Rat hepatic stellate cell expression of α2-macroglobulin is a feature of cellular activation: implications for matrix remodelling in hepatic fibrosis

C. A. KAWSER, J. P. IREDALE, P. J. WINWOOD and M. J. P. ARTHUR
University Medicine, University of Southampton, Southampton SO16 6YD, Hampshire, U.K.

ABSTRACT

1. Hepatic stellate cells are key mediators of hepatic fibrosis. We have studied hepatic stellate cell expression of the collagenase and general protease inhibitor α2-macroglobulin after activation in tissue culture and in response to certain cytokines.

2. Hepatic stellate cells isolated by Pronase–collagenase digestion were activated by culture on uncoated plastic. By Northern analysis hepatic stellate cells undergoing activation (5 days) expressed α2-macroglobulin mRNA and α2-macroglobulin could be immunolocalized to hepatic stellate cells from 5 to 15 days of culture.

3. By ELISA of cell culture supernatants hepatic stellate cell secretion of α2-macroglobulin was found to increase from 2.78 ± 1.13 ng ml⁻¹ µg⁻¹ DNA per 24 h at 5 days of culture (n = 8) to 13.55 ± 4.64 ng ml⁻¹ µg⁻¹ DNA per 24 h at 15 days of culture (n = 7). Stimulation of hepatic stellate cells with interleukin-6 at 5 days caused a significant increase in α2-macroglobulin expression as did exposure to Kupffer-cell conditioned medium. However, exposure of hepatic stellate cells to interleukin-1, transforming growth factor-β1 and tumour necrosis factor-α had no significant effect.

4. During profibrotic liver injury plasma α2-macroglobulin levels were found to increase to between 850% and 250% of the control value (100%) after bile duct ligation (72 h to 13 days respectively), and to 1166% and 1106% of the control value during progressive CCl₄-induced fibrosis (24 h to 4 weeks respectively).

5. These data suggest that hepatic stellate cells are a potential source of the potent protease inhibitor α2-macroglobulin, expression of which may inhibit matrix remodelling during progressive fibrosis.

INTRODUCTION

Liver fibrosis results from a relative imbalance between the synthesis and degradation of matrix proteins. In progressive liver fibrosis there is an increase in the absolute amount of matrix and its composition changes such that fibrillar collagens (types I and III) are predominant [1]. Hepatic stellate cells (HSCs, lipocytes, fat-storing or Ito cells) are now known to be central to this process as both a source of matrix proteins and matrix-

Key words: fibrosis, hepatic stellate cell, α2-macroglobulin, matrix.
Abbreviations: HSC, hepatic stellate cells; IL, interleukin; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; TGF, transforming growth factor; TNF, tumour necrosis factor.
Correspondence: Dr J. P. Iredale.
degrading metalloproteinases [2–8]. During liver injury HSCs undergo a phenotypic transformation from a retinoid and lipid-filled perisinusoidal cell to a myofibroblast-like cell, a process termed activation. Activated HSCs proliferate and secrete collagens and other matrix proteins and are the major source of the fibrillar matrix that characterizes fibrosis [1].

Studies of HSCs have been facilitated by the observation that during primary culture on uncoated tissue culture plastic, many of the features of cellular activation observed in vivo during fibrotic injury (e.g. α-smooth muscle actin expression, collagen I and III expression and cellular proliferation) are reproduced. It has now been established that during activation to a myofibroblastic phenotype HSCs may express gelatinase A and stromelysin [7–10], both enzymes with degradative activity against components of the normal matrix. In addition, HSCs may express interstitial collagenase, a metalloproteinase with specific activity against the fibrillar collagens [2–4,11,12]. Interstitial collagenase would be expected to mediate matrix remodelling even in advanced fibrosis. In studies undertaken to measure interstitial collagenase activity during progressive liver fibrosis in vivo, including CCl4-induced and alcoholic liver injury, collagenase activity decreases as fibrosis progresses [13–15]. Studies on murine schistosomiasis have indicated that although collagenase activity decreases, the absolute level of secreted collagenase, determined by ELISA, does not show a corresponding decrease [16–18]. Furthermore, in these studies there is evidence that the protease scavenger, α2-macroglobulin, mediated this diminution in collagenase activity [19]. α2-Macroglobulin expression by HSCs has been reported [20]. This suggests that HSCs may regulate the activity of matrix-degrading metalloproteinases through α2-macroglobulin expression in addition to the specific tissue inhibitors of metalloproteinases (TIMPs)-1 and -2 [11,12,21,22].

