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Induction of MHC Antigens by Tumour Cell Lines in Response to Interferons

A.M.E. Nouri, R.F. Hussain, A.V.L. Dos Santos, D.J. Gillott and R.T.D. Oliver

The induction of major histocompatibility complex antigens by interferons (IFN) on 17 established tumour cell lines was investigated by radio binding. One bladder (Fen) and two testis lines (Tera I and Ha) lacked class I antigens and IFN-γ failed to induce their expression. However, IFN-γ upregulated these antigens on lines expressing low class I antigens (Tera II and EP2102) with little or no significant effect on high class I expressing lines (T24 and RT112). In one bladder line (Wil) IFN-γ, whilst failing to alter monomorphic class I, upregulated polymorphic HLA-A2 and A3 antigens. None of the 17 lines expressed class II antigens, but could all be induced by IFN-γ except T24, TccSup, Tera II and Lan lines. This defect was not due to the absence of IFN-γ receptor, since under the same conditions intracellular adhesion molecule 1 was upregulated. IFN-α, whilst failing to have any effect on class II, induced class I antigens. IFN-β showed no activity on either class I or II antigens when used alone. However, in combination, it inhibited IFN-γ induced class II antigens. Thus, it may be possible to study cells from fresh tumours to preselect the minority of patients who might benefit from cytokine therapy.
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INTRODUCTION

DURING THE past decade it has become clear that a small minority of durable complete remissions occur after the use of cytokines [interferon (IFN) α and interleukin (IL) 2] for treating human cancer [1]. *In vivo* studies with cloned neomycin-resistant gene-labelled tumour infiltrating lymphocytes from some of these patients provide the most compelling evidence supporting the concept that these tumour rejections are mediated by immune T lymphocytes. There is a pressing need for methods of preselecting this subgroup of patients to reduce the unnecessary use of expensive and toxic drugs for patients who will not benefit.

The recognition and response to foreign antigens requires the participation of major histocompatibility complex (MHC) class I and II antigens acting as associative molecules for presentation of antigens to T cells [2]. This stimulated interest as to whether abnormality in expression of these antigens may be a factor for tumour escape from cytolytic T cell attack.

It has become apparent that a wide variety of aberrant expression of MHC antigens can be demonstrated on both experimental and human tumour cells. This and the demonstration that transfection of the missing class I gene into a mouse lymphoma led to loss of tumorogenicity and induced protection against untransfected cells [3], has stimulated speculation as to whether these changes may be major factors in tumour escape from immune surveillance.

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Given that IFN-α upregulates HLA class I [4] and IFN-γ upregulates both class I and II antigens [5, 6], and the reports demonstrating that the response of IL-2 in melanoma [7, 8] and intravesical BCG in superficial bladder cancer [9] correlate with augmented class II expression, it was decided to investigate the effect of IFNs on HLA expression on tumour lines, as it is known that they show similar patterns of aberrant HLA expression as seen using immunocytochemistry on tumour sections [10, 11].

This paper reports the preliminary attempt to use IFNs to develop *in vitro* assays that might be used to select the patients whose tumour responds to treatment.

MATERIALS AND METHODS

Development of cell lines

Surgically removed tumour biopsy specimens were used for developing cell lines. After cutting tissues finely and passing through a sterile sieve, the larger fragments were forced through the sieve using sterile syringe plunger and after washing they were cultured in RPMI containing 10% fetal calf serum (FCS) and incubated overnight at 37°C. The non-adherent cells and cell debris were removed and replaced by fresh medium. The adherent cells were fed until confluence (1–2 × 10^6 per 25 cm^2 flask) and were expanded by trypsinisation and subculturing at lower (0.5 × 10^6 per 25 cm^2 flask) seed in new culture flasks.

Binding assay

Tumour cells (10^4 per well) were treated with IFNs for 48 h in flat-bottomed microtitre plates and appropriate concentrations of specific monoclonal antibodies (50 μl per well, in three replicates) containing 0.02% sodium azide were added and incubated for 45 min at room temperature. After three washes, 50 μl of diluted (in RPMI plus 10% FCS and 0.02% azide) iodinated rabbit-antimouse antibody (50 000 cpm per well, Amersham) were added and incubation was continued for a further 45 min. Following three washes, the cells were lysed with 100 μl per well of 2% Triton X100 in water and the degree of radioactivity in the supernatants was measured using a gamma counter.

