed pouch and incubated at 25 °C for 1 h on a vertically rotating platform. Slides were washed once in high salt TBS-t for 5 min followed by two washes of TBS-t and two washes of TBS. Slides were dipped in molecular biology-grade water, placed in a metal slide carrier, dried in a centrifuge for 7 min at 500 × g, and stored in the dark until scanning.

Standard One-color Antibody Microarrays—To control for the universal secondary antibodies a one-color antibody array was tested. These antibody arrays were manufactured and hybridized with the DNP-labeled protein mixture described above. The universal secondary antibody step was excluded, and the DNP was detected as above. Once washed and dried the antibody microarrays were quantified using median fluorescence intensity (MFI).

Quantification of Hybridized Antibody Microarrays—Slides were scanned on an Axon 4000B scanner using GenPix Pro 4.1 (Axon Instruments, Union City, CA) following standard protocols. Laser intensity was set to provide optimal signal intensity with the least amount of background and no saturated pixels in the antibody spots. The median background and signal intensity were exported into an Excel spreadsheet, and signal intensity was calculated by subtracting background from signal intensity. Normalized spot intensity was calculated by taking the ratio of the antigen to antibody signals. The
mean normalized spot intensity was calculated by averaging median spot intensities. To allow for comparison with Western blot analysis results, the control was set as 100%, and intensity was calculated as a normalized percentage of control.

Western Blot Analysis of Macrophage Proteins—Total cellular protein (100 μg) was run under denaturing conditions on a 4–12% BisTris NuPage precast two-dimensional gel at a constant 200 V for 35 min (Invitrogen). The gel was blotted onto nitrocellulose (Invitrogen) using a TransPhor (Bio-Rad) semidry transfer apparatus in 2× NuPage transfer buffer with 20% methanol at a constant 10 V for 1 h. The blot was washed in TBS-t and blocked for 1 h in blocking buffer (5% dry milk powder in TBS-t). The blocked membrane was placed onto the Miniblotter 28 (Immunetics Inc., Boston, MA), and the membrane was washed with 25 ml of TBS-t using the wash manifold. Antibodies recognizing IL-1β (R&D Systems), IL-5 (R&D Systems), IL-6 (R&D Systems), MIP-1α (R&D Systems), MIP-1β (R&D Systems), and glyceraldehyde-3-phosphate dehydrogenase (1:10,000) (AbCam, Cambridge, MA) were diluted 1:500 in blocking buffer except where noted, and 58 μl were added to each well. Each antibody had three replicate lanes per experiment. Primary antibodies were incubated for 1 h on a horizontally rotating platform at 25 °C, and 25 ml of TBS-t was flushed through the lanes using the washing manifold. The membranes were removed from the Miniblotter 28 and washed for 5 min in high salt TBS-t (500 mM NaCl) followed by a 5-min wash in TBS-t. Secondary antibodies conjugated with horseradish peroxidase (HRP) (Zymed Laboratories Inc.) were diluted 1:5,000 in blocking buffer, placed on the washed membranes, and incubated for 30 min at 25 °C. Secondary antibody was removed, and the blots were washed three times in TBS-t for 5 min. The membranes were washed once in TBS, and proteins were detected using enhanced chemiluminescence (ECL+) and Hyperfilm ECL (Amersham Biosciences). Band and background intensities were quantified using UnScanIt (Silk Scientific, Orem, UT), and background intensity was subtracted from band intensity. Mean and S.D. of normalized band intensity were calculated and plotted using PrismGraph (GraphPad Software, Inc., San Diego, CA). Miniblotter 28 results were also verified using standard Western blots (data not shown).

RESULTS

Overview of the Antibody Array—A general overview of the steps involved in setting up the internally controlled antibody microarray is diagramed in Fig. 1. Antibodies were diluted in spotting buffer and spotted onto a solid substrate on epoxy ES slides by the non-contact arrayer. The array was then treated with the blocking buffer followed by incubation with labeled protein lysate. The lysate was directly labeled with the DNP-SE hapten (i.e., DNP or fluorescein). Excess lysate was removed by washing the slides. The amount of antibody spotted was determined by using a universal secondary antibody. The cell lysate proteins labeled with the hapten were detected with the fluorescently labeled Cy5-anti-DNP anti-hapten antibody along with the detection of the universal antibody with fluorescently labeled Cy3-streptavidin. The slides were washed and dried, and using a confocal laser scanner, fluorescence intensity of the antigen and the normalizing antibody was determined. The normalized amount of antigen expression was determined as a ratio of the amount of antibody.