α2-Macroglobulin is a major plasma globulin and functions as a plasma inhibitor of the coagulation and fibrinolytic systems [23]. It has a molecular mass of 725 kDa and consists of four polypeptide subunits of 185 kDa, each held together by disulphide bonds [24]. It binds to and inactivates proteases from the four major classes including the metalloproteinase class (e.g. stromelysin and interstitial collagenase) [25]. Indeed, α2-macroglobulin has been directly demonstrated to react rapidly with both collagenase and a further matrix metalloproteinase (MMP), stromelysin, and result in their inhibition [26,27]. In so doing, α2-macroglobulin would be expected to diminish matrix turnover. However, because α2-macroglobulin has activity against the serine protease plasmin, which has been implicated in profibrotic metalloproteinase activation, its presence will also inhibit the activation cascade of stromelysin and interstitial collagenase. α2-Macroglobulin is secreted by hepatocytes as a feature of the acute phase response, and is regulated at the level of the gene via the glucocorticoid response element and the interleukin (IL)-6 response element [28–30].

Because of the key position of HSCs in matrix turnover in fibrogenesis we have studied expression of α2-macroglobulin during HSC activation and have characterized HSC α2-macroglobulin secretion in response to certain cytokines. Our results suggest that α2-macroglobulin expression is a feature of HSC activation. Moreover, we have demonstrated that secretion of α2-macroglobulin at all stages of HSC activation may be further augmented by the influence of cytokines and particularly IL-6.

**METHODS**

**Isolation in culture of rat HSCs**

HSCs were isolated from normal rat liver by sequential in situ perfusion with collagenase and Pronase as described previously [6]. Buoyant HSCs separated from the resulting cell suspension over a discontinuous two-layer Nicodenz (Nigard, Birmingham, U.K.) gradient prepared as described by Hendriks et al. [31]. Purified HSC and Kupffer cell fractions were centrifuged and resuspended in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Paisley, Scotland, U.K.) containing 20% fetal calf serum, penicillin (10 m-units/ml), streptomycin (10 µg/ml) and gentamycin (32 µg/ml). Cell viability and total yield were determined with a haemocytometer using Trypan Blue dye exclusion (final concentration 0.05%). Cells were plated at a density of 2 × 10⁶ cells per well on uncoated 35-mm plastic wells in 1.5 ml of media. Culture media were changed every 48 h. HSCs were identified as more than 95% pure by their characteristic lipid droplets on phase-contrast microscopy and by endogenous vitamin A fluorescence.

**Collection of cell conditioned media**

Before collection of cell conditioned media the tissue culture plates were washed 3-fold with serum-free DMEM, followed by a 3 h preincubation step in serum-free media. After this period the media were discarded and the cells were incubated with 1.5 ml of fresh serum-free media (containing antibiotics and 0.1% BSA) with or without cytokines or the regulatory factors used in the experiments. After 24 h the media were collected, clarified by centrifugation and stored at −20 °C until assayed.

**Collection of cell extracts for DNA assay**

After collection of the media, corresponding tissue culture plates were washed once with PBS and then incubated in 1ml of PBS. Cells were sonicated and the lysed cellular extract collected and stored at −20 °C until assayed for DNA.
DNA assay
The total DNA content of cell sonicates was assayed by the spectrofluorometric method of LaBarca and Paigen [32]. Total DNA content was used to normalize α2-macroglobulin secretion which was expressed per microgram of cellular DNA.

Temporal secretion and regulation by cytokines
α2-Macroglobulin was assayed on days 2 (not activated), 5 (undergoing activation), 9–11 (activated) and 15 (highly activated) after HSC isolation and culture on plastic. Cells from each isolation were used for two or more time points. At specific time points (see results below), cytokines were added to the cell culture media in serum-free conditions for a 24 h incubation period. The following recombinant human cytokines were used: IL-6, 1000 units/ml (Genzyme Fine Chemicals Ltd, Suffolk, U.K.); IL-1, 300 units/ml; transforming growth factor (TGF)β1, 10 ng/ml; tumour necrosis factor (TNF)α, 300 units/ml (Boehringer, Lewes, Sussex, U.K.). These cytokine preparations were demonstrated to be effective in our group ([33] and C. A. Kawser and P. W. Kowalski-Saunders, unpublished work).

