Effects of polystyrene surface chemistry on the biological activity of solid phase fibronectin and vitronectin, analysed with monoclonal antibodies

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SUMMARY

The conformation and biological activities of fibronectin (FN) and vitronectin (VN) coated on different plastic surfaces were investigated using cell adhesion and a panel of domain-specific monoclonal antibodies (mAbs). The adhesion of BHK fibroblasts was markedly better on FN coated on hydrophilic tissue culture polystyrene (TCPS) than on hydrophobic, untreated polystyrene (PS). mAbs A17 and 3E3, which inhibit the binding of BHK cells to the RGD-containing sequence within the cell binding region of FN, also bound preferentially to FN on TCPS. In contrast, two anti-FN mAbs, which have no effect on cell adhesion (A35 and A3), bound preferentially to the conformation of FN on the more hydrophobic PS. Mouse melanoma cells utilise an additional cell-binding site in the Hep II domain of FN and their preference for FN coated on TCPS was less marked than that of BHK cells. This reduced preference was again mimicked by the binding of a mAb, A32, which inhibits the binding of B16 cells to the Hep II domain of FN. In contrast, BHK cell adhesion to VN did not display a preference for TCPS over PS. The cell-binding activity of adsorbed VN was matched by the binding of a cell adhesion-inhibitory mAb, A18, which, unlike mAbs A17 and A32, displayed slightly increased binding to VN coated on PS, rather than TCPS.

When the denaturing effect of coating FN and VN to PS in the presence of urea was investigated, similar correlations between BHK cell adhesion and the binding of inhibitory mAbs were observed. Urea treatment of FN significantly reduced both BHK cell adhesion and the binding of both cell adhesion-inhibitory mAbs, whereas the binding of A35 and A3 was unaffected.

There was no significant effect of urea treatment of VN upon either BHK cell adhesion or mAb binding.

A larger panel of anti-FN mAbs was used, together with the anti-VN mAbs, to determine whether there were differences in mAb recognition of FN and VN adsorbed on three different brands of TCPS. The mAbs segregated into four reactivity patterns, of which A17, A32, A35 and A18 respectively were representative. Significant differences were observed in mAb recognition of FN and VN adsorbed to different brands of TCPS. These may reflect differences in the ability of these surfaces to support optimal growth of different cell types.

The effect of divalent cations upon adsorbed FN and VN was also investigated. Divalent cations changed the conformation of coated VN such that the binding of mAb A18 was reduced, whereas that of mAb A27 (which does not inhibit cell adhesion), was unaffected. This suggests a role for divalent cation binding to VN in cell adhesion processes. The binding of anti-FN mAbs was unaffected by cation treatment.

Our results indicate that mAbs can be used to determine the conformational status of surface-adsorbed proteins and to predict or analyse their biological activities under different conditions. In particular, they can be used to analyse at the molecular level the cell adhesive capacity of adsorbed protein for cells which utilise different integrin receptors, and the conformational outcome of various chemical treatments.

INTRODUCTION

Fibronectin (FN) and vitronectin (VN) are cell-adhesive glycoproteins present in mammalian plasma (and serum) at similar concentrations (200-400 µg/ml). For FN, the major cell-adhesive activity centres around an Arg-Gly-Asp (RGD) sequence in a central domain of the molecule, with input from other more amino-terminal sequences within the same domain (Pierschbacher and Ruoslahti, 1984; Nagai et al., 1991). An additional domain (the C-terminal heparin-binding domain, Hep II, containing an alternatively spliced connecting segment), has also been shown to exhibit cell adhesive activity for certain cell types (Humphries et al. 1986; McCarthy et al., 1986). VN also contains an RGD

sequence in its cell-binding domain, but different flanking sequences confer specificity for cellular receptors. A different integrin receptor binds to each of the FN cell adhesion domains and to VN. At present, only one cell-binding region in VN has been described. Apart from adhesion of cells, both FN and VN exhibit many other molecular binding activities, including affinity for heparin and collagens.

The conformation of different domains of these molecules responds individually to different physical and chemical stimuli. Ligand- or cation-binding tends to cause local disturbances in conformation (Khan et al. 1988; Ankel et al. 1986) which may influence other binding activities (Osterlund et al. 1985; Katchalski-Katzir and Kenett, 1988). Binding to surfaces also induces conformational changes in both FN and VN, resulting in "activation" of the RGD cell adhesion domain in FN (Akiyama and Yamada, 1987; Narasimhan et al. 1988), and "activation" of the heparin-binding region of VN (Tomasini and Mosher, 1988). Treatment with chemical denaturants such as urea results in a more profound, yet reversible conformational change in a large proportion of the FN molecule, which destroys many of its binding activities (Markovic and Engel, 1983). VN responds to urea treatment with similar "activation" of heparin-binding as observed on surface adsorption (Hayashi et al. 1985; Barnes et al., 1985). Unlike the case with FN, this conformational change is not reversible, and no deleterious effects upon VN binding activities have been reported.

Conformational changes which occur on surface-binding of FN and VN are important considerations in the effectiveness of a particular surface for cell adhesion. Cell adhesion to synthetic polymer implants in vivo and to culture surfaces in vitro are dependent upon surface-adsorbed FN and VN (Grinnell and Feld, 1982; Underwood and Bennett, 1989; Steele et al. 1991, 1992, 1993). A knowledge of the individual conformational effects upon the cell adhesion domains of these molecules on different surfaces is essential to the intelligent design of surfaces in contact with cells, whether the desired outcome is to encourage cell adhesion or to discourage it.

Physical techniques such as Fourier transform infrared spectroscopy (Pitt et al. 1987, 1989), electron spin resonance (Narasimhan and Lai, 1989), total internal reflection spectroscopy (Andrade et al. 1984), ellipsometry (Jonsson et al. 1987) and fluorescence energy transfer (Wolff and Lai, 1989), have been used to show that surface adsorption of FN and VN causes some degree of general unfolding or "opening up" of their three-dimensional structures. Analyses comparing the binding of FN between hydrophobic and hydrophilic surfaces have shown that conformational changes are greater on the hydrophobic surface (Andrade et al. 1984; Jonsson et al. 1987; Pitt et al. 1987). This has been shown to result in poorer adhesive properties for BHK fibroblasts and human umbilical endothelial cells on FN coated on hydrophobic surfaces (Grinnell and Feld, 1982; Steele et al. 1993). In contrast, Pitt et al., (1989) showed that surface binding of VN produced similar conformational changes on each surface. This was an unexpected finding and led the authors to postulate that the energy driving VN adsorption may be in the molecular structure of the protein itself, rather than in the characteristics of the polymer/water interface (which is held to be the driving force for adsorption of most proteins). This was borne out by our findings that VN coated onto hydrophobic or hydrophilic surfaces, in the absence of other proteins, was equally potent for BHK cell adhesion (Steele et al. 1993).

