QUALITATIVE AND QUANTITATIVE DIFFERENCES IN SPREADING OF HUMAN FIBROBLASTS ON VARIOUS PROTEIN COATS. MODULATION BY TREATMENT OF THE CELLS WITH AMINES

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SUMMARY

In contrast to established cell lines, normal human skin fibroblasts spread on their own fibronectin. The present investigation has examined whether human fibroblasts, like established cell lines, would be capable of spreading on substrata coated with proteins with different reactivity towards the cell surface. Coverslips were coated with human serum, fibronectin, α2-macroglobulin-trypsin, a polyspecific anti-fibroblast antibody and a polyspecific anti-calf-serum antibody. The attachment of the cells to these substrata was of the same extent. Spreading was examined qualitatively using phase, interference contrast and reflection contrast optics on live cells, as well as scanning electron microscopy on fixed cells. To quantitate the maximum degree of cell spreading a semi-automated system was used, which measured the cell perimeter on a large number of cells. The distributions of the degree of cell spreading on the five substrata were compared statistically. The qualitative and quantitative differences observed on the various substrata could be further differentiated by adding various amines to the cells during 60 min spreading or during a 30 min preincubation before spreading. No strict correlation could be found between the effect of the amines on attachment or on spreading and their presumed effects on cellular transglutaminases. The results clearly indicate that the spreading of human fibroblasts can be modulated by the nature of the substratum and that, by using quantitative methods, these differences in behaviour can be measured accurately.

INTRODUCTION

Attachment and spreading of cultured cells on various substrata have been extensively studied, but the molecular mechanisms involved in these processes remain to be elucidated. Fibronectin is undoubtedly the best characterized spreading factor, with potential relevance in vivo (Grinnell & Hays, 1978b; Hahn & Yamada, 1979). The importance of fibronectin for spreading is further strengthened by the finding that a fairly good correlation exists between the ability of a cell to deposit fibronectin on a tissue culture substratum and its ability to spread on this substratum (Grinnell & Feld, 1979; Pena & Hughes, 1978). For normal human skin fibroblasts, which rapidly produce their own fibronectin (Grinnell & Feld, 1979), spreading in serum-free medium is considered to be mediated almost exclusively by the interactions of the cells with their own fibronectin (Grinnell & Feld, 1979, 1980). For...
many established cell lines that show poor fibronectin deposition, various other molecules, unrelated to fibronectin, but which can nevertheless induce rapid attachment and spreading, have been described. All these compounds share the property of interacting with the cell surface: either by their electric charge, such as polycationic ferritin (Grinnell & Hays, 1978a), by their carbohydrate specificity, such as lectins (Oppenheimer-Marks & Grinnell, 1981; Grinnell & Hays, 1978a; Prinz & Von Figura, 1978) or glycosidases (Rauvala, Carter & Hakomori, 1981; Carter, Rauvala & Hakomori, 1981), by their antibody specificity (Wylie, Damsky & Buck, 1979; Hsieh & Sueoka, 1980; Hughes, Pena, Clark & Dourmashkin, 1979) or by their binding to a specific cell surface receptor (Rubin, Höök, Öbrink & Timpl, 1981; Michl, Pieczonka, Unkeless & Silverstein, 1979).

These findings suggested that the spreading of established cell lines occurred when the appropriate cell surface molecules were triggered by substratum-bound ligands. Further evidence that alternative or complementary mechanisms to fibronectin existed was obtained when spreading of these cells in or on collagen gels could be visualized (Grinnell & Bennett, 1981; Harper & Juliano, 1981).

The present investigation was undertaken in order to determine whether, as described for established cell lines, the spreading of human fibroblasts could be modulated by the nature of the substratum. Differences occur in the spreading behaviour of the cells on different protein coats, to which they attach readily and would indeed suggest that alternative mechanisms of spreading are present.

As discussed, cellular and plasma fibronectin have been previously identified as potent spreading factors (Hahn & Yamada, 1979; Grinnell & Hays, 1978b). While the spreading activity in serum coats is considered to be due to the presence of fibronectin (Grinnell & Hays, 1978b), still other components might provide spreading activity on the serum coat (Thom, Powell & Rees, 1979; Laterra, Ansbacher & Culp, 1980; Hedman et al. 1979). α₂-macroglobulin–trypsin complexes provide a good substratum for the attachment of human fibroblasts but receptor-facilitated attachment or spreading does not occur (Cassiman et al. 1981b).

