Beta-cell gene expression and functional characterisation of the human insulinoma cell line CM

M G Baroni[1], M G Cavallo[1], M Mark[2], L Monetini[1], B Stoehrer[2] and P Pozzilli[3,4]

[1]Institute II Clinica Medica, University La Sapienza of Rome, Rome, Italy

[2]Boehringer Ingelheim Pharma, Biberach, Germany

[3]Department of Diabetes and Metabolism, St Bartholomew's Hospital, London, UK

[4]University of Rome Tor Vergata and Campus Biomedico, Rome, Italy

(Requests for offprints should be addressed to P Pozzilli, Department of Diabetes and Metabolism, St Bartholomew's Hospital, London EC1 7BE, UK)

Abstract

Animal insulinoma cell lines are widely used to study physiological and pathophysiological mechanisms involved in glucose metabolism and to establish *in vitro* models for studies on β-cells. In contrast, human insulinoma cell lines are rarely used because of difficulties in obtaining and culturing them for long periods.

The aim of our study was to investigate, under different experimental conditions, the capacity of the human insulinoma cell line CM to retain β-cell function, particularly the expression of constitutive β-cell genes (insulin, the glucose transporters GLUT1 and GLUT2, glucokinase), intracellular and secreted insulin, β-cell granules, and cAMP content.

Results showed that CM cells from an early-passage express specific β-cell genes in response to glucose stimulation, in particular the insulin and GLUT genes. Such capacity is lost at later passages when cells are cultured at standard glucose concentrations. However, if cultured at lower glucose concentration (0·8 mM) for a longer time, CM cells re-acquire the capacity to respond to glucose stimulation, as shown by the increased expression of β-cell genes (insulin, GLUT2, glucokinase). Nonetheless, insulin secretion could not be restored under such experimental conditions despite the presence of intracellular insulin, although cAMP response to a potent activator of adenylate cyclase, forskolin, was present indicating a viable system.

In conclusion, these data show that the human insulinoma cell line CM, at both early-passage and late-passage, posseses a functional glucose-signalling pathway and insulin mRNA expression similar to normal β-cells, representing, therefore, a good model for studies concerning the signalling and expression of β-cells. Furthermore, we have previously shown that it is also a good model for immunological studies. In this respect it is important to note that the CM cell line is one of the very few existing human β-cell lines in long-term culture.

Journal of Endocrinology (1999) **161,** 59–68

Introduction

Animal insulinoma cell lines like RINm5F (Gylfe & Hellman 1980, Meglasson *et al.* 1986, Trautman & Wollheim 1987), Ins-1 (Frödin *et al.* 1995, Verspol *et al.* 1995), or HIT (Meglasson *et al.* 1986, Hill *et al.* 1987) are widely used to study both physiological and pathophysiological mechanisms involved in glucose metabolism and to establish *in vitro* models for the β-cell damage occurring in insulin-dependent diabetes (IDDM) (Atkinson & Maclaren 1994). In contrast, human insulinoma cell lines are rarely used because of difficulties in obtaining and culturing them for long periods (Adcock *et al.* 1975).

We have isolated and established a long-term culture of a human insulinoma cell line denominated CM that was originally obtained from peritoneal ascites of a patient affected by a primary pancreatic insulinoma (Gueli *et al.* 1987). This cell line grows spontaneously *in vitro*. So far, the CM line has been studied mainly for its antigenic properties with the purpose of establishing an *in vitro* model to investigate the immune mechanisms leading to β-cell destruction in IDDM (Cavallo *et al.* 1996).

Studies on early-passaged cells provided evidence that insulin and C-peptide were detectable in the supernatants of CM cell culture (Cavallo *et al.* 1992), and since then the CM cells have undergone several passages. The CM cell line is routinely cultured in standard medium containing high glucose concentration (11 mM). High glucose in the culture medium and the number of passages are factors known to affect insulin secretion, as described for other insulinoma cell lines (Robertson *et al.* 1992).

0022–0795/99/0161–0059

One characteristic of a functioning β-cell system is the adequate response to the physiological stimulus of glucose. Reports from different insulinoma cell lines differ remarkably in that either relatively high glucose concentrations have to be used to stimulate insulin secretion or the quantitative output of insulin is rather low (Chick *et al.* 1973, Gazdar *et al.* 1980). The effect of glucose on insulin synthesis has been studied by Robertson *et al.* (1992), showing that long-term exposure of the HIT-15 cell line to high concentrations of glucose (11 mM) determines a significant decrease in insulin secretion and gene expression. However, insulin mRNA, insulin content and secretion could be restored by culturing the cells in medium containing low glucose concentrations.

Another system involved in the secretory process of insulin is the cAMP system that can be stimulated by either direct stimulation of adenylate cyclase or inhibition of phosphodiesterase. Again, variable results are reported for the insulinoma cell lines described (Hill *et al.* 1987). Furthermore, this system is involved in the stimulus-secretion coupling of various physiological stimuli of insulin secretion such as glucagon and glucagon-like peptide-1 (GLP-1). For this reason these two alternative pathways were investigated with the CM cell line.

The aim of our study was to investigate, under different experimental conditions, the capacity of CM cells from long-term culture to retain β-cell function, in particular the expression of constitutive β-cell genes (insulin, the glucose transporters GLUT1 and GLUT2, glucokinase (GCK)), intracellular and secreted insulin, β-cell granules and cAMP content.

Materials and Methods

Cell culture

The CM cell line grows routinely as an adherent semiconfluent monolayer. Cells were cultivated in 5% CO_2/95% humidified air at 37 °C in RPMI 1640 medium (Gibco BRL, Eggenstein, Germany; without glucose, without L-glutamine) supplemented with 5% fetal calf serum, 2 mM L-glutamine, 1% antibiotics cocktail (10 000 units penicillin+10 mg/ml streptomycin) and 11 mM glucose. Cells were split weekly using trypsin-EDTA, and fed every two days by changing the medium. Prior to all experiments, cells from passage 70 that were routinely grown at 11 mM concentration, were split and gradually transferred in medium containing lower concentrations of glucose until the culture was settled at 0·8–1 mM glucose. CM cells were then cultured for another 8 weeks before being used in all the studies. Cells were used after reaching confluency.

