Anal. Chem. **2002,** *74,* 927−933

Accelerated Articles

Immunoassay with Multicomponent Protein Microarrays Fabricated by Electrospray Deposition

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Two new techniques were recently developed in these laboratories for fabrication of protein microarrays: electrospray deposition of dry proteins and covalent linking of proteins from dry deposits to a dextran-grafted surface. Here we apply these techniques to simultaneously fabricate 1200 identical microarrays. Each microarray, 0.6 × 0.6 mm^2 in size, consists of 28 different protein antigens and allergens deposited as spots, 30−40 μm in diameter. The ability of the microarrays to detect IgG antibodies in plasma samples from mice immunized with different sets of antigens and IgE antibodies in human plasmas was examined using ELISA. Comparison of the microarray-based ELISAs with standard ELISAs in microtiter plates established that both techniques provided identical responses in 88% of all the antibody/antigen interactions tested. Both techniques showed similar antibody detecting sensitivity defined by the maximum dilution of serum at which a reliable signal distinguishable from the background was obtained.

Any technology for fabricating protein microarrays requires two steps: (i) deposition of proteins in an active state and (ii) their immobilization. Arrays of substances can be deposited in serial or parallel fashion. Serial robotic microspotting methods have been used to fabricate the first antibody arrays by Ekins et al.[1,2] and later were applied to the manufacture of microarrays of antigens.[2−7] However, these serial methods encounter problems in production of a large number of complex microarrays since the number of motions needed to manufacture N array with M elements each is proportional to $N \times M$ and such large-scale motions cannot be carried out rapidly without sacrificing precision.[8] On the other hand, parallel methods allow rapid creation of many identical arrays simultaneously on one substrate. Until recently, only one such parallel technology has been described that is applicable to fabricating protein microarrays, microcontact printing.[9,10] Though microcontact printing provides astonishing resolution, the amount of the protein transferred from stamp to substrate cannot be precisely controlled and the technique is inefficient for conservation of expensive proteins. Recently we reported another parallel technique, electrospray deposition (ESD), which overcomes some of the disadvantages of the microcontact printing technology. ESD relies on generation of dry charged products (ions and nanoclusters of protein molecules) from microdroplets of protein solutions electrosprayed in a dry (less than 30% humidity) atmosphere. These dry charged products are then deposited simultaneously in many positions on a suitable

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10.1021/ac010970k CCC: $22.00
Published on Web 01/29/2002

conducting substrate under control of electrostatic forces.[11,12] The technology is rapid, is highly economical in the amount of proteins needed (e.g., 1 μL of solution can be distributed between 100 000 spots in 20−30 s), and can generate highly miniaturized arrays with spots as small as several micrometers in diameter. Parameters of the ESD process have been studied and conditions found under which protein activity is retained.[12]

One major advantage of the ESD technology for fabricating microarrays is that dry proteins are deposited. Klibanov et al. showed that storage of proteins as wet deposits at intermediate humidities of 60−80% results in rapid loss of their activity and solubility.[13] But these are precisely the conditions recommended to prevent clogging the spotting tips in microspotting.[8] By contrast, ESD allows deposition at low relative humidity, A <20−30%, with dehydration of proteins so rapid that no major change in conformation occurs. For example, we have shown that microdroplets dry within a millisecond time interval[14] so that solutes are deposited in a dry state provided the distance between the tip and the substrate is sufficient (>20 mm). Deposition of biopolymers in the dry form was demonstrated by direct imaging of the deposited molecules using tunneling[15] and force microscopy.[14] Dry protein deposits can then be stored at room temperature for a long time without loss of activity provided carbohydrate protectants are added and the humidity is less than 20−30%.[16,17] Thus, ESD effectively solves one major problem in fabricating protein microarrays, namely, preserving protein structure and function during fabrication. In contrast to microcontact printing also, the amount of the deposited protein is easily controlled.

We recently reported a method for immobilizing proteins in dry deposits, covalently linking proteins to an aluminum substrate via an oxidized dextran spacer.[18] Using microarray-based ELISA, we demonstrated that this procedure combines high sensitivity and low background in the case of several antigen−antibody pairs. In this paper, we demonstrate that a combination of ESD and immobilization via oxidized dextran can be applied to fabrication of microarrays from a large variety of protein antigens and allergens. The performance of these new microarrays containing a variety of different protein molecules in a single assay is compared with that of standard ELISAs (ST-ELISA) in analysis of antibodies from human and mouse sera. The new microarrays detect antibodies as effectively as ST-ELISAs, with obvious advantages in terms of conserving materials and rapidity of fabrication.

