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Microimmunoassay Using a Protein Chip: Optimizing Conditions for Protein Immobilization

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Optimizing conditions for the microarraying of protein antigens onto glass slides were studied. Various vendors, surface functional groups, buffers, and fixatives were evaluated to enhance protein binding. A total of 125 pg of human immunoglobulin was detectable with this assay system, suggesting that protein microarray can be applied for routine immunodiagnosis.

In addition to the functional analysis of the proteome, protein microchips might be used in simultaneous immunodiagnosis of various infectious diseases (1, 8). To achieve the highest sensitivity of immunodiagnosis with protein chips, a sufficient amount of antibody in serum should bind to antigens immobilized on the chip. Therefore, it is necessary to develop efficient immobilizing techniques which increase binding of the antigens. It is also necessary to immobilize the protein antigens on a glass slide in a way that preserves their immunological characteristics exposing linear or conformational epitopes. Several methods of protein immobilization on glass slides have been described previously (1, 2, 3, 4, 5, 6, 7, 9, 10). Although extensive studies with protein microarrays have been performed (11, 12, 13, 14, 15), fundamental analyses comparing the conventional methods easily accessible to a laboratory have not been studied. This study was undertaken to evaluate the technology easily accessible and compatible with conventional laboratory procedures.

The glass slides were purchased from Cel Associates, Inc. (slide C, lots 09280 and 04160; Houston, Tex.), Xenopore Corp. (slide X, lots MA93541 and GF03191; Hawthome, N.J.), Genpak Inc. (slide G; Stony Brook, N.Y.), and TeleChem International Inc. (slide T, lots 001229 and 001213; Sunnyvale, Calif.). Silylated or silanated slides were obtained from each manufacturer. Epoxy-coated microscope slides were obtained from Xenopore Corp. Human immunoglobulin M (IgM), IgG, or biotinylated protein L were dissolved in a phosphate-buffered saline (PBS) buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4) or in a carbonate buffer (20 mM Na_2CO_3, 30 mM $NaHCO_3$, pH 9.6) at concentrations of 0 to 60 μg/ml.

A manual microarrayer (V&P Scientific Inc., San Diego, Calif.) was used to microarray proteins in accordance with the manufacturer's instructions. A manual printing device delivered approximately 20 nl of protein to the slides, yielding spots approximately 200 to 300 μm in diameter (678 spots per cm^2). Replicator pins with a diameter of 0.457 mm were used for printing proteins. After printing proteins, the replicator pins were washed serially with a solution containing 1% sodium dodecyl sulfate, distilled water, and absolute ethanol as instructed by the manufacturer. The pins were dried completely before immersion into the protein solution, and the protein antigens were microarrayed in triplicate.

After microarraying polyclonal human IgM (Calbiochem-Novabiochem Corp., San Diego, Calif.), polyclonal human IgG (Calbiochem-Novabiochem Corp.), or biotinylated protein L (Pierce Inc., Rockford, Ill.) onto the separate slides, the slides were incubated for 18 h at 4°C in a humid chamber. The slides were washed once with PBS, and to quench the unreacted groups on the slide they were immersed in PBS containing 3% skim milk for 1 h at room temperature. The slides were then caged in a 50-ml conical tube, and they were centrifuged at 400 $\times g$ for 3 min. Either a 1:40 dilution of biotinylated anti-human IgM (12.5 μg/ml in 30 μl) or a 1:40 dilution of biotinylated anti-human IgG (12.5 μg/ml in 30 μl) was incubated with each slide for 1 h in a humid chamber and then was washed with PBS. To detect biotinylated protein L immobilized onto the slide, PBS was used instead of biotinylated anti-Ig. Cy3-labeled streptavidin (1:40 dilution in 30 μl of PBS) was incubated with each slide for 1 h and was washed with PBS. Cy3-labeled streptavidin, biotinylated anti-human IgG, and biotinylated anti-human IgM were purchased form Kirkegaard & Perry Laboratories Inc. (Gaithersburg, Md.). The slides were dried by centrifugation and were scanned with a GMS 418 scanner (Affymetrix Inc., Santa Clara, Calif.).

ScanAlyze (version 2.35; Stanford University) was used to analyze the scanned images stored as 16-bit TIFF files. The antigens were microarrayed in triplicate, and the mean of these triplicate values was used in a nonlinear regression analysis. The nonlinear regression curves were generated with SigmaPlot (version 6.0; SPSS Science, Chicago, Ill.). The regression curves were fitted with following equation: $f(x) = a/\{1 + \exp[-(x - x_0)/b]\}$, where a, b, and $x0$ were calculated for each curve by software. R^2 was calculated for each curve to verify the regression curves and are indicated in the figures. Two-tailed Student's t test was used to compare triplicate values of fluorescence intensities.