Insulin secretion and insulin content

Insulin secretion of CM cells was examined in response to glucose, forskolin (Sigma, St Louis, MO, USA) and L-arginine (Serva, Deisdenhofen, Germany). For this purpose cells grown in media containing either 0·8 mM or 11 mM glucose were seeded in 12-well plates (Corring Costar, Milano, Italy; 100 000 cells/well) and used after two and three days respectively, when they had reached confluency. Cells were washed once with glucose-free Krebs-Ringer-bicarbonate-Hepes buffer (KRBH: 134 mM NaCl, 3·5 mM KCl, 1·2 mM KH_2PO_4, 0·5 mM $MgSO_4$, 1·5 mM $CaCl_2$, 5·0 mM $NaHCO_3$, 10·0 mM Hepes, 0·1% BSA; pH 7·4) and incubated for 4 h at 37 °C with KRBH (0·5 ml/well) supplemented with the different test compounds. In addition, experiments were performed after seeding cells in plates that were coated with the extracellular matrix protein, fibronectin (10 μg/cm^2). In another set of experiments cells were preincubated for 24 h with transforming growth factor (TGF)-β_1 (PBH, Hannover, Germany) or butyric acid sodium salt (Fluka, Neu-Ulm, Germany) before they were stimulated with the above mentioned test compounds.

The insulin content of the incubation medium was then either directly analysed by radioimmunoassay (DPC/Biermann, GmbH, Bad Nauheim, Germany; Coat-a-count human insulin-RIA; Linco Research Inc., St Louis, MO 63304, USA: Ultra sensitive insulin RIA) or concentrated with centricon-3 prior to analysis (Amicon, Stonehouse, UK; 3000 molecular weight cut-off). To determine the insulin content of CM cells, they were stimulated as described above. Afterwards, cells were incubated for 15 min at room temperature with 300 μl/well extraction solution composed of 1·5% HCl (37%), 18·5% H_2O and 80% ethanol (95%). The same volume of 0·1 M NaOH was added before the insulin content of the extract was analysed by radioimmunoassay. The same experiments were performed with the rat insulinoma cell line Ins-1 (passage 40, kindly provided by Dr C Wollheim, Geneva, Switzerland).

Immunostaining for insulin

CM cells were frozen in culture medium and cryostatic sections were coated onto poly-L-lysine (Sigma) slides and fixed in ice-cold acetone for 10 min. The endogenous peroxidase activity was blocked with 1 ml H_2O_2 (30%) in a coplin jar of methanol for 20 min. After washing in PBS the sections were pre-incubated with normal rabbit serum (Dako, Copenhagen, Denmark) (1:20 in PBS buffer) for 15 min. The sections were washed again with PBS buffer and incubated with guinea-pig anti-bovine insulin (1/500 dilution in PBS) overnight at 4 °C.

The sections were then washed in PBS 4 times and incubated with rabbit anti-guinea-pig IgG-peroxidase-conjugated (Dako) (1/100 dilution) for 20 min at room temperature. After 4 washes in PBS the enzyme reaction was developed using the diaminobenzidine (Sigma) substrate and H_2O_2 (25 μl) for 3 min at room temperature. The sections were rinsed in tap water, counterstained in

haematoxylin for 1 min, and mounted in DePeX (BDH, Merck, Milan, Italy). Mouse pancreas sections were used as positive control and normal guinea pig pre-immune serum (Dako) was taken as negative control.

Electron microscopy

Ultrastructural analysis of CM cells was performed according to Groudin & Beaudoin (1996). Briefly, cells were pelleted and fixed in 2·5% glutaraldehyde prepared in 0·1 M sodium cacodylate buffer (pH 7·4) for 1 h, and postfixed in 2% osmium tetroxide in the same buffer at room temperature. Cells were dehydrated in ethanol and embedded in Epon 812. Thin sections were double stained in uranyl acetate and lead hydroxide.

RNA expression studies

Total cellular RNA was extracted from $2–3 \times 10^7$ CM cells with the guanidinium-isothiocyanate method (Chomczynski & Sacchi 1987). For Northern blotting, 10 μg total RNA were fractionated on 1·5% agarose-formaldehyde gel and transferred onto nylon filters. For Slot-blot analysis, 5 μg total RNA were immobilised on nylon filters using a Slot Blot Minifold apparatus. All experiments were performed in duplicate.

The probes for the insulin gene, the glucose transporter genes GLUT1, GLUT2, GLUT3, GLUT4, the glucokinase gene, and the β-actin gene were labelled by the random priming method with ^{32}P-dCTP. Nylon filters (Amersham International, Amersham, Bucks, UK) were hybridised with all probes following the manufacturer's instructions. After hybridisation, the filters were visualised by autoradiography. The relative abundance of messenger RNA for each gene was quantified by densitometric scanning, comparing the [expressed gene]/[β-actin] ratio at time 0 to the ratio at 48 h.

cAMP content

CM cells, grown in media containing either 0·8 mM or 11 mM glucose, were seeded in 12-well plates (Costar; 100 000 cells/well) and used after three days when they reached confluency. They were washed once with 1·0 ml/well KRBH. New prewarmed buffer, supplemented with 11 mM glucose and 0·8 mM isobutyl-methyl-xanthine (Sigma), was added in a total volume of 1·0 ml/well. Test compounds were dissolved and diluted in dimethyl sulphoxide in the case of forskolin (Sigma), or KRBH if GLP-1 (Saxon Biochemicals GmbH, Hannover, Germany), glucagon (Sigma), or exendin-4 (Sigma) were used as stimulators. Cells were incubated for 10 min at room temperature before the incubation medium was decanted and were lysed for 15 min with 200 μl/well 1 M HCl. The lysate was then neutralised with the same volume of 1 M NaOH and the cAMP content determined by a ^{125}I-cAMP radioimmunoassay (DuPont NEN Research Products, Bad Homburg, Germany). As a control system the Ins-1 cell line was used under the same assay conditions.

