A mycoplasmal protein influences tumour cell invasiveness and contact inhibition \textit{in vitro}

CHRISTIAN SCHMIDHAUSER*, ROBERT DUDLER, THOMAS SCHMIDT
Institut für Pflanzenbiohogie, Universität Zürich, Zollikerstrasse 107, 8008 Zürich, Switzerland
and ROGER W. PARISH†
Botany Department and Centre for Protein and Enzyme Technology, La Trobe University, Bundoora, Victoria 3083, Australia
* Present address: Cell and Molecular Biology Division, Lawrence Berkeley Laboratory, California 94720, USA
† Author for correspondence

Summary

Fab fragments of a monoclonal antibody directed against p37, a protein associated with the surface of FS9 mouse sarcoma cells, were previously found to inhibit the highly invasive behaviour of FS9 cells \textit{in vitro}. We show that p37 originates from \textit{Mycoplasma hyorhinis}. Infecting various cell lines with the mycoplasma consistently increased their invasiveness when confronted with chicken heart fibroblasts using Abercrombie's confronted explant technique. Conversely, removing the mycoplasmas or blocking p37 with specific Fab fragments reduced invasiveness. Analysis of individual cell collisions using time-lapse filming showed that the addition of Fab fragments to cells infected with \textit{M. hyorhinis} greatly increased the level of contact inhibition. The antibody also reduced the invasiveness of transformed cells that did not express the p37 antigen. Hence, a cellular protein or proteins that are structurally related to p37 apparently influence invasive behaviour.

Key words: mycoplasm, cell invasiveness, contact inhibition.

Introduction

The primary causes of malignancy are the loss of normal control of cell motility and growth. The discovery of oncogenes has been crucial in unravelling the molecular mechanisms responsible for the loss of growth control. However, very little is known about the molecular changes that are responsible for the ability of malignant cells to metastasize or invade surrounding tissue (Fidler, 1978; Fidler and Hart, 1982; Weiss, 1985; Liotta et al. 1986; Parish et al. 1987). In order to identify changes in motile behaviour independently of changes in growth and changes in the production of enzymes that digest the extracellular matrix it is necessary to study the cells in tissue culture. Abercrombie and Heaysman (Abercrombie, 1979) found that malignant cells failed to respond in a normal manner to collision with non-malignant cells. The motility of a normal fibroblast is largely inhibited when it makes contact with other normal fibroblasts. However, the motility of malignant fibroblasts is much less affected by contact with normal cells. Abercrombie (1979) suggested that this 'failure of heterotypic contact inhibition' underlies the ability of tumour cells to invade other tissues. Studies on many cell types have shown that a strong correlation exists between the ability to invade and to metastasize \textit{in vitro} and the failure to exhibit heterotypic contact inhibition \textit{in vitro} (Abercrombie and Heaysman, 1976; Heaysman, 1978; Abercrombie and Turner, 1978; Stephenson and Stephenson, 1978; Abercrombie, 1979; Paddock and Dunn, 1986).

We are studying the role of cell surface proteins in this \textit{in vitro} invasive behaviour of cells using Abercrombie's confronted explant assay. Monovalent polyclonal and monoclonal antibodies directed against p37, a protein associated with FS9 cells, inhibited the invasive behaviour of the cells (Steinemann et al. 1984a, b). p37 was recently found to be a constituent of a contaminating mycoplasma, \textit{Mycoplasma hyorhinis} (Dudler et al. 1988). The protein was purified, the terminal amino acids sequenced and oligonucleotides used to clone the gene. On the basis of the amino acid sequence the protein has a molecular weight of $4.35 \times 10^3$ after removal of a signal sequence. The p37 gene is part of an operon coding for three proteins. These proteins have structural similarity to the components of periplasmatic binding protein.
dependent transport systems of gram-negative bacteria, suggesting that p37 is part of a high-affinity transport system in *M. hyorhinis*, a gram-positive bacterium.

This work shows that *M. hyorhinis* increases the invasivity of all cell lines to which it has been added and that p37 is involved. Thus, an antibody directed against p37 is involved. Thus, an antibody directed against invasivity of all cell lines to which it has been added and that p37 reduces heterotypic contact inhibition. The antibody also reduced the invasiveness and affected the collision response of transformed cells that did not express the p37 antigen.