Multivariant lectins and antibodies, containing specificities against cell surface components have previously been shown to induce rapid spreading of a variety of cell lines (Grinnell & Hays, 1978a; Prinz & Von Figura, 1978; Hughes et al. 1979; Oppenheimer-Marks & Grinnell, 1981; Carter et al. 1981), even in the absence of fibronectin. In the present investigation, a polyspecific antibody preparation, aNHF, containing specificities directed against surface components of human fibroblasts was used (Verlinden, Van Leuven, Cassiman & Van den Berghe, 1981). Finally, anti-newborn-calf-serum antibodies (Van Leuven, Cassiman & Van den Berghe, 1978) were used as a control for the antibody specificities against serum components also present in the aNHF; in the absence of any serum component they can be considered as rabbit immunoglobulins G (IgG) known to provide a poor substratum for cell spreading (Giaever & Ward, 1978).

In order to obtain maximum information on the effect of these substrata on cell spreading we elected to study a time point at which differences in the degree of cell spreading would still be apparent and at which modification of the substratum
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would be minimal. Previous investigations have shown that the 60 min time point is informative (Brugmans et al. 1981) and that the substrata are minimally modified (Cassiman et al. 1981b). However, at this time point variation in the degree of spreading of the cells on any coat is still observed. Therefore a semi-automated system was used to quantitate cell spreading on large numbers of cells (Brugmans et al. 1981). The quantitative results obtained in this fashion allowed us to compare the distributions of the degree of cell spreading on the various substrata statistically. The morphological properties of the cells on the different substrata were followed dynamically under interference contrast and under reflexion contrast optics as well as by the scanning electron microscopy.

To differentiate further spreading of the cells on the different substrata, amines were added either during spreading or before the cells were preincubated for 30 min. The amines were chosen for their effect on the recycling of certain surface receptors (methylamine, MA; mono-dansylcadaverine, m-DNSC; Van Leuven, Cassiman & Van den Bergh, 1980; King, Hernaez-Davis & Quatrecasas, 1980) and on the activity of cellular transglutaminases (m-DNSC, MA, Bacitracin, cystamine, histamine; Davies et al. 1980; Levitzki, Willingham & Pastan, 1980; Folk, 1980). Chloroquine was used as a control for this group.

The results of our investigations indicated that while the attachment of human fibroblasts to the different substrata occurred to the same extent, qualitative and quantitative differences in the extent of cell spreading could be observed, depending on the nature of the substratum. The differences between the substrata were further stressed by the differential effects of the amines. Finally, on the same substratum, amines with a presumed single target in the cells, still had quantitatively different effects on cell spreading.

MATERIALS AND METHODS

Cell culture

Normal human diploid skin fibroblast cultures were obtained and grown as described (Cassiman et al. 1979). The medium of the secondary cultures consisted of Dulbecco's Modified Eagle's Medium (DME-Gibco) supplemented with 10 % (v/v) heat-inactivated newborn calf serum, 15 mM-M-(tris(hydroxyethyl)methyl-2-amino)ethanesulphonic acid (TES) and 15 mM-4-(2-hydroxyethyl)-piperazine ethanesulphonic acid (HEPES) buffered at pH 7.4 and 1 g/l NaHCO3. The cultures were split 1:2 twice a week and monitored regularly for mycoplasma contamination, by DAPI staining.

Cell suspension

Forty eight hours prior to assay, the cells were seeded in 75 cm² flasks at 1.5 x 10⁶ cells per flask in full medium. Immediately before use, the cells were washed with 5 ml 0.02 % EDTA in Tris-buffered saline (pH 7.4) and incubated for 10 min in a 5 ml solution containing 2 mg/ml Dispase II (Boehringer) and 0.02 % EDTA in Tris-buffered saline (Cassiman, Brugmans & Van den Bergh, 1981a). The cell suspension thus obtained was washed three times in serum-free culture medium without NaHCO3 (assay medium, pH 7.4). For some experiments the cells were washed in phosphate-buffered saline (PBS) or Ca²⁺/Mg²⁺-free PBS (CMF-PBS). The suspensions were then filtered through a double layer of Nytex (20 μm pore size), counted in an electronic particle counter (Coulter) and adjusted to 4 x 10⁴ cells/ml. Aliquots (0.5 ml) were plated on coated, round glass cover slips (φ 13 mm, Chance no. 1) in multiwell culture trays (Linbro) and incubated at 37 °C for the indicated times.
Substratum coating

Round coverslips (φ 13 mm, Chance no. 1) were placed in multiwell trays (Linbro) and were incubated during 30 min at 37 °C with 750 μl protein solution in assay medium, washed twice with assay medium and used in the different attachment and spreading assays.