Results

Insulin secretion and insulin content of CM cells

Glucose in concentrations of 1, 5, 11, and 22 mM was added to confluent cells. After 4 h at 37 °C the insulin secreted into the medium was determined. There was no insulin secretion in response to increasing glucose concentrations. Even under conditions when CM cells were preincubated in high (11 and 22 mM) glucose medium, insulin secretion was not detected. In contrast, cells of the rat insulinoma cell line Ins-1 showed a substantial increase in insulin secretion in response to increasing concentrations of glucose under the same experimental conditions (Fig. 1). For further experiments, CM cells were subcultured for three weeks in 'normoglycemic' medium (5 mM glucose). They were stimulated with 11 mM glucose and 0·1 mM forskolin in KRBH for 4 h at 37 °C. Supernatants were then concentrated about 15-fold and the insulin content was determined by RIA with an approximate 10-fold greater sensitivity compared with the assay used previously. In spite of the fact that forskolin led to an increase in cAMP concentration, and the use of a highly sensitive assay, no insulin secretion was detected. In contrast, stimulation of Ins-1 cells with forskolin under identical conditions led to a 2-fold increase in insulin secretion (basal: 30·68 μIU/ml; stimulated: 60·46 μIU/ml). To evaluate whether the missing insulin secretion of CM cells was due to a defect in the secretory process, the intracellular insulin content of the cells was examined. As shown in Fig. 2 insulin was indeed measurable at a concentration of approximately 70 μIU/ml. However, this concentration was 10- to 20-fold lower than in the rat insulinoma cell line Ins-1. Because the data clearly showed the presence of insulin within the CM cells, further studies were performed investigating the influence of differentiation factors upon insulin secretion of these cells. In one experiment, cells grown in media containing 0·8 mM and 11 mM glucose were seeded in plates coated with the extracellular matrix protein, fibronectin. Fibronectin is known to be involved in cell–matrix interactions and can support both the differentiation and survival of cells by signal transduction pathway (Aoshiba *et al.* 1997). In another set of experiments, cells were preincubated for 24 h with other differentiation factors, such as 1 mM butyric acid sodium salt or 1 and 10 ng TGF-β_1 (Pouillart *et al.* 1992, Arias & Bendayan 1993, Hao & Palmer 1995), before being stimulated with 5 mM glucose and 0·1 mM forskolin or 10 mM L-arginine. L-Arginine leads to membrane depolarisation and hence induces insulin secretion in pancreatic beta cells (Larsson & Ahren 1995, Mc Clenaghan *et al.* 1996).

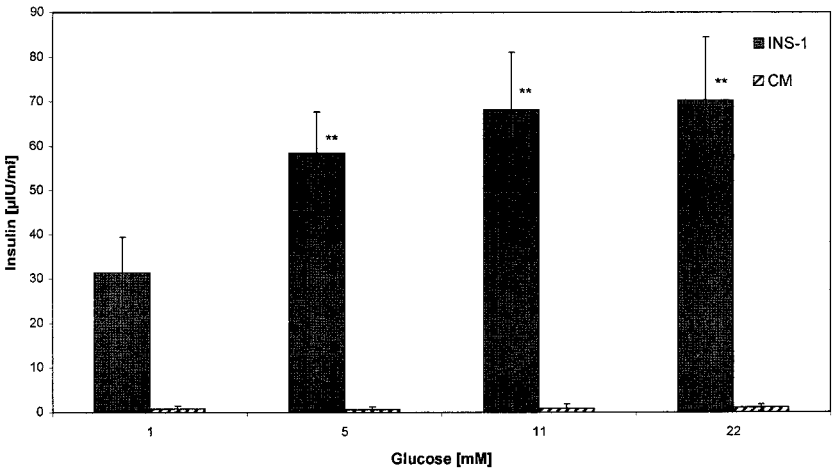

Figure 1 Insulin secretion from CM cells compared with Ins-1 cells. Cells were stimulated with different concentrations of glucose for 4 h. The values shown are means ± S.D. of 3 measurements. $^{**}P<0{\cdot}01$ for changes versus 0·8 mM glucose.

Neither compounds nor pretreatment led to a significant increase in insulin secretion, although an ultrasensitive human insulin radioimmunoassay was used for analysis.

Immunostaining for insulin

CM cells exposed to 11 mM glucose concentration did not show any staining (Fig. 3B). However, the presence of insulin in CM cells after culture in 0·8 mM glucose was demonstrated by immunoperoxidase staining (Fig. 3A). The same positive results were obtained when the CM cells cultured in 0·8 mM glucose were exposed to 11 mM glucose for 48 h, although the staining was weaker than CM cells cultured in 0·8 mM glucose. Finally, images by electron microscopy of late-passaged CM cells as opposed to early-passaged CM cells (Gueli *et al.* 1987) did not show the presence of typical secretory granules of β-cells stained with immunogold at any glucose concentration. This suggests that the mechanisms by which insulin is stored in granules are altered in CM cells.

RNA expression studies

Three sets of experiments were performed for most genes.

Experiment 1: a preliminary study of the RNA expression of the insulin, GLUT2 and GLUT1 genes was performed in CM cells from an early-passage (~30th passage) cultured at physiological (5·5 mM) glucose concentration. At time 0 glucose concentration was changed to 0·8 mM, 11 mM, and 22 mM. RNA was extracted at time 0 (5·5 mM) and after 48 h. This time was chosen after earlier experiments demonstrated the maximal expression of genes after 48 h. The insulin gene mRNA

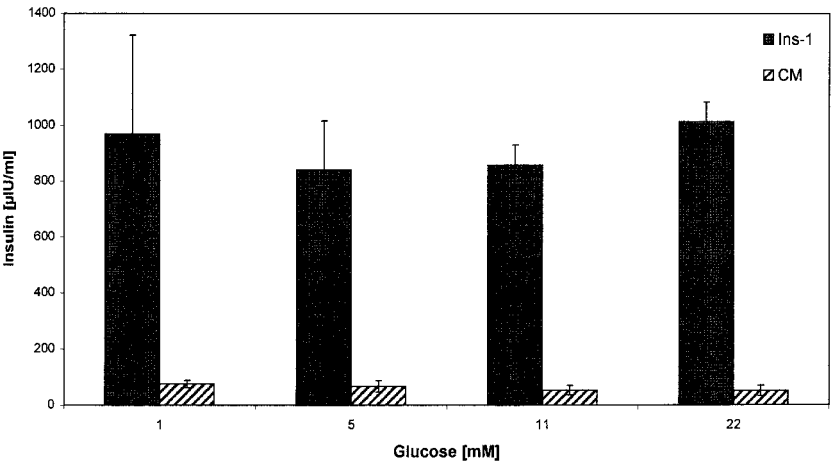

Figure 2 Insulin content of CM cells compared with Ins-1 cells. Cells were stimulated with different concentrations of glucose for 4 h. Mean values ± S.D. refer to 4 measurements.