Although it is possible to detect overall changes in protein conformation upon surface binding using physical methods, these are not designed to discriminate between small, localised effects which may alter the biological activity of particular domains. The aim of this study was to investigate the conformational integrity of the two FN cell adhesion domains and the VN cell adhesion domain, upon adsorption to hydrophobic as opposed to hydrophilic plastic surfaces. Our approach was to investigate the cell adhesion of BHK cells and mouse melanoma B16 cells to FN and VN coated on these surfaces. The former cell type has integrin receptors for the RGD domains of FN and VN, whereas the latter cell type also expresses the integrin receptor for the Hep II domain of FN (Mould et al. 1990). We compared cell adhesion with the binding of a panel of domain-specific monoclonal antibodies (mAbs) to FN and VN. This panel included mAbs which in binding to FN or VN, inhibit the ligand binding of each of the three cell adhesion receptors, as well as other mAbs which bound to other domains. Thus we were able to determine whether such mAbs could be used to analyse the response of different cell types to FN and VN coated on different surfaces.

MATERIALS AND METHODS

Polystyrene tissue culture plasticware (TCPS) was from Falcon, Nunc and Flow (Linbro, cat. no. 76-032-05). The 96-well microtitration plates, not treated for tissue culture (PS) were from Flow (Linbro/Titertek, cat. no. 76-232-05), polyvinyl, U-shaped, 96-well ELISA plates were from Dynatec and 35 mm polystyrene bacteriological culture dishes (PS) were from Bunzl, Australia. Bovine FN was from Sigma or Calbiochem. Bovine VN was affinity purified from serum using a mAb affinity column as described (Underwood and Bennett, 1989). All chemicals were of Analar grade. Individual experiments were repeated at least twice. Statistical analyses of data were done using Student's t-test, Analysis of Variance and Student-Newman-Keul's test, where applicable.

Monoclonal antibodies

mAbs to FN and VN were prepared from Balb/c mice immunised with extracellular matrix from cultured bovine corneal endothelial cells, as previously described (Underwood et al. 1990). Use of animals was in accordance with CSIRO and NH&MRC/AAC code of practice. mAbs were purified from ascites fluids by affinity chromatography on Protein A-Sepharose (Pharmacia) as previously described (Underwood et al. 1983). Characteristics of the mAbs used in this study have been described elsewhere. VN mAbs were A18 and A27 (both G1). These mAbs bind to distinct epitopes on bovine VN. A18 inhibits BHK cell adhesion to VN, whereas A27 does not (Underwood et al. 1990). Twelve anti-FN mAbs were used: A32 and A35 map to the C-terminal heparin binding region of FN (Hep II) and A17 maps to the central cell-binding domain. A17 is a strong inhibitor of BHK cell adhesion to FN. A32 is a strong inhibitor of mouse melanoma B16 cell adhesion to the isolated Hep II domain and acts synergistically with A17 to inhibit B16 adhesion to complete FN (Underwood et
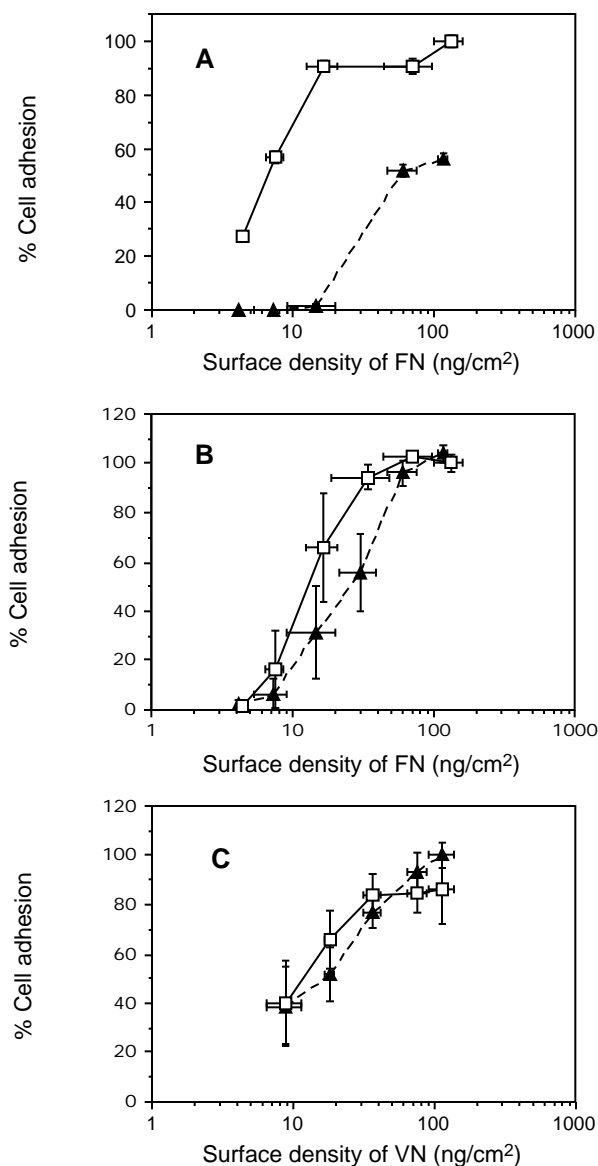
Fig. 1. Effect of surface chemistry (PS versus TCPS) on cell adhesion to adsorbed FN and VN. Linbro PS 96-well titration plates and Nunc TCPS tissue culture plates were coated with serial two-fold dilutions of FN or VN for 1 h at 37°C. The starting concentration was 4 μg/ml. The amount of surface adsorbed protein (ng/cm^2) was estimated using ^{125}I-labelled proteins. After blocking unoccupied protein-binding sites, 1.5×10^4 cells were added per well and incubated at 37°C for 1.5 h. Unattached cells were washed off, and attached cells were fixed and stained with methylene blue. Cell adhesion is expressed as a percentage of the absorbance obtained with BHK cells at the highest concentration of FN used (A and C), or with B16-F1 cells at the highest concentration of FN used (B). Points are the means of 4 experiments (ng protein/cm^2) or 6 replicates (% adhesion). Error bars are 95% confidence limits calculated using Student's *t*-test. (A) BHK cell adhesion to FN coated on TCPS(□) and PS (▲). (B) B16-F1 cell adhesion to FN coated on TCPS (□) and PS (▲). (C) BHK cell adhesion to VN coated on TCPS (□) and PS (▲).

al. 1992). A22 and A23 map to a more N-terminal region near the collagen binding site (see Underwood et al. 1992 for details). A3, A4 and A7 have been tentatively mapped to the Hep II

domain. A19, A24, A25 and A26 have not been mapped. A3, A4, A7, A17, A19, A22, A23, A26, A32 and A35 are all G1s; A24 is a G2b and A25 is a G2a. MAb 3E3, reactive with a 15 kDa fragment of the cell-binding domain containing RGD, described by Pierschbacher et al. (1981), was purchased from Telios Pharmaceuticals (San Diego).