EXPERIMENTAL SECTION

Reagents. Dextran, M_r = 20 000, was a product of Loba-Chemie. $NaBH_4$ and $NaCNBH_3$ were products of Jensey Co. ELF-97 cytological kit was purchased in the Molecular Probes, Inc. (Eugene, OR). Human serums with standard amounts of IgE were taken from a Diaplus IgE IFA kit, a product of the Diaplus Co. Glycerol of molecular biology quality was purchased in the ICN Biomed. Inc. All the following reagents were from Sigma: *p*-nitrophenyl phosphate; (γ-aminopropyl)triethoxysilane (APTES); mouse IgG; alkaline phosphatase (AP) from bovine intestinal mucosa; AP conjugated anti-mouse IgG (F_c-specific, from goat); anti-mouse IgG (polyclonal, against whole molecule, from goat); monoclonal antibody to human IgE, anti-hIgE, (mouse ascite liquid); AP conjugated anti-hIgE from goat (ϵ-chain specific); bovine serum albumin; ovalbumin; goat's serum albumin; horseradish peroxidase; papain; β-galactosidase from *Escherichia coli*; concanavalin A from jack bean; sperm whale myoglobin; horse myoglobin; soy bean trypsin inhibitor; avidin; hen egg white lysozyme; yeast hexokinase; urease from jack bean; yeast alcohol dehydrogenase; acetylcholine esterase from electric eel; tyrosinase from mushrooms; casein; and Freund's adjuvant. A set of allergens (latex protein, allergens from German roach, two honey bee venom samples, and allergens from mite *Dermatophagoide pteronyssinus* and from mite *Dermatophagoide farinae*) was kindly provided by Dr. M. C. Swanson. All other chemicals were of analytical grade or purer.

Materials. Aluminized (0.5−0.6-μm layer of Al on one side film) 50-μm-thick Mylar film was used as a substrate for microarray fabrication. A ruby mica 50 μm thick was used to manufacture the mask.

Methods. (1) Preparation of Substrate for ESD. Aluminized surfaces of plastic were treated for 20−30 s in a plasma discharge at low air pressure,[19] rinsed with doubly distilled (d/d) water, and dried. Then the aluminum surface was aminated for 30 min in APTES solution (0.25 mL of APTES in 5 mL of d/d water, pH 7, prepared 1 h before using to allow APTES hydrolysis[20]), rinsed with d/d water, dried, and baked at 90−100 °C for 9−12 h in a nitrogen atmosphere. After baking, the pieces were immediately brought into contact with a solution of oxidized dextran (30−50 mg/mL, 4% of oxidized monomers, prepared as described in ref 21) for 1 h at room temperature, rinsed with d/d water, dried, and stored at −20 °C in dry air or under nitrogen.

(2) Preparation of Proteins. Weighed protein samples were dissolved in d/d water to obtain a concentration of 0.2 mg/mL, and their electrical conductivities were measured. Three solutions (honey bee venom 1 (HB1), a-hIgE ,and a-mIgG) with conductivities exceeding 100 μS/cm were dialyzed against d/d water, and protein concentration was determined in the dialyzed solutions using a quartz crystal microbalance.[12] Concentrated sucrose solution was then added to each protein to give a 10-fold excess over the dry mass of protein. To prevent autolysis of papain a stoichiometric amount of $HgCl_2$ was added to its solution.

(3) Fabrication of Microarrays. Microarrays were fabricated by ESD as described in ref 11 with minor modifications to the mask. Our present experiments used a mask made of mica sheet.

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An array of 1200 conic holes (spaced by 1.5 mm, entrance of 150 μm, output of 30−60 μm) was drilled in the mica by means of a rotating tungsten wire with a conic tip. The mask was glued to a rigid glass frame, which could be firmly and reproducibly fixed in the holder of the ESD device. A micrometer 3-D stage was used to hold the substrate film. The film was placed onto a disk made of a polished sintered metal. Pumping air from beneath the disk attached the substrate film firmly to the surface. After deposition of each protein, the substrate was moved to a new position by micrometer screws. Deposition was performed in a plastic chamber[12] filled with dry air (20−30% humidity). The distance between the ES capillary tip and the substrate surface was ~60 mm. A current of 20−25 nA was produced by applying +3 to +6 kV to a platinum electrode placed inside a glass capillary with a tip of outer diameter 20−30 μm. These conditions were found to be optimal for preserving functional activity of proteins.[12] Depositing each protein at 1200 positions took 3−5 min. This interval includes the time spent for changes and washing and refilling the ES capillary, manual displacement of substrate with respect to the mask, and adjustment of the electrical parameters for each protein solution. A total volume of 1 μL of each protein solution was electrosprayed. The volume sprayed was measured directly by observing changes in position of the meniscus in the ES capillary under a low-power microscope. Each deposited microarray was inspected using a low-power stereomicroscope. Microarrays with defects (e.g., missing spots) were discarded.