Assay of α2-macroglobulin
α2-Macroglobulin was measured by indirect ELISA using a rabbit anti-rat α2-macroglobulin antibody (the kind gift of Professor J. Gauldie, Ontario, Canada). Swine anti-rabbit IgG peroxidase conjugate was used as a secondary antibody with o-phenylene diamine (Sigma) as a detection substrate. The intra- and inter-assay coefficients of variation for the ELISA were 2.8% and 15% respectively.

Detection of mRNA for α2-macroglobulin
RNA was extracted from HSCs after 5 days in primary culture on plastic by the method of Chomczynski and Saachi [34]. Five micrograms of total HSC RNA was fractionated on a 1% agarose RNA denaturing gel and transferred to a nylon membrane (Hybond N, Amersham International, U.K.) by vacuum transfer apparatus. Membranes were probed with a random primed 3.4 kb HindIII derived fragment from the rat α2-macroglobulin cDNA (the kind gift of George H. Fey, Scripps Clinic and Research Foundation, La Jolla, CA, U.S.A.). The cDNA was labelled using the Amersham megaprime random priming kit (Amersham, U.K.) using [32P]ATP. Hybridization was undertaken overnight at 42 °C. Membranes were washed in 0.2% SDS and 0.2 x SSC at 42 °C then exposed to Kodak X-mat films for 48–72 h.

Immunolocalization of α2-macroglobulin
HSCs in primary culture on plastic at 5 days of culture were utilized for immunolocalization studies. Cells were preincubated in 5 μmol of monensin for 3 h in order to acidify the Golgi body and promote intracellular protein accumulation. Cells were then washed three times in PBS and fixed in 4% paraformaldehyde. After this cells were permeabilized with 0.1% Triton followed by a methanol wash to dry the plates. The plate was then rehydrated in PBS and α2-macroglobulin antibody or non-immune IgG (control) at a 1 to 50 dilution in PBS/5% BSA was added to the plate. After this the plate was incubated with a fluorescein isothiocyanate-labelled secondary antibody. Each step of the protocol was interspersed with three washes of PBS, each lasting 5 min. All antibody incubations were for 30 min. After the final wash cover slips were mounted on Citifluor (City University, London, U.K.). Photographs were taken using Kodak EES film with a 400 × magnification by Leitz fluorescent microscope.

Collection of Kupffer-cell conditioned media
Kupffer cells were isolated as described previously [35] and plated at equal density in 75-cm² culture flasks. Cells were washed after 48 h with replacement of medium supplemented with 10% serum and antibiotics. Subsequently these media were collected after 24 h exposure, for 3 consecutive days, clarified by centrifugation and kept at −20 °C until used. Experiments involving HSC exposure to Kupffer-cell conditioned medium were undertaken on day 5 of culture. Forty-eight hours after plating, HSCs were washed and then exposed to filter-sterilized Kupffer-cell conditioned medium supplemented with 10% fetal calf serum and the media was changed each day until day 5 of culture. On day 5 cells were washed to remove the serum and the serum-free medium was added as described previously. Media and sonicated cell extracts were collected after 24 h for all conditions.

Rat models of fibrotic liver injury
Fibrotic liver injury was induced in cohorts of rats by intraperitoneal injection of CCl₄ (with appropriate olive oil controls) and bile duct ligation (with appropriate sham-operated controls), as described previously [12]. In the CCl₄ model of fibrosis, livers from two treated and one control animal were harvested for histological analysis at 6, 24 and 72 h after initial injection (acute injury), and then at 1 week (early fibrosis) and 4 weeks (established fibrosis) of treatment. Harvest of the 1- and 4-week samples took place 3 days after the last injection. At the time of liver harvest, 2 ml of heparinized blood was withdrawn from the right ventricle and a plasma sample stored at −20 °C pending assay by ELISA. In the bile-duct ligation model of liver injury, harvest of three treated and one control liver with accompanying plasma samples was undertaken at 16, 24 and 72 h (acute injury), and at 7 and 13 days (early biliary fibrosis) after bile duct ligation.
RESULTS

Northern analysis for α2-macroglobulin mRNA

Five-day-old rat HSCs in primary culture were found to contain mRNA for α2-macroglobulin by Northern analysis, as indicated by a positive signal at 5 kb (Figure 1).