IgG was diluted in PBS and was microarrayed onto a silylated slide or an epoxy slide (Fig. 1A). The epoxy slide dis-

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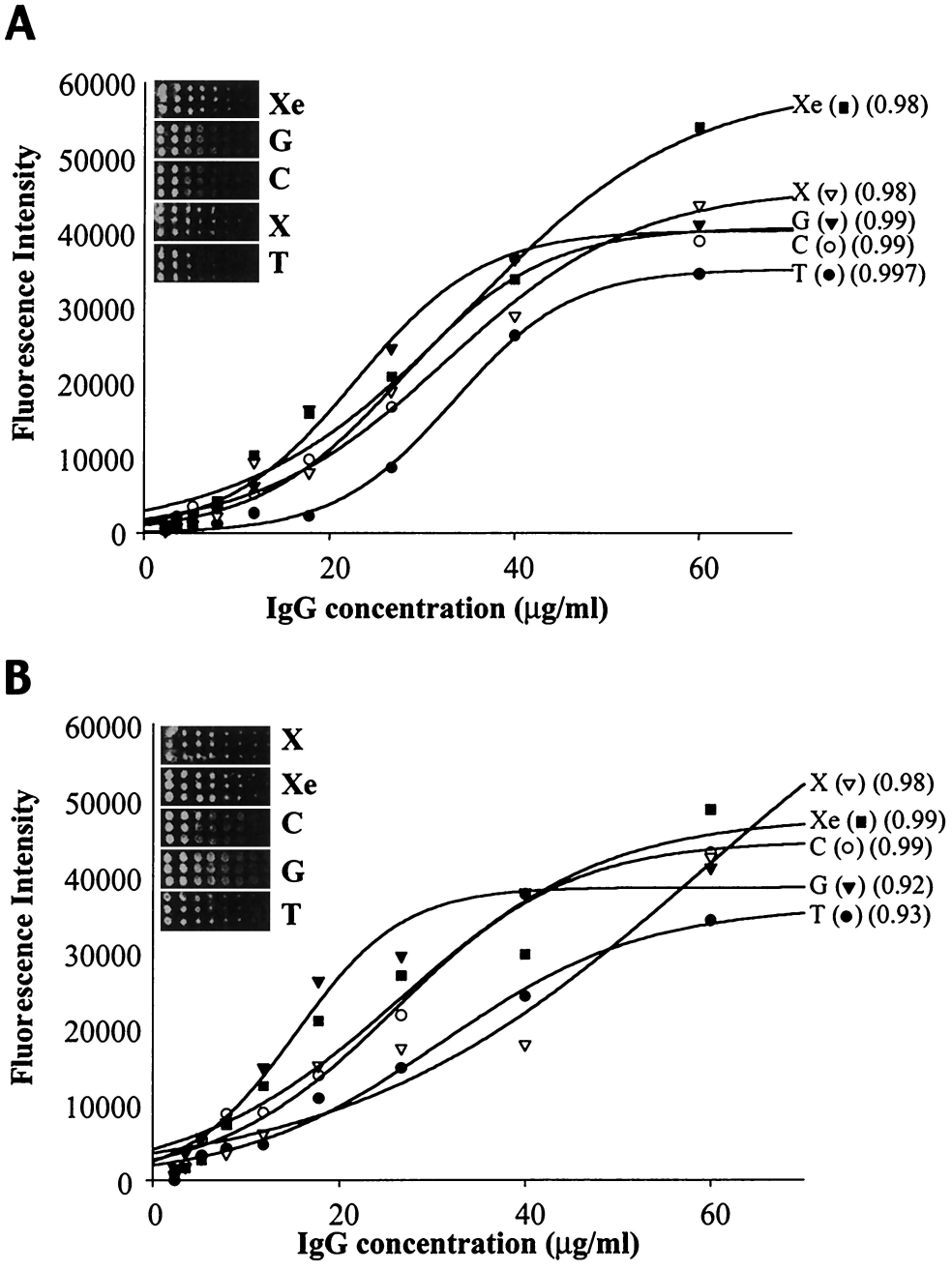

FIG. 1. Comparison of protein binding on silylated glass slides from various vendors. Human IgG was solubilized in PBS (A) or in carbonate buffer (B). Bound IgG was detected with biotinylated anti-human IgG and Cy3-conjugated streptavidin. Numbers in parentheses indicate the R^2 values of nonlinear regression curves. X, Xenopore Corp.; G, Genpak Inc.; C, Cel Associates Inc.; T, TeleChem Inc.; Xe, epoxy slide from Xenopore Corp.

played superior binding capacity above any silylated slide when the antigen concentration was higher than 40 μg/ml ($P < 0.01$). Significantly higher signals were obtained with slide C than with slide T when the protein concentration was 40 μg/ml ($P < 0.01$). The diameter of spots was small when protein was used in low concentrations, except for that of slide G. Protein spots were smeared when highly concentrated proteins (60 μg/ml) were spotted onto slide X or Xe.