The solutions used to coat the coverslips contained: 5% (v/v) human serum (HuS), 150 μg/ml α₂macroglobulin saturated with trypsin (α₂M-T, Van Leuven, Cassiman & Van den Berghe, 1979); 50 μg/ml fibronectin (FnB) (Collaborative Research); rabbit anti-human whole fibroblast antibody (aNHF) (1:3-2:8 % IgG, Verlinden et al. 1981); rabbit anti-newborn-calf-serum antibody (aNCS) (1:3-2 % IgG, Van Leuven, Verbruggen, Cassiman & Van den Berghe, 1977). We have previously shown that under the assay conditions used, these proteins coat the substratum and are stable during the time of the assay procedure (Cassiman et al. 1981).

Cell attachment

The cell suspensions were prepared as described above, but 2 h before dissociation the medium was replaced with culture medium, without leucine (Gibco) and without serum but containing 10 μCi/ml [³H]leucine (sp. act. 1 Ci/mmol, the Radiochemical Centre, Amersham). A portion (0.5 ml) of a suspension containing 4 x 10⁵ cells/ml was plated at the appropriate temperature and for 60 min on the different substrata. The number of cells remaining attached, after two washes with assay medium, was estimated by counting the radioactivity associated with the substratum (Brugmans, Cassiman & Van den Berghe, 1978), and was expressed as the percentage of a control incubated for 2 h at 37 °C under comparable conditions (90% attached cells under microscopic inspection).

Cell spreading

Cell suspensions plated on coverslips were examined at the appropriate time period under an inverted microscope and their degree of spreading was scored qualitatively by two investigators independently. Most of the coverslips were also fixed in 2.5% glutaraldehyde in 0.1 M-cacodylate buffer (pH 7.4) for 15 min and stained for 5 min with acridine orange (0.01% in PBS). The glass coverslips were mounted in PBS and photographed under a fluorescent microscope (Leitz, Dialux 20). The photonegatives were processed in an interactive automated system and the cell perimeter was used as the parameter to measure the degree of cell spreading (Brugmans et al. 1981). The results of the measurements were plotted as frequency distributions. Since most of these distributions were not normally distributed, the Kolmogorov-Smirnov two-sample test was used for statistical comparison of two samples (Siegel, 1956). Minimum and maximum differences between two cumulative frequency distributions were tested for samples containing more than 30 measurements. This test is fairly insensitive in comparison to the Student's t-test. Significant differences at P < 0.01 or < 0.001, therefore, indicate fairly large differences in the shape of the distributions.

Morphological studies of cell spreading

For microcinematographical recordings, the cells were dissociated as described, washed five times in assay medium and 100,000 cells were plated in 2 ml assay medium on square coverslips coated as described above and mounted in tissue culture chambers (Labtek). The cells were filmed for 1 h on an inverted microscope (Leitz, Diavert) fitted with a black and white SIT camera (Tektec) and the images, 4 frames/s, were recorded on a video time-lapse recorder (GYYR). Phase optics were 32 x /N.A.O. 40-condensor factor x 1.25, interference contrast (same conditions as phase) and reflection contrast, objective 50 x oil/N.A. 1.00, with a mercury light source and filter IL54L mm.

For scanning electron microscopy the same conditions were used as during quantitative cell spreading studies. The cells were fixed in 3% glutaraldehyde in cacodylate buffer for 2 h, followed by 1% osmium tetroxide in PBS (pH 7.4) for 1 h and processed as described (Van der Schueren, Cassiman & Van den Berghe, 1976).
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**Products**

Mono-dansylcadaverine (m-DNSC), chloroquine and Bacitracin were from Sigma; methylamine was from Merck; histamine and cystamine dihydrochloride were from Aldrich. Fresh solutions were made at the appropriate concentrations in assay medium and buffered at pH 7.7.