Immunocytochemistry

Exponentially growing cells were trypsinised and aliquoted (10^4 cells per well) onto sterile multichamber microscope slides (Gibco) and incubated for 48 h at 37°C, after which the cells were air dried, fixed in acetone for 5 min and kept at −40°C until use. The expression of test antigens was determined using a previously described technique [10].

IFNs and monoclonal antibodies

IFN-α (Welferon), β and γ were obtained from Wellcome, Bioferon and Biogen, respectively. The monoclonal antibodies (Mabs) were W6/32 (detects all beta 2m-associated HLA-A,B,C antigens [12]), HC10 (detects non-beta 2m-associated HLA-A, B,C antigens [13]), BBM.1 (detects beta 2-m [12]), ME1 detects HLA-B7 (gift from A McMichael Lab, Oxford), BB7.2 (detects HLA-A2 and Aw69 [11]), GAP-A3 (detects HLA-A3, [14]), L243 (detects DR [15]) and RR1/1 (detects intercellular adhesion molecule-1 [16]).

Biochemical analysis of MHC antigens

Metabolic labelling of cell lines using radioactive ^{35}S-methionine was carried out as described by Jones *et al.* [17]. Labelled cells were lysed in a buffer containing Nondiet P40 before preclearance with protein-A. Test antigens were precipitated using appropriate Mab-conjugated protein-A-Sepharose beads (Pharmacia). After washing, antigens were eluted by boiling in reducing sodium dodecyl sulphate (SDS) sample buffer, prior to estimation of radioactivity. Lysates were electrophoresed using the Biorad mini-protean II gel system, employing 15% SDS polyacrylimide gels. After drying, the gels were exposed to Kodak X-Omat AR film at −70°C.

Cell lines

Cell lines Fen, Ha and Lan were in-house established lines from tumour biopsy samples of patients with transitional cell carcinoma, teratoma and seminoma, respectively. For T24, J82, RT4, TccSup, Scaber, 5637, RT112, SKV14, TccDes (bladder) and Tera I, Tera II (testis) lines [18] for EP2012 [19], for Wil [20], MCF7 (breast) from the human cell culture bank (Mason Research Institute Rockville) and T47D (breast) from ECACC (catalogue no. 85102201).

Statistical analysis

Analysis of results were carried out using the paired *t*-test.

RESULTS

Development of cell lines

From the total of 100 tumour biopsies, four permanent cell lines, two bladder (Wil and Fen) and two testis (Lan and Ha) were established and subcultured more than 30 times. Morphological and cell surface antigen profile demonstrated the epithelial origin of the cells (results not shown).

Assessment of class I antigen expression

The amount of increased class I antigen expression induced in reponse to IFN-γ treatment was used to subdivide the cell lines into three groups (Table 1): (1) lines which showed no binding, i.e. values equal or below the background counts (< 100 cpm) e.g. Fen and Ha. (2) Lines which after IFN treatment showed increased binding over the spontaneous levels, referred to as 'low expressor', e.g. Tera II and EP2012. (3) Cell lines which in response to IFNs showed no change over already high spontaneous levels, referred to as 'high expressor', e.g. T24 and RT112.

Effective doses of IFNs

Two testis and two bladder lines were used to establish optimum concentrations of IFNs, results of which are presented in Table 1. The Tera II line was spontaneously a low class I expressor and IFN-γ at 100 U/ml upregulated this by more than 10-fold, whereas at concentrations as high as 1000 U/ml, there was no effect on class II antigen expression. In the case of Ha the situation was reversed. Whilst IFN-γ failed to induce class I on this class I negative line, it induced a 10-fold increase in class II antigens. Using this approach, optimum concentrations for IFN-α, β and γ were found to be 1000, 2000 and 100 U/ml, respectively and were used for subsequent experiments. The viability of cells at these concentrations show no sign of toxicity (results not shown).

The levels of class I and II antigen expression in response to IFN-γ by different lines were investigated, the results of which are presented in Tables 2 and 3. Class I antigen expression on non-testis lines ranged from [mean (S.D.)] 566 (161) to 2064 (407) cpm and IFN-γ stimulation did not produce significant increases in any of the lines except T47D. Class I negative line (Fen) again remained unaffected. None of the lines were positive

Table 1. Expression of class I and II antigens on two bladder and two testis cell lines treated with and without IFN-γ