(4) Immobilization of Proteins in Microarray. Immobilization was performed as described in ref 18. The substrate film with dry protein deposits was cut into pieces, 3 × 3 mm^2, containing four identical microarrays each and the pieces were glued with a double-sided Scotch to special holders. The holders with the microarrays were placed in a Petri dish with 100% humidity inside where sucrose/protein dry spots immediately turned into microdroplets.[18] During 20-min exposure to the humid atmosphere, the covalent links via Shiff bases were formed between amino groups of proteins and carbonyl groups of the oxidized dextran molecules on the substrate surface. To reduce the Shiff bases and to make the links stable, the microarrays were placed for 10 min in a 5% cyanoborohydride solution containing 1% BSA. This solution was then changed with 5% solution of cyanoborohydride without BSA, and microarrays were kept there for another 10 min. After washing with physiological solution (0.15 M NaCl, pH 7.2−7.5), the microarrays were additionally treated with the blocking solution (1% dry milk in 100 mM TRIS, pH 7.2) and then rinsed 2−3 times with the washing solution (0.05% Tween 20 dissolved in the physiological solution).

(5) Microarray-Based ELISA (MB-ELISA). After immobilization and blocking, the microarrays were placed in the wells of a standard 96-well plate, each filled with 100 μL of analyzed plasma diluted with the blocking solution, and incubated there at 37 °C for 2 h. The solutions inside the wells were mixed thoroughly with magnetic stirrers. After incubation in the primary antibody, the microarrays were rinsed with washing solution 3 times and allowed to react overnight at 4 °C with 100 μL of secondary antibody conjugated with alkaline phosphatase diluted with the blocking solution, as recommended by the manufacturer. After a final 3−4 rinses with the washing solution, the microarrays were detached from the holders and placed face down for 1−2 h onto 10−20-μL droplets of fresh ELF-97 cytological kit solution prepared as recommended by the manufacturer. The microarrays were then washed twice with distilled water and dried.

(6) Detection in MB-ELISA. Fluorescent products were observed using a conventional fluorescent microscope equipped with a digital CCD camera (Pixera Corp.). The intensities of fluorescence of spots were measured using the ARRA program[22] developed by Dr. A. Deev in the Institute of Theoretical and Experimental Biophysics, Pushchino, Russia. It allows one to average gray levels in an array of circular areas with the position of each such area adjusted automatically or manually. In case of significant background fluorescence, this was subtracted as described below.

(7) Subtraction of Background upon Measurements of Fluorescence Intensity with a CCD Camera. In our experiments with a series of gray filters, it was found for a wide range of light intensities that the inverted output signal (digital brightness) of our CCD camera can be represented as

$$U = A + B \log I \tag{1}$$

where A is a numerical constant dependent on exposure time and $B = 143 \pm 6$ is an exposure-independent constant. Since the average intensity of fluorescence from a spot is the sum of fluorescence of ELF-97 product, I_s, and the background intensity, I_b, the signal measured in the dot area is equal to

$$U_s = A + B \log(I_s + I_b) \tag{2}$$

The background intensity, I_b, can be calculated from the signal, U_b, measured outside the spot area:

$$I_b = 10^{(U_b - A)/B} \tag{3}$$

From (2) and (3) we can calculate the corrected brightness, U_{corr}, for each spot in which the background intensity is subtracted:

$$U_{corr} = A + B \log I_s = B \log (10^{U_s/B} - 10^{U_b/B}) \tag{4}$$

Thus, only one experimental parameter independent of the exposure time is needed to account for the background.

(8) Standard ELISA with pNPP Substrate. Protein antigens were adsorbed onto a standard 96-well ELISA plate from 100 μL of solutions containing 20 μg/mL protein in physiological solution during an overnight incubation at 4 °C. All other procedures were as described above for fabricating microarrays except for omission of the cyanoborohydride treatment and use of the soluble substrate in the measurement of AP activity. At the final step, 100 μL of pNPP solution (1 mg/mL pNPP, 5 mM $MgCl_2$, 200 mM TRIS-HCl, pH 9.5) was added to each well. After incubation with stirring for 30 min at 37 °C, the reaction was stopped by addition of 100 μL of 2 M NaOH to each well. Optical density of the product was measured with a multiscan device at $\lambda = 405$ nm.

(9) Mice Immunization. Four groups of mice each containing three animals were immunized. Three mice (a−c) in the first

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group were immunized with concanavalin A (Con A). The second group was immunized with a mixture of equal amounts of Con A and trypsin inhibitor (TrI). The third group was immunized with a mixture of three proteins: Con A, TrI, and sperm whale myoglobin (Mbw). The fourth group was immunized with a mixture of 10 proteins: horse myoglobin (Mbh), lysozyme (HEWL), β-galactosidase (βGa), hexokinase (HK), avidin (Avi), tyrosinase (Trs), peroxidase (HRP), urease (Ure), alcohol dehydrogenase (ADH), and acetylcholine esterase (AcE). Two injections were made intraperitoneally at an interval of 3 weeks. The first contained 0.12 mg of total protein dissolved in 0.25 mL of water mixed with an equal volume of complete Freund's adjuvant. The second contained 40 μg of total protein dissolved in 80 μL of water mixed with an equal volume of incomplete Freund's adjuvant. Animals were decapitated 2 weeks after the second immunization, and serum plasma was prepared. Plasma was stored at −25 °C with 50% glycerol added.