Immunostaining for α2-macroglobulin

α2-Macroglobulin could be immunolocalized to HSCs after 5, 11 and 15 days in primary culture on plastic (Figure 2). Positive staining of these cells was observed in a perinuclear distribution consistent with accumulation of the immunolocalized protein in the Golgi apparatus. Control non-immune IgG staining was uniformly negative (Figure 2).

α2-Macroglobulin secretion by HSCs: effect of duration of culture

α2-Macroglobulin secretion by HSCs was confirmed by ELISA and was found to increase with duration of culture and morphological evidence of cellular activation which we and others have demonstrated is associated with expression of collagen I and α-smooth muscle actin. On day 2, α2-macroglobulin was not detected in serum-free conditioned media, by day 5 it was detectable at 2.78 ± 1.13 ng·ml⁻¹·µg⁻¹ DNA per 24 h (n = 8), by the second week of culture (9–11 days) it increased to 16.23 ± 4.33 ng·ml⁻¹·µg⁻¹ DNA per 24 h (n = 7) (P = 0.01 by Mann–Whitney compared with day 5) and remained raised at 15 days of culture [13.55 ± 4.64 ng·ml⁻¹·µg⁻¹ DNA per 24 h (n = 9) (P = 0.01 by Mann–Whitney compared with day 5)].

Table 1 Secretion of α2-macroglobulin by rat HSCs cultured on plastic: effect of IL-6 at different time points in culture

<table>
<thead>
<tr>
<th>Days</th>
<th>Plastic (control)</th>
<th>IL-6 treated</th>
<th>% of control</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>2.40 (0.86)</td>
<td>3.72 (1.27)</td>
<td>155</td>
<td>P &lt; 0.05*</td>
</tr>
<tr>
<td>9–11</td>
<td>15.27 (4.09)</td>
<td>19.82 (3.57)</td>
<td>130</td>
<td>P &lt; 0.05*</td>
</tr>
<tr>
<td>15</td>
<td>8.09 (1.73)</td>
<td>17.98 (2.83)</td>
<td>222</td>
<td>P &lt; 0.05*</td>
</tr>
</tbody>
</table>

Effect of cytokines on secretion of α2-macroglobulin by rat HSCs

Stimulation of rat HSCs with IL-6 was found to significantly enhance α2-macroglobulin secretion at each stage of activation in paired cultures (Table 1). In a...
The data reported here confirm that rat HSCs are a potential source of α2-macroglobulin, a potent protease inhibitor. We have demonstrated that HSCs contain mRNA for α2-macroglobulin and that this mRNA is increased in response to stimulation by IL-6. α2-Macroglobulin has been immunolocalized to the cytoplasm of cultured HSCs. In addition, secretion of immunoreactive α2-macroglobulin has been demonstrated during HSC activation by culture on plastic and is increased in response to IL-6. Moreover, two mechanistically different models of hepatic fibrosis have been demonstrated to be associated with increases in plasma α2-macroglobulin levels. This provides further evidence for the general role of HSCs in the process of matrix turnover, not only as a source of matrix-degrading metalloproteinases, but also their inhibitors. We have previously described release of TIMP-1 and TIMP-2 by activated HSCs and suggested that these proteins may inhibit matrix degradation [11,12,21,22]. We now report that HSC-derived α2-macroglobulin may also play a role in the inhibition of matrix degradation during fibrogenesis.