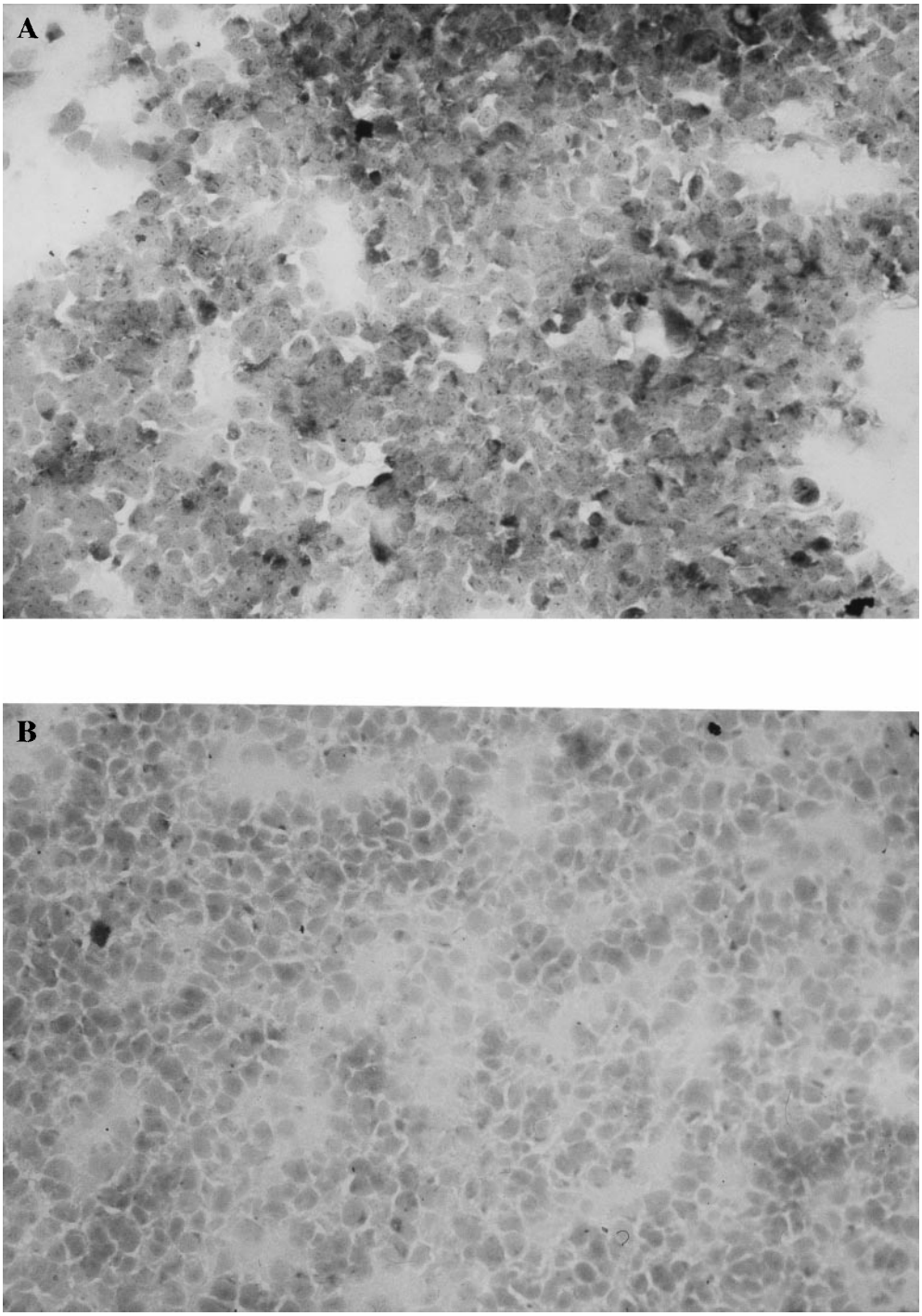

Figure 3 Immunoperoxidase staining of CM cells after incubation with anti-insulin polyclonal antibody. (A) CM cells cultured in 0·8 mM glucose showing immunoperoxidase staining. (B) CM cells cultured in 11 mM glucose showing no immunoperoxidase staining.

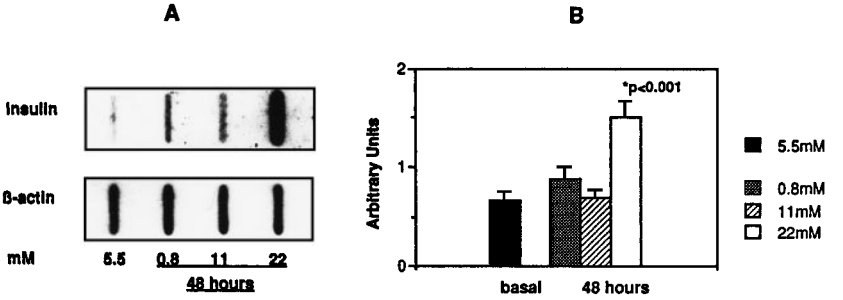

Figure 4 Insulin gene expression in early-passaged CM cells cultured in 5·5 mM glucose. Arbitrary units are means ± S.D. of the insulin/β-actin mRNA ratio. (A) P^{32}-labelled shot blots for insulin and B-actin genes. (B) Differences between basal and 48 hours expression of insulin gene at different glucose concentrations.

from experiment 1 showed a significant increase after 48 h at 22 mM glucose (Fig. 4). The GLUT1 gene mRNA in experiment 1 did not show any significant change at all glucose concentrations (data not shown). The GLUT2 gene mRNA showed a modest increase at 11 and 22 mM over basal values (data not shown). These results confirmed that early-passaged CM cells have the capacity to respond to glucose stimulation.

Experiment 2: the RNA expression was measured in late-passaged CM cells cultured in 5·5 mM glucose for 4–6 weeks. At time 0 the glucose concentration was changed to 0·8 mM, 11 mM, and 22 mM; RNA was measured at time 0 and after 48 h. The insulin gene expression did not show any significant changes after 48 h (data not shown). Also GLUT1 and GLUT2 genes mRNA expression did not show any significant change at any glucose concentration (data not shown).