IFN-γ (μ/ml)	Tera II		Ha		Fen		RT112	
	Class I	Class II	Class I	Class II	Class I	Class II	Class I	Class II
NT	207 (16)	59 (34)	72 (19)	134 (88)	108 (30)	58 (23)	1635 (189)	43 (11)
1	258 (46)	47 (4)	72 (27)	316 (30)	68 (12)	225 (4)	1930 (199)	48 (7)
10	1426 (17)	44 (27)	111 (21)	1368 (242)	82 (17)	1090 (43)	2287 (348)	148 (47)
100	2243 (197)	83 (32)	42 (16)	1589 (150)	76 (12)	1104 (51)	2092 (124)	230 (58)
1000	2243 (134)	52 (10)	64 (16)	1538 (103)	64 (18)	1183 (61)	1809 (140)	221 (33)

Results are expressed as mean (S.D.) of three replicates. IFN-γ was added at the beginning of 48 h culture.
NT = no treatment.

Table 2. Class I and II antigen expression by bladder and breast cell lines before and after IFN-γ treatment

	Class I		Class II	
	NT	IFN-γ	NT	IFN-γ
RT4	566 (161)	772 (123)‡	72 (22)	212 (92)†
Scaber	920 (109)	999 (58)‡	92 (12)	619 (24)*
RT112	1020 (57)	902 (64)‡	73 (14)	261 (28)†
T24	1786 (91)	2162 (234)‡	60 (12)	57 (18)‡
J82	2064 (407)	2284 (236)‡	53 (19)	1021 (122)*
TccSup	1680 (67)	1619 (198)‡	39 (7)	110 (53)*
Wil	1208 (67)	1194 (113)‡	44 (6)	1227 (118)*
Fen	64 (5)	76 (16)‡	58 (23)	1337 (34)*
SKV14	1627 (288)	2146 (118)‡	43 (18)	1484 (183)*
5637	1138 (110)	1150 (204)‡	65 (3)	1292 (143)*
MCF7	1186 (77)	1080 (59)‡	62 (14)	960 (114)*
T47D	1105 (77)	1438 (182)‡	93 (17)	1575 (174)*

Results are expressed as mean (S.D.) in cpm of three replicates.
*, † and ‡ indicate P values of 0.001, 0.01 and not significant, respectively.

for class II but 13/17 could be induced by IFN-γ to produce values ranging from 212 (92) to 1575 (174) cpm. T24 and TccSup, EP2012, Lan and Tera II lines failed to respond at all.

Among the testis lines, Ha and Tera I were negative for class I and IFNs failed to correct this deficit. However, IFN-γ upregulated these antigens on Tera II and EP2102 (i.e. low expressors) lines, but failed to induce class II antigens.

Table 3. Class I and II antigen expression by testis cell lines before and after IFN-γ treatment

	Class I		Class II	
	NT	IFN-γ	NT	IFN-γ
Tera I	63 (12)	65 (30)‡	40 (9)	677 (90)*
Tera II	240 (49)	1560 (139)*	59 (34)	93 (26)‡
EP2102	660 (108)	1222 (149)*	92 (72)	130 (32)‡
Lan	799 (76)	680 (32)‡	48 (8)	89 (22)‡
Ha	84 (14)	60 (37)‡	54 (12)	1005 (20)*

Results are expressed as mean (S.D.) in cpm of three replicates.
*, † and ‡ indicate P values of 0.001, 0.01 and not significant, respectively.

To establish whether the absence of IFN-γ receptor could explain the lack of MHC antigen inducibility, three cell lines Fen, RT112 and Wil were analysed for another inducible antigen. Levels of ICAM-1 before and after IFN-γ stimulation were 323 (29) and 512 (43) ($P = 0.001$); 76 (7) and 322 (15) ($P = 0.001$) and 110 (10) and 284 (21) ($P = 0.001$) cpm, respectively, indicating that the lack of response was not due the absence of IFN-γ receptor.

Intracellular expression of class I and II antigens

One other possible explanation for the absence of MHC antigens on tumour lines may have been due to defective antigen transport mechanism(s). In order to exclude this possibility, cells were grown on microscope slides, acetone fixed to expose internal antigens and stained. Consistent with the results of binding, Fen, Ha and Tera I lines showed negative staining, not only with W6/32, but also HC10 and BBM.1 antibodies indicating the absence of both light and heavy chains of class I antigens.

Effect of IFN-α and-β on class I and II expression

At optimum concentration, whilst IFN-α showed a similar pattern of activity to IFN-γ in upregulating class I antigens, it failed to have any effect on class II antigens (Table 4). On the other hand, IFN-β showed no activity on either class I or class II antigens (Table 5).