HSCs are the key effector cells in the process of fibrosis, becoming activated during liver injury (a process mimicked by culture on plastic) to express procollagen-I and -III and contractile filaments, at which stage they become phenotypically indistinguishable from myofibroblasts and are quantitatively the major source of the fibrotic matrix which characterizes fibrosis [1]. Although HSC activation by culture on plastic does not wholly reflect the cellular and molecular events which occur during this process in vivo, this model is both widely accepted and used to mimic this process in cell culture. It is therefore significant that expression of α2-macroglobulin increased as HSCs became activated by culture on plastic, precisely because this suggests that this protease scavenger may promote fibrosis by inhibiting matrix degradation when these same cells are activated during liver injury. Furthermore, the demonstration that HSC expression of α2-macroglobulin increases in response to IL-6 (the major acute phase cytokine) [36–39] suggests that during profibrotic injury, in which there is an acute phase component with IL-6 release, secretion of α2-macroglobulin may be further enhanced with a consequent diminution in matrix remodelling. These findings are consistent with previous work in whole murine liver, which has demonstrated a decrease in

**Table 2** Secretion of α2-macroglobulin by primary cultures of rat HSCs after 5 days on plastic: effects of different cytokines

Values are means (S.E.). *By Mann–Whitney test of cytokine-treated cultures relative to paired control cultures at each time point (n = 6 at each time point, NS = not significant).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Plastic (control)</th>
<th>Cytokine treated</th>
<th>% of control</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>2.5 (0.86)</td>
<td>4 (1.29)</td>
<td>160</td>
<td><em>P &lt; 0.05</em></td>
</tr>
<tr>
<td>IL-1</td>
<td>2.2 (0.91)</td>
<td>2.9 (1.42)</td>
<td>130</td>
<td>NS</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>2.5 (0.86)</td>
<td>3.6 (1.3)</td>
<td>140</td>
<td>NS</td>
</tr>
<tr>
<td>TNFα</td>
<td>2.2 (0.91)</td>
<td>2.1 (1)</td>
<td>95</td>
<td>NS</td>
</tr>
</tbody>
</table>

By Mann–Whitney test relative to control, n = 6. α2-Macroglobulin secretion was enhanced 2-fold in response to Kupffer-cell conditioned medium: control, 2.54 ± 0.92 ng·ml⁻¹·µg⁻¹ DNA per 24 h versus treated, 4.4 ± 1.04 ng·ml⁻¹·µg⁻¹ DNA per 24 h (P < 0.05 by Mann–Whitney test relative to control, n = 5).

**Effects of Kupffer-cell conditioned medium on α2-macroglobulin secretion by rat HSCs**

Kupffer-cell conditioned medium was added to cultured rat HSCs on day 5 as described in the Methods. α2-Macroglobulin secretion was enhanced 2-fold in response to Kupffer-cell conditioned medium: control, 2.54 ± 0.92 ng·ml⁻¹·µg⁻¹ DNA per 24 h versus treated, 4.4 ± 1.04 ng·ml⁻¹·µg⁻¹ DNA per 24 h (P < 0.05 by Mann–Whitney test relative to control, n = 5).

**Secretion of α2-macroglobulin during fibrotic injury in vivo**

Experimental liver injury was confirmed histologically; at 24 h after bile duct ligation there was evidence of bile ductule proliferation which become more distinct at 72 h and at 7 and 13 days was accompanied by a mononuclear cell infiltrate and early fibrotic change. At 24 h after CCl₄ intoxication there was perivenular hepatocyte degeneration associated with a neutrophilic infiltrate; by 7 days there was evidence of early fibrotic change and this progressed to an established mature fibrosis with bridging collagen bands at 4 weeks.

α2-Macroglobulin levels were found to be increased above control levels in the plasma of all treated animals from the 24-h time point in both of the fibrotic liver injury models studied. Twenty-four hours after bile duct ligation, plasma α2-macroglobulin levels in treated animals (n = 3), expressed as a percentage of those observed in controls (n = 1, arbitrarily given the value 100%), were raised at 440%; at 72 h, 7 days and 13 days, α2-macroglobulin in treated animals was raised at 370% and 250% of control values respectively. During CCl₄-induced hepatic fibrosis, plasma α2-macroglobulin levels were also raised in treated animals (n = 2) relative to controls (n = 1, arbitrarily given the value 100%), being 1166%, 716%, 728% and 1106% of control values at 24 h, 72 h, 7 days and 4 weeks respectively.
collagenase activity during progressive experimental schistosomiasis despite little change in the total amount of secreted collagenase as determined by ELISA [16–19]. In these studies α2-macroglobulin was implicated as mediating collagenase inhibition [19].