A comparison of the different expression of GLUT1, GLUT2, and insulin genes between early- and late-passaged cells is shown in Fig. 5.

Experiment 3: late-passaged CM cells were cultured in 0·8 mM glucose for several weeks (8 weeks). The glucose concentration was changed to 5·5 mM, 11 mM, and 22 mM; RNA was measured after 48 h. A significant increase in the insulin gene RNA expression at 22 mM glucose after 48 h was detected (Fig. 6). In this experiment, a small but significant increase in the expression of the GLUT2 gene was observed at 22 mM glucose compared with the basal value (Fig. 7). GLUT3 RNA levels were very low at all glucose concentrations, whereas GLUT4 RNA levels were undetectable, as expected.

Finally, glucokinase gene expression was measured in experiments 2 and 3. In experiment 2 there was no significant increase in GCK expression after 48 h (data not shown); in experiment 3 the increase in expression did not reach significant levels, despite showing an increase at all glucose concentrations (Fig. 8).

In summary, these data show that CM cells from an early-passage express specific β-cell genes in response to glucose stimulation, in particular the insulin and GLUT2 genes. Such capacity is lost at later passages when cells are cultured at standard glucose concentrations; however, if cultured at a lower glucose concentration

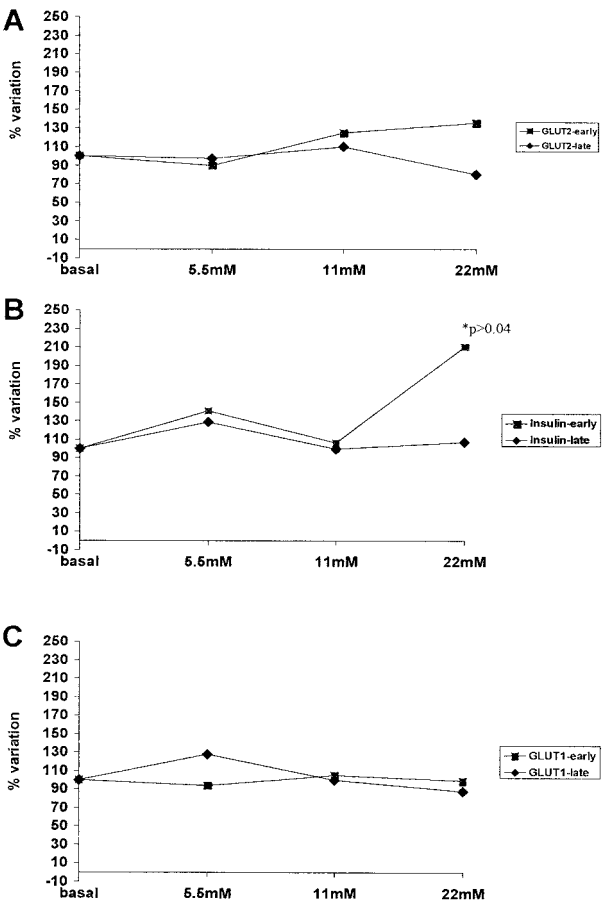

Figure 5 Comparison of the different expression of GLUT2 (A), insulin (B) and GLUT1 (C) genes between early- and late-passaged cells. Data are presented as percentage variation of gene expression compared with the basal value. *Insulin gene expression of early-passaged cells vs late-passaged cells at 22 mM (*t*-test).

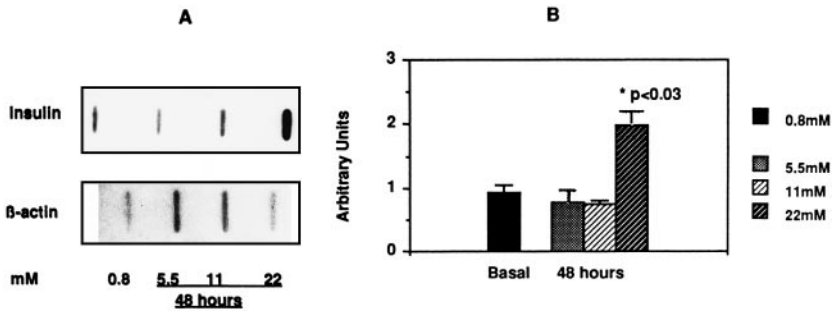

Figure 6 Insulin gene expression in late-passaged CM cells cultured in 0·8 mM glucose. Arbitrary units are means ± S.D. of the insulin/β-actin mRNA ratio. (A) P^{32}-labelled slot blots of insulin and B-actin genes. (B) Differences between basal and 48 hours expression of insulin gene at different glucose concentrations.

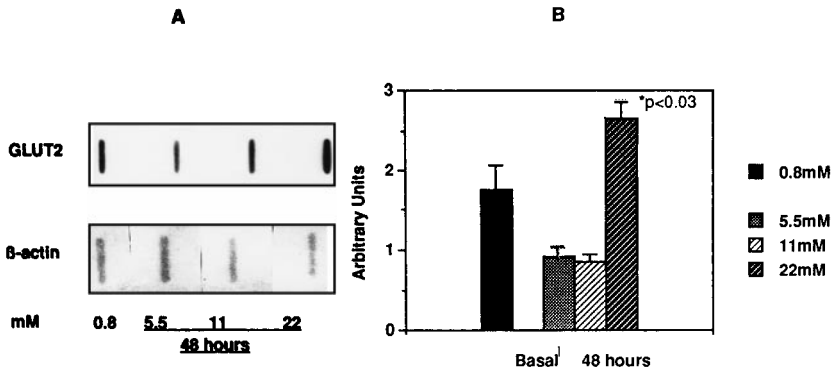

Figure 7 GLUT2 gene expression in late-passaged CM cells cultured in 0·8 mM glucose. Arbitrary units are means ± S.D. of the GLUT2/β-actin mRNA ratio. (A) P^{32}-labelled slot blots of GLUT2 and B-actin genes. (B) Differences between basal and 48 hours expression of GLUT2 gene at different glucose concentrations.