**RESULTS**

**Attachment to different substrata**

Secondary cultures of human skin fibroblasts dissociated with Dispase attached readily to coverslips coated with either human serum, fibronectin, α2M-T, polyclonal antibodies or IgG controls. After 60 min at 37 °C more than 80% of the cells had attached to these different substrata (Table 1). The rates of attachment on the IgG preparations were very similar to those previously published for serum, α2M-T and fibronectin (Brugmans & Cassiman, 1980; Cassiman et al. 1981a, b).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Trypsin</th>
<th>PBS</th>
<th>CMF-PBS</th>
<th>EDTA</th>
<th>4 °C</th>
<th>pH 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>HuS</td>
<td>98.6 ± 14.8</td>
<td>49.7</td>
<td>ND</td>
<td>71</td>
<td>21.9</td>
<td>37.3</td>
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<tr>
<td>PbN</td>
<td>84.8 ± 17.3</td>
<td>100</td>
<td>114</td>
<td>81</td>
<td>25</td>
<td>115</td>
<td></td>
</tr>
<tr>
<td>α2M-T</td>
<td>79.2 ± 4.8</td>
<td>97</td>
<td>100</td>
<td>93</td>
<td>29</td>
<td>121</td>
<td></td>
</tr>
<tr>
<td>αNHF</td>
<td>102.4 ± 12.6</td>
<td>78</td>
<td>107</td>
<td>86</td>
<td>32</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>aNCS</td>
<td>90.6 ± 17.4</td>
<td>77</td>
<td>107</td>
<td>95</td>
<td>15</td>
<td>107</td>
<td></td>
</tr>
</tbody>
</table>

Attachment of [3H]leucine-labelled cells to the different substrata was measured after 60 min as described in Materials and Methods. The control values obtained with Dispase-dissociated cells are expressed as the percentage of the label bound to a plastic substratum after 2 h at 37 °C. The other experimental values are expressed as the percentage of this Dispase control. All values represent the mean of at least two duplicate experiments. The standard deviation of the control is indicated.

ND, not done.

Since Dispase II is not routinely used for cell attachment or spreading studies the values for attachment after dissociation with 0.05% crystalline trypsin are given for comparison in Table 1. With the exception of human serum, which showed less than 50% attachment of the Dispase control as previously discussed (Cassiman et al. 1981a), the attachment to the other substrata was comparable to that of Dispase-treated cells. Also, plating the cells in phosphate-buffered saline, instead of assay medium, gave very similar results. In contrast to results described for other cell lines (Takeichi & Okada, 1972; Seglen & Gjessing, 1978; Damluji & Riley, 1979; Grinnell, 1976), the attachment of Dispase-dissociated cells was strikingly independent of the presence of divalent cations in the attachment medium (Table 1). The process was inhibited, however, by low temperature to the same extent on all the substrata, while lowering the pH to 6 inhibited only the attachment on human serum (Table 1).
It is clear from these results that the attachment of human fibroblasts to the various substrata occurred rapidly and that only the attachment to human serum could be distinguished from attachment to the other substrata by two parameters: its sensitivity to dissociation by trypsin and to acid pH.

Fig. 1. Frequency distributions of the cell perimeter of cell spreading on different substrata. Dispase-dissociated human fibroblasts were plated for 60 min at 37 °C in serum-free medium on glass coverslips coated with the five substrata: (x—x) aNCS; (Δ—Δ) α4M-T; (▲—▲) HuS; (□—□) FbN; (○—○) aNHF. After fixation and staining, the cell perimeter was determined for at least 200 cells on each substratum. The solid line is the theoretical distribution that would be obtained if the five substrata were to be combined in a single coat.

Spreading on various substrata

Spreading of Dispase-dissociated human fibroblasts on the five substrata, was analysed qualitatively and quantitatively over a 60 min time period. In some experiments the cells were allowed to proceed beyond this time limit in order to examine whether at later time points (2–3 h) more striking changes in their morphology would occur.

Quantitative results. The cell perimeter, shown previously to characterize the degree of cell flattening and polarization on the substratum (Brugmans et al. 1981),
Fig. 2. Scanning electron micrographs of spreading cells. Dispase-dissociated human fibroblasts were plated for 60 min at 37 °C in serum-free medium on coated glass coverslips. After glutaraldehyde fixation and gold coating the cells were examined under a Philips SEM 500. A. On 5% human serum. B. On fibronectin. C. On α2-macroglobulin–trypsin. D. On polyspecific anti-fibroblast antibody. Bars, 10 μm.
was measured after 60 min for at least 200 cells on each substratum. Since one preparation always contains cells of different degree of spreading, frequency distributions of the cell perimeter were computed. Statistical comparisons were made only between distributions and not their means. In assay medium, the cells spread well on fibronectin, serum and polyspecific antibody, and poorly on α5M-T and IgG (Fig. 1). When spreading was examined in PBS, similar results were obtained.

In agreement with previous investigations, low temperature (4 °C), chelators (EDTA, EGTA), 2 M-urea and 5 mM-iodoacetamide inhibited spreading of cells that were allowed to attach for 10 min at 37 °C before treatment with the appropriate agent. The presence of 0.1 mM-cycloheximide, 0.2 mM-urea and 16 mM-KCN during the 60 min assay were without effect on the degree of cell spreading (results not shown).

