The proliferation of multiple myeloma colonies (MY-CFUc) in vitro is independent of prognosis and is not associated with mutated N- or K-ras alleles in human bone marrow aspirates

BC Millar. JBG Bell. R Barfoot and M Everard

Section of Academic Haematology. The McElvain Laboratories. Institute of Cancer Research. Sutton. Surrey. UK.

Summary. During the period September 1987 to March 1993 the proliferation of myeloma cells as colonies (MY-CFUc) in vitro was examined in bone marrow aspirates from 43 patients with multiple myeloma and two patients with Waldenström's macroglobulinaemia. Twenty-four samples from 45 patients, of whom three were at presentation, four were in complete remission (CR), six had achieved a partial response (PR) and 11 had progressive disease (PD).produced MY-CFUc in vitro. The same bone marrow aspirates or one taken within 2 months of that assessed for MY-CFUc were used in the polymerase chain reaction (PCR). Genomic DNA was analysed for mutations in N- and K-ras by slot blotting of the amplified products from the PCR with 32P-labelled probes and by direct sequencing. No mutations were detected in N- or K-ras proto-oncogenes at codons 12, 13 or 61 in any sample. Eleven of the patients from whom MY-CFUc were produced remain alive with a median survival of 73 months (range 15-75 months).MY-CFUc have been cultured from 19 of these 24 patients on subsequent occasions, of whom nine remained alive. The absence of mutations in MY-CFUc in vitro at the time of sampling for mutated ras alleles, biopsy samples from four patients have produced MY-CFUc in vitro on subsequent occasions, of whom one patient remains alive. The data show that the proliferation of MY-CFUc in vitro occurred independently of disease status and was not indicative of prognosis. The failure to detect mutated N- or K-ras alleles in any sample suggests that if such mutations were present in the cells which form colonies in vitro they represented less than 0.1% of the tumour burden and did not affect the survival of this group of patients.

Keywords: myeloma: MY-CFUc: N-ras: K-ras: prognosis

During the past 8 years we have had some success in culturing multiple myeloma as colonies (MY-CFUc) in vitro from human bone marrow aspirates (Millar et al., 1988). Although MY-CFUc can be cultured from approximately 70% of patients at some time during treatment, in remission and or at relapse the likelihood of growing the tumour in vitro is dependent on the individual patient and cannot be predicted from the level of bone marrow infiltration with plasma cells (Bell et al., 1990; Maitland et al., 1990). Furthermore, the growth of MY-CFUc is not necessarily associated with progressive or fulminating disease (Bell et al., 1990; Maitland et al., 1990). Since there are a number of patients from whom it has not been possible to grow MY-CFUc in vitro at any stage of disease, there is clearly scope for refining the culture procedure or determining whether there are specific genetic mutations in some myeloma cell populations which facilitate tumour cell proliferation in vitro in the absence of added growth factors. However, the significance of such mutations will only be of clinical value if their presence is associated not only with proliferative activity in vitro but also with prognosis.

Although multiple myeloma is characterised by the presence of excess plasma cells in the bone marrow, the identification of circulating isotypic lymphocytes (Ruiz-Arguelles et al., 1984), as well as the production of colonies in vitro consisting of cells which are lymphoplasmacytoid (Millar et al., 1988), suggests that the major proliferative compartment occurs in less differentiated B cells. Also, the increased clonogenicity in vitro following cytotoxic chemotherapy, despite a decrease in tumour burden, suggests that multiple myeloma may be regulated by a homeostatic control mechanism in vivo (Maitland et al., 1990). Furthermore, the failure to increase MY-CFUc formation in vitro by the addition of cyclophosphamide or verapamil to the VAMP regime (vincristine. adriamycin and methylprednisolone) suggests that the potential increase in tumour proliferative capacity after chemotherapy is dependent on the nature of the cytotoxic insult (Bell et al., 1990).

The concept that previous chemotherapy might influence subsequent attempts to culture myeloma cells in vitro is likely to produce constraints in addition to the possible requirement for added growth factors which may be required to stimulate cell division. Mobilisation of putative precursors which have evaded chemotherapy may be a prerequisite for the successful culture of MY-CFUc in vitro in some instances. Thus, in attempting to assess the relationship between colony formation in vitro with genetic mutations, the likelihood of a particular mutation effecting proliferation may be influenced by the drugs to which the patient has been subjected.