**Materials and methods**

**Cell cultures and medium**

All mammalian and avian cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) containing 10% heat-inactivated foetal calf serum (Inotech, Switzerland) at 37°C in 5% CO₂ in air. Total protein preparations were prepared by washing cells in PBS (0.01 M Na,K-phosphate, pH 7.2, 0.15 M NaCl) and adding 1% Triton X-100 in PBS to the pellet. Nuclei were removed by centrifugation at 10,000 g for 10 min and the supernatant used for SDS–polyacrylamide gel electrophoresis and Western blotting.

The cell lines used were mouse L929 cells, NIH/3T3 cells, Rat-1 cells (all three fibroblast cell lines were obtained from Dr H. Binz, Institute for Immunology and Virology, University of Zurich; Rat-1 cells are a 3T3-like fibroblast line isolated from Fisher rat embryos); and mouse fibrosarcoma FS9 cells (Abercrombie, 1979; obtained from Dr G. A. Dunn, MRC Cell Biophysics Unit, London).

*M. arginini* and *M. ocale* were isolated from the cell cultures by Dr R. H. Leach, Mycoplasma Reference Facility, NCTC, London. They were grown at 37°C to log phase in media containing 1.8% (w/v) mycoplasma broth base (Oxoid), 0.7% (w/v) yeast extract (Oxoid), 20% mycoplasma-screened horse serum (Gibco), 0.02% (w/v) thallous acetate (Fluka, Switzerland), Na-benzylpenicillin (Sigma) at 200 units ml⁻¹, and 0.2% (w/v) L-arginine (Sigma). Phenol Red was added to a final concentration of 0.0025% (w/v) in order to visualize growth of mycoplasma by a colour change from red to dark red. *M. hyorhinis* GDL, a gift from Dr R. H. Leach, was grown under the same conditions, except that L-arginine was replaced by 0.1% glucose. Growth was monitored by the pH-dependent colour change from red to bright orange. Mycoplasmas were harvested by centrifugation at 10,000 g for 20 min and then washed three times in PBS. (Note: most cell lines were tested for p37 the moment they were obtained. The exceptions were the FS9 and L929 lines, which were obtained in 1981; the L929 cells have remained free of p37, i.e. *M. hyorhinis*.)

**Gel electrophoresis and Western blotting**

A discontinuous SDS–polyacrylamide gel electrophoresis system (Laemmli, 1970) employing 3% polyacrylamide in the stacking gel and 12% or 15% in the separating gel was used to separate the proteins. Separated proteins were transferred electrophoretically onto nitrocellulose filters (Towbin et al. 1979) in 0.025 M Tris–HCl, 0.193 M glycine, 20% methanol (v/v), pH 8.35, at 4°C. After treatment with peroxidase-conjugated rabbit anti-mouse antibody (Nordic, Immunological Laboratories), diluted 1:600, the filters were stained for peroxidase activity using 0.5 mg ml⁻¹ 3',3-diaminobenzidine tetrahydrochloride (DAB) in 0.015% H₂O₂.

**Preparation of monoclonal antibodies**

Purified antibodies from ascites fluid were digested with mercaptoethanol (Sigma) into Fab and Fc fragments. Digestion was done for 16 h at 37°C with a IgG:enzyme ratio of 100:1 in phosphate buffer (0.1 M, pH 7.2) containing cysteine (0.01 M) and EDTA (0.002 M) to activate the enzyme. After dialysis against water (48 h) and sodium acetate buffer (0.1 M, pH 5.5), fragments were applied to a carboxymethyl (CM)-cellulose column. Elution was with a 0.1 M to 0.9 M sodium acetate (pH 5.3) gradient. The fractions were collected and analysed by SDS–PAGE.

**Confronted explant test**

Circular acid-washed coverslips were placed in 24-well microtiter plates. Heart ventricles of 7- to 9-day-old chicken embryos were cut into small pieces (0.5 mm³), transferred to fresh medium (DMEM, 10% FCS) and then placed on the coverslips. Clumps of cultured tumour cells grown to high density were placed on the coverslip 1 mm distant from the chicken heart explants. Confronted explants were then incubated at 37°C to allow the cells to migrate outwards from the explant. Fab fragments (0.1 mg ml⁻¹) were included in the culture medium. Twenty-four hours after the cells from the two explants had made their first contact, the cultures were fixed with 3% formaldehyde. Measurements were made to compare migration distances achieved by a given population between the confronted explants with migration distances achieved by unconfronted cells of the same population moving into free space (Abercrombie and Heaysman, 1976; Abercrombie, 1979; Stephenson, 1982).