Qualitative results. The dynamic aspects of the spreading were followed by phase and interference contrast optics during 60 min. The formation of cell contacts with the different substrata was examined cinematographically under reflection contrast optics. After 1 h the cells were also examined by scanning electron microscopy (Fig. 2).

Examination under phase and interference contrast optics of live cells indicated that the nature of the coat as such did not induce alternative morphological mechanisms of spreading. In summary, the attached cells sent out very dynamic filopodia, which after contacting the substratum formed small lamellipodia. Concomitantly with a flattening of the cell body these lamellipodia extended, showing characteristic ruffling activity at their periphery. On human serum, filopodia and several lamellipodia with an intense ruffling activity were observed initially. Later, the cells underwent a considerable change in shape, often resulting in a U-shape, followed by a rearrangement of the cell processes and slight retraction of the cell body.

On fibronectin, the initial small ruffling lamellipodia extended rapidly so that the whole periphery became involved in the spreading, and during spreading a rapid and extreme flattening of the cells occurred (Fig. 2B).

On α5M-T, the cells stayed round, sometimes surrounded by filopodia and small lamellipodia in the immediate vicinity of the cells (Fig. 2C). On αNHF, the cells formed multiple extensions, which branched extensively and ruffled actively. The

Fig. 3. Reflection contrast images of spreading cells. Fibroblasts plated in serum-free medium on coated glass coverslips were examined under reflection optics during 5 h. Photomicrographs of the live cells were taken at regular intervals. The pictures shown were taken at time points best illustrating the differences observed in the morphology of the cells on the different substrata.

A. On human serum after 5 h spreading. The presence of a dark ring containing some black areas suggestive of focal contacts is typical. B. On fibronectin after 60 min. A lamellipodium stains darkly at its onset. Closer to the cell body points of presumed focal contact at which bundles of fibres insert are visualized (arrows). The cell body is darkly stained and contains bundles of filaments suggesting an extreme flattening of the cells on the substratum. C. On α5M-T after 3.5 h. The cell has a mottled aspect. The absence of black rings or areas of focal contact is striking. D. On αNHF after 8 h. The cells contain many dark concentric rings. As in human serum, a peripheral dark band is present. In addition, typical arborizations extend from the cell periphery. Bar, 10 μm.
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continuous formation of filopodia resulted in a typically star-shaped cells, completely surrounded by branched filopodia (Fig. 2D).

Reflection contrast studies were undertaken in order to examine the cell-substratum contacts in more detail. As previously reported (Izzard & Lochner, 1976; Gingell, 1981), the contribution of the thin peripheral cytoplasm in the image formation was often quite extensive and sometimes even prohibited a simple interpretation of the images in terms of cell-substratum contacts. The typical morphology of the cells on each coat (Fig. 3) confirmed, however, that the cells displayed differences in behaviour on each substratum.

Effect of amines on the attachment to various substrata

Fibroblast suspensions, dissociated with Dispase, washed in assay medium and adjusted to $4 \times 10^4$ cells/ml in assay medium were plated on the five different substrata in the presence of a variety of amines at concentrations shown previously to affect endocytosis (Van Leuven et al. 1980). When no clear cellular effect had been described, the concentration was arbitrarily taken at 10 mM.

| Table 2. Attachment of NHF on various substrata: effect of the addition of amines |
|-----------------------------------------------|----------------|----------------|----------------|----------------|
| Control                                      | m-DNSC         | MA             | Chloroquine     | Histamine      |
| HuS                                          | 85.7 ± 9.09    | 74.6           | 85             | 80             |
| FbN                                          | 102.7 ± 5.5    | 86.6           | 96.3           | 83             |
| α4M-T                                        | 93 ± 13.2      | 91.4           | 83.8           | 87             |
| aNHF                                         | 112.7 ± 6.7    | 50.5           | 83             | 80             |
| aNCS                                         | 92 ± 21.8      | 50             | 98.9           | 94             |

Attachment of Dispase-dissociated normal human fibroblasts (NHF) on coated glass coverslips was measured at 60 min. Amines were added to the cell suspensions at time 0. The concentrations of the amines were: 0.2 mM-m-DNSC; 20 mM-MA; 0.5 mM-chloroquine; 10 mM-histamine. The results are expressed as a percentage of the attachment of the control value (as in Table 1). Each value represents the mean of at least two duplicate experiments. ND, not done.

