Detection of circulating antibodies against c-myc protein in cancer patient sera

K. Ben-Mahrez\(^1\), D. Thierry\(^2\), I. Sorokine\(^1\), A. Danna-Muller\(^1\) & M. Kohiyama\(^1\)

\(^1\)Institut Jacques Monod, Université Paris VII, 2 place Jussieu, 75251 Paris Cedex 05; \(^2\)Service de Radio-pathologie, Institut Curie, 26 rue d’Ulm, 75231 Paris Cedex 05, France.

Summary

We have partially purified an archaebacterial protein of 84 kD which shares common epitopes with the human c-myc protein as shown by its cross-reactivity with a commercialized anti-human c-myc antiserum.

An antiserum raised against the 84 kD protein recognizes a 60 kD protein from HL-60 nuclei. This protein is also recognized by the anti-human c-myc antiserum.

Using this archaebacterial protein as antigen for Western blot analysis, we found that the human c-myc oncogene product could be immunogenic and that it is possible, in some spontaneously occurring human tumours, to detect antibodies against the c-myc gene product in the serum of cancer patients.

It has been shown that cancer patients may develop a cell-mediated and humoral response to various associated antigens. This recognition process is difficult to identify because the responses expressed by both patients and healthy individuals are not specific. The target structures for immune recognition that have been characterized do not show real tumour specificity in most experiments, but the presence of antibodies during malignancies may be clinically important for prognosis and follow up (Mastrangelo et al., 1984). In contrast to recent progress in the cellular immunology of cancer, the role of humoral immunity in oncogenesis remains poorly understood.

‘Serum enhancement’ and ‘blocking antibody’ theories suggest that B cell activation and antibody production could promote tumour growth (Smith & Landy, 1970). On the other hand, numerous studies deal with the fact that specific antibodies can inhibit tumour cell growth in vitro or in vivo. It has been shown that the level of circulating antitumour antibodies may be modulated by tumour treatment. The fact that antibodies may be effective in preventing the development of metastasis rather than blocking the growth of the primary tumour has also stressed the positive aspects of humoral antitumour immunity (Seto et al., 1983; Witz, 1977; Vaage & Agarwal, 1974). Chemically induced tumours are often immunogenic: the host responds specifically in vivo to the tumour by generating a state of concomitant antitumour immunity (North, 1985).

Among spontaneously occurring human tumours, the specific immune recognition of antigenic tumour cells may be restricted to virally induced tumour and in particular to those associated with DNA viruses (Klein & Klein, 1985). Although several tumour antigens have been claimed to be specific, and analysed for their role in immunological responses in tumour-bearing host (Sulitzeanu, 1985), the immunogenicity of cellular oncogene products has not yet been reported. Among the latter is the c-myc family of oncogenes, the expression of which has been demonstrated in many tumours and cancer cell lines (Slamon et al., 1984; Sikora et al., 1985).

We have previously described the presence in the archaebacterium Halobacterium halobium genome of DNA fragments which hybridize with a v-myc probe. A comparison of appropriate H. halobium genomic clones with the v-myc gene showed regions of significant homology (Ben-Mahrez et al., 1988a). Moreover, hybridization experiments showed that these bacteria possess two RNA molecules homologous to the v-myc oncogene (Ben-Mahrez et al., 1988b).

Experiments on the immunogenicity of the myc oncogene product are restricted by the fact that the purified protein is not available in quantity. We report here a solution to the problem of protein limitation, using the properties of a protein from H. halobium which reacts with an anti-human c-myc antiserum. Preliminary experiments suggest that the myc oncogene product could be immunogenic during the transformation process and that it is possible to detect antibodies against the myc protein in the serum of some cancer patients.

Materials and methods

Strain and culture

H. halobium CCM2090 was cultured in classical halophilic medium as described previously (Sehgal & Gibbons, 1960) to an absorbance of 1.4 at 600 nm.

Eukaryotic cell cultures

HL-60 cells were cultured as previously described (Grosso & Pitot, 1984). MCF-7 and OD262 cells were cultured in 10% foetal calf serum in MEM medium, at 37°C in 5% CO₂ (Kozbor & Croce, 1984).

Antibodies

Polyclonal antibodies raised against a human c-myc synthetic peptide were purchased from Oncor Inc. USA, anti-IgG antiserum linked to peroxidase was from Miles Scientific.

Cancer patients and healthy individuals sera

Blood samples from cancer patients and healthy individuals were obtained with informed consent. Serums were stored at −20°C prior to test.

Human c-myc synthetic peptides

Two different peptides were chemically synthesized by Toray Research Center (Japan). The E peptide sequence is: His-Gln-His-Asn-Tyr-Ala-Ala-Pro-Pro-Ser-Thr-Ary-Lys (amino acids 305 to 318 of the human c-myc protein). The F peptide corresponds to the carboxy-terminal amino acid sequence of the human c-myc protein (Colby et al., 1983): Arg-Lys-Arg-Glu-Gln-Leu-Lys-His-Lys-Leu-Glu-Gln-Leu-Arg-Asn-Ser-Cys-Ala.

Preparation of DNA free extracts from H. halobium

Twenty-five g of frozen H. Halobium cells were resuspended in 25 ml of buffer containing 25 mM HEPES pH 7.5, 3 mM KCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 2 mM

*Present address: CEA, IPSN, DPS, SHR, BP 6 92260 Fontenay Aux Roses Cedex, France.

Correspondence: M. Kohiyama.

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diethiothreitol and disrupted using a Potter homogenizer at 4°C. After centrifugation (100,000 g, 60 min) at 4°C, soluble and fast sedimenting materials were separated. The fast sedimenting materials containing cell envelopes and most of the nucleic acid were sonicated.