**Time-lapse recording of cell behaviour**

Primary explants of chick heart ventricle from 7- to 9-day-old embryos were cultured in DMEM with 10% FCS at 37°C. Outwandering cells were trypsinized 2 days later and mixed with a cell suspension of tumour cells in the log phase of growth. The mixed cultures were seeded on acid-washed coverslips and allowed to settle for 1–2 h before use. Cell behaviour was recorded at 37°C using Zeiss Universal phase-contrast microscopy equipment. Kodak Plus-X film was used in a Bolex H-16 camera at frame intervals of 30 s, determined by a Wild-Paillard Variotimer. The selection of the cells for analysis was governed by the requirements that they should remain within the imaged area for at least an hour and that either they should be free from contact with other cells or they should collide with only one other cell (Dunn and Paddock, 1982; Paddock and Dunn, 1986). More details are given in Results.

**Immunocytochemistry**

Cells were grown overnight on acid-washed coverslips in DMEM containing 10% FCS. After a rinse with PBS followed by fixation with freshly prepared formaldehyde (3% in PBS, 15 min), the cells were washed twice with PBS and incubated in hybridoma supernatant for 1 h at 37°C. After washing with PBS (twice, 15 min), FITC-labelled rabbit anti-mouse IgG (1:100, Nordic, Immunological Laboratories) in PBS containing 1% BSA (bovine serum albumin) was applied for 30 min at 37°C. After washing as described above, coverslips with attached cells were placed upside down onto a drop of 70% glycerol in PBS. p-Phenylenediamine was used to prevent fading. Fluorescence was monitored using a Zeiss Universal microscope.
Results

p37 is transmitted between cell lines

p37 is inducible in the non-invasive L929 mouse cells. Immunofluorescence studies (Schmidhauser, 1987) and Western blotting demonstrated that the L929 cells expressed p37 after inoculation with sterile filtered (filtered through 0.22 μm filters) FS9 culture supernatant (Fig. 1C). This observation suggested that either infectious agents, such as viruses and mycoplasmas, or cellular factors, such as autocrine regulators, may be responsible for the appearance of p37 in L929 cell cultures. We wished to study this phenomenon in detail, using Western blotting to screen for the appearance of p37 in infected/induced cell cultures.

p37 could be similarly induced in all cell lines subsequently tested. The appearance of p37 in 3T3 cells is shown in Fig. 1E. Rat-1 cells already carried p37 (Fig. 1F).

The presence of mycoplasma infection in cell cultures was determined using the DAPI DNA-staining technique. Mycoplasmas showed up clearly in the fluorescence microscope. Most of the cell lines we studied were positive for mycoplasmas, irrespective of their invasive behaviour or the presence of p37. Although previous treatments with anti-PPLO agent had had no effect on p37 levels and mycoplasma contamination, addition of BM-cycline (Boehringer-Mannheim) to cells resulted in the disappearance of mycoplasmas, as monitored by DAPI staining. Moreover, Western blotting showed that p37 disappeared from cultures treated with BM-cycline (Schmidhauser, 1987).

![Fig. 1. SDS–polyacrylamide gel and Western blot analysis of total protein preparations from various mammalian cell lines and Mycoplasma hyorhinis. The p37-specific antibody DD9 was used for Western blotting. A. L929 mouse fibroblasts. B. FS9 mouse fibrosarcoma cells. C. L929 cells grown in FS9 cell culture supernatant. D. Mouse 3T3 cells. E. 3T3 cells grown in FS9 cell culture supernatant. F. Rat-1 cells. G. M. hyorhinis GDL, gel stained with Coomassie Blue. H. M. hyorhinis GDL, Western blot.](image)

Removal and addition of p37 to cells modifies their invasiveness in vitro

Since p37 could be added to other cell lines as well as eliminated from FS9 cells, we wished to determine whether the appearance of the protein could influence the in vitro invasiveness of these cells as measured by Abercrombie’s confronted explant assay.

An invasion index could be calculated for every con-
The invasion index is defined as the distance of the cell movement between the explants (i.e. the explants where no interpopulation contact occurs) divided by the distance the cells move at the sides of the explants where no interpopulation contact occurs (Abercrombie and Heaysman, 1976; Abercrombie, 1979; Stephenson, 1982).