Cell culture and adhesion assays

BHK fibroblasts (BHK cells) were cultured in DMEM (Gibco, Life Technologies Inc.), containing 10% foetal calf serum (FCS, Gibco), 10% tryptose phosphate broth (Flow), 100 μg/ml penicillin and 50 μg/ml streptomycin sulphate (Sigma). B16 F1 mouse melanoma cells (B16 cells) were kindly supplied by I. J. Fidler (Houston, Texas). They were grown as BHK cells, omitting the tryptose phosphate broth. For cell adhesion experiments, BHK and B16 cells in the logarithmic phase of growth were used. Cell adhesion was measured as previously described using 35 mm bacteriological polystyrene dishes (standard assay, Underwood et al. 1990), or 96-well plates (Underwood et al. 1992), coated with FN or VN for 2 h at 37°C as described below.

Coating of surfaces and binding assays

FN and VN were coated onto the wells of 96-well plates of the above types in phosphate-buffered saline (PBS) or 8 M urea in PBS for 1-2 h at room temperature or 37°C, or for 2 days at 4°C, 50 μl per well. Concentrations ranged from 0.05 to 20 μg/ml. Following coating, plates were either used for mAb-binding experiments, or estimation of adsorbed FN or VN. After coating FN or VN for 2 days at 4°C, some wells were treated with 0.05 M Tris/HCl, pH 7.4, containing either 0.01 M CaCl$_2$, 0.01 M MgCl$_2$, 0.01 M EDTA or no addition, for varying periods of time at 37°C.

Antibody binding was measured by ELISA as previously described (Underwood, 1985; Underwood et al. 1992). Estimation of the absolute amounts of adsorbed FN or VN on the plates used in ELISA assays was done as follows. FN and VN were labelled with ^{125}I using the chloramine T method and stored at –70°C for up to 4 weeks (Underwood and Steele, 1991). The non-adsorbable fraction, resulting from the radiolabelling procedure, was determined by sequential adsorption to Dynatec polyvinyl at the time of use, as described previously (Underwood and Steele, 1991). Samples of FN and VN containing 2×10^5 cts/min of radiolabelled proteins were adsorbed to the various TCPS and PS plates for 2 days at 4°C. The wells were washed with PBS and then incubated with 1% BSA for 1 h at 37°C. After removal of BSA solution, the wells were cut out of the plate using a hot wire device and adsorbed ^{125}I was measured directly in a gamma counter (Cobrat II). Surface density of FN and VN was expressed as ng/cm^2, from the surface area of contact within the wells (0.63 cm^2) after correcting for the non-adsorbable fraction.

RESULTS

Comparison of hydrophobic and hydrophilic polystyrene surfaces for cell adhesion to adsorbed FN and VN

Effects on cell adhesion to adsorbed FN and VN

Fig. 1 shows a comparison between B16 cells and BHK cells for the effect of polystyrene surface chemistry upon cell attachment to FN and VN. The surfaces used were Nunc TCPS and Linbro PS. For similar surface-coated concentrations of FN, BHK cell adhesion was clearly greater on the TCPS surface over the whole FN coating range (Fig. 1A). The effect of surface chemistry was different for B16
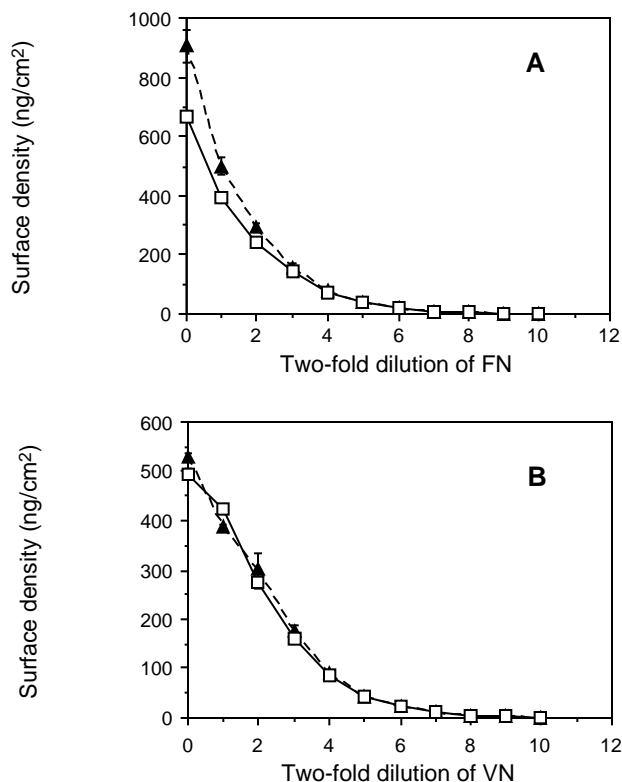
Fig. 2. Effect of surface chemistry (PS versus TCPS) on adsorption of FN and VN under ELISA conditions. Linbro PS 96-well titration plates and Nunc TCPS tissue culture plates were coated with serial two-fold dilutions of FN or VN for 2 days at 4°C, starting at 20 μg/ml. All dilutions contained 2×10^5 cpm of ^{125}I-labelled FN or VN, as appropriate. Surface concentration of adsorbed protein (ng/cm^2) was calculated after the blocking step, from cts/min bound, taking the surface area of contact of 50 μl per well as 0.63 cm^2 and correcting for the non-adsorbable fraction of label. Results are expressed as the means of 4 replicates. Error bars are 95% confidence limits calculated using Student's *t*-test. (A) FN coated on TCPS (□) and PS (▲). (B) VN coated on TCPS (□) and PS (▲).

cells, where attachment was greater on the TCPS surface at low FN-coating concentrations, but this difference "disappeared" at higher concentrations (Fig. 1B). In contrast, BHK cell adhesion to VN displayed no significant preference, between substrates (Fig. 1C). Repeated experiments either showed no preference, as in Fig. 1C, or a preference for the PS substrate rather than TCPS. B16 cell adhesion on VN was not tested. The range of coating densities of each of FN and VN was below the saturation limit for monolayers of 'side on' molecules as estimated by Pitt et al., (1987, 1989: 0.36 μg/cm^2 and 0.21 μg/cm^2 for FN and VN, respectively). Thus multilayering of coated protein would not have occurred within this range.