Table 4. Effects of combination of IFNs on induction of class I and II antigens by tumour cell lines

	NT	γ100	α1000	γ100 + α1000
Class I				
Tera II	177 (6)	1731 (27)	1326 (54)	1572 (144)
Ha	74 (12)	74 (10)	76 (10)	76 (15)
T24	2092 (299)	2635 (357)	2173 (132)	2500 (228)
TccSup	1590 (105)	1689 (271)	1641 (321)	1770 (216)
Class II				
Tera II	60 (10)	95 (29)	56 (8)	71 (11)
Ha	76 (15)	1166 (185)	83 (15)	949 (67)
T24	60 (12)	71 (11)	51 (9)	59 (17)
TccSup	39 (7)	60 (7)	41 (13)	71 (8)

Results are expressed as mean (S.D.) in cpm. Interferon (U/ml) was added at the beginning of the 48 h culture.

Table 5. Effects of combination of IFNs on induction of class I and II antigens by tumour cell lines

	EP2102	Fen	RT112
Class I			
NT	347 (52)	69 (26)	1056 (28)
γ100	566 (33)	62 (16)	1421 (138)
γ100+ β1000	713 (112)	60 (14)	1431 (14)
γ100+ β2000	772 (74)	56 (9)	1294 (40)
Class II			
NT	52 (8)	62 (10)	66 (7)
γ100	40 (17)	1411 (110)	208 (45)
γ100+ β1000	56 (37)	670 (128)	164 (20)
γ100+ β2000	37 (6)	542 (60)	115 (13)

Results are expressed as mean (S.D.) in cpm. IFNs (U/ml) were added at the beginning of the culture.

Combined effects of cytokines on MHC antigens

Effects of IFN-α and -γ on class I and II antigen expression alone and in combination were investigated, the results from four representative lines are presented in Table 4. As expected, both IFNs upregulated class I antigens on Tera II with little or no effect on other lines. The combination of two IFNs did not alter the levels of expression, indicating that there is a finite maximum level of expression. The results for combined IFN-β and -γ on three lines are summarised in Table 5. The addition of β did not have a significant effect on IFN-γ stimulated class I antigen expression, however, class II antigen induction was inhibited by as much as 62%. Thus, the values for IFN-γ activated Fen and RT112 with and without IFN-β were 542 (60), 115 (13) and 1411 (110) ($P = 0.01$) and 208 (48) ($P = 0.01$) cpm, respectively, indicating the selective down regulation of class II antigens by IFN-β.

Selective upregulation of polymorphic class I antigens

Using Wil cell line, the effects of IFN-γ on upregulation of HLA-A,B,C, A2, A3, B7 and class II was investigated, the results of which are presented in Table 6. As can be seen, IFN-γ showed no significant upregulation of monomorphic class I antigens (as detected by W6/32 antibody) whilst polymorphic A2 and A3 antigens were upregulated by up to 3-fold. Under the same conditions, HLA-B7 the missing class I antigen (see discussion) could not be induced indicating that missing MHC antigen expression cannot be corrected by IFN-γ.

Table 6. Expression of class II and monomorphic and polymorphic class I antigens by Wil line with and without IFN-γ

	Untreated	Treated	*P* values
Class I	1208 (67)	1194 (113)	NS
Class II	59 (3)	1227 (118)	0.0001
HLA-A2	421 (87)	773 (49)	0.001
HLA-A3	851 (59)	1138 (128)	0.01
HLA-B7	61 (4)	73 (7)	NS

Results are expressed as mean (S.D.) in cpm of three replicates.
NS denotes not significant.

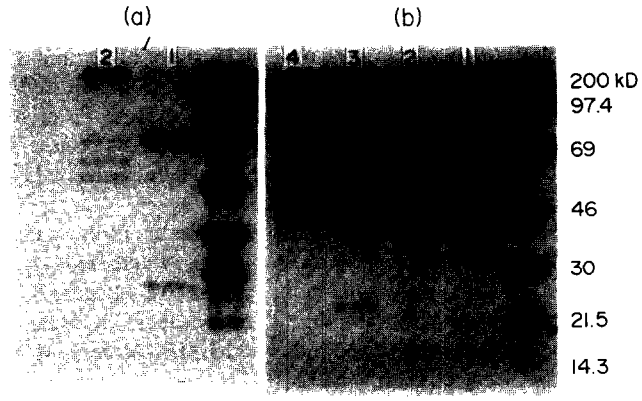

Fig. 1. Immunoprecipitating bands from: (a) Fen activated lymphocytes using W6/32 (lane 1) and HB55 (lane 2). Values denote Mwt markers. (b) Fen tumour cells using W6/32 (lanes 1 and 2) and HB55 (lanes 3 and 4). Lanes 1 and 3 are from unstimulated and lanes 2 and 4 from IFN-γ stimulated cells.