FS9 cells invade CHF but not vice versa (non-reciprocal invasion) and the invasion indices calculated were consistent with those described by Steinemann et al. (1984a,b).

The FS9 invasion index (0.7725, Fig. 2), estimated from 24 confronted explants, demonstrated the high invasive potential of these mouse sarcoma cells. On the other hand, the chicken heart fibroblasts gave an index of only 0.2075 (Fig. 2). The effect of Fab derived from the monoclonal antibody DD9 directed against p37 was apparent in the confronted explant system at concentrations as low as 0.1 mg ml⁻¹. The invasion of CHF by FS9 cells in the area between the explants was strongly inhibited (invasion index: 0.3600, Fig. 2). When Fab derived from control IgG or from anti-L929 plasma membrane rabbit antiserum preabsorbed with FS9 cells was used, no effect on FS9 cell invasiveness was observed (Steinemann et al. 1984a,b). The non-invasive control cell line L929 had an invasion index of 0.2450 (Fig. 2), estimated from 15 explants. This was similar to the invasion index estimated for non-transformed chicken heart fibroblasts. There was no significant change in invasive behaviour following the addition of 0.1 mg ml⁻¹ DD9 Fab to L929 cells in the confronted explant system (invasion index: 0.2328, Fig. 2).

Treatment of FS9 cells with BM-cycline resulted in the loss of p37. FS9 cells treated in this way had an invasion index of 0.6020 (Fig. 1). This indicates that these cells are still much more invasive than L929 cells but significantly less invasive than untreated FS9 cells. The addition of specific Fab (0.1 mg ml⁻¹) directed against p37 to p37-free FS9 cells reduced the invasion index significantly (to 0.3906, Fig. 2). We also examined the effect of non-specific Fab on the invasive behaviour of p37-free FS9 cells. No significant modifications of the invasion index occurred (Fig. 2).

BM-cycline-treated L929 cells (mycoplasma-free) had an invasion index of 0.3039 (Fig. 2) estimated from 18 explants, i.e. elimination of mycoplasmas did not significantly alter the low invasion index of these cells. L929 cell explants, which had been grown in FS9 culture supernatant, demonstrated significantly increased indices (0.4333, 21 assays, Fig. 2). Addition of p37-specific Fab (DD9, 0.1 mg ml⁻¹) significantly reduced the indices of 10 explants, the averaged index of 0.2741 being equivalent to the background level (Fig. 2). Non-specific Fab had no effect on the invasive behaviour of M. hyorhinis-infected L929 cells (Fig. 2). The increase in the invasion index of the latter cells was rather small compared to the increase obtained with M. hyorhinis-infected 3T3 cells (see below). However, DAPI staining indicated a lower level of mycoplasmas associated with the L929 cells (not shown).

Normal NIH/3T3 cells had an invasion index of 0.5090 (Fig. 3), calculated from 20 confronted explants. They exhibited some non-reciprocal invasion but were clearly less invasive than FS9 cells although more invasive than L929 cells. 3T3 cells grown in medium conditioned with sterile filtered FS9 culture supernatant expressed p37 (Fig. 1E). When confronted with CHF these p37-positive 3T3 cells demonstrated a significantly higher invasion index (0.7788, Fig. 3) than the normal 3T3 cells. The relatively large standard deviation indicated that the invasive behaviour of both normal 3T3 cells and p37-positive 3T3 cells varied considerably. When DD9 Fab (0.1 mg ml⁻¹) was added to the confronted explant assay, the invasion index of the p37-positive 3T3 cells was greatly reduced, namely to 0.3360 (Fig. 3). Five assays were carried out. Unexpectedly, when Fab was added to normal 3T3 cells (i.e. lacking p37), their invasion index was reduced significantly to 0.1767 (Fig. 3). This was even lower than the invasion index of CHF. Nevertheless, the data were derived from three tests only. Addition of non-specific Fabs did not significantly change invasivity.