Comparison of hydrophobic and hydrophilic polystyrene surfaces for mAb binding to adsorbed FN and VN

Effects on adsorption of FN and VN

Nunc hydrophilic TCPS plates and Linbro hydrophobic PS plates were compared for FN and VN adsorption, using ^{125}I-labelled tracers, and incubation conditions as for

ELISA assays. The results in Fig. 2 show that there was significantly higher binding of FN to PS at coating concentrations above 5 μg per ml. Below this concentration there was no difference between the two surfaces (Fig. 2A). It is noteworthy that at FN concentrations above 5 μg/ml, the surface density on both surfaces is higher than 0.36 μg/cm^2, suggesting that either the orientation of the molecules is changing from a 'side on' orientation to a more 'end on' one or that multilayering is occurring. VN displayed similar adsorption characteristics to both surfaces over the whole concentration range tested (Fig. 2B). With this molecule, monolayer saturation was exceeded at coating concentrations between 2.5 and 5 μg/ml. The shorter period of adsorption of FN and VN for cell adhesion experiments yielded very similar surface densities of each molecule to either surface (Steele et al., 1993) and monolayer saturation was not exceeded.

Effects on mAb binding to adsorbed FN

The binding of mAbs to FN coated on Nunc TCPS as opposed to Linbro PS is shown in Fig. 3A. A17 and 3E3, which are directed to different epitopes of the RGD-containing domain of FN, both showed higher binding to FN on the TCPS surface at coating concentrations below 5 μg/ml, reflecting BHK cell adhesion to these surfaces (Fig. 3A cf. Fig. 1A). There is therefore an interesting correlation between the effect of the surface upon binding of the mAbs which react with the cell-binding region (A17 and 3E3) and the attachment of BHK cells, which attach to FN via this region. At higher FN coating concentrations than those used in the cell adhesion experiments, the preference for the TCPS surface by these mAbs disappeared. This may be due to changes in conformation at higher packing densities, as described by Pitt et al., (1987), loss of effects of surface chemistry due to multilayering (Cantarero et al., 1980; Fabrizius-Homan and Cooper, 1991), or to steric hindrance effects. All the mAbs (anti-FN and anti-VN) exhibited absorbance plateaus within the highest 2 to 4 coating concentrations on either surface (Fig. 3A & B), whereas the amount of coated proteins continued to increase within this range (cf. Fig. 2). This suggests that multilayering and/or steric hindrance of mAb binding was occurring at these higher concentrations.

The binding of A32, directed to a cell-binding site in the Hep II domain, reflected the adhesion of B16 cells, with higher binding to FN on the TCPS surface at low coating concentrations and a more rapid convergence at higher concentrations (Fig. 3A cf. Fig. 1B). Conversely, two mAbs which bind to the Hep II domain, but which have little effect on cell adhesion, showed higher binding to FN coated on the PS surface than the TCPS surface (A35 and A3, Fig. 3A) over the whole concentration range.

Effects on mAb binding to adsorbed VN

Similar results to A35 and A3 were observed with the anti-VN mAbs (Fig. 3B). Both the inhibitor of cell adhesion, mAb A18, and the non-inhibitory A27, showed slightly higher binding to the PS surface. Amounts of coated material were similar for each surface under either assay format, indicating a slight preference by both antibodies for the conformation displayed on PS even at high surface den-
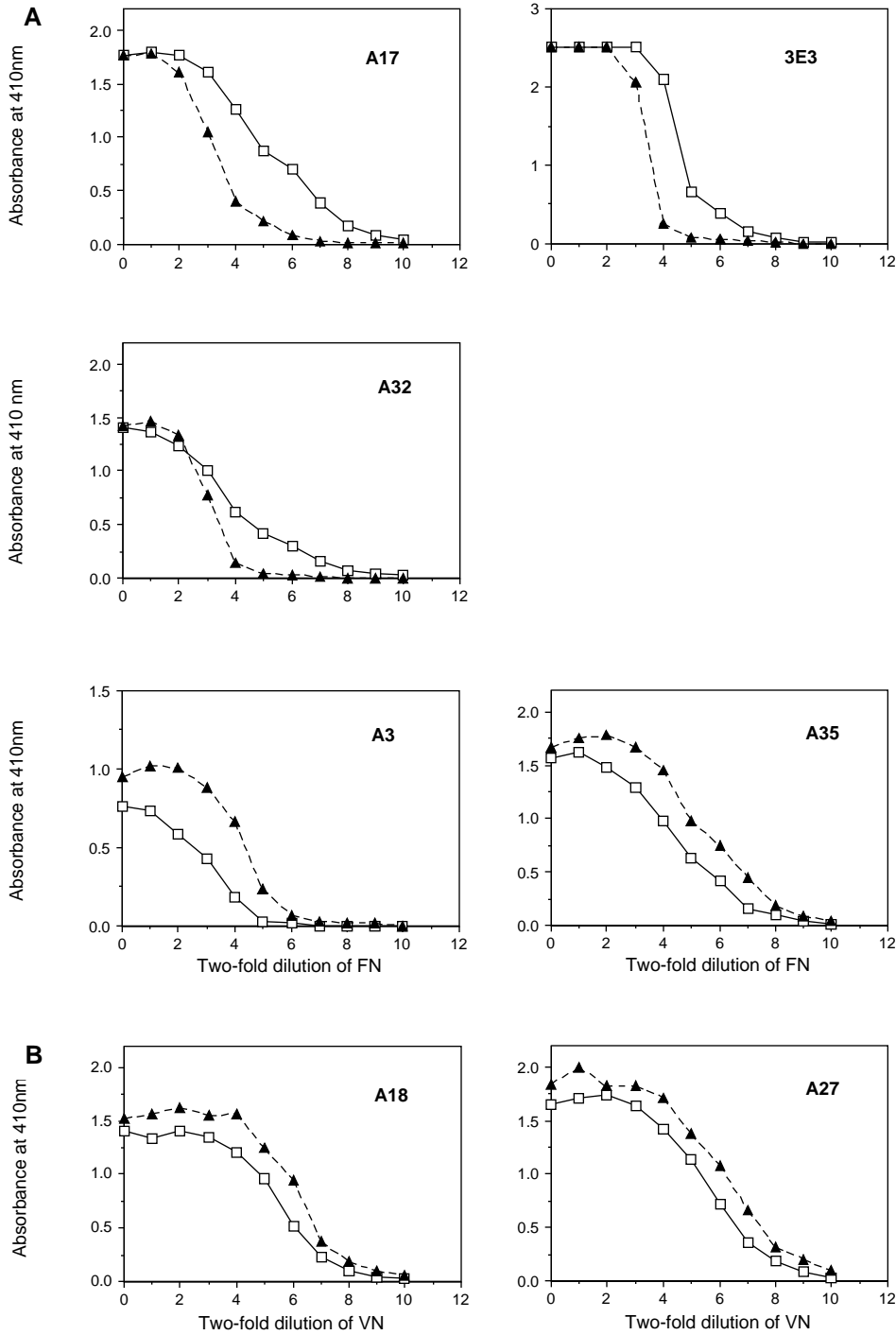
Fig. 3. Effect of surface chemistry (PS versus TCPS) upon mAb binding to coated FN and VN. Linbro PS 96-well titration plates, and Nunc TCPS tissue culture plates were coated with serial two-fold dilutions of FN (A) or VN (B) for 2 days at 4˚C. The starting concentration was 20 μg/ml. After blocking unoccupied protein-binding sites, the indicated mAbs were added at 1 μg/ml and mAb binding was measured by ELISA. Results are the absorbance values from one experiment, expressed as the means of duplicates. (▲) PS plates; (□) TCPS plates.