RESULTS AND DISCUSSION

In our previous paper,[18] we reported a new technique for protein immobilization that combines dry ES deposition with covalent binding of the deposited proteins to an aluminum surface via dextran spacer. This technology is applied here to design high-density multiantigen (allergen) microarrays and to establish that the technology can be applied to a diverse set of proteins differing in structure (monomers, such as HEWL and multisubunit proteins such as Ure), in biological function (enzymes, binding proteins), in stability, and in reactivity of protein groups. A schematic of chip composition is presented in Figure 1A. A total of 28 different proteins were deposited in 6 × 6 arrays as spots, 30−40 μm in diameter, spaced by 125 μm. Each microarray occupied an area of 0.6 × 0.6 mm^2.

Qualitative Analysis of Mice Plasma with MB-ELISA. With ELF-97 as a substrate of the AP reaction, ELISA on microarray results in a system of fluorescent spots of different brightnesses. Figure 1B presents one example of a MB-ELISA of plasma from a mouse immunized with three antigens (Con A, TrI, Mbw). Comparison with the scheme in Figure 1A shows that spots of all these antigens are visible, indicating that MB-ELISA can readily detect specific IgG antibodies in the mouse serum. Even after immunization of mice with a set of 10 antigens simultaneously, positive signals are observed for most of these antigens (Figure 1C).

Apart from the spots of antigens used in immunization, we see a few additional spots on each microarray. These are not artifacts of MB-ELISA since the pattern of interactions found was confirmed (see Table 1) in standard ELISAs (ST-ELISA). The additional spots can be divided into three groups. The first consists of control spots the pattern of which depends on the type of plasma and on type of antibodies assayed. For example, three spots (a-mIgG, mIgG, and a-hIgE developed in mouse) are visible on all the microarrays when mouse plasma is tested for IgG antibodies, while only one spot (a-hIgE) is visible in assays of human plasma for IgE antibodies (see Figure 1F). Since mIgG can directly interact with a-mIgG conjugated with AP, intensities of the mIgG and a-hIgE spots do not depend on plasma dilution. The intensity of a-mIgG spot is also independent of plasma dilution at all the dilutions used in our experiments but for another reason. The total concentration of mIgG in normal plasma is so high (typically[23] ∼10 mg/mL) that even at the highest dilution (1:300 000) its concentration (30 ng/mL) can saturate nearly all the available a-mIgG sites. As seen in Figure 3, the brightness of a-mIgG spots is high and weakly dependent on the mIgG concentration when the latter exceeds 30 ng/mL.

The origin of the second group of additional spots on microarrays can be explained by cross-reactivity of antibodies to homologous antigens or generation of additional specific antibodies upon immunization of mice. Thus, an additional fluorescent spot in Figure 1B belongs to horse myoglobin, structurally homologous to Mbw. The ST-ELISA data presented in Table 1 also reveal cross-reactivity between Mbh and plasmas of all the mice immunized with Mbw. Other examples of plasma cross-reactivity can be seen in the data presented in Table 1. Both MB-ELISA and ST-ELISA reveal antibodies against urease in plasmas of nearly all mice immunized with Con A. Conversely plasmas of all the mice immunized with Ure contain antibodies against Con A. Since both these proteins are prepared from the same jack bean source, we suggest that these proteins either have the same impurities or similar epitopes (a true cross-reactivity of antibodies).

The third group of additional spots is specific for each mouse and can be considered as a "fingerprint" of plasma reactivity, characterizing the immunological status inherent in each animal. Examples of additional interactions revealed by MB-ELISA and confirmed by ST-ELISA are presented in Table 1. Enhanced reactivity of plasma from mouse 3a to βGa, that of 2c mouse to ADH, that of 3c mouse to Avi as well as a number of additional interactions can be readily seen.

Though antibodies to nearly all the antigens were detected in serum of the mice immunized with 10 proteins simultaneously, they are all seen only at low dilution, together with several other false positives observed in Figure 1C. On dilution of the sera, all these false positives disappear, but the number of detectable antibodies decreases as well (Figure 1D, E). As the agreement between MB-ELISA and ST-ELISA shows (see Table 1), these observations are not due to artifacts of the assay but reflect a true immune response in animals immunized with multiple antigens such that high-affinity antibodies are produced against a limited number of antigens while many nonspecific antibodies can be revealed in plasma at low dilution. The positive response to papain found in the plasma of all the mice from the fourth group may present an example for such nonspecific affinity.