In addition, IgG was diluted in carbonate buffer and was printed onto the silylated or epoxy slide (Fig. 1B). When the protein was used at a concentration of 60 μg/ml, the epoxy slides displayed higher binding capacity than any other silylated slide ($P < 0.01$), except for silylated slide X ($P = 0.2$). Slide C showed higher binding capacity for 40 μg of protein/ml than slide T ($P < 0.01$) and slide X ($P < 0.001$). The buffers used to solubilize the antigens did not affect the binding capacity of various silylated slides, except for that of slide C (Fig. 1A and B). The carbonate buffer increased the binding of

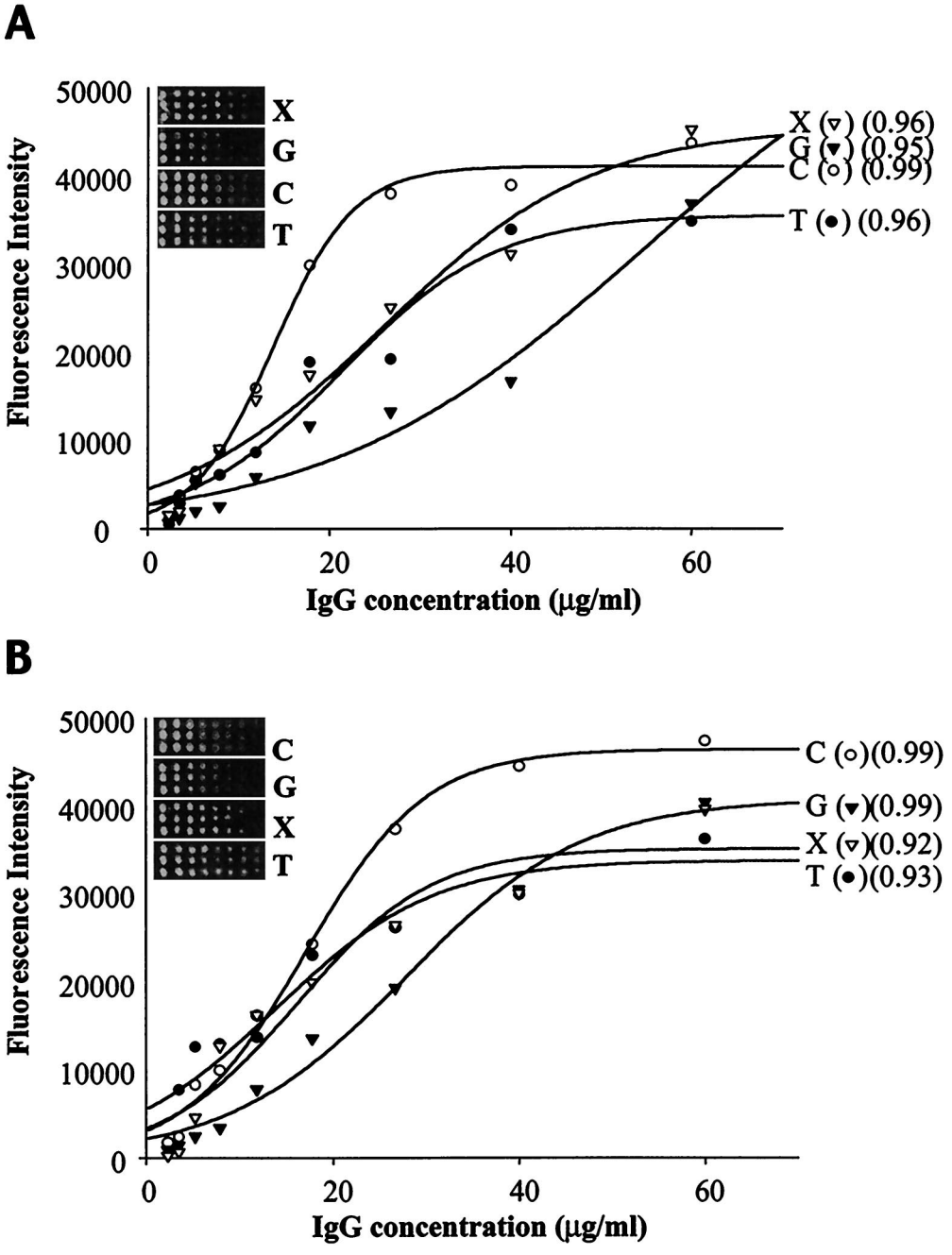

FIG. 2. Comparison of protein binding capacity of silanated glass slides from various vendors. Human IgG was solubilized in PBS (A) or in carbonate buffer (B). Numbers in parentheses indicate the R^2 values of nonlinear regression curves. See the legend to Fig. 1 for abbreviations.

protein (60 μg/ml) onto silylated slide C by 10% compared to that of PBS ($P = 0.08$).

IgG samples diluted in PBS were printed onto silanated slides (Fig. 2A). Slide C and slide X displayed no significant differences of binding capacity for 60 μg of protein/ml in PBS. When used with 60 μg of protein/ml in PBS, slides C and X displayed higher fluorescent intensities than slide T ($P < 0.01$) or slide G ($P < 0.01$). Slide C displayed the highest level of protein binding with 27 μg of protein/ml. When IgG samples diluted with carbonate buffer were microarrayed onto the silanated slide (Fig. 2B), slide C showed higher binding capacity than slides G ($P < 0.01$), T ($P < 0.01$), and X ($P < 0.05$). When silanated slide G was used for printing a protein solubilized with carbonate buffer (Fig. 2B), the binding capacity was 10% higher than that when PBS was used (Fig. 2A; $P < 0.05$). PBS increased the binding capacity of silanated slide X by 14% compared to that with carbonate buffer (Fig. 2A and B, $P = 0.08$). Fluorescent intensity of proteins (27 or 60 μg/ml) was significantly higher when it was printed on silanated slide C (Fig. 2) than when it was printed on silylated slide C (Fig. 1; P

< 0.05) irrespective of solubilizing buffers. No significant differences were observed between silanated and silylated slides from other vendors.