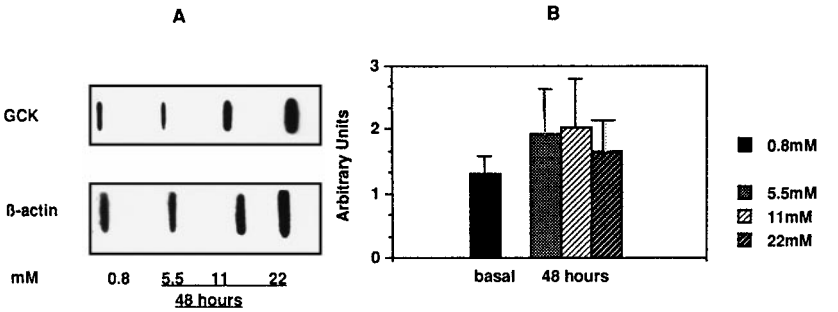

Figure 8 Glucokinase gene expression in early-passaged CM cells cultured in 0·8 mM glucose. Arbitrary units are means ± S.D. of the glucokinase/β-actin mRNA ratio. (A) P^{32}-labelled slot blots of GCK and B-actin genes. (B) Differences between basal and 48 hours expression of GCK gene at different glucose concentrations.

(0·8 mM) for a longer time, CM cells re-acquire the capacity to respond to glucose stimulation.

cAMP content

To characterise the CM cell line, cells were stimulated with different types of test compounds. One of them was forskolin, a direct activator of adenylate cyclase (Wiedenkeller & Sharp 1983, Malaisse *et al.* 1984, Hermansen 1985). Forskolin stimulated the cAMP production 70-fold above the basal value (Fig. 9).

Stimulation of cAMP production was also tested with GLP-1, glucagon, and exendin-4 (a 39 amino acid peptide that shares 53% structure homology with GLP-1(7–36)

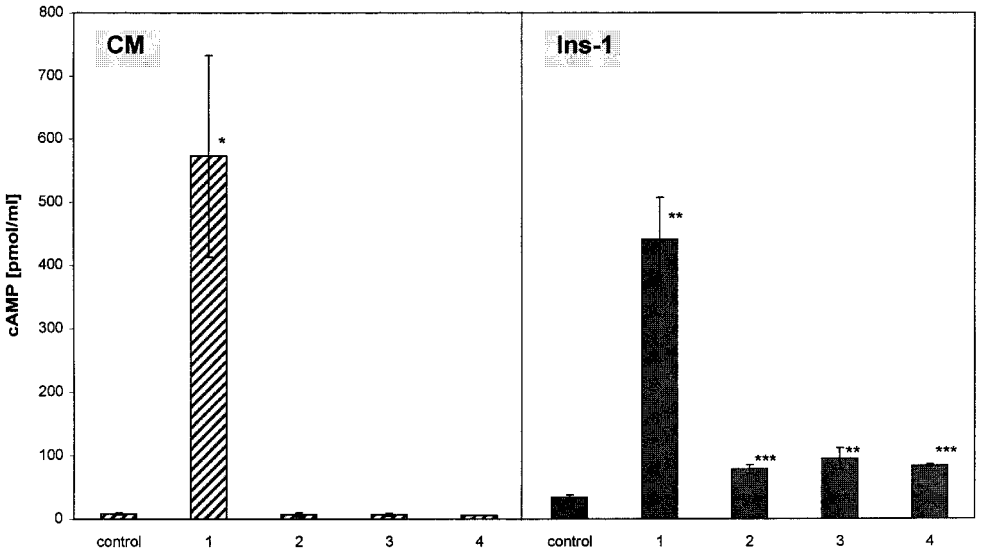

Figure 9 cAMP content of CM cells compared with Ins-1 cells after stimulation with: (1) forskolin (0·1 mM); (2) GLP-1; (3) glucagon; (4) exendin-4; (n. 2, 3 and 4 each 0·1 μM). The values shown are means ± S.D. of 3 or 4 measurements *P<0·05, **P<0·01, ***P<0·001 compared with control.

amide), all of which act via G-protein coupled receptors. Both exendin-4 and GLP-1(7–36) amide compete specifically with the GLP-1 receptor from different cell types. Similar to GLP-1, exendin-4 has a pronounced effect on the production of cAMP (Göke *et al.* 1993). Neither compound led to an increase in cAMP (Fig. 9).

Control experiments were performed with the rat insulinoma cell line Ins-1 under comparable conditions. With this cell line each of the stimuli tested led to significant increases in cAMP concentrations indicating a viable adenylate cyclase system as well as an intact GLP-1 and glucagon receptor and receptor coupling (Fig. 9).

Discussion

The aims of this study were to characterise the insulinoma cell line CM with respect to the expression of constitutive β-cell genes and to evaluate the functional characteristics of these cells under different stimuli.

The results showed that CM cells are capable of responding to glucose stimulation enhancing the expression of mRNAs specific for insulin and GLUT. However, analogous to what has been shown by studies on other insulinoma cell lines (Robertson *et al.* 1992) CM cells tend to lose the capacity of secreting insulin when cultured continuously in the presence of high glucose concentrations and fail to respond consistently to glucose stimulation. Nevertheless, when the CM cells were cultured in 0·8 mM glucose for several weeks, the mRNA response to glucose stimulation was re-acquired although it was less pronounced compared with early-passaged CM cells.

Both radioimmunoassay and immunostaining showed that insulin was present in the CM cells; however, typical secretory granules studied by electron microscopy were not detected. Furthermore, insulin was undetectable in supernatants of cell cultures. These data, together with the studies on constitutive β-cell genes confirm that the biosynthetic pathway for insulin is conserved in CM cells, but storage of insulin (intracellular granules) and secretion are somehow impaired.

The loss of insulin response to glucose by CM cells cultured in high glucose medium is reminiscent of the impaired insulin secretion present in non-insulin-dependent diabetics where first phase insulin secretion is lost when hyperglycaemia is present (Brunzell *et al.* 1976). In animal models the role of glucose toxicity has been extensively studied, showing that normalisation of blood glucose in pancreatectomized rats restored normal insulin response (Rossetti *et al.* 1987). It is interesting to note that GLUT2 gene expression, although present, shows a modest response to glucose stimulation. The role of GLUT2 in human islets is still debated and it may not be the key gene involved in glucose sensing in humans (Schuit 1997). Furthermore, this is a common feature of insulinomas, where GLUT1 and/or GLUT3 transporters constitute the predominant types for glucose transport (Yamamoto *et al.* 1990, Seino *et al.* 1993).