NIH/3T3 cells are reportedly non-tumorigenic, so the relatively high invasion index of M. hyorhinis-free cells was unexpected. However, metastatic potential/invasiveness and tumorigenicity/transformed state may be distinct properties of cells. Furthermore, Greig et al. (1985) have shown there are subpopulations of NIH/3T3

![Fig. 2. The effects of M. hyorhinis and p37-specific Fab fragments on the invasion indices of FS9 and L929 cells. Chicken heart fibroblasts (CHF) were used as the control explant. The number of confronted explants measured is indicated. a. CHF, using FS9 cells as the second explant (24). b. FS9 cells (24). c. FS9 cells+Fab (18). d. Mycoplasma-free FS9 cells (14). e. Mycoplasma-free FS9 cells+Fab (16). f. Mycoplasma-free FS9 cells+non-specific Fab (13). g. L929 cells (15). h. L929 cells+Fab (11). i. Mycoplasma-free L929 cells (18). j. Initially mycoplasma-free L929 cells infected with M. hyorhinis (21). k. Initially mycoplasma-free L929 cells infected with M. hyorhinis+Fab (10). l. Initially mycoplasma-free L929 cells infected with M. hyorhinis+non-specific Fab (13). The bars indicate standard deviations. Statistical analysis of the invasion indices using a t-test for two independent means was carried out to determine the significance of the differences between treatments. The results of the analysis were: for differences between b and c, t=8.43, t0.05=2.02; d and e, t=5.48, t0.05=2.05; d and f, t=0.72, t0.05=2.06 (not significant); b and d, t=3.59, t0.05=2.02, g and h, t=1.99, t0.05=2.06 (not significant); k and j, t=3.14, t0.05=2.04; k and i, t=1.35, t0.05=2.06 (not significant); l and j, t=0.32, t0.05=2.05 (not significant); i and j, t=3.06, t0.05=2.03.](image-url)
M. hyorhinis cells infected with M. hyorhinis + non-specific Fab. The invasion index (to 0.2800, Fig. 3) did not differ much from that of the original Rat-1 cells (Fig. 4). Rat-1 cells infected with M. hyorhinis + Fab (4). Rat-1 cells infected with M. hyorhinis + Fab (4). Rat cells were infected with M. hyorhinis. The invasion index was comparable to that of 3T3 cells. As expected, Rat-1 fibroblasts grown for 10 days in the inducing medium (normal medium conditioned with sterile filtered FS9 culture supernatant) showed no significant increase in invasion index. The invasion index of these Rat-1 cells (0.5070, Fig. 3) did not differ much from that of the original Rat-1 cells (0.4435, Fig. 3). Once again, a dramatic and significant reduction in the invasion index (to 0.2800, Fig. 3) resulted from including 0.1 mg ml⁻¹ anti-p37 Fab in the assay, whereas non-specific Fabs had no effect. The chicken heart fibroblasts had low invasion indices in all experiments (Fig. 2) and the duration of the confronted explant assays would not have been sufficient for them to become infected with M. hyorhinis. The invasion indices shown in Figs 2 and 3 were statistically analysed using a t-test for two independent means. Fab-treated cells were compared with untreated cells and M. hyorhinis-infected cells were compared with non-infected cells.

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Time-lapse film analysis of cell collisions shows that p37 influences contact inhibition of locomotion

Collisions of single FS9 cells and single chicken heart fibroblast cells were filmed. For each collision, the path of a series of points representing the sequential positions of the calculated centre of the nucleus was obtained. Points represented 1-min intervals (see Paddock and Dunn, 1986, for details). The positional data were input to a PDP 11/44 computer using a digitizing tablet (Summographics bitpad). Changes in motion were analysed by a program developed by Drs A. Brown and G. Dunn (Paddock and Dunn, 1986). The contact acceleration index was calculated for a sample of collisions and the effects of treatments (±Fab, ±M. hyorhinis) were determined. The contact acceleration index was defined in detail by Paddock and Dunn (1986) and may be described as the mean longitudinal component of acceleration due to collision. The presence of contact inhibition is indicated by a significantly negative value, since a colliding cell shows an excess of longitudinal deceleration over a free cell. A selection for velocity larger than 0.5 μm min⁻¹ over the entire 20-min interval was made. The more negative the contact acceleration index, the greater the inhibition of locomotion.