The presence of histamine or chloroquine had no significant or differential effect on the attachment of the cells to the five substrata (Table 2). Methylamine inhibited the attachment to rabbit IgG up to 50% of the control, but had much less effect on the other substrata. Mono-dansylcadaverine was the most powerful inhibitor of all the amines tested. Its greatest effect was observed on a serum coat (29.1% of the control) and its smallest effect on fibronectin (60.3% of the control).

Since some of the primary amines may exert their effect only after intracellular accumulation (Van Leuven et al. 1980), cell layers were preincubated for 30 min before dissociation with three amines shown to accumulate intracellularly (Van Leuven et al. 1980), and the attachment of the cells to the five substrata was examined in the absence of amines (Table 3). Preincubation with 0.2 mM-m-DNSC, 20 mM-MA and 0.05 mM-chloroquine did not noticeably affect the dissociation of the cell layer; the same number of single cells per culture flask was obtained. Only pretreatment
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with m-DNSC decreased the attachment on all the substrata, to about 60\% of the controls. This effect was thus less pronounced than the presence of the amine in the attachment medium as shown above. Combined pretreatment and addition during the assay gave the same results as addition only.

Table 3. Attachment of NHF on various substrata: effect of preincubation with primary amines

<table>
<thead>
<tr>
<th>Substrata</th>
<th>Control ± S.D.</th>
<th>m-DNSC</th>
<th>MA</th>
<th>Chloroquine</th>
</tr>
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<tbody>
<tr>
<td>HuS</td>
<td>85.7 ± 9.00</td>
<td>67.6</td>
<td>96.8</td>
<td>106.2</td>
</tr>
<tr>
<td>FbN</td>
<td>102.7 ± 5.5</td>
<td>65.2</td>
<td>101</td>
<td>124.8</td>
</tr>
<tr>
<td>aM-T</td>
<td>91.3 ± 13.2</td>
<td>61.2</td>
<td>100</td>
<td>119</td>
</tr>
<tr>
<td>aNHF</td>
<td>112.7 ± 6.7</td>
<td>69.2</td>
<td>86</td>
<td>106</td>
</tr>
<tr>
<td>aNCS</td>
<td>92 ± 21.8</td>
<td>72.8</td>
<td>70</td>
<td>135.8</td>
</tr>
</tbody>
</table>

Attachment of Diapase-dissociated NHF on coated glass coverslips at 60 min. Amines were added in assay medium 30 min before dissociation of the cell layers at the following concentrations: 0.2 mM-m-DNSC; 20 mM-MA; 0.05 mM-chloroquine. The results are expressed as a percentage of the control (as in Table 1); each value represents the mean of duplicate experiments. The mean and standard deviation of the control are indicated.

Table 4. Spreading of NHF on various substrata: effect of the addition of amines

<table>
<thead>
<tr>
<th>Substrata</th>
<th>Control</th>
<th>m-DNSC</th>
<th>MA</th>
<th>Chloroquine</th>
</tr>
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<tr>
<td>HuS</td>
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<td>101.5</td>
<td>106.7</td>
<td>ND</td>
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<tr>
<td>FbN</td>
<td>121.4</td>
<td>103.8*</td>
<td>111.7</td>
<td>124.5</td>
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<td>aM-T</td>
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<td>aNHF</td>
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<td>69.7*</td>
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<td>110.4</td>
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<td>aNCS</td>
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<td>69.1</td>
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</tbody>
</table>

Dispase-dissociated NHF were allowed to spread on coated glass coverslips for 60 min at 37 °C in assay medium with or without amines added at time 0 at the following concentrations: 0.2 mM-m-DNSC; 20 mM-MA; 0.05 mM-chloroquine. The results are expressed as the mean perimeter of the cells. At least 200 cells were examined in different experiments.

* Significantly different from the control at P < 0.001.

ND, not done.

Effect of amines on cell spreading

The effect of various amines on the degree of cell spreading was examined by adding the amine at time zero to spreading cells, by preincubation during 30 min prior to dissociation or by a combination of both. Addition of 0.2 mM-monomodansylcadaverine reduced spreading significantly on fibronectin and on polyspecific antibodies. Methylamine reduced the spreading only on polyspecific antibodies to the same extent as m-DNSC, while chloroquine was without effect on any of the substrata (Table 4). Bacitracin (0.2 mM) and histamine (10 mM) inhibited spreading only on aNHF, while cystamine (10 mM) inhibited spreading on serum, fibronectin and aNHF. After preincubation with this first group of amines even greater effects on cell spreading were observed, which could be stimulatory as well as inhibitory.
(Table 5). On serum, only chloroquine was without effect. On fibronectin, both m-DNSC and methylamine increased the degree of spreading. In contrast to this, Bacitracin and chloroquine inhibited spreading on the polyspecific antibodies, while on IgG, m-DNSC, MA and chloroquine stimulated, and Bacitracin inhibited, spreading (Table 5). It may seem odd that the effect of chloroquine on fibronectin-spreading is not significant while the mean perimeter is at least as long as the one for dansylcadaverine-treated cells (Table 5). However, it does illustrate the fact that the mean degree of spreading is not a reliable parameter for the comparison of two distributions.