In addition the gene for glucokinase, which has a high K_m for glucose and catalyses a rate-limiting step in glycolysis (Meglasson & Matschinsky 1986) showed a blunted response to glucose in late-passaged CM cells grown in 5·5 mM glucose. Glucose-induced alterations in another component of glucose sensing, with or without

GLUT2 deficiency, may be implicated in insulin release defect.

In agreement with other studies on insulinoma cell lines (Hill *et al.* 1987) stimulation of CM cells with forskolin, a direct activator of adenylate cyclase (Wiedenkeller & Sharp 1983, Malaisse *et al.* 1984, Hermansen 1985) led to a greatly enhanced cAMP production exceeding basal values, indicating that CM cells possess a viable adenylate cylase system. In contrast glucagon or GLP-1, both of them acting via G-protein coupled receptors (Schuit & Pipeleers 1986, Drucker *et al.* 1987, Gefal *et al.* 1990, Thorens 1992, Dillon *et al.* 1993, Thorens *et al.* 1993, Wheeler *et al.* 1993) showed no stimulatory effect on cAMP production. Both are known to be very potent stimulators of the adenylate cyclase system and hence should lead to a significant increase in cAMP content. Furthermore, exendin-4, a peptide which binds to the GLP-1 receptor and exerts agonistic activity (Göke *et al.* 1993), did not affect the cAMP production. The reason neither of these compounds led to an increase in cAMP may be due to the fact that the coupling of GLP-1 and of glucagon receptors to the adenylate cyclase system is defective in CM cells. Reports from other β-cell lines have shown that an increase in cAMP levels potentiates insulin secretion. Hill *et al.* (1987) reported that stimulation of cAMP production with forskolin results in a potentiation of the first phase of insulin release in HIT cells. Although we found a pronounced increase in cAMP levels after stimulation with forskolin, we were not able to detect an increase of insulin secretion in the CM cell line. In addition our attempts to increase insulin secretion by support of cell differentiation did not show the expected effect. So, neither coating cell plates with the extracellular matrix protein, fibronectin (Aoshiba *et al.* 1997), nor stimulating cells with differentiation factors like butyric acid sodium salt or TGF-β_1 led to positive results (Pouillart *et al.* 1992, Arias & Bendayan 1993, Hao & Palmer 1995).

Several conclusions can be drawn from our studies. First, the finding of reversible insulin mRNA expression and protein biosynthesis associated with culturing CM cells in low glucose concentration points towards intracellular events for the search of the deleterious effects of chronic hyperglycaemia in β-cells, commonly referred to as glucose toxicity or glucose exhaustion. Secondly, the human insulinoma cell line CM, both in early-passaged and in late-passaged cells, shows a functional glucose signalling pathway and insulin mRNA expression similar to normal β-cells, Therefore, it could serve as a good model for signalling and expression studies of β-cells under different experimental conditions. Finally, the CM cell line could be a useful tool for cellular engineering studies, in order to create an insulin-secreting cell-line that resembles the performance of native β-cells. In this respect it is important to note that the CM cell line, as a result of the difficulties in obtaining human β-cell lines capable of continuous growth *in vitro*, is one of the very few existing human β-cell lines in long-term culture.

Acknowledgements

This study was supported by grant no. E252 from Telethon Italy (to M G B), the Fondazione Cenci-Bolognetti, Istituto Pasteur, Rome (to M G B and L M), by grants from the University of Rome La Sapienza Ateneo 60% (P P) and the Joint Research Board of the St Bartholomew's Hospital, London (P P).

References

Adcock R, Austin M, Duckworth WC, Solomon SS & Murrell LR 1975 Human islet cell adenoma: metabolic analysis of the patients and of tumor cells in monolayer. *Diabetologia* **11** 527–535.

Aoshiba K, Rennard SI & Spurzem JR 1997 Fibronectin supports bronchial epithelial cell adhesion and survival in the absence of growth factors. *American Journal of Physiology* **273** L684–L693.

Arias AE & Bendayan M 1993 Differentiation of pancreatic acinar cells into duct-like cells *in vitro*. *Laboratory Investigation* **69** 518–530.

Atkinson MA & Maclaren NK 1994 Mechanisms of disease: the pathogenesis of insulin-dependent diabetes mellitus. *New England Journal of Medicine* **331** 1428–1436.

Brunzell JD, Robertson RP, Lerner RL, Hazzard WR, Ensinck JW, Bierman EL & Porte Jr D 1976 Relationships between fasting plasma glucose levels and insulin secretion during intravenous glucose tolerance tests. *Journal of Clinical Endocrinology and Metabolism* **42** 222–229.

Cavallo MG, Baroni MG, Toto A, Gearing AJ, Forsej T, Andreani D, Thorpe R & Pozzilli P 1992 Viral infection induces cytokine release by beta cells. *Immunology* **75** 664–668.

Cavallo MG, Dotta F, Monetini L, Dionisi S, Previti M, Valente L, Toto A, Di Mario U & Pozzilli P 1996 Beta-cell markers and autoantigen expression by a human insulinoma cell line: similarities to native beta cells. *Journal of Endocrinology* **150** 113–120.

Chick WL, Lauris V, Soeldner JS, Tan MH & Grinsberg M 1973 Monolayer culture of a human pancreatic beta cell adenoma. *Metabolism* **22** 1217–1224.

Chomczynski P & Sacchi N 1987 Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* **162** 156–159.

Dillon J, Tanizawa Y, Wheeler MB, Leng XH, Lignon BB, Rabin DU, Yoo-Warren H, Permutt MA & Boyd AE III 1993 Cloning and functional expression of the human glucagon-like peptide-1 (GLP-1) receptor. *Endocrinology* **133** 1907–1910.

Drucker DJ, Philippe H, Mojsov S, Chich Wl & Habener JF 1987 Glucagon-like peptide 1 stimulates insulin gene expression and increases cyclic AMP levels in a rat islet cell line. *Proceedings of the National Academy of Sciences of the USA* **84** 3434–3438.

Frödin M, Sekine N, Roche E, Filloux C, Prentki M, Wollheim CB & Van Obberghen E 1995 Glucose, other secretagogues, and nerve growth factor stimulate mitogen-activated protein kinase in the insulin secreting beta-cell line, Ins-1. *Journal of Biological Chemistry* **270** 7882–7889.