sity, indicating that the conformational differences were maintained through potential multilayering or reorientation. The graphs in Fig. 3 show data from a single experiment; the points are the means of duplicates and therefore do not carry meaningful errors. This ELISA was done three times, each repeat giving the same result. The observed differences in ELISA absorbance between the TCPS and PS surfaces are, therefore, considered significant. BHK cell adhesion to VN showed some variability, sometimes displaying a preference for the PS surface, sometimes displaying no difference, but never showing a preference for TCPS. This indicates a similar overall response of BHK cells and anti-VN mAbs.

Effects of disturbing conformation of FN and VN, by treatment with urea, upon cell adhesion and mAb binding to ligands on PS

FN coated on PS was a poorer substrate for cell adhesion than FN coated on TCPS, whereas no such differences were observed with VN (see Fig. 1). It was therefore of interest to determine the effect of urea treatment of ligands, which should result in greater denaturation than does the PS sur-
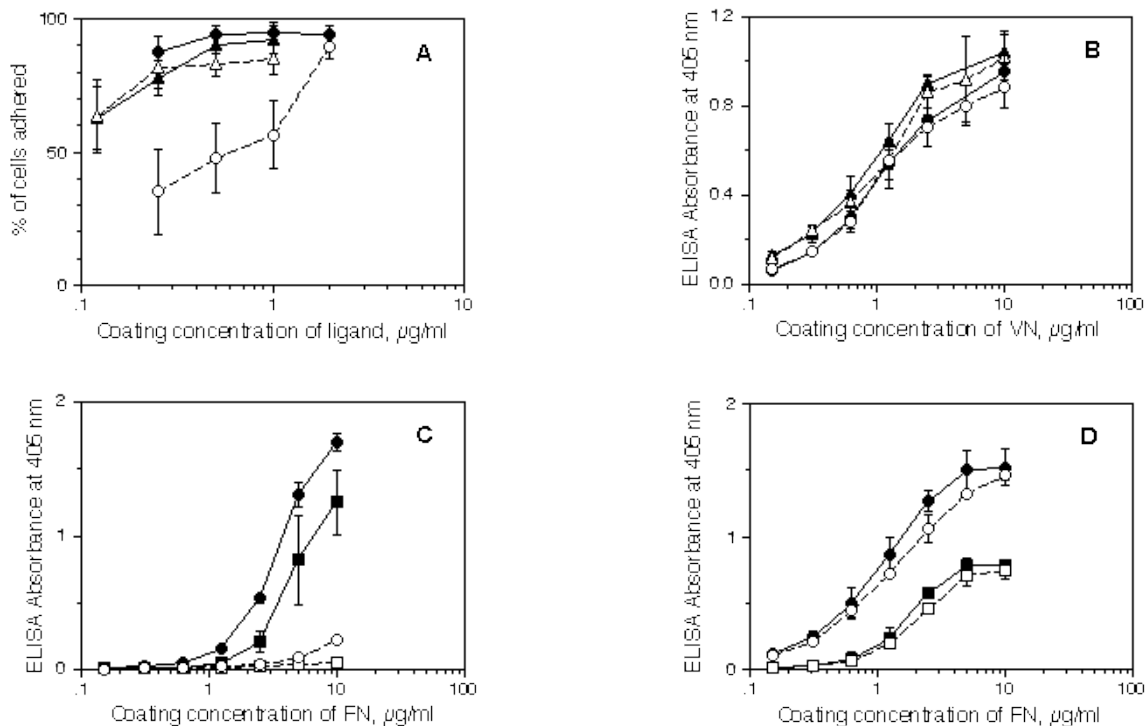
Fig. 4. Effect of coating FN and VN in urea upon subsequent BHK cell adhesion and mAb binding. 35 mm PS dishes or 96-well plates were coated with serial dilutions of FN or VN in PBS or 8 M urea/PBS for 2 h at 37°C. Following blocking of unoccupied protein-binding sites, BHK cells were added to the dishes and incubated for 1 h at 37°C. Non-adherent cells were counted in a haemacytometer. Results are expressed as the percentage of cells which adhered to the surface after 1 h incubation at 37°C, averaged over 5 replicates. mAbs were added to the 96-well plates at 1 µg/ml and mAb binding was measured by ELISA as for Fig. 3. Points are the means of triplicate absorbance readings. Error bars in each panel are the 95% confidence limits of the means, estimated using Student's *t*-test. Unbroken lines are measurements on untreated ligands, broken lines are measurements on urea-treated ligands. (A) BHK cell adhesion on FN (●○) and VN (▲△). (B) Binding of anti-VN mAbs A18 (●○) and A27 (▲△). (C) Binding of anti-FN mAbs A17 (●○) and A32 (■□). (D) Binding of anti-FN mAbs A35 (●○) and A3 (■□).

face, upon subsequent BHK cell adhesion and mAb binding. The results of these experiments are shown in Fig. 4. There was no significant difference between the adhesion of BHK cells to VN coated in the presence or absence of urea (Fig. 4A). Similarly urea treatment produced no difference in the binding of either anti-VN mAb (Fig 4B). Conversely, FN coated in the presence of urea was a relatively poor substrate for BHK cell adhesion compared with untreated FN (Fig. 4A). mAbs A17 and A32, which inhibit cell adhesion, also showed poor binding to urea-treated FN (Fig. 4C). The two non-inhibitory anti-FN mAbs, A35 and A3, showed similar binding to urea-treated or untreated FN (Fig. 4D). Thus, as for the differences observed between the PS and TCPS surfaces, cell adhesion and relevant mAb binding showed similar responses to treatment of ligand with urea.