Labeling of Proteins—The isolated peritoneal macrophages were stimulated by LPS, LPS/IFNγ, or immune complexes in vitro as described above, and cell lysates were collected for protein analysis by the antibody array. The proteins in the cell lysates as well as the recombinant proteins were labeled with DNP-SE. Because removal of the unbound DNP-SE is critical for a low background (data not shown) the abilities of SM-2 macrophores or gel filtration chromatography to remove unconjugated DNP-SE from the macrophage lysate proteins were compared. As shown in Table I the SM-2 beads effectively removed all but 0.2% of the free DNP while retaining 91.4% of cellular protein. Size exclusion chromatography

| TABLE I Comparison of free DNP removal techniques |
|-------------------------------------------------|
| Gel filtration chromatography and SM-2 macrophores | used to remove free dinitrophenol (DNP) from labeled macrophage lysate. Percent protein recovery, DNP (A348) absorbance, and free DNP (A348) are shown along with S.D. in parentheses (n = 4). |
| Percent protein recovery | DNP label (A348) | Free DNP (A348) |
|-------------------------|-----------------|----------------|
| Gel filtration          | 78.1 (5.3)      | 0.036 (0.002)  | <0.005         |
| SM-2 Bio-Beads          | 91.4 (5.9)      | 0.046 (0.001)  | <0.005         |
removed all but 0.3% of the free DNP; however, only 78.1% of the protein was recovered. DNP absorbance corresponded to the amount of protein recovered. Thus, the SM-2 beads were much more effective at removing the unbound DNP-SE than size exclusion techniques and were used in the subsequent array studies.

**Characterization of the Antibody Array Using Standard Proteins**—The antibody array limits of detection and cross-reactivity were determined using a mixture of recombinant protein standards diluted in BSA. The standard curve was determined from 0 to 5000 pg/ml using DNP-labeled recombinant standards diluted in BSA (Fig. 2A), and the limit of detection was determined (Fig. 2B). The effect of using the antibody for normalization was determined on the standards, and IL-1β is shown (C). Finally, the standards were tested individually at 1000 pg/ml to determine cross-reactivity. The antibody and antigens were detected using strepavidin-Cy5 and Cy3-anti-DNP, respectively. A representative figure of IL-1β is show in D. Error bars show S.D. from at least three independent experiments.

**Fig. 2. Characterization of the antibody array using standard proteins.** The standard curve, lower limit of detection, comparison to MFI (not normalized), and cross-reactivity were determined using standard proteins. The standard curve was determined from 0 to 5000 pg/ml using DNP-labeled recombinant standards diluted in BSA (A), and the limit of detection was determined (B). The effect of using the antibody for normalization was determined on the standards, and IL-1β is shown (C). Finally, the standards were tested individually at 1000 pg/ml to determine cross-reactivity. The antibody and antigens were detected using strepavidin-Cy5 and Cy3-anti-DNP, respectively. A representative figure of IL-1β is show in D. Error bars show S.D. from at least three independent experiments.
tested antigens, and a representative array with IL-1β spiking is shown in Fig. 2D.

Characterization of Protein Expression from Mouse Macrophages by the Antibody Array—The antibody microarray contained five specific antibodies (IL-1β, IL-5, IL-6, MIP-1α, and MIP-1β) and two control antibodies (trypsin inhibitor and carbonic anhydrase) that were spotted in quadruplicate on epoxy ES glass slides as illustrated in Fig. 3. Several nonspecific isotype control antibodies were also spotted with a range of different additives such as gelatin, bovine serum albumin, or trehalose. The use of gelatin but not bovine serum albumin or trehalose caused smearing and nonspecific fluorescence. In Fig. 3A the entire antibody array is shown with the Cy5 staining of the antigen, Cy3 staining of the antibody, and the combination of the fluorescent channels. The white box indicates the expanded region shown in Fig. 3B. Fig. 3B shows a single set of antibodies in the antigen channel with detection of the proteins in the macrophage lysate after simulation with the various agonists. Looking at the brightness of the antigen channel spots there are differences in expression levels. For example, the IL-1β spot intensity increases in macrophages treated with LPS. Despite spotting the same amount of antibody, antibodies from different host animals had different fluorescence intensities (data not shown).