Assay of IgE Antibodies in Human Plasma. The same antigen (allergen) containing microarrays were used to assay human plasmas for the presence of IgE antibodies and for IgE specificity. Figure 1F presents an illustration of analysis of a plasma obtained from one of the authors who experienced a severe allergic reaction to papain 20 years ago and has never been in contact with the protein since. It is seen that this plasma contains an appreciable amount of IgE antibodies specific to papain. Papain spots showed no positive reaction in assays with plasmas of two other co-authors of the present paper. Thus, ELISA with antigen (allergen) microarrays readily reveals both IgG and IgE antibodies in animal and human plasmas and may be used in rapid qualitative analysis of blood for the presence of specific antibodies of different classes.

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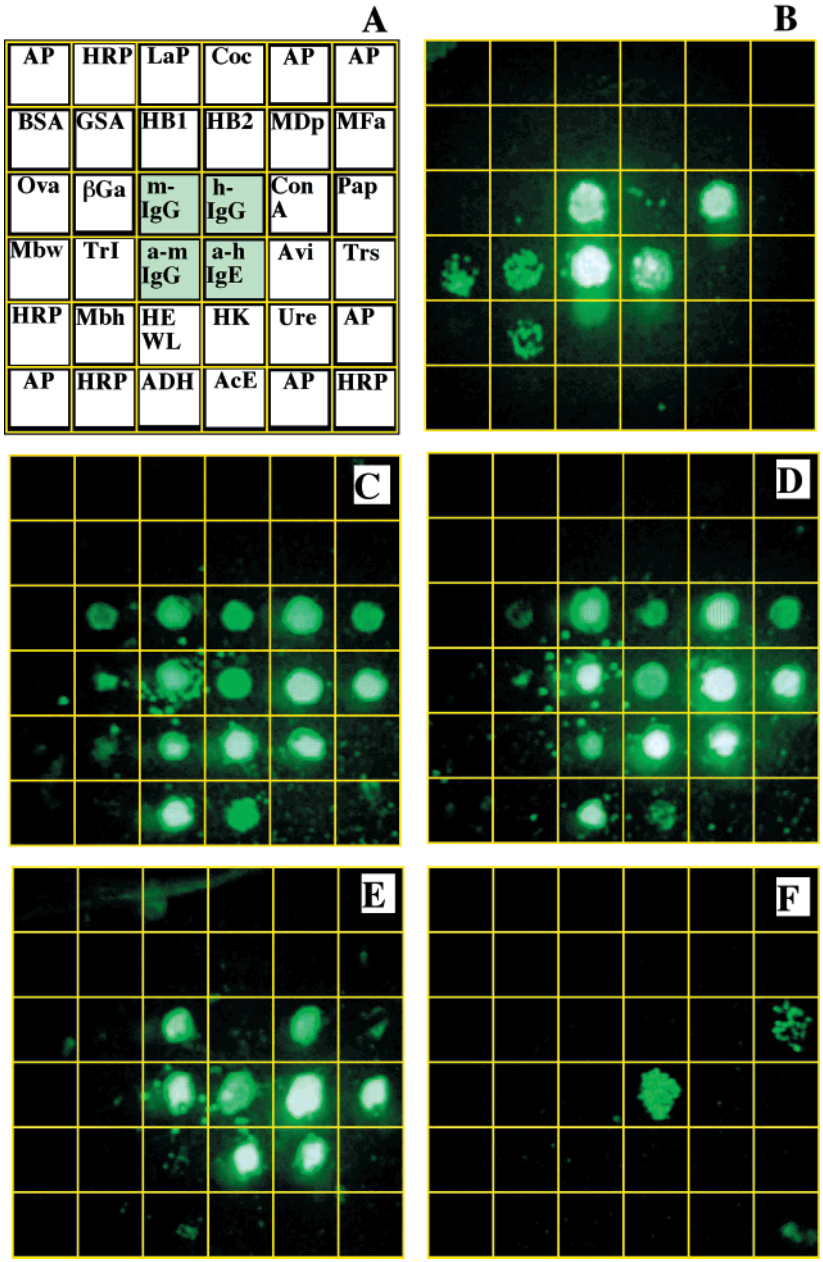

Figure 1. Microarray-based ELISA of mouse and human plasmas. Panel A shows the location of 28 antigens (allergens) on microarrays. Panel B shows distribution of fluorescent ELF-97 product after assay of plasma from mouse 3b (immunized with Con A, TrI, and Mbw) diluted 1:300 000. Panels C−E show results of assay of plasma from mouse 4b (immunized with Mbh, HEWL, βGa, HK, Avi, Trs, HRP, Ure, ADH, and AcE) diluted 1:300; 1:3000, and 1:30 000, respectively. Panel F presents an example of using the microarray in an allergenic test to detect specific IgE antibodies in a human serum diluted 1:6.