Comparison of different brands of TCPS for binding of mAbs to adsorbed FN and VN

Since different anti-FN mAbs could distinguish readily between FN coated on TCPS and PS, it was of interest to determine whether they could be used to distinguish the subtler differences between different brands of TCPS. For this purpose a larger panel of anti-FN mAbs was used, together with the two anti-VN mAbs. Three different brands of TCPS (Falcon, Nunc and Linbro) were compared for

their antibody-binding characteristics at similar coating concentrations of FN and VN. In these experiments, the TCPS surfaces were also compared with Dynatec flexible polyvinyl, as this surface is specifically designed for ELISAs and has high protein-binding characteristics. The protein-binding characteristics of this surface are hydrophobic in nature, compared with the hydrophilic TCPS surfaces. The results are shown in Fig. 5.

mAb binding to FN is shown in Fig. 5 A-C. The mAbs could be grouped into three types. Those with titres showing no significant difference between different surfaces are shown in Fig. 5A (*P* > 0.05, Analysis of Variance). This group contained A17, active against the RGD containing cell-binding domain. The second group showed significantly reduced binding to FN coated on Dynatec polyvinyl and Falcon TCPS, as compared to Nunc TCPS and Linbro TCPS (Fig. 4B, *P* < 0.05, Analysis of Variance and Student-Newman-Keul's test). This group contained A32, active against a cell-binding site in the Hep II domain. The third group showed highest binding to FN on Dynatec polyvinyl plates, with significantly lower titres on the TCPS surfaces (Fig. 5C, *P* < 0.05, Analysis of Variance and Student Newman Keul's test). This group contained A35, which binds to the Hep II domain but has little effect on cell adhesion. Note that the mAbs displaying the three different binding patterns on the TCPS as opposed to PS sur-
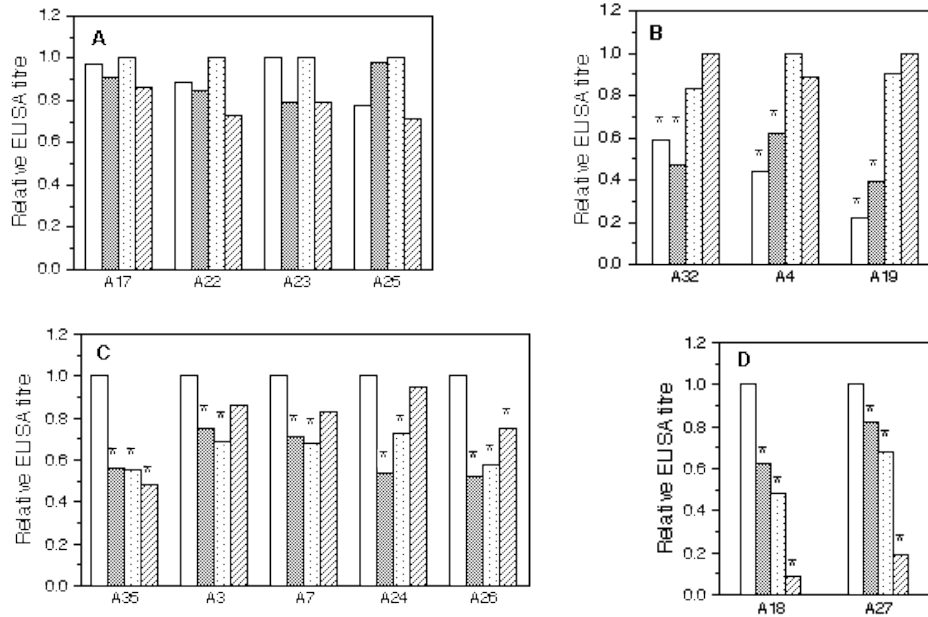
Fig. 5. Comparative ELISA titres of mAbs on FN and VN coated on different plastic surfaces. 96-well plates were coated with FN or VN in PBS. Coating concentrations ranged between 0.075 and 20 µg/ml (FN) or 0.06 and 0.5 µg/ml (VN). Coating time was 2 h at room temperature. Antibodies were used at 1 µg/ml and binding was measured by ELISA. For each mAb the titres are expressed as a proportion of the absorbance on the plate giving the highest value for that particular mAb. These proportions were averaged over 4 coating concentrations within the linear portion of the binding curve. (A), (B) and (C) show anti-FN mAbs grouped according to reactivity patterns. (D) shows anti-VN mAbs. □, Dynatec polyvinyl; ▨, Falcon TCPS; ⬚, Nunc TCPS; ▨, Linbro TCPS. * over the bar indicates significantly lower absorbance than the maximum ($P < 0.05$, Analysis of Variance and Student-Newman-Keul's test).

faces (Fig. 3) are segregated into these three separate groups (Fig. 4). The fact that the different anti-FN mAb groups displayed maximum titres on different brands of plastic indicates that these differences are not solely due to different plates maintaining different surface concentrations of FN, but are a genuine reflection of conformational differences.

Titres of VN mAbs are shown in Fig. 5D. Both anti-VN mAbs displayed similar binding characteristics, maximum on VN-coated Dynatec polyvinyl, with significantly less binding to TCPS. mAb binding to VN coated on Linbro TCPS was significantly lower than to the other two TCPS surfaces (Fig. 5D, $P < 0.05$, Analysis of Variance and Student-Newman-Keul's test). Since the two anti-VN mAbs showed similar reactivities, the differences between plate types could be explained by either differences in coating densities or by differences in conformation. We favour the former explanation, since in comparisons of adsorption of ^{125}I-labelled VN to Linbro TCPS versus Nunc TCPS, there is less initial adsorption of VN to the Linbro surface, and more dissociation in subsequent ELISA incubation steps (unpublished observations).

The effect of coating FN and VN in the presence of 8 M urea upon mAb binding to these surfaces is shown in Fig. 6. By and large the effects of urea correlated with the mAb groupings based on response to plate type and were similar to those described for ligands on PS (cf. Fig. 4). The least effect of urea on anti-FN mAb-binding was shown by group C (Fig. 6C). Within this group, three mAbs were virtually unaffected by urea treatment of FN ($P > 0.05$, Student's t-test), while two mAbs-showed reductions in titre of 50-60% after urea treatment on two surfaces only (Falcon and Nunc). The preservation of mAb-binding activity after urea treatment is consistent with this group of mAbs displaying the highest titres on Dynatec polyvinyl (Fig. 5C).

The polyvinyl surface is hydrophobic with high protein-binding capacity and is likely to result in greater denaturation of bound proteins than the more hydrophilic TCPS surfaces. Thus, mAbs which exhibit higher binding to antigen coated on polyvinyl are less likely to be affected by denaturation of the antigen by urea than are mAbs showing higher binding on hydrophilic surfaces. Group B mAbs displayed the greatest sensitivity to urea treatment of FN (Fig. 6B). All titres were reduced to below 40% of the original, and on three out of the four surfaces, titres were reduced to below 20%. This is consistent with these mAbs showing low titres on polyvinyl plates. Their epitopes are likely to be partially denatured by antigen binding on the polyvinyl surface, and to be more severely denatured by urea. Group A mAbs displayed intermediate effects (Fig. 6A). They showed considerable sensitivity to urea treatment of FN while showing little selectivity between plate types (compare Fig. 6A with Fig. 5A). This suggested that the epitopes of these mAbs were not denatured by binding to the plastic surfaces, but were denatured by urea. The anti-VN mAbs bound equally well to VN coated in the presence or absence of urea (Fig. 6D), and in this respect were similar to anti-FN group C.