Table 5. Spreading of NHF on various substrata: effect of preincubation with primary amines

<table>
<thead>
<tr>
<th>Substrata</th>
<th>Control</th>
<th>m-DNSC</th>
<th>MA</th>
<th>Bacitracin</th>
<th>Chloroquine</th>
</tr>
</thead>
<tbody>
<tr>
<td>HuS</td>
<td>102.7</td>
<td>93.5*</td>
<td>85.8*</td>
<td>75.6*</td>
<td>104.6</td>
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<tr>
<td>FbN</td>
<td>109</td>
<td>115**</td>
<td>121.3**</td>
<td>104.6</td>
<td>118.1</td>
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<tr>
<td>α2M-T</td>
<td>67.1</td>
<td>62.9**</td>
<td>65.3</td>
<td>62.4</td>
<td>63.6</td>
</tr>
<tr>
<td>αNHF</td>
<td>98.7</td>
<td>104.3</td>
<td>109.7**</td>
<td>88*</td>
<td>77*</td>
</tr>
<tr>
<td>αNCS</td>
<td>59.5</td>
<td>66.4*</td>
<td>67.8**</td>
<td>56.7**</td>
<td>69.2**</td>
</tr>
</tbody>
</table>

NHF were incubated at 37 °C in assay medium containing 0.2 mM of the amine for 30 min. At this time the cells were dissociated with Dispase and plated on coated glass coverslips. The circumference was measured after 60 min spreading in assay medium.

* P < 0.001; ** P < 0.01.

The stimulatory effect of dansylcadaverine pretreatment on cell spreading is illustrated further by examining the effect of different concentrations of this chemical on cell spreading. Increasing the concentrations of m-DNSC from 0.05 to 0.8 mM shifted the frequency distribution towards the greater cell circumference (Fig. 4). At higher concentrations (1–10 mM), the preincubated cell layers could no longer be dissociated into single cells (at least 50% decrease in total cell count). Preincubation of the cells for 24 h with 0.1 M-cystamine stimulated the spreading on serum, fibronectin and IgG significantly, while no effect was observed on α2M-T and αNHF. When pre-treatment with any of the amines was combined with additions during spreading, the effect of addition only was always observed.

Pretreatment with primary amines increases the intralysosomal pH and as a consequence lysosomal degradation is decreased (Hollemans et al. 1981; Van Leuven et al. 1980). This effect of intracellular pH change was further examined by adjusting the pH of the assay medium between 5 and 8.5. Between pH 6.5 and 7.5 no significant difference in the degree of spreading was observed on the different substrata. At an even more acid pH inhibition occurred, although attachment was still comparable to that in the controls (Table 1), while at pH 8 a significant increase in cell spreading was observed on serum and on fibronectin (results not shown).

Qualitative examination of the spreading cells, did not reveal major changes in the morphology of the spreading cells under the influence of amines (results not shown).
Fig. 4. Effect of preincubation with dansylcadaverine on the spreading of cells on fibronectin. Fibroblast cell layers incubated in serum-free medium containing 0 (■—■), 0.05 (○—○), 0.2 (□—□) and 0.8 (×—×) mM-m-DNSC, for 30 min prior to their dissociation by Dispase. The cells were plated on a fibronectin coat and their perimeter was determined after 60 min spreading on this coat in serum-free medium. A gradual shift of the frequency distribution curve towards greater cell circumference is observed with increasing concentration of the compound. Higher concentrations could not be tested since they affected the dissociation of the cells. At least 100 cells were scored at each concentration.

DISCUSSION

Nature of the substratum

The attachment of Dispase-dissociated fibroblasts to the five substrata chosen shows some difference in extent but is of the same order of magnitude and follows similar kinetics. The attachment, therefore, does not allow us to discriminate between these substrata on a qualitative or a quantitative basis.