The data on changes in motion were summarized diagrammatically in an accelogram (Fig. 4) (Paddock and Dunn, 1986). Each accelogram was constructed from a sample of accelerations, all obtained using the same time interval of 10 min after the initial contact. The samples were gathered together so that they had a common origin at the centre of a circle of radius 1. The accelograms show the changes in motion in relation to the initial direction of motion. Each acceleration was oriented so that its corresponding vector (displacement prior to contact during 10 min) would have pointed horizontally to the right had it been included in the figure. Hence, the change in motion in relation to the initial direction of motion is shown in the accelograms. For example, the cell with the vector A in the accelogram shown in Fig. 4A has changed direction, following collision, by 45° to the left and has increased its speed by a factor of 1.5. The accelograms clearly show the dramatic effect of the p37-specific antibody on the contact inhibition of locomotion of FS9 cells (Fig. 4).

p37-positive FS9 cells had a contact acceleration index of -0.26110 (Table 1). They represent the least contact-inhibited FS9 cells. When Fab (0.1 mg ml⁻¹) directed against p37 was present these cells showed a strongly altered contact behaviour, contact inhibition being significantly different from zero); and FS9mf and FS9mf+Fab, t=2.35, 0.05=2.08; for FS9 and FS9mf, t=1.05 (which is not significantly different from zero); and FS9mf and FS9mf+Fab, t=0.73 (also not significant at the 90% confidence level).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Number of cells analysed (n)</th>
<th>Contact acceleration index</th>
</tr>
</thead>
<tbody>
<tr>
<td>FS9</td>
<td>10</td>
<td>-0.26110±0.010890</td>
</tr>
<tr>
<td>FS9+Fab</td>
<td>12</td>
<td>-0.77292±0.06106</td>
</tr>
<tr>
<td>FS9mf</td>
<td>20</td>
<td>-0.37286±0.05987</td>
</tr>
<tr>
<td>FS9mf+Fab</td>
<td>11</td>
<td>-0.45321±0.15268</td>
</tr>
</tbody>
</table>

A selection for velocities larger than 0.5 μm min⁻¹ has been made. Standard deviations are indicated. Statistical analysis was carried out as described in Fig. 2. For differences between FS9 and FS9+Fab, t=4.27, 0.05=2.09; for FS9+Fab and FS9mf+Fab, t=2.35, 0.05=2.08; for FS9 and FS9mf, t=1.05 (which is not significantly different from zero); and FS9mf and FS9mf+Fab, t=0.73 (also not significant at the 90% confidence level).
Fig. 4. Accelograms showing the effects of p37-specific Fab fragments on the contact inhibition of locomotion of FS9 cells during collisions with chicken heart fibroblasts. Time-lapse film-analysis was used and analysis time extended from 10 min prior to the first contact between the FS9 cell and a chicken heart fibroblast until 10 min after contact. Each acceleration is represented by a straight line with its origin in the centre of the diagram. A. FS9 cells infected with *M. hyorhinis*. B. FS9 cells infected with *M. hyorhinis* + Fab. C. Mycoplasma-free FS9 cells. D. Mycoplasma-free FS9 cells + Fab.
greatly increased. The difference between the two treatments was highly significant.

The contact acceleration index was significantly lower (i.e. less negative) in mycoplasma-free FS9 cells treated with Fab compared to Fab-treated FS9 cells. This result was unexpected as both treatments resulted in a similar invasion index (Fig. 2). The other results were not significant at the 90% level, although averaged contact acceleration indices suggested tendencies consistent with the invasion index data. Thus, mycoplasma-free FS9 cells appeared to be more contact inhibited than FS9 cells carrying *M. hyorhinis* and Fab further increased their level of contact inhibition. Differences between treatments are apparent in Fig. 4, where the behaviour of individual cells is shown. (The size of the samples may need to be increased some tenfold to obtain statistically significant differences.)

We have not yet made a detailed analysis of the effects of the treatments on the chicken heart fibroblasts. Preliminary results indicated that, following their collisions with FS9 cells, the contact inhibition was somewhat lower (accompanied by a higher standard deviation) than reported by Paddock and Dunn (1986). However, we have now found that collisions between the leading lamellae of FS9 cells and the sides of chicken heart fibroblasts do not significantly affect the locomotion of the latter cells. 'Head-to-head' collisions between the two cell types (or collisions between the leading lamellae of fibroblasts and the sides of FS9 cells) do result in strong contact inhibition of the chicken heart fibroblasts. The presence of *M. hyorhinis* on the FS9 cells did not change the behaviour of the chicken heart fibroblasts.

We have not analysed the effects of the various treatments on the homotypic contact inhibition of FS9 cells.