Comparison of Sensitivities of Microarray-Based and Standard ELISA on Microtiter Plates. Panels C−E of Figure 1 show that the fluorescence intensity of the spots decreases with plasma dilution and disappears at a certain dilution. This threshold dilution can be used as a measure of sensitivity in MB-ELISAs. To compare this with that of ST-ELISAs, we measured the latter for all the plasmas and a number of antigens. The sensitivity in ST-ELISA was estimated from the dilution at which the optical density (OD) becomes reliably (at least 1.5 times) higher than in the controls. Two types of controls were used. In assays of plasmas with proteins to which the mice had been previously immunized, the control OD was obtained in an experiment with plasma from a nonimmunized mouse properly diluted. Otherwise, the OD of a blank pNPP solution was used as a control.

Table 1 summarizes our data. Qualitative agreement between the microarray data and those obtained with ST-ELISA is readily seen. Testing 73 combinations of protein antigens and mice plasmas in the table shows that in 88% of cases positive and negative responses of both techniques coincide and that in 80% of cases a comparable sensitivity is also obtained. Assays of plasmas with the most antigens to which mice were immunized (shown in boldface figures in Table 1) reveal similar sensitivities of both techniques in 90% of cases. Only for one antigen, HRP, was a highly positive response in ST-ELISA accompanied by a very low response on the microarray. The origin of this difference is not yet clear. One possible explanation for a small signal on microarray consists of a limited ability of the peroxidase to form covalent bonds with oxidized dextran due to its small number of

Table 1. Comparison of Sensitivities of ELISA on Microarays and on Standard Microtiter Plates[a]

mouse plasma[b]	proteins												
	Con A	TrI	Mbw	Mbh	HEWL	βGa	HK	Avi	Trs	HRP	Ure	ADH	AcE
1a	**4/4**[c]	−/0	−/0	−/0	−/0	−/0	−/0	−/0	−/0	−/0	2/2	−/0	−/0
1b	**4/4**	−/0	1/0	−/0	−/0	−/0	−/0	−/3	−/0	−/0	1/1	−/0	−/0
1c	**4/4**	−/0	−/0	−/0	−/0	−/0	−/0	−/0	−/0	−/0	2/3	−/0	−/0
2a	**4/4**	**3/3**	−/2	−/0	−/0	−/0	−/0	−/0	−/0	−/0	−/0	−/0	−/0
2b	**4/4**	**3/3**	1/2	−/0	−/0	−/0	−/0	−/0	−/0	−/0	1/0	−/0	−/0
2c	**4/4**	**3/3**	−/0	−/0	−/0	−/0	−/0	−/0	−/0	−/0	1/2	4/2	1/2
3a	**4/4**	**3/4**	**2/3**	1/3	−/0	4/2	−/0	−/0	−/0	−/0	1/1	−/0	1/2
3b	**4/4**	**3/4**	**3/4**	3/4	−/0	−/0	−/0	0/2	−/0	−/0	−/0	−/0	1/2
3c	**4/4**	**4/4**	**3/4**	3/3	−/0	−/0	−/0	1/3	−/0	−/0	2/2	−/0	2/2
4a	1/1	−/0	0/0	**0/0**	**4/4**	**2/2**	**0**[d]**/2**	**2/4**	**2/4**	**0/1**	**3/3**	**0/0**	**0/0**
4b	2/3	−/3	0/1	**1/1**	**4/4**	**2/1**	**2/3**	**4/4**	**4/4**	**4/0**	**4/3**	**2/2**	**3/2**
4c	3/4	−/0	−/0	**0/0**	**4/4**	**1/2**	**0**[d]**/2**	**4/4**	**4/4**	**3/1**	**3/4**	**0/0**	**1/0**

[a] The figures denote the maximum dilution of plasma at which a signal distinguishable from background can be seen: 4, 3, 2, and 1 correspond to dilutions of 1:300 000; 1:30 000; 1:3000, and 1:300, respectively. The number before a slash denotes the result for ST-ELISA on a microtiter plate; the one after the slash is that for the microarray result. A zero denotes the absence of the signal at all dilutions. A minus indicates that no assay was performed. [b] Mice 1a, 1b, and 1c were immunized with Con A; mice 2a, 2b, and 2c, with a mixture of Con A and TrI; mice 3a, 3b, and 3c, with a mixture of Con A, TrI, and Mbw; mice 4a, 4b, and 4c, with a mixture of Mbh, HEWL, βGa, HK, Avi, Trs, HRP, Ure, ADH, and AcE. [c] Boldface figures are for the results of assays of plasmas of immunized mice with the antigens to which they were immunized. Regular font is for antigens not used in the mouse immunization. [d] High value of control OD in ST-ELISA with plasma from a nonimmunized mouse.

amino groups and their shielding by glycoside chains on the surface of the HRP molecule. The other two cases of a substantial difference were observed in assays of HK interaction with 4a and 4c plasmas when OD measured in ST-ELISA was indistinguishable from the control. However, the control OD were unusually high in these cases, indicating that plasma of the control nonimmunized mouse also possessed some antibodies against HK.