Quantification of the fluorescence intensities was done, and the data are shown in Fig. 4. CD-1 mouse macrophage cells were stimulated with LPS, LPS/IFNγ, or immune complexes in serum-free medium. Total cellular protein expression was analyzed by antibody array. As labeling and hybridization controls, carbonic anhydrase and trypsin inhibitor were spiked into the cellular lysate. Spiked control expression of trypsin inhibitor and carbonic anhydrase remained relatively stable throughout the entire experiments except for the LPS treatment group that had about a 50% lower relative intensity. Interestingly the relative intensity of trypsin inhibitor and carbonic anhydrase was similar in all experiment despite 5-fold more trypsin inhibitor. A different affinity for their antigen is the most likely cause of this discrepancy.
The antibody array analysis shows that the levels of IL-1β and IL-6 are increased in the macrophages stimulated with LPS but not by LPS/IFN-γ or immune complexes. IL-5 appeared to be decreased by all treatment groups when compared with control. MIP-1α expression was unchanged by LPS; however, LPS/IFN-γ and immune complexes slightly decreased the protein expression of this cytokine. MIP-1β expression remained unchanged throughout the different experimental groups.

A comparison of the internally controlled antibody array to a similar array without the internal control is shown in Table II. The standard one-color antibody microarray lacks the universal secondary antibody and controls for protein binding and the effect of normalizing against the amount of antibody spotted. There was no statistical difference between the mean fluorescence intensity of the standard and internally controlled microarrays. The intra-assay variability or the variability that occurs between the replicates on the same slide indicates either hybridization differences or antibody spotting density differences. The majority of the standard one-color intra-assay variability was around 10% with only IL-5 being greater because of nonspecific background. When the amount of antibody spotted was accounted for, the intra-assay variability dropped by 50% so nearly all analytes had less than 10% variability. The interassay variability or the variability seen between the different arrays indicates slide-to-slide variability as well as antibody spotting differences. The standard one-color antibody array showed variability (11 and 39%). When the amount of antibody spotted was accounted for, it dropped to between 6 and 13%. These data indicate that controlling for the amount of antibody deposited on the slide decreases the amount of intra- and interslide variability.

**Table II**

|                | Standard one-color | Internally controlled |
|----------------|--------------------|-----------------------|
|                | Intra-assay variability | Interassay variability | Intra-assay variability | Interassay variability |
| IL-1β          | 9                  | 18                    | 4                      | 9                     |
| IL-5           | 23                 | 39                    | 11                     | 13                    |
| IL-6           | 7                  | 11                    | 6                      | 6                     |
| MIP-1α         | 11                 | 18                    | 3                      | 7                     |
| MIP-1β         | 13                 | 27                    | 3                      | 8                     |

Comparison of Antibody Array and Western Blot—Western blot analysis of the macrophage protein expression was done using the same antibodies to verify the antibody microarray results. To normalize for differences in technology, all expression values were normalized as a percentage of the unstimulated cells. The expression patterns were similar for the Western blot analysis and the antibody array (Fig. 5).

The expression of IL-1β after LPS stimulation of the macrophages was reduced in Western blot analysis with an increase of 241 versus 598% in the antibody array as compared with the expression in the unstimulated cells. In contrast, a 148% increase in IL-1β expression in the Western blot analysis of LPS/IFN-γ and immune complex-treated macrophages was not detected in the antibody array. Both Western blotting and the antibody microarray detected decreased expression of IL-5 of 60 and 52%, respectively, after LPS stimulation as
compared with unstimulated cells. However, the antibody microarray detected further decreases in expression of IL-5 after LPS/IFN-γ or immune complex stimulation that was not detected by Western blot analysis. The expression pattern of IL-6 was similar after LPS stimulation using both methods, whereas the other treatment groups remained elevated in antibody microarray determinations but were slightly down-regulated via Western blot analysis. In conclusion, it appears that the antibody array results closely mimic those seen with Western blot analysis. Furthermore the antibody arrays are a sensitive method for measuring levels of some cytokines.