All eukaryotic organisms use GTP-binding proteins to control cellular processes which are involved in the transduction of signals initiated by growth factors and lymphokines. ras is a family of highly sequence-conserved proteins which have functional similarities with other GTP-binding proteins. In the human genome, three ras genes have been identified: H-ras-1, K-ras-2 and N-ras (Ellis et al., 1981; Parada et al., 1982; Hall et al., 1983). A similar mechanism has been proposed for all GTP-binding proteins by which they exist in two interconvertible forms. When bound to GDP they are inactive, whereas they become functionally active when bound to GTP. In addition, they can recycle from GDP to GTP through intrinsic GTPase activity. Since activated versions of ras, in which mutations have led to the constitutive production of the GTP-binding form, have been found in many human solid tumours, including melanomas, teratocarcinomas, neuroblastomas and gliomas (for review see Bard, 1986), it has been suggested that the abnormal growth of some cancers is due to the continuous activation of the signalling cascade initiated by the aberrant protein.

The role of mutated ras in haematological malignancies remains controversial. In multiple myeloma, mutations have been found in N-ras and K-ras, particularly at codon 61 in
N-ras, with frequencies ranging from 12% to 47% of the patient populations studied (Paquette et al., 1990; Matozaki et al., 1991; Portier et al., 1992; Tanaka et al., 1992). In a study by Neri et al. (1989) mutations in N-ras occurred in only a fraction of the neoplastic clone, suggesting that they may result from either the selective loss or the selective acquisition of mutated alleles during tumour development. In two other studies mutations in K- and N-ras occurred with greater frequency in patients with fulminating disease (67%) than in patients with less aggressive disease (27%) (Portier et al., 1992; Corradini et al., 1993) suggesting that the development of mutated ras alleles occurs in late or terminal stage multiple myeloma. In none of these studies did the authors attempt to culture cells which had mutated ras alleles in vitro.

Mutated H-ras has not been detected in clinical samples of myeloma, although high levels of H-ras protein (p21) correlate with poor prognosis (Tsuchiya et al., 1988). Also, the greater expression of H-ras p21 in plasma cells from patients with multiple myeloma rather than monoclonal gammapathy of undetermined significance (MGUS) suggests that the overexpression of H-ras proto-oncogene may be involved in the transformation of the malignant plasma cell (Danova et al., 1990).

The data presented represent a retrospective study of a group of patients with multiple myeloma from whom monoclonal cells were available which had been cryopreserved from the same sample that was used for MY-CFUc. The aim of the study was to determine whether patients from whom MY-CFUc were cultured in vitro had a poorer prognosis than those from whom cells did not proliferate in vitro and whether colony formation in vitro was associated with detectable levels of mutated N- or K-ras in the monoclonal cell fraction.

Materials and methods

Clinical samples

Patients were recruited at random as they presented or were undergoing treatment for multiple myeloma. All patients had given written consent, following the Royal Marsden Hospital's Ethics Committee approval, to take part in laboratory-based studies. The criterion for entering the study was that sufficient mononuclear cells (MNCs) were available from the same bone marrow aspirate, or one taken within 2 months of that used for colony growth, for isolation of DNA for experiments involving the polymerase chain reaction (PCR). Among these 13 patients from whom sequential samples were taken for PCR studies no further treatment had occurred during the intervening period. There had been no changes in haematological or clinical parameters during the intervening period in these patients. Samples were accrued during the period from September 1987 to March 1993 and aliquots frozen as pellets in liquid nitrogen. The proliferation of myeloma cells as colonies (MY-CFUc) was examined at the time of bone marrow biopsy.

Bone marrow was aspirated from the posterior iliac crest under local anaesthesia and heparinised as described previously (Millar et al., 1988). The biopsy sample from one patient was taken from a pleural effusion.

Clinical status

A complete remission (CR) was defined as the absence of measurable paraprotein and bone marrow infiltration by myeloma cells of <5%. A partial response (PR) was defined as a paraprotein level reduced by 50% and improvement in all other clinical features sustained for greater than 1 month. Clinical staging at the time of diagnosis was done according to Durie and Salmon (1981).