We have gone on to demonstrate that α2-macroglobulin levels in plasma increase in both CCl₄ and bile duct ligation fibrotic liver injuries, implying a role for α2-macroglobulin in these models also. In both models the majority of the plasma α2-macroglobulin is most probably derived from hepatocytes, reflecting an acute phase response, although the prolonged release observed in the bile duct ligation model (up to 13 days after injury) may, in part, reflect secretion from a progressively increasing number of activated HSCs.

α2-Macroglobulin has also been demonstrated to bind and act as a carrier to or inactivate cytokines [40–44]. The observation that inhibition of TGFβ₁ activity may be mediated by α2-macroglobulin [45] suggests a potential alternative role for this protein. TGFβ₁ is powerfully profibrogenic and is expressed in liver fibrosis [46–49]. It is therefore possible that co-expression of α2-macroglobulin with TGFβ₁ may result in a down-regulation of fibrogenic activity. However, studies in other organ systems suggest that TGFβ₁ activity may be irreversibly inhibited, rather that the TGFβ₁/α2-macroglobulin complex may act synergistically [42–44]. A full dissection of the relative importance of these two mechanisms is beyond the scope of this study. However, the existing data on collagenase activity in hepatic fibrogenesis, and the temporal pattern of expression of α2-macroglobulin described here and in previous studies, is consistent with the hypothesis that matrix degradation is being inhibited.

The response of HSCs to IL-6 was quantitatively smaller than that witnessed for hepatocytes under similar conditions (results not shown), and, in contrast to hepatocytes, no response was seen to IL-1 and TNFα by HSCs. This suggests that IL-1 and TNFα do not regulate HSC α2-macroglobulin expression at a concentration at which they are active in hepatocytes, or that HSCs are expressing other regulatory factors which block or attenuate the response to these cytokines. This consideration apart, the absolute level of α2-macroglobulin secreted may be less important than local concentration in the pericellular milieu. We have recently demonstrated that HSCs express MT-MMP, a membrane-bound metalloproteinase which activates the progelatinase A [50]. We have also demonstrated that HSCs express components of the plasmin activating system and cell receptors which will focus enzyme activity to the pericellular area [51]. The implication is that focused activation of MMPs in the immediate extracellular milieu is important. Therefore local release of α2-macroglobulin by HSCs might be expected to have a significant effect on down-regulating local matrix degradation.

Kupffer cells are known to produce a number of cytokines during injury/activation [52]. TGFβ₁ and platelet-derived growth factor have been demonstrated to be important in HSC activation and secretory function during injury [1]. In addition, an as yet uncharacterized low-molecular-mass HSC stimulating factor (LSF) is known to affect HSC gene expression [53]. It is therefore of interest that Kupffer-cell conditioned medium also stimulates α2-macroglobulin expression. The absence of a response under identical conditions to recombinant TGFβ₁ suggests that Kupffer-cell-derived TGFβ₁ alone is not responsible for this effect. The magnitude of HSC response to Kupffer-cell conditioned medium was similar to that observed for IL-6 and may implicate this cytokine. These data highlight another route through which HSC α2-macroglobulin secretion may be modulated during fibrotic liver injury.

Our data indicate that rat HSCs express the gene for and secrete α2-macroglobulin; moreover such gene expression is a feature of HSC activation by primary culture on plastic. In addition, HSC α2-macroglobulin secretion increases in response to IL-6. These data suggest that HSC-derived α2-macroglobulin may be important in inhibiting matrix degradation during fibrosis characterized by HSC activation in vivo. Furthermore, this effect is likely to be enhanced in those profibrotic processes that are characterized by an acute phase response and Kupffer cell activation.

ACKNOWLEDGMENTS

At the time this work was undertaken C.A.K. was a WHO PhD student and P.J.W. was a Wellcome Trust Clinical Training Fellow. J.P.I. is an MRC Clinician Scientist and gratefully acknowledges the support of the British Liver Trust. This work was also supported by a grant from the Wellcome Trust (grant no 038712/Z/93/A).

REFERENCES

metalloproteinase that degrades basement membrane (type IV) collagen. J. Clin. Invest. 84, 1076–1085


Received: 12 January 1998/18 March 1998; accepted 26 March 1998