**DISCUSSION**

The results from this study show for the first time that an internally controlled color antibody array utilizing a single antibody has the potential to accurately assess proteins in complex fluids. Furthermore comparison studies with Western blot analysis reveal that the internally controlled antibody array is not only accurate but may have a larger range of linearity over Western blot analysis in terms of measuring the levels of protein expression. Reciprocal labeling experiments showed no difference in the expression patterns of the stimulated mouse macrophage lysate. However, there was a 14% increase in background signal intensity when Cy3-anti-DNP was used to detect the antigen. Additionally experiments performed without the standardizing antibody showed no statistical difference in signal intensity but had a greater standard deviation both inter- and intra-assay. However, this result may be limited to the use of antibody microarrays that use hapten-labeled proteins.

Another major advantage of this type of antibody array is the use of a single antibody for capture and quantification of the proteins. Many currently existing antibody arrays utilize a sandwich ELISA technique that requires two specific antibodies: one to capture the protein and another antibody for detection. This sandwich ELISA technique can be semiquantitative, but the requirement for two specific antibodies for each protein detected has limited the number of proteins that can be assessed at a given time. Our single antibody system can be scaled up to allow simultaneous assessment of several proteins.
hundred proteins in a high throughput manner. Thus, this technology has the potential to screen complex mixtures of proteins without any a priori assumptions of which proteins are up-regulated in disease states.

Several technological limitations exist with the currently existing antibody arrays. One problem associated with microarrays in general is the lack of internal controls. This has been partially overcome in our studies by using the spotted antibody as a control for antigen binding. The antibody alone controls for amount of antibody spotted and allows for multiple comparisons across different antibody arrays lots.

Protein tagging using DNP-SE to label the amino terminus and lysine residues has a 2-fold purpose. First, ~5% of protein amino acids are lysine residues, which are ~98% solvent-accessible. This lysine acts as a form of signal amplification over use of a labeled capture antibody that recognizes a single epitope. Second, a spectrophotometer can be used to control for labeling differences. This allows an additional level of control, which demonstrates if the labeling reaction was successful. A final control applied was the use of standard proteins (i.e. carbonic anhydrase and trypsin inhibitor) to normalize for labeling and hybridization differences. These proteins are readily available as SDS-PAGE standards and can be made at any concentration needed. Two standards are the absolute minimum for comparison, and additional standards have been recently used as controls. One interesting result was the similar amount of normalized antigen concentration for trypsin inhibitor and carbonic anhydrase despite a 5-fold difference in concentration. Despite their similar size the normalized antigen concentration is probably due to differences in antibody affinity. Thus, the use of the internally controlled system with a range of different antibodies against the same protein may be used to compare binding characteristics.

The removal of dinitrophenol, fluorophores, and other hydrophobic organic molecules has been accomplished using SM-2 macroporous beads (14, 15). When compared with size exclusion chromatography, the use of SM-2 Bio-Beads is a rapid method that can easily be scaled to high throughput labeling reactions. This removal of free DNP is critical in reducing the degree of background staining.

This internally controlled system is adaptable for different labeled proteins. Several different permutations of hapten or fluorochrome labeling of the cellular lysate were tested. Directly attaching the fluorochrome Cy3 or Cy5 to cellular protein caused a marked increase in background fluorescence (data not shown). One possibility to account for the high nonspecific background caused by direct Cy3 labeling is a change in the solubility of the proteins.

Our studies demonstrate that this single antibody array technology is superior for larger arrays (i.e. >50 antibodies) where it becomes difficult to titrate individual secondary antibodies and protein standards. Although the use of this single antibody array for normalization allows for quantification, a micro-ELISA-based system would be more useful for repeat quantification studies once specific proteins of interest have been identified by the single antibody array. The technology described in this study decreases overall standard deviation when compared with hapten-based labeling.