Gazdar AF, Chick WL, Oie HK, Siems HL, King DL & Weir GC 1980 Continuous clonal insulin and somatostatin secreting cell lines established from a transplantable rat islet cell tumor. *Proceedings of the National Academy of Sciences of the USA* **77** 3519–3523.

Gefal D, Hendrick GK, Mojsov S, Hebener JF & Weir GC 1990 Glucagon-like peptide 1 analogues: effect on insulin secretion and adenosine 3′, 5′-monophosphate formation. *Endocrinology* **126** 2164–2168.

Göke R, Fehmann HC, Linn T, Schmidt H, Krause M, Eng J & Göke B 1993 Exendin-4 is a high potency agonist and truncated exendin-(9–39)-amide an antagonist at the glucagon-like peptide 1-(7–36)-amide receptor of insulin-secreting beta-cells. *Journal of Biological Chemistry* **268** 19650–19655.

Groudin G & Beaudoin AR 1996 Immunocytochemical demonstration of a novel selective pathway of secretion in the exocrine pancreas. *Journal of Histochemistry and Cytochemistry* **44** 357–368.

Gueli N, Toto A, Palmieri G, Carmenini G, Delpino A & Ferrini U 1987 *In vitro* growth of a cell line originated from a human insulinoma. *Journal of Experimental and Clinical Cancer Research* **6** 281–285.

Gylfe E & Hellman B 1980 Glucose-stimulated sequestration of Ca^{2+} in clonal insulin-releasing cells. Evidence for an opposing effect of muscarinic-receptor activation. *Biochemical Journal* **233** 865–870.

Hao W & Palmer JP 1995 Recombinant human transforming growth factor-β does not inhibit the effects of interleukin-1β on pancreatic islet cells. *Journal of Interferon and Cytokine Research* **15** 1075–1081.

Hermansen K 1985 Forskolin, an activator of adenylate cyclase, stimulates pancreatic insulin, glucagon, and somatostatin release in the dog: studies *in vitro*. *Endocrinology* **116** 2251–2258.

Hill RS, Oberwetter JM & Boyd III AE 1987 Increase in cAMP levels in beta-cell line potentiates insulin secretion without altering cytosolic free calcium concentration. *Diabetes* **36** 440–446.

Larsson H & Ahren B 1995 Effects of arginine on the secretion of insulin and islet amyloid polypeptide in humans. *Pancreas* **11** 201–205.

Mc Clenaghan NH, Barnett CR, O'Harte FPM & Flatt PR 1996 Mechanisms of amino acid-induced insulin secretion from the glucose-responsive BRIN-BD11 pancreatic B-cell line. *Journal of Endocrinology* **151** 349–357.

Malaisse WJ, Garcia-Morales P, Dufrane SP, Sener A & Valverde I 1984 Forskolin-induced activation of adenylate cyclase, cyclic adenosine monophosphate production and insulin release in rat pancreatic islets. *Endocrinology* **113** 2015–2020.

Meglasson MD, Manning CD, Najafi H & Matschinsky FM 1986 Glucose transport by radiation-induced insulinoma and clonal pancreatic beta-cells. *Diabetes* **35** 1340–1344.

Meglasson MD & Matschinsky FM 1986 Pancreatic islet glucose metabolism and regulation of insulin secretion. *Diabetes Metabolism Reviews* **2** 163–214.

Pouillart P, Cerutti I, Ronco G, Villa P & Chany C 1992 Enhancement by stable butyrate derivatives of antitumor and antiviral actions of interferon. *International Journal of Cancer* **51** 596–601.

Robertson RP, Zhang HJ, Pyzdrowski L & Walseth T 1992 Preservation of insulin mRNA levels and insulin secretion in HIT cells by avoidance of chronic exposure to high glucose concentrations. *Journal of Clinical Investigation* **90** 320–325.

Rossetti L, Shulman GI, Zawalich W & DeFronzo RA 1987 Effect of chronic hyperglycemia on *in vivo* insulin secretion in partially pancreatectomized rats. *Journal of Clinical Investigation* **80** 1033–1044.

Schuit FC 1997 Is GLUT2 required for glucose sensing? *Diabetologia* **40** 104–111.

Schuit FC & Pipeleers D 1986 Differences in adrenergic recognition by pancreatic A and B cells. *Science* **232** 875–877.

Seino Y, Yamamoto T, Kazumoto I, Imamura M, Kadowaki S, Kojima H, Fujikawa J & Imura H 1993 Abnormal facilitative glucose transporter gene expression in human islet cell tumors. *Journal of Clinical Endocrinology and Metabolism* **76** 75–78.

Thorens B 1992 Expression cloning of the pancreatic beta cell receptor for the gluco-incretin hormone glucagon-like peptide 1. *Proceedings of the National Academy of Sciences of the USA* **89** 8641–8645.

Thorens B, Porret A, Buhler L, Deng SP, Morel P & Widmann C 1993 Cloning and functional expression of the human islet GLP-1 receptor. Demonstration that exendin-4 is an agonist and exendin-(9–39) an antagonist of the receptor. *Diabetes* **42** 1678–1682.

Trautmann ME & Wollheim CB 1987 Characterization of glucose transport in an insulin secreting cell line. *Biochemical Journal* **242** 625–630.

Verspol EJ, Tollkühn B & Kloss H 1995 Role of tyrosine kinase in insulin release in an insulin secreting cell line (INS-1). *Cellular Signalling* **7** 505–512.

Wheeler MB, Lu M, Dillon JS, Leng XH, Chen C & Boyd AE III 1993 Functional expression of the rat glucagon-like peptide-1 receptor, evidence for coupling to both adenylyl cyclase and phospholipase-C. *Endocrinology* **133** 57–62.

Wiedenkeller DE & Sharp GWG 1983 Effects of forskolin on insulin release and cyclic AMP content in rat pancreatic islets. *Endocrinology* **113** 2311–2313.

Yamamoto T, Seino Y, Fukumoto H, Koh G, Yano H, Inagaki N, Yamada Y, Inoue K, Manabe T & Imura H 1990 Over-expression of facilitative glucose transporter genes in human cancer. *Biochemical and Biophysical Research Communications* **170** 223–230.

Received 13 March 1998
Revised manuscript received 26 October 1998
Accepted 3 November 1998