Spreading is more informative and indicates quantitative differences in the extent of cell spreading on the five substrata. Moreover, from the dynamic and static morphological observations, it is evident that within 60 min the cells acquire an aspect that is typical for each substratum (Figs. 2, 3).

Our results, therefore, do suggest that the substrata chosen in the present investigation elicit different behaviour from the fibroblasts when they are plated on these substrata, and that these differences can be observed qualitatively and measured quantitatively.

Whether the different substrata exert a differential effect on fibronectin production
or deposition is unknown. The parallel between our observations and those described for established cell lines (Grinnell & Hays, 1978a; Carter et al. 1981; Hughes et al. 1979), and the fact that only monomolecular layers of proteins are formed on a sub-stratum (Rosenberg, 1960), do however suggest that difference in fibronectin deposition is not the primary cause of the observed differences in the morphology and the degree of cell spreading. A plausible explanation for these observations would be that the rapid spreading of established cell lines and human fibroblasts on fibronectin or on any other substratum occurs through the triggering of the same group of cell surface molecules. This interaction would then set off intracellular events resulting in the organization of the cytoskeleton (Bragina, Vasiliev & Gelfand, 1976; Lloyd, Smith, Woods & Rees, 1977; Badley, Woods, Smith & Rees, 1980).

**Effect of amines**

The initial attachment of the cells to the different substrata is quite insensitive to treatment with most amines. Unlike attachment, however, spreading of human fibroblasts is quite sensitive to amine treatment. A comparison of the effect of the individual amines (Table 6) indicates that cystamine and dansylcadaverine have the greatest effect, whereas chloroquine and histamine have very little effect at the concentrations tested.

<table>
<thead>
<tr>
<th>Table 6. Summary of the effect of amines on cell spreading</th>
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</thead>
<tbody>
<tr>
<td><strong>Addition</strong></td>
</tr>
<tr>
<td>HuS</td>
</tr>
<tr>
<td>DNSC</td>
</tr>
<tr>
<td>MA</td>
</tr>
<tr>
<td>Bacitracin</td>
</tr>
<tr>
<td>Chloroquine</td>
</tr>
<tr>
<td>Cystamine</td>
</tr>
<tr>
<td>Histamine</td>
</tr>
</tbody>
</table>

=, No effect; \, inhibition; /, stimulation.

Values incorporated in the table are extracted from Tables 4–6 and from the text. Arrows in parentheses indicate significance at $P < 0.01$; the others are significant at $P < 0.001$.

ND, not done.

The addition of amines, when effective only decreases the degree of spreading without exception, while preincubation may increase or decrease spreading. These results further stress the differences in cell spreading on the different substrata, as already discussed in a previous section. One would indeed expect that all the amines would have a similar effect on the cells if the mechanisms by which they spread on these substrata were the same. This evidence, added to the morphological description and to the results available in the literature, suggests that the spreading of human fibroblasts on protein coats can and does occur through different mechanisms. One of these mechanisms might be the deposition of fibronectin in the absence of any protein coat.
Finally, our results indicate that, with the exception of dansylcadaverine, the amines used in these experiments do not inhibit attachment even when spreading is significantly reduced. These observations are in agreement with those of Grinnell & Hays (1979a), Butters, Devalia, Aplin & Hughes (1980) and Carter et al. (1981), and suggest that cell attachment and cell spreading are dissociable processes, which are likely to be due to different types of interactions and bonds, and hence must be regulated by different mechanisms.

**Mechanisms of action of the amines**

As indicated in Results, increase in intralysosomal pH during spreading cannot be considered as the primary cause of the effect of the primary amines, since increasing the extracellular pH and treatment with chloroquine are without consequence. Cellular transglutaminases (TG) would be inhibited by most amines at the concentrations tested, whereas chloroquine would be ineffective (for a review see Folk, 1980). As illustrated, variable results were obtained with the amine treatments. Our investigations, therefore, do not allow us to rule out transglutaminases or include them in the spreading phenomenon and we await more precise information on the site(s) of action of the primary amines before our results can be interpreted in terms of molecular mechanisms.

Recently, Aplin & Hughes (1981) reported the effects of a similar group of primary amines on the spreading of BHK cells. In agreement with our findings their results did not show a correlation between cell spreading and the presumed effect of the amines on transglutaminase activity. Again, we cannot rule out the possibility that the amines interfere with the production or deposition of fibronectin or other cellular components such as proteoglycans (Laterra et al. 1980) by human fibroblasts, but, as discussed, the spreading behaviour observed on the different substrata suggests that other mechanisms may be involved.

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Spreading of human fibroblasts


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