Cell culture

Asynchronous cultures of RPMI-8226 and HL60 cell lines were propagated by methods described previously (Millar et al., 1988). Mononuclear cells were separated over Ficoll–Hypaque (Boyum, 1968) and assayed for myeloma colonies (MY-CFUc). Details of the methods have been reported previously (Millar et al., 1988). Briefly, MY-CFUc were assayed by plating 1 x 10⁶ MNCs per plate using heavily irradiated HL60 cells as an inhibiting layer to prevent the proliferation of granulocyte–macrophage colonies (GM-CFUc) (Montes Borinaga et al., 1990). Samples of MNCs were also plated in the absence of an HL60 underlay. Overlays containing MNCs were added to underlays in either 0.5 ml of a-medium supplemented with 20% fetal calf serum and 0.2% agar (final concentration) (agar/agar) or in 0.2 ml of medium without agar (agar/liquid). All cultures were set up in triplicate, incubated for 21 days at 37°C in an atmosphere of 5% carbon dioxide, 10% oxygen and 85% nitrogen and counted using an inverted microscope. Colonies consisted of >50 cells. Cells from agar/liquid cultures were harvested, stained for cytoplasmic immunoglobulins characteristic of the patients' M protein using mouse anti-human immunoglobulins conjugated to fluorescein isothiocyanate (FITC) and examined by fluorescence microscopy. Colonies were removed from agar/agar plates, collected by cytospin (1500 r.p.m. for 5 min; Shandon, UK), stained with May–Grünwald–Giemsa and examined microscopically. Plasma cells were identified by strong cytoplasmic staining, the presence of a perinuclear 'hof' and an eccentric nucleus or nuclei.

Molecular studies

Polymerase chain reaction

High molecular weight DNA was extracted from aliquots of 5 x 10⁶ MNCs, RPMI-8226 and HL60 cells (Sambrook et al., 1989). The concentration of DNA in the final solution was measured and used at a concentration of 1 µg 100 µl⁻¹ reaction volume. Oligomer primers for K-ras exons 1 and 2 were supplied by Orwell, (Edinburgh, UK) and biotinylated primers for N-ras exons 1 and 2 were synthesised in house (Dr H King, Chester Beatty Laboratories, Institute of Cancer Research, London) (see Table 1).

The PCR reaction mixture consisted of 1 µg of DNA, 0.2 mM each of dGTP, dTTP, dATP and dCTP (Perkin Elmer, UK), 0.15 µM each of the relevant upstream and downstream primers, 2.5 units of Bio Taq, 1 x PCR buffer and 4 mM magnesium chloride (Bioline, London, UK). The reaction volume was adjusted to 100 µl with dimethyl pyrocarbonate (DEPC)-treated water. Samples were heated at 94°C for 20 s followed by 35 cycles consisting of 94°C for 20 s, 55°C for 30 s and 72°C for 30 s in a Hybaid Omnimere (Hybaid, London, UK). DNA from RPMI-8226 and HL60 cells were used as controls. A 5 µl aliquot of the PCR product was run on a 2% agarose minigel using HaellII fragments of pX174 as reference standards (Gibco BRL, UK). Purity of the PCR product was confirmed by the presence of a single band between marker positions 118 and 194 bp.

Slot blotting for ras mutations

ras mutations were analysed using the human ras Mutla-lyser probe panels for K-ras codons 12, 13 and 61 and for N-ras codons 12, 13 and 61. The protocol for this was done according to the manufacturer's instructions (Clontech Labs., Cambridge Bioscience, UK). Probes were end-labelled with [γ-³²P]ATP, as directed, using T4 polynucleotide kinase.

To determine the sensitivity of the assay, DNA from RPMI-8226 cells was mixed with placental DNA in known amounts and subjected to PCR. The products were analysed by Southern blotting.