After centrifugation (5,000 g, 10 min), the upper phase was treated for 1 h at 37°C with DNase I (10 µg ml⁻¹ in 0.01 M MgCl₂) and tested by Western blot analysis for the presence of human c-myc like protein.

**Purification of the 84 kD protein**

The 84 kD protein was assayed by Western blot analysis using the anti-c-myc antiserum. The extract treated by DNase I and containing the c-myc like protein (1,000 mg of protein) was passed through a column of DEAE-cellulose (DE52) (3 x 15 cm) previously equilibrated with 20 mM sodium phosphate pH 7.3, 100 mM KCl and 10% (v/v) glycerol. Under these conditions, the 84 kD protein was found in the non-adsorbed and washed fractions which were then chromatographed on a column of hydroxylapatite (3 x 10 cm) previously equilibrated with 10 mM sodium phosphate pH 6.8, 100 mM KCl and 10% (v/v) glycerol. After washing, the 84 kD protein was eluted with 300 ml of 0.3 M Na₂HPO₄.

**Preparation of purified HL-60 nuclei**

HL-60 nuclei were purified according to the method of Lebkowski & Laemmli (1982).

**Western blot analysis**

The *H. halobium* or eukaryotic cell proteins were precipitated by trichloroacetic acid (6%) and the pellet was resuspended, after washing with acetone, in 10 mM sodium phosphate pH 7.0, 1% sodium dodecyl sulfate (SDS) and 1% 2-mercaptoethanol and heated at 100°C for 5 min. After electrophoresis on SDS-10% polyacrylamide gel, the proteins were electrophoretically transferred onto Millipore nitrocellulose at 60 volts for 2 h 30 min at 4°C. The transfer buffer consisted of 25 mM Tris-HCl pH 8.5, 192 mM glycine and 20% (v/v) methanol. After transfer, the blot was washed for 2h in buffer X (50 mM Tris-HCl pH 7.5, 200 mM NaCl, 0.1% Tween 20 and 0.25% gelatin) and incubated overnight at 4°C in buffer X containing the antibodies. Then, the blot was rinsed 3 times for 5 min in buffer X and incubated for 2h at 4°C with anti-IgG antiserum linked to peroxidase in buffer X.

After 3 rinses in buffer X without gelatin, the blot was revealed by incubating for 45 min in buffer containing 15 mg 4-chloro-1-naphthol (Sigma), 5 ml methanol, 25 ml of buffer (50 mM Tris-HCl pH 7.5 and 200 mM NaCl) and 20 µl H₂O₂ (9%).

**Preparation of an antiserum directed against the 84 kD protein**

A rabbit was immunized with 100 µg protein in Freund's complete adjuvant by injection at multiple s.c. sites. A booster immunization, containing 100 µg protein in Freund's incomplete adjuvant was administered after 3 weeks. Bleedings were performed 15 days after injection.

**Results**

**C-myc related protein of *H. halobium***

Figure 1 demonstrates that a protein of 84 kD present in fast sedimenting materials cross reacted with the anti-human c-myc antiserum. Such a cross-reacting protein was not seen in the soluble fraction.

We have examined whether this antiserum specifically recognizes the human c-myc protein. Using extracts of a human promyelocytic leukaemia cell line, HL-60, which is known to express c-myc (Grosso & Pitot, 1984), it was found that the anti-human c-myc reacted with two proteins of 60 kD and 42 kD (Figure 2a).

The presence of dimethylsulfoxide in HL-60 cell cultures is known to inhibit the synthesis of the c-myc protein (Grosso & Pitot, 1984). The disappearance of both the 60 kD and 42 kD proteins was observed after 48 h dimethylsulfoxide treatment (Figure 2b). It has already been reported that a 47 or 49 kD protein has a similar epitope to the c-myc protein and is subject to dimethylsulfoxide treatment (Faletto et al., 1985; Persson et al., 1984a). As the c-myc protein is a nuclear protein, but the 47 kD protein is not (Persson & Leder, 1984b), HL-60 nuclei were purified, extracted and tested with the anti-human c-myc antiserum. Only a peptide of 60 kD was detected by Western blot analysis (Figure 3c).

In order to confirm the identity between the 60 kD protein and the c-myc protein, two other human cell lines were examined, a breast carcinoma cell line MCF-7 which is negative (Kozbor & Croce, 1984) and an ovary carcinoma cell line OD262 which is positive, for c-myc expression.

Figure 4 demonstrates that the 60 kD protein was detected both in HL-60 and OD262 but not in MCF-7. From these results it was concluded that the 60 kD protein recognized by the antiserum (Oncor Inc.) is the human c-myc gene product.

**Polyclonal antiserum against the 84 kD protein**

In order to show further similarities in epitopes between the 84 kD protein of *H. halobium* and the c-myc protein, a polyclonal antibody against the 84 kD protein was prepared. For this purpose the halophile protein was purified from bacterial extracts (see Materials and methods). One major protein band of 84 kD was obtained when analysed on
polyacrylamide gel under denaturing conditions. A rabbit antiserum was raised against this preparation.

We then examined whether the antiserum against the 84 kD protein could recognize the c-myc protein by Western blot analysis. Figure 3 demonstrates that a 60 kD protein in nuclear extracts of HL-60 was revealed by our antiserum in almost the same way as the anti-c-myc antiserum, and that the loss of the c-myc protein provoked by dimethylsulfoxide treatment paralleled the absence of the 60 kD band in Western blot analysis