Effects of divalent cation treatment of coated ligands upon mAb binding

Cellular receptors for FN and VN are known to require bound divalent cations for activity. It is not known whether similar cation binding properties are required by the ligands themselves and in a cell adhesion assay it is difficult to distinguish between the two effects. We tested the effects of divalent cations on mAb binding to FN and VN, coated on Dynatec polyvinyl, to see whether any effects would correlate with mAbs which influence cell adhesion. The results for VN are shown in Fig. 7. It can be seen that there was
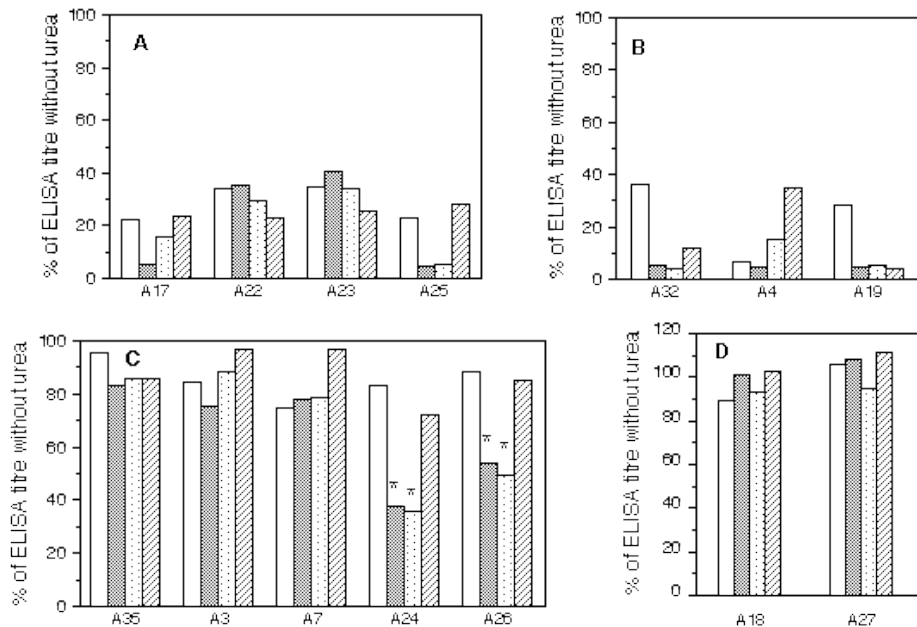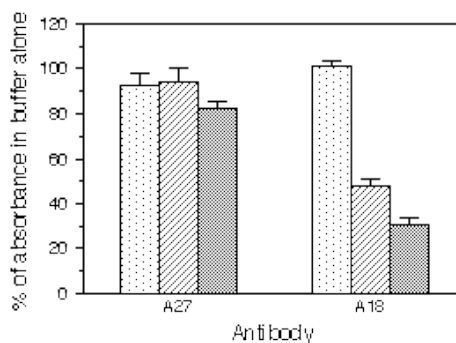
Fig. 6. Effect of coating FN and VN in urea on different plastic surfaces upon ELISA titre of mAbs. 96-well plates were coated with FN or VN in PBS or 8 M urea/PBS and ELISA assays were done as for Fig. 5. For each mAb the titres on urea-treated plates are expressed as percentages of the absorbances obtained in the absence of urea. The percentages were averaged over 4 coating concentrations within the linear range. Panels and key as for Fig. 5. * indicates titre significantly different from 100% ($P < 0.05$, Student's t-test). In (A) and (B) all titres were significantly different from 100%.

no effect on the binding of either mAb by removal of divalent cations with EDTA. The binding of A27 was similarly unaffected by Mg^{2+} treatment and marginally reduced by Ca^{2+}. In contrast the binding of A18 was affected by divalent cation treatment. Both Ca^{2+} and Mg^{2+} significantly reduced A18 binding, Ca^{2+} being more effective than Mg^{2+}. The effect was manifest on the ligand rather than on the mAb-epitope interaction, as the treatment was removed before mAb application. Since the effects on the two mAbs were different, the effect is likely to be a conformational one rather than a quantitative dissociation phenomenon. (The concentration range of VN used was in the linear range of both mAb-binding curves and concentration differences would have been detected by A27 as well as A18.) The conformational effect on the coated ligand is a slow acting one, requiring 16 h of cation treatment. This may reflect a requirement for dissociation and re-association with the surface for the cations to effect a conformational change. No differences in binding of any of the anti-FN mAbs were detected between any treatments of coated FN (data not shown).

Fig. 7. Effect of cation treatment of coated VN upon subsequent mAb binding. Dynatec polyvinyl 96-well plates were coated with VN in PBS for 2 days at 4°C. Coating concentrations were 0.03, 0.06 and 0.125 µg/ml. After coating, wells were treated with 0.05 M Tris/HCl containing 0.01 M EDTA, ▨; 0.01 M $MgCl_2$, ▨; 0.01 M $CaCl_2$, ▨; or no additions, for 24 h. mAbs were used at 0.1 µg/ml and mAb binding was measured by ELISA. Results are shown as the % of absorbance on the treated surface compared to the untreated control, averaged over the three coating concentrations. Error bars are the standard errors of the means.

DISCUSSION

The determination of active conformation of cell adhesive domains of adsorbed FN and VN using mAbs

We have demonstrated in this report that binding of mAbs, which are specific for the cell-binding regions of FN, mimics cell adhesion to FN coated on hydrophilic TCPS as opposed to hydrophobic PS surfaces. Thus, BHK cells, which depend on the Arg-Gly-Asp (RGD) cell-binding domain of FN for cell adhesion (Humphries et al., 1986; Underwood et al. 1992), display greater adhesion to FN coated on a TCPS surface than a hydrophobic PS surface, despite the fact that similar surface concentrations of FN are adsorbed to both under cell adhesion conditions (Steele

et al. 1993). This has also been demonstrated previously for these cells (Grinnell and Feld, 1981), and for human umbilical endothelial cells (Steele et al. 1993). mAbs which inhibit cell adhesion to this site (A17 and 3E3) also show preferential binding to FN coated on TCPS over the same range of coating density. B16 mouse melanoma cells, which have receptors for the Hep II cell-binding site as well as the RGD site (Mould et al., 1990), show a lesser degree of dependence on the nature of the polystyrene surface, displaying a preference for TCPS only at relatively low FN coating concentrations. This behaviour is again mimicked by the binding of mAb A32, which inhibits a cell-binding site in the Hep II domain (Underwood et al. 1992). Other mAbs, which are not directed to these cell-binding epitopes

(e.g. A3 and A35), do not display increased binding to FN coated on TCPS – rather they show a slight preference for FN adsorbed to the hydrophobic surface.