**Discussion**

The results show that the invasive behaviour (invasive index) of cells in the confronted explant assay is modified by the presence of *M. hyorhinis*. The *in vitro* invasivity of cells was increased following infection with *M. hyorhinis* and decreased when the mycoplasma had been removed by BM-cycline treatment. The p37 protein on the surface of *M. hyorhinis* was apparently directly or indirectly involved in the invasion index data. Thus, mycoplasma-free FS9 cells appeared to be more contact inhibited than FS9 cells carrying *M. hyorhinis* and Fab further increased their level of contact inhibition. Differences between treatments are apparent in Fig. 4, where the behaviour of individual cells is shown. (The size of the samples may need to be increased some tenfold to obtain statistically significant differences.)

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We have not analysed the effects of the various treatments on the homotypic contact inhibition of FS9 cells.

The reduction in invasivity (invasion index) following the removal of *M. hyorhinis* from cells was less than that resulting from the presence of the p37-specific monoclonal antibody. This suggested that, while p37 increased the intrinsic invasivity of the cells, the intrinsic invasivity itself could be inhibited by the antibody. This inhibition implies that the antibody recognizes an epitope or epitopes on the surface of mycoplasma-free cells and that these epitopes are directly or indirectly involved in invasive behaviour. The antibody reduced the invasiveness of mycoplasma-free FS9 and 3T3 cells to levels comparable to the very weakly invasive L929 cells. The antibody had no effect on the *in vitro* invasiveness of L929 cells.

The influence of the antibody was specific, antibodies that did not bind to p37 had no effect on invasive behaviour (see also Steinemann et al. 1984a,b).

The effect of the specific antibody on the behaviour of BM-cycline-treated cells does not appear to be due to residual *M. hyorhinis* infection. No p37 could be detected using immunoblotting and cell cultures have remained mycoplasma-free. The protein A assay was used to titrate the DD9-specific antigens on the surface of mycoplasma-free FS9 cells. Maximum binding was 15–20% of binding to mycoplasma-infected cells and the titration curves showed prozone effects (results not shown). Fixation in formaldehyde destroyed the binding of DD9 antibody by cells and no antigen was detectable using immunoblotting. Hence, the identity of the putative cellular antigen is still unknown. The antibody does not affect cell viability and movement is not slowed. The most likely interpretation is that the antibody recognizes an epitope(s) on the cell surface, and we are now using an immunoselection procedure as a means of isolating the relevant gene (Seed and Aruffo, 1987).

Knowledge of the function of p37 in *M. hyorhinis* might provide information about the mechanism by which it influences the behaviour of cells *in vitro*. Sequence data suggest that p37 is part of a ternary protein complex involved in transport across the membrane (Dudler et al. 1988). The complex resembles the periplasmic transport systems (permeases) of gram-negative bacteria. p37 would be equivalent to the bacterial periplasmic protein that tightly binds the solute being transported and can associate with the membrane-bound complex of several permeases. The mammalian P-glycoprotein, coded by the multidrug resistance (*mdr*) gene, is structurally very similar to the bacterial permeases. Elevated expression of the *mdr* gene is correlated with resistance of tumour cells to certain drugs, the P-glycoprotein acting as an energy-dependent export pump. The normal role of P-glycoprotein is unknown. p37 might influence transport in cancer cells by binding to the P-glycoprotein or similar proteins. Subtle changes in the transport of certain substrates may interfere with the signal transduction that normally follows heterotypic cell collisions.

There is no evidence that the alteration of cellular invasive behaviour *in vitro* is a general property of mycoplasmas. *M. arginini* and *M. orale* failed to influence the invasivity of cells and carried no epitope recognised...
by the monoclonal antibody DD9. p37 DNA does not hybridize with DNA from these two mycoplasmas (Dudler et al. 1988). Nevertheless, polyclonal antibodies directed against an N-terminal 16 amino acid peptide of p37 do bind to antigens in M. orale and M. arginini. Hence, these mycoplasmas may have proteins resembling in function the p37 of M. hyorhinis. It is intriguing to speculate that certain mycoplasmas might influence metastasis in immunologically weakened individuals. We have not yet checked human mycoplasmas for cross-reactivity with the p37-specific antibody.

Although our results implicate p37 in the invasive behaviour of cells, we have as yet no direct proof. The protein may, for example, function only in combination with the mycoplasmal transport complex or subtly alter the interaction of the mycoplasma with the cell surface. Clearly, it would be useful to study the effects of purified p37; however, purification is difficult and we have only obtained a few micrograms using immuno-affinity chromatography (Dudler et al. 1988). At present we are mutagenising the p37 gene so it can be expressed in bacteria and adequate amounts of the protein can be obtained.

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