Biochemical analysis of MHC antigens on bladder and testis lines

In order to verify the binding results, biochemical analyses of MHC antigens were carried out using metabolic labelling technique, the results of which are presented in Fig. 1(a and b) and Fig. 2. As can be seen, activated lymphocytes from Fen (peripheral blood cells) expressed both class I (as seen by the presence of β2m band, lane 1) and class II antigens (as seen by the presence of α and β chain bands, lane 2). In the case of the Fen tumour line, whilst the cells were negative for class I before (Fig. 1b, lane 1) and after IFN-γ stimulation (Fig. 1b, lane 2), class II antigens were induced (Fig. 1b, lane 4). Similar patterns of precipitating bands were observed in the case of Ha cell line again indicating the inability of IFN-γ to upregulate missing class I whilst inducing class II antigens.

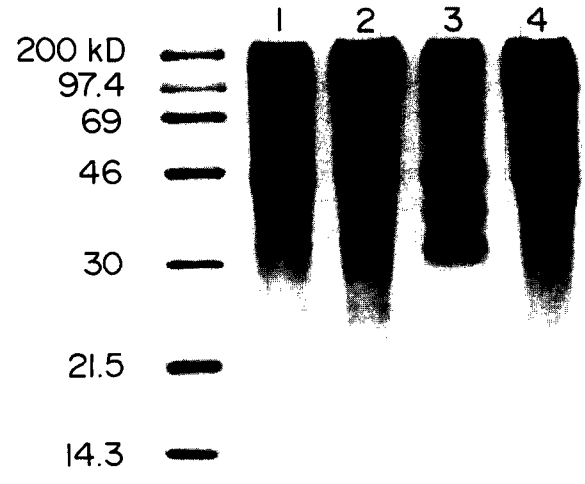

Fig. 2. Immunoprecipitating bands from Ha tumour line using W6/32 (lanes 1 and 2) and HB55 (3 and 4). Lanes 2 and 4 are from unstimulated and lanes 1 and 3 from IFN-γ stimulated cells.

DISCUSSION

The results of this investigation have demonstrated: (a) the total absence of class I antigens on 3 of 17 tumour lines and the inability of IFNs to correct this deficit, (b) both IFN-α and γ but not β upregulated class I antigens only on lines (3 of 17) expressing spontaneously low levels of antigens (c) class II antigens were induced by IFN-γ on all the cell lines except T24, TccDes, Tera II, EP2012 and Lan.

These findings add an additional dimension to our increasing knowledge of the abnormalities in MHC class I and II antigens on human tumours that could confer a selective advantage for the tumour to escape from immune T cell attack directed against tumour specific antigen(s) and indicate that, in addition to over expression of class II antigens [21], there are tumours where these antigens are not inducible. The frequency of non-inducible class II antigens in this series of bladder tumour lines (2/10) is similar to our observation on tumour sections using immunocytochemistry (1/11 of cases [10]) and those reported by Smith *et al.* in colon cancer tissues [11].

It is increasingly evident that decreased expression in some instances leading to total loss of HLA class I antigens is a common event in human cancers such as lymphoma [22], colorectal [11] and breast carcinomas [23]. Our own studies in bladder cancer demonstrated that 14 out of 18 tumours had diminished or total loss of class I antigens [10] and that IL-2 dependent tumour infiltrating lymphocytes were established more frequently from tumour biopsy specimens expressing normal levels of class I antigens [24].

For class II, the situation is more variable and reports are conflicting. With few exceptions, most cells are negative for these antigens, but they can be induced by IFN-γ. In melanoma, Natali *et al.* [21] reported that increasing expression of class II antigens on tumours was associated with increased malignancy. In contrast, Cohen *et al.* [7] reported that expression of class II antigens was associated with a more frequent response to IL-2 therapy. A similar observation has been made by Prescot *et al.* [9] who showed that patients responding to intravesical BCG therapy had a higher incidence of class II antigen expression and release of IFN-γ in their urine [25]. A possible explanation for these divergent views came from the observation of Alexander *et al.* [26] who demonstrated that the excess class II antigen was aberrant on both metastatic and non-metastatic melanoma, but only the metastatic melanoma was defective in presenting antigen to autologous T cells and that this defect could be corrected by transfecting in a normal version of the aberrant gene [27].