Direct sequencing of PCR products

K-ras exon 1 and exon 2
Aliquots of 50 µl of PCR products were run on a 2% low melting point agarose gel (Ultrapure or Nuseive, Gibco BRL, UK) for 1.5–2 h at 140 V. The appropriate band was cut out from the gel and purified using
the 'Magic' PCR preparation DNA system (Promega Corporation, USA). Purified PCR products were directly sequenced using Sequenase 2.0 DNA sequencing kit (United States Biochemicals, Cambridge Bioscience, UK) and incorporation of $[^{32}P]_{S}$ATP, using the upstream primer for exon 2 of K-ras and the downstream primer for exon 1 of K-ras. Samples were run on a 6% denaturing polyacrylamide gel, dried and exposed to Kodak X-OMAT for 24–44 h before development.

N-ras exons 1 and 2 As purification of the PCR products for N-ras exons 1 and 2 was impractical using the 'Magic' PCR preparation system, the PCR mastermix was modified to include a biotinylated upstream primer. The resulting biotinylated PCR products were sequenced by separation on a low melting point gel, the bands removed and melted at 65°C followed by incubation at 50°C with streptavidin-coated Dynal beads (Dynal, UK) for 30 min with constant agitation. The biotinylated PCR products attached to the beads were removed by magnet and the supernatant discarded. The beads were washed in 1 x Tris–boric acid–EDTA buffer (TBE; Gibco BRL, UK) and re-extracted by magnet. The resultant pure double-stranded DNA attached to the beads was resuspended in 200 μl of 0.15 M sodium hydroxide and incubated for 5 min at room temperature. The beads were reattatched to the magnet and the supernatant discarded leaving pure single-stranded DNA attached to the beads. Sequencing was done using a Sequenase 2.0 kit (United States Biochemicals) and incorporation of $[^{32}P]_{S}$ATP using the downstream primers of both exons. After adding the stop solution, the magnetic beads were removed from the assay mixtures by magnet. Samples were run on a 6% denaturing polyacrylamide gel and treated as above.

Results

Bone marrow aspirates were available from 45 patients. of whom 43 had multiple myeloma (MM) and two had Waldenström's macroglobulinaemia (W). A second biopsy was available from three patients (two MM and one W), taken a year (two MM patients) and 4 years (one W) after the first sample. The stage at presentation, sex and isotype together with the clinical status of each patient at the time when bone marrow was available for study are shown in Table I. The median time at which samples were received from the time of diagnosis was 12 months (range 0–11 years 5 months). No attempts were made to enrich MNCs for plasma cells since this had not proved necessary in our previous studies on the clonogenicity of myeloma cells in vitro (Millar et al., 1988). Twenty-four samples from 45 patients produced MY-CFUc which were confirmed by morphology and staining for the light chain (Table III). In the majority of samples the clonogenicity of the myeloma cell population ranged from 0.0057% to 0.093%, excepting that of MY-CFUc from patients 16 and 18, who had progressive disease and in whom it was higher, namely 1.0% and 0.3% respectively. At the time of testing three patients were at presentation, six had achieved PR, four were in CR after intensive therapy and 11 had progressive disease. Sixteen of these patients had bone marrow infiltration with plasma cells of at least 10% and seven had an infiltration of 5% or less.

Preliminary experiments to optimise the conditions for PCR were undertaken with the myeloma cell line RPMI-8226 (Matsuoko et al., 1967) and the promyelocytic leukaemic line HL60 (Collins et al., 1977). DNA from these cell lines was subjected to PCR and examined by slot blotting with $[^{32}P]$-labelled probes and direct sequencing for mutations in K-ras exons 1 and 2 and N-ras exons 1 and 2. In RPMI-8226 cells, no mutations were detected in N-ras and K-ras. A mutation was found in K-ras exon 1, codon 12, in which GGT was replaced by GCT (Figure 1). Only one allele was mutated. This is different from the mutation reported previously (Neri et al., 1988). Since the RPMI-8226 cell line was established in 1967 (Matsuoko et al., 1996) culture conditions in different laboratories may have enabled different subpopulations to dominate the original parental cell line, or new mutations may have arisen during the culture period in vitro, resulting in no overt change of phenotype. Dilution experiments in which placential DNA was mixed with DNA from RPMI-8226 cells in known amounts and examined by slot blotting after PCR showed that the mutated allele was detectable in samples containing 10% RPMI-8226 DNA (Figure 2). This sensitivity is similar to that noted by Farr et al. (1988) using the HT 1080 cell line, which is heterozygous for N61 lysine. In HL60 cells other workers (Bo et al., 1984) have shown that there is a point mutation in N-ras codon 61 in which CAA is replaced by CTA. Examination of PCR products by slot blotting and direct sequencing confirmed this finding (data not shown). No other mutations were found either in K-ras exons 1 and 2 or in N-ras exon 1. Only one allele was mutated.