CONCLUDING REMARKS

In conclusion, we have developed a single antibody-based protein array technology that appears at least as sensitive as Western blot analysis and has internal controls to assess the specificity of the protein-antibody binding. This approach can be easily automated in a high throughput manner and thus has the potential to provide a discovery platform to detect the presence of hundreds of proteins in complex biofluids such as blood in disease processes. Additionally using the amount of antibody spotted as an internal control can be applied to both the sandwich ELISA and pairwise comparison antibody microarray methods.

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REFERENCES

1. Templin, M. F., Stoll, D., Schrenk, M., Traub, P. C., Vohringer, C. F., and Joos, T. O. (2002) Protein microarray technology. Trends Biotechnol. 20, 160–166
2. Haab, B. B., Dunham, M. J., and Brown, P. O. (2001) Protein microarrays for highly parallel detection and quantitation of specific proteins and antibodies in complex genomes. Genome Biol. 2, RESEARCH0004
3. Huang, R. P. (2001) Detection of multiple proteins in an antibody-based protein microarray system. J. Immunol. Methods 255, 1–13
4. Lesaicherre, M. L., Yue, R. Y., Chen, G. Y., Zhu, S. Q., and Yao, S. Q. (2002) Inhine-mediated biotinylaton of proteins and its application in a protein microarray. J. Am. Chem. Soc. 124, 8768–8789
5. MacBeath, G., and Schreiber, S. L. (2000) Printing proteins as microarrays for high-throughput function determination. Science 289, 1760–1763
6. Sreekumar, A., Nyati, M. K., Varambally, S., Barrette, T. R., Ghosh, D., Lawrence, T. S., and Chinnaiyan, A. M. (2001) Profiling of cancer cells using protein microarrays: discovery of novel radiation-regulated proteins. Cancer Res. 61, 7585–7593
7. Lin, Y., Huang, R., Santanam, N., Liu, Y., Parhasarahathy, S., and Huang, R. P. (2002) Profiling of human cytokines in healthy individuals with vitamin E supplementation by antibody array. Cancer Lett. 187, 17
8. Macario, A. J., and Conway de Macario, E. (1975) Antigen-binding properties of antibody molecules: time-course dynamics and biological significance. Curr. Top. Microbiol. Immunol. 71, 125–170
9. Froese, A., and Sehon, A. H. (1975) Kinetics of antibody-hapten reactions. Contemp. Top. Mol. Immunol. 4, 23–54
10. Leijh, P. C., van Zwet, T. L., ter Kulle, M. N., and van Furth, R. (1984) Effect of thioglycolate on phagocytic and microbicidal activities of peritoneal macrophages. Infect. Immun. 46, 448–452
11. Cohn, Z. A. (1978) Activation of mononuclear phagocytes: fact, fancy, and future. J. Immunol. 121, 813–816
12. Chen, T., Lei, M. G., Suzuki, T., and Morrison, D. C. (1992) Lipopolysac-
charide receptors and signal transduction pathways in mononuclear phagocytes. *Curr. Top. Microbiol. Immunol.* 181, 169–188

13. Warner, R. L., Bless, N. M., Lewis, C. S., Younkin, E., Beltran, L., Guo, R., Johnson, K. J., and Varani, J. (2000) Time-dependent inhibition of immune complex-induced lung injury by catalase: relationship to alterations in macrophage and neutrophil matrix metalloproteinase elaboration. *Free Radic. Biol. Med.* 29, 8–16

14. Spack, E G., Packard, B., Wier, M. L., and Edidin, M. (1988) Hydrophobic adsorption chromatography to reduce nonspecific staining by rhodamine-labeled antibodies. *Anal. Biochem.* 158, 233–237

15. Junk, G A., Richard, J. J., Grieser, M. D., Witiak, D., Witiak, J. L., Arguello, M. D., Vick, R., Svec, H. J., Fritz, J. S., Calder, G. V. (1974) Use of macroreticular resins in the analysis of water for trace organic contaminants. *J. Chromatogr.* 99, 745–762