The aldehyde on the silylated slides reacted readily with primary amines on the proteins forming a Schiff's base linkage. Since most of the proteins display many lysines on their surfaces and more reactive α-amines at their NH_2 termini, the proteins can be immobilized on the silylated glass slide. Silanated slides contain linear primary amines attached covalently to the glass surface. The amines carry a positive charge at a neutral pH, allowing attachment of the protein through the formation of ionic bonds with negatively charged amino acid residues. Therefore, the isoelectric point of the protein is important in its binding to the silanated slides. In this experiment, the carbonated buffer increased binding of proteins. Elevating the pH above the isoelectric point might increase negative charges on the protein surface. Although the statistical significance was borderline, the protein in carbonate buffer bound to the silanated slide better than it did in PBS.

The binding of biotinylated protein L and IgM was analyzed simultaneously to see whether the fixatives interfere with biotin-streptavidin interactions or antigen-antibody interactions because of denaturating molecules. Biotinylated protein L and IgM were dissolved in carbonate buffer, printed on silanated slide C, and immobilized with various fixatives. The fixatives acetone (Merck & Co. Inc., Rahway, N.J.), 4% formalin (Merck & Co. Inc.) in PBS, and methanol (Merck & Co. Inc.) were incubated with each glass slide at room temperature for 5 min after the proteins were adsorbed. Fixation with 1% glutaraldehyde (Merck & Co. Inc.) in PBS was performed at room temperature for 1 h. Formalin decreased fluorescence intensity by 36% (IgM) or 9% (protein L) compared to that with PBS ($P < 0.05$). This implies that formalin interferes with biotin-streptavidin interactions and IgM–anti-IgM interactions. Acetone increased fluorescent intensity of IgM by 11% ($P < 0.05$), suggesting that denaturation of IgM by acetone is not essential in antigen-antibody interactions.

Simultaneous diagnosis of infectious diseases requires microarraying many strain-specific, species-specific, and type-specific antigens. The economical aspect of a test kit is also important in the development of diagnostic kits that use protein chips in routine laboratory diagnosis. For this reason, efficacy of protein immobilization and acceptable prices should be evaluated at the same time. I think that the commercial side can produce economical diagnostic kits by using an easy fabrication method. Thereby, wide use of simultaneous diagnosis using multiple antigens on a chip can enhance sensitivity of immunodiagnosis.

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