In experiments with patients' samples, amplified PCR products of K- and N-ras from 48 samples of bone marrow and one pleural effusion were subjected to hybridisation with $[^{32}P]$-labelled oligonucleotide probes and slot blotting. Samples of RPMI-8226 and HL60 cells were included as controls for mutations in K- and N-ras. No positive signals were detected for mutated alleles in either K- or N-ras in any patient's sample. Because mutations in myeloma cells have been reported by other workers (Paquette et al., 1990; Matozaki et al., 1991; Portier et al., 1992; Tanaka et al., 1992). PCR products from all samples in which background activity had been detected by hybridisation and slot blotting were subjected to direct sequencing. From the total of 288 slot blots, 18 samples were sequenced for N-ras exon 2. 13 for N-ras

| N-ras exons 1 and 2 | Clinical response at assessment |
|---------------------|--------------------------------|
| CR                  | Male 25                          |
|                    | Female 20                         |
|                    | Clinical stage at diagnosis       |
|                    | IA 10                            |
|                    | IIIA 6                           |
|                    | IIIB 22                          |
|                    | Waldenström's 5                  |
|                    |                    | No. of patients |
|                    | Isotype                         |
|                    | CR 4                            |
|                    | PR 17                           |
|                    | PD 22                           |
|                    | Presentation 5                  |
|                    | Mutated                          |
|                    | Not mutated                      |
|                    | Isotype                         |
|                    | CR 4                            |
|                    | PR 17                           |
|                    | PD 22                           |
|                    | Presentation 5                  |
|                    | Mutated                          |
|                    | Not mutated                      |

Table I

| K-ras  | Downstream primer |
|--------|-------------------|
| 1      | 5'-TGT, TGG, ATCA, TTG, GTCA, CA |
| 2      | 5'-TAA, ACC, CAC, CTA, TAA, TGG, TG |

Table II

The above table shows the clinical details of patients at time in vitro studies.
Table III  Proliferation of myeloma (MY-CFUc) colonies in vitro

| Patient no. | Clinical status | Infiltration in BM (%) | MY-CFUc per 10^6 MNCs | Clonogenicity* | Proliferation from subsequent samples | Survival (months) |
|-------------|-----------------|------------------------|------------------------|----------------|--------------------------------------|------------------|
| 1-4         | PR              | 11                     | 4                      | 36             | +                                    | 26*              |
| 2           | PR              | 10                     | 1                      | 10             | +                                    | 72               |
| 3-4         | PD              | 35                     | 2                      | 5.7            | -                                    | 15               |
| 4-5         | PR              | 15                     | 9                      | 60             | -                                    | 16               |
| 5-6         | CR              | >10                    | 3                      | 30             | +                                    | 73               |
| 6           | PD              | >10                    | 1                      | 10             | +                                    | 74               |
| 7-8         | CR              | 1                      | 4                      | 400            | +                                    | 60*              |
| 8           | PD              | <5                     | 3                      | 60             | +                                    | 73               |
| 9-10        | PD              | <5                     | 1                      | 20             | -                                    | 8*               |
| 10          | PR              | 30                     | 280                    | 930            | +                                    | 62               |
| 11          | PD              | 18                     | 1                      | 5.5            | -                                    | 10*              |
| 12          | PR              | >5                     | 3                      | 60             | +                                    | 75               |
| 13          | Pres.           | 50                     | 2                      | 40             | +                                    | 3*               |
| 14          | PR              | 5                      | 4                      | 80             | +                                    | 75               |
| 15          | Pres.           | 10                    | 67                     | 670            | +                                    | 9*               |
| 16          | PD              | 2                      | 200                    | 10000          | +                                    | 75*              |
| 17-19       | CR              | 5                      | 24                     | 480            | +                                    | 4*               |
| 18          | PD              | 10                    | 300                    | 3000           | +                                    | 73               |
| 19          | Pres.           | >10                    | 4                      | 40             | +                                    | 76               |
| 20-22       | PD              | 18                    | 20                     | 111            | +                                    | 7*               |
| 21          | PD              | 20                    | 19                     | 95             | -                                    | 10*              |
| 22          | CR              | <5                     | 13                     | 260            | +                                    | 22*              |
| 23          | PD              | 47                    | 84                     | 178            | +                                    | 6*               |
| 24          | PD              | 50                    | 110                    | 550            | +                                    | 5.5*             |