The attachment of BHK cells to VN was either similar on both PS and TCPS or slightly increased on PS, in marked contrast to attachment to FN. Both anti-VN mAbs, A18 (which inhibits the adhesion of BHK cells to VN), and A27 (which is non inhibitory), showed slightly increased binding to VN on the hydrophobic PS surface. Similar amounts of VN adsorb to both surfaces under cell adhesion (Steele et al., 1992) or ELISA conditions.

Overall, for both adhesive proteins, where inhibition of cell adhesion had been demonstrated for a particular mAb and a particular cell type, the relative binding reactivities of the mAb to ligand coated on different surfaces reflected the relative cell-adhesive characteristics of ligand coated on those surfaces. This was also borne out by the response of cells and mAbs to urea-treated ligands. BHK cells adhered poorly to urea-treated FN. The mAbs which inhibited cell adhesion to FN also bound poorly to urea-treated FN, whereas mAbs which bound to other epitopes were unaffected by urea treatment. Similarly, neither BHK cell adhesion nor mAb binding to VN were affected by urea treatment.

When a broader panel of mAbs was used to compare FN on the Nunc TCPS surface with two other TCPS surfaces and a hydrophobic ELISA surface, differences between the TCPS surfaces were detectable. For the anti-FN mAbs, the reactivities were separable into three groups. One group showed no significant differences in mAb binding between any of the surfaces. A second group displayed higher binding on Nunc and Linbro TCPS than to Falcon TCPS, and the third group showed higher binding to the hydrophobic surface than to the TCPS surfaces. It should be noted that the different reactivities of these mAb groups do not correlate with the proteolytic domains of FN, but reflect individual disturbances of epitopes within the domains. Interestingly, the effect of deliberately denaturing the conformation of FN with urea treatment upon the binding of the mAbs varied amongst these groups, in a manner which is consistent with the hypothesis that binding of FN to a hydrophobic surface results in greater conformational change ("denaturation") than binding to a hydrophilic one (Grinnell and Feld, 1981, 1982; Andrade et al. 1984; Jonsson et al. 1987; Pitt et al. 1987). We show that those mAbs which bound better to the hydrophilic (less denaturing) TCPS surfaces were drastically affected by urea treatment, whereas those mAbs which preferred the hydrophobic (more denaturing) surface were the least affected by urea. Some epitopes which were conformationally stable upon surface adsorption (group A, Fig. 5) were nevertheless considerably affected by urea treatment, demonstrating that the chemical denaturation was more drastic than that due to surface adsorption. This was also demonstrated by the effects of urea treatment of FN upon subsequent cell adhesion. Surface adsorption of FN to hydrophobic PS surfaces yielded reasonable cell adhesive capacity and binding of A17, whereas urea treatment drastically reduced both of these activities. The effects of urea were not due simply to quantitative differences in protein adsorption. Results of other workers using physical measurements have shown that urea treatment of FN denatures the regions of type III homology (cell binding and C-terminal heparin binding domains), while the terminal regions of type I and II homology are relatively resistant (Markovic and Engel, 1983). Urea treatment of FN in solution is reversible, the molecule reverting to its original conformation (except for a few localised changes), after removal of urea by dialysis (Markovic and Engel, 1983). Our results with surface adsorption in the presence of urea indicate that once surface adsorbed, the denatured conformation is "frozen" upon subsequent removal of urea.

Comparison of the effects of surface chemistry upon FN with that upon VN

The biological consequences of "denaturation" of VN by surface binding and urea treatment appear to be quite different from those of FN. Although mAb A18 recognises a conformational cell-binding epitope (it does not react in western blots whereas A27 does; Underwood et al. 1990), its binding profile on the different plastic surfaces was the same as that of A27. For both these mAbs, a preference for VN coated on the ELISA hydrophobic surface was noted and mAb binding was also considerably reduced on the Linbro TCPS surface compared with the other two TCPS surfaces. Neither of the epitopes of these two mAbs were denatured by urea treatment, in contrast to the response of the FN mAbs which inhibit cell adhesion. This supports the notion that "denaturation" of the VN molecule by surface binding is similar to "denaturation" by urea and may be an intrinsic property of the molecule itself rather than of the binding surface (Pitt et al. 1989). The requirement for denaturation of VN for many of its biological activities (see review, Tomasini and Mosher, 1991), may underlie the differences in effects on biological activities of denaturation of FN and VN and explain why urea treatment of VN does not reduce BHK cell adhesion.

Another difference between FN and VN was observed in the response to divalent cations. Cell adhesion experiments could not distinguish between the effects of divalent cations on the ligand and the cell receptor. mAb binding could be independently measured after removal of the cation. Treatment of FN coated on Dynatec polyvinyl plates with Ca^{2+} did not affect the binding of any of the twelve anti-FN mAbs studied. With VN, however, the binding of A18 was significantly reduced after cation treatment, particularly Ca^{2+}. This indicates a Ca^{2+}-induced conformational change in the region of A18 binding, which is also the region involved in cell adhesion. Cellular binding to FN and VN occurs through different integrin receptors. Binding of cations by EF-hand motifs in the integrin beta subunits of each receptor is essential for receptor function (Edwards et al. 1988; Loftus et al. 1990). Cell adhesion to VN, however, shows a requirement for higher cation concentration than adhesion to FN (Edwards et al. 1987). Different models involving different mechanisms of Ca^{2+} binding to receptor or ligand were discussed by Edwards et al. (1987), but the experimental evidence at that time could not distinguish between them. Our finding that Ca^{2+} perturbs the binding of a mAb which affects cell adhesion, indicate that Ca^{2+} binding to the VN ligand, as well as to the receptor, may be involved in cell adhesion to VN. Functional changes

in the conformation of a number of different proteins have been detected with mAb probes in other studies (Le Vine et al. 1988; Katchalski-Katzir and Kenett, 1988; Loftus et al. 1990), and Ca^{2+} binding is a demonstrated requirement for the activity of another cell-adhesive glycoprotein, thrombospondin (Lawler et al. 1988).

It may seem surprising that mAbs to both FN and VN were able to detect differences between brands of TCPS, which have essentially similarly prepared surfaces. It is a common, but generally unreported finding that some cells in culture display a preference for a particular brand of TCPS. This may be related to surface conformational or quantitative differences in adsorbed cell-adhesive proteins, such as we have detected with our mAb panel.

CONCLUSION

This report demonstrates that a carefully selected panel of mAbs against FN and VN, which includes antibodies affecting biological activity, can be used to determine the conformational status of surface adsorbed protein and to predict or analyse at the molecular level its biological activity under different conditions. In particular, it can be used to analyse the cell adhesive behaviour of adsorbed protein on different surfaces, for cells which utilise different integrin receptors. The use of these mAbs can also permit the detection of conformational changes in proteins adsorbed to surfaces that have had various chemical treatments, and cases where the proteins are adsorbed from complex biological mixtures, rather than a purified preparation.

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