Despite these differences, we can conclude that both techniques showed a very similar sensitivity in assays of plasmas of immunized mice. Threshold dilutions (titers) measured using both techniques agree well despite their 1000-fold variation for different antigens. We conclude then that MB-ELISA can be used not only to qualitatively analyze plasmas for the presence of a set of antibodies but also to quantitatively compare titers of different antibodies in the same manner as ST-ELISA does.

Comparison of Concentration Dependencies. Though threshold dilution could be used to quantify antibodies in mouse plasmas, it would be practically more convenient if the amount of antibodies could be characterized in one assay with a single microarray by measuring the intensity of the spot fluorescence. Using different dilutions of 4a, 4b, and 4c plasmas, we compared fluorescence intensity in MB-ELISA with the OD in ST-ELISA and found that the dependence on dilution is similar. Figure 2 illustrates such a similarity in dependence on dilution. Before further discussion we need to explain why the logarithm of OD is used to represent the data of ST-ELISA. As shown in Methods, the digital brightness is a linear function of the logarithm of fluorescence intensity. Therefore, spot brightness is proportional to the logarithm of the quantity of the fluorescent product in the spot, in contrast to the optical density, which changes in direct proportion to concentration of the soluble product. Thus, one needs to take the logarithm of the OD in ST-ELISA to make the signal scales comparable for the two detection techniques. Turning back to Figure 2, one sees that both signals behave remarkably similarly at low dilutions. However, there is a marked difference between Log (OD) and digital brightness at high dilutions, where the brightness shows more abrupt changes as compared to the OD. Despite this difference, both methods demonstrate similar

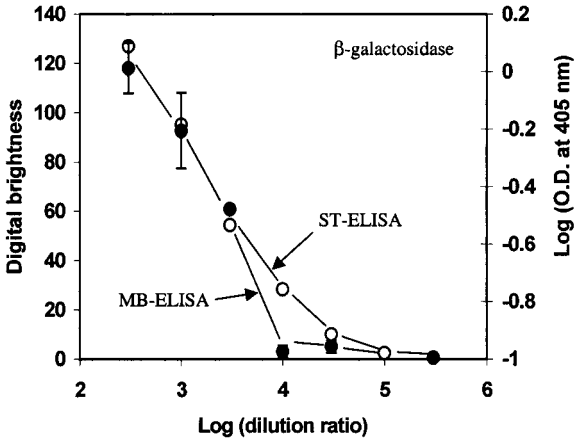

Figure 2. Comparison of MB-ELISA and ST-ELISA responses to β-galactosidase antigen as a function of dilution of serum from mouse 4a. Digital brightness is averaged over four identical spots in four microarrays. Optical density of pNPP product was measured at λ = 405 nm.

sensitivity since positive fluorescence of βGa spots and reliable difference from the background in ST-ELISA occur at the same dilution, 1:30 000.

To further compare the MB-ELISA and ST-ELISA techniques, the intensity of fluorescence of the a-mIgG spots was measured after reaction with m-IgG in solutions of known concentrations and detection of the bound antibody with the a-mIgG−AP conjugate. The same a-mIgG antibodies were also adsorbed on the standard polystyrene microtiter plate and ST-ELISA protocol was applied. As shown in Figure 3, the logarithm of fluorescence intensity (digital brightness) of the a-mIgG spots is closely similar in dependence on mIgG concentration to that of the Log (OD) in ST-ELISA. The two curves in Figure 3 are similar in form but are shifted, reflecting the slightly higher sensitivity of ST-ELISA (0.3 ng/mL) as compared to MB-ELISA. The minimum concentration detectable on microarray with ELF-97 as substrate is ~3 ng/mL in accordance with our previous data.[18]

Two differences in MB-ELISA and ST-ELISA techniques might account for the differences in dilution and concentration depend-