As far as understanding the mechanism(s) involved in down regulation of class I and II expression and the response to IFNs are concerned, our studies have made three observations. In contrast to a previous report indicating that the defective class I antigen expression might either be due to the loss of beta-2m or free heavy chain of class I [28], in this study, class I negative lines were negative for both these molecules. Second, the absence of cell surface antigen was not due to defective transport mechanism, since acetone fixation, which allows detection of intracellular antigens, did not alter the pattern of staining. Third, the failure to correct missing class I or to induce class II antigens was not due to the absence of IFN receptors, since under the same conditions ICAM-1 was upregulated.

Our results indicated that only 2 of 5 testis, 0 of 10 bladder and 1 of 2 breast lines showed a significant upregulation of class I antigens in response to IFN-α and -γ. However, in the one instance tested (Wil) there was a significant upregulation of polymorphic A2 and A3 but no effect on monomorphic or the lost class I polymorphic B7 antigens. For those lines with total absence of class I and, in the one case, with selective loss of a single polymorphic determinant, neither IFN-α nor -γ were able to induce re-expression. Northern blot analysis of the cell line has shown absence of mRNA for B44 transcript (results not shown) indicating that the defect might either be at the transcriptional or DNA levels.

Taking the recent report that early bladder cancer is responsive to IFN-α [29] and the ease of obtaining exfoliated cells from urine, it may be possible to serially study urinary cells for their response to IFNs *in vitro* and correlate the results with response to treatment. There are two recent observations in support of this approach. Firstly, Prescot *et al.* reported on the correlation of clinical response of bladder tumours to BCG, the degree of induction of class II on tumour biopsies [9] and the level of IFN-γ release into the urine and secondly, our own recent studies showing a correlation between the degree of class I expression on tumour biopsies and the development of tumour infiltrating lymphocytes after treatment of the tumour cells *in vitro* with IL-2 [24].

The findings reported in this paper on the variable degree of alteration of HLA class I and II after IFN stimulation taken with the above mentioned studies provide real hope that it may be possible to develop a practical *in vitro* assay to predict the minority of patients whose tumours will respond to cytokine therapy.

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Stability of K-*ras* Mutations Throughout the Natural History of Human Colorectal Cancer

Lorena Losi, Jean Benhattar and Jose Costa

We have used a rapid, non-radioactive and sensitive method based on allele-specific amplification using the polymerase chain reaction for the identification of K-*ras* mutations in archival tissues of colorectal carcinomas. Our purpose was to determine whether or not K-*ras* mutation provides, when present, a tumour marker throughout the natural history of the disease. We have studied 35 patients who developed recurrent cancer. In 71% of these patients a *ras* mutation in codons 12 or 13 was observed in the primary tumour. For each of these cases an identical *ras* mutation was found in the DNA from the local or distant recurrence. In the 29% of cases where no *ras* mutation was observed in the primary tumour, no newly acquired *ras* mutation appeared in the recurrent tumour. The time interval between primary tumours and recurrences varied from 3 to 60 months. Our results indicate that K-*ras* mutation provides a stable tumour marker throughout the natural history of colorectal cancer.
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INTRODUCTION

THE FAMILY of *ras* genes is frequently found mutated in human tumours [1–2]. The protein product of these genes acquires transforming potential when a substitution of a single aminoacid occurs at a critical position [3]. The incidence of mutated *ras* genes varies strongly among different tumour types. The highest incidences are found in adenocarcinoma of the pancreas [4, 5], lung [6], thyroid tumours [7, 8], myeloid leukaemia [9], and colorectal cancer [10, 11]. In colorectal tumours, a K-*ras* mutation is present in approximately 50% of cases and occurs in codons 12, 13 and 61. Study of the clonal composition of human colorectal tumours has shown that all tumours examined, including very small adenomas, have a monoclonal composition [12]. *ras* mutations are acquired at a relatively early stage of carcinogenesis [13, 14] and they can serve, when present, as clonal markers of the tumour cell population. Local recurrences and metastases are very frequent events in colon cancer [15]. Specific *ras* mutations found in recurrences could also serve to establish their clonal origin with respect to a primary tumour when the mutation found in both tissues is identical.