*Number of clonogenic cells per 10^6 plasma cells. *Time between sample used for PCR studies and *death or until present day (July 1994). Patients' samples subjected to direct sequencing of PCR products shown at: *for N-ras exon 1; *for N-ras exon 2.

Figure 1  Direct sequencing of amplified products of K-ras codon 12 from the DNA of RPMI-8226 cells, showing the deduced sequence of GCT (alanine) replacing GGT (glycine). Sequencing was done from the 3'-primed end of the sequence.

Figure 2  Hybridisation of 32P-labelled synthetic oligomer probes of K-ras codon 12 to amplified PCR products of DNA from RPMI-8226 cells. The concentrations of products from the cells were 100%, 60%, 50%, 30%, 10%, 5%, 1%, 0.5%, 0.1%, 0.05% and 0.0%. A, Arginine; B, alanine; C, glycine.
exon 1 and five for K-ras exon 2. The samples which were directly sequenced from patients whose mononuclear cells had produced MY-CFUc in vitro are identified in Table III. Wild-type alleles were found in all samples examined by direct sequencing, but there were no mutated alleles.

The survival of patients used in this study is shown in Table IV. Among patients who are alive and from whom MY-CFUc were grown in vitro at the times when tests were done for mutated N-or K-ras alleles, four have had no further treatment. They remain in remission 62 (patient 10), 73 (patient 5), 74 (patient 6) and 75 months (patient 12) after sampling. Six of the remaining patients received intensive therapy with high-dose melphalan within 7 months after samples were used in these studies, and one patient received intensive therapy with busulphan 13 months after the samples were taken. Two of these seven patients now have progressive disease 16 months and 73 months after samples had been used for studies to detect mutated ras alleles. Subsequent bone marrow aspirates from 19/24 of these patients have produced myeloma colonies in vitro, including nine patients who remain alive.

Among patients whose cells did not produce MY-CFUc in vitro, ten remain alive, including the two patients with Waldenström's macroglobulinaemia. In this group of patients only one has had intensive therapy with high-dose melphalan since the samples were used in vitro. Tumour cells from four of these patients have subsequently produced myeloma colonies in vitro since this study, of whom one patient remains alive.

### Discussion

We have explored the possibility that patients from whom MY-CFUc can be cultured have a poorer prognosis than those whose tumour does not proliferate in vitro. We have also investigated whether the detection of mutations in N- and K-ras at exons 1 and 2 in bone marrow aspirates was associated with our ability to grow MY-CFUc in vitro from the same samples or one taken within 2 months of that assessed for colony formation. Studies to detect activated oncogenes or quantitate the expression of specific protooncogenes in multiple myeloma may provide information about the pathogenesis of the disease and may provide targets for new treatment strategies if they are associated with poor prognosis. Despite the low levels of plasma cells in eight samples, MY-CFUc were grown from 24 samples from 45 patients, including those that had infiltration with plasma cells of 5% or less, and included four patients who were in complete response at the time of testing. We have previously shown that in patients who respond to induction chemotherapy with VAMP the residual myeloma cell population has enhanced proliferative activity in vitro, suggesting that tumour growth is regulated by a homeostatic control mechanism(s) (Maitland et al., 1990) which may be modulated by specific treatment regimens (Bell et al., 1990).

Furthermore, the median time to clinical CR is less than required for the disappearance of MY-CFUc from their bone marrow (Maitland et al., 1990) or for the detection of the malignant clone by molecular techniques (Bell et al., 1993). Among these patients, the four who were in CR at the time of testing had had treatment with high-dose melphalan 25 months (patient 5), 27 months (patient 7), 1 month (patient 17) and 4 months (patient 22) previously. Patient 17 was diagnosed as having progressive disease 7 weeks after the samples were taken for in vitro studies. However, it may be too simplistic to suggest that the production of MY-CFUc in vitro was due to clonal activation in this instance since a second patient (no. 5) has remained in CR for 73 months without further treatment even though MY-CFUc have been grown in vitro on four subsequent occasions. This observation is not an isolated instance (BC Millar and JBG Bell, unpublished observations). It suggests that immune modulation in vivo may also contribute to the suppression of tumour cell proliferation and that in some instances this growth-inhibitory effect is absent in vitro.

In no instance were mutated N- or K-ras detected from either samples which produced colonies in vitro or those that did not. While the failure to detect mutated ras alleles in samples which had 5% or less infiltration with plasma cells but which produced MY-CFUc in vitro is likely to be due to the sensitivity of the assay, this must be assessed in relation to the clonogenicity of the myeloma cell population from individual patients. Since a single mutated allele in a mixed population was not detectable below the level of 10% (also reported by Farr et al., 1988), the possibility that cells which are clonogenic in vitro carry a mutation in N- or K-ras cannot be excluded. However, such a proposal suggests that cells which may have a mutation represent less than 0.1% of the tumour burden in most instances. This level of mutation would not have been detectable in most instances in purified populations of plasma cells given the level of tumour infiltration in the samples and the clonogenicity of the cells. Also, since cells other than identifiable plasma cells may be part of the malignant clone, purification of the plasma cell compartment could remove putative clonogenic cells, which may be capable of both proliferation and differentiation in vitro. While examination of individual colonies would resolve the possibility that some cells which are clonogenic in vitro carry a mutated ras allele, the data (Table III) suggest that they would represent a minor contribution to the tumour burden in vivo.

Furthermore, since MY-CFUc have been grown from subsequent bone marrow aspirates from 19/24 patients whose tumour proliferated in vitro in the initial scan, including 9/11 who remain alive, the data suggest that even if occult mutations in N- or K-ras had effected proliferation in vitro they did not contribute to resistance to chemotherapy or effect early relapse or survival. Also the observation that samples from four patients whose tumour did not produce MY-CFUc in vitro in the initial assay yielded MY-CFUc on subsequent occasions could indicate the development of additional genetic changes, clonal activation, the absence of in vivo inhibitory constraints or merely differences in the quality of putative progenitor cells in the bone marrow aspirates.

In other haematological malignancies, the role of mutated ras remains controversial. Although mutations have been found in patients with leukaemias, there was no correlation between the presence of mutations and cytological features or immune phenotype (Ahuya et al., 1990). Also, in patients with acute myelogenous leukaemia (AML) ras mutations were not detected in four patients in relapse even though they had been detected at presentation (Farr et al., 1988), suggesting that ras mutations arise as part of the oncogenic process but may be subsequently lost as other mutations occur within the malignant clone. In AML, even in situations in which mutations in N-ras were detected in primary colonies in vitro, their expression was heterogeneous (Bashey et al., 1992).
suggesting that the expression of this oncogene had occurred after the initial development of malignancy. The authors made no comment regarding the potential proliferative advantage that such mutations might elicit in the tumour cell population in vivo. This caution is justifiable since, unless mutated alleles are present throughout the tumour, it is difficult to determine whether mutations in a subpopulation cultured in vitro reflect enhanced growth potential in vivo. In B-cell dyscrasias there is evidence that the involvement of oncogene expression in the malignant phenotype is dependent on the stage of differentiation. In acute lymphocytic leukemia (ALL), patients have been reported to have point mutations in ras oncogenes (Neri et al., 1988), whereas mutations in ras have not been detected in patients with non-Hodgkin's lymphoma or chronic lymphocytic leukemia (Neri et al., 1988; Selvanayagam et al., 1988).

Our data suggest that mutated N- or K-ras alleles are unlikely to be of clinical importance in multiple myeloma as is the case with non-Hodgkin's lymphoma and chronic lymphocytic leukemia. Our ability to culture the tumour in vitro independently of the disease status or prognosis of individual patients suggests that immunoregulatory mechanisms contribute to tumour control in vivo, and genetic changes in some individuals may necessitate the provision of additional growth factors in vitro, as has been reported by other workers (Kawano et al., 1988).

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