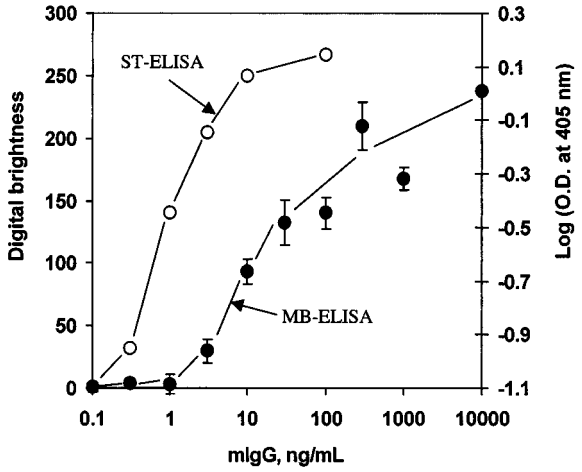

Figure 3. Fluorescence brightness of a-mIgG spots after MB-ELISA and optical density in ST-ELISA in microtiter wells (with adsorbed a-mIgG) as a function of concentration of primary antibody, m-IgG, in solution. In ST-ELISA, mIgG was dissolved in 0.5% BSA on the physiological solution; in MB-ELISA, mIgG was dissolved in 100 mM TRIS-HCl (pH 7.2−7.5) containing 1% dry defatted milk. Brightness at 10 000 ng/mL was corrected to account for background as described in Methods. See legend to Figure 2 and Methods for more details.

ences in Figures 2 and 3. First, accumulation of the insoluble ELF-97 product in the MB-ELISA is distinct from the reaction producing soluble product in ST-ELISA. The insoluble product precipitates only when the solubility level is reached in a competition between the rate of the product generation and its removal due to diffusion. This may explain the more abrupt changes in fluorescence seen at high dilutions in Figure 2. The second factor responsible for the difference in the responses may be due to the way antigens are linked to the surfaces. Adsorption-induced structural changes,[24] different orientation of immobilized molecules,[25] and different density of antigen adsorbed to surface[26] in ST-ELISA together with covalent modification of antigens upon their binding to the oxidized dextran on microarrays may account for changes in the affinities of immobilized antigens and, hence, for differences in sensitivity of ST-ELISA and MB-ELISA.

Storage Stability of Protein Microarrays. Our antigen (allergen) microarrays show excellent storage stability: no difference in response was detected after 8 months of storage over silica gel at a temperature of −25 °C.

CONCLUSION

We have shown in this paper that a set of diverse proteins differing in structure, activity, and physical properties can be deposited under dry conditions and then covalently bound to substrate to permit fabrication of a large number of identical microchips simultaneously. The microarrays produced in this way are stable to long-term storage and can be applied for qualitative and quantitative analysis of animal and human sera using ELISA protocols. It should be emphasized that, using the same amount of material in a single well of a 96-well microtiter plate, our microarrays represent a gain in sample utilization efficiency of 36-fold in the data shown. But this gain will increase as the number of spots in the arrays increases: in principle, arrays with over 1000 spots can be fabricated. Another way of stating the advantage is that, in a standard assay, roughly 1 μg of a protein is needed per well; in the microarray format, only 100 pg of the same protein would be needed per spot. This corresponds to an economy in sample protein utilization of 10 000-fold. Though protein antigens and allergens are used exclusively in these experiments, we foresee no obstacles for utilizing other antigens and allergens, including polysaccharides, DNA, and drugs. With proper adjustment of the immobilization technique, microarrays should be capable of fabricated from all these substances.

ABBREVIATIONS

ELISA, enzyme-linked immunosorbent assay; ST-ELISA, standard ELISA on microtiter plates with soluble product of AP reaction; MB-ELISA, microarray-based ELISA with ELF-97 substrate of AP reaction; ES, electrospray; ESD, electrospray deposition; OD, optical density; pNPP, *p*-nitrophenyl phosphate; APTES, (γ-aminopropyl)triethoxysilane; mIgG, mouse IgG; a-mIgG, anti-mouse IgG; a-mIgG−AP, anti-mouse IgG conjugated with alkaline phosphatase; BSA, bovine serum albumin; Ova, ovalbumin; GSA, goat's serum albumin; AP, alkaline phosphatase; HRP, horseradish peroxidase, LaP, latex protein, Coc, allergens from german roach; HB1, honey bee venom 1; HB2, honey bee venom 2; MDp, allergens from mite D. Pter.; MDf, allegens from mite D. farin.; Pap, papain, βGa, β-galactosidase; Con A, concanavalin A; Mbw, sperm whale myoglobin; Mbh, horse myoglobin; TrI, soy bean trypsin inhibitor; Avi, avidin; HEWL, hen egg white lysozyme; HK, yeast hexokinase; Ure, urease from canavalia beans; ADH, yeast alcohol dehydrogenase; AcE, acetylcholine esterase from electric eel; Trs, tyrosinase from mushrooms.

ACKNOWLEDGMENT

We sincerely thank Dr. Neville Kallenbach (New York University) for the critical reading of the manuscript and the valuable comments. We also express our gratitude to Dr. O. S. Morenkov (Institute of Cell Biology, Pushchino) for his help in immunizing mice and to Dr. A. V. Pichugin and to Dr. O. Pankrashina (Institute of Immunology of the Russian Academy of Medical Sciences) for help in performing ST-ELISA.

Received for review August 31, 2001. Accepted December 19, 2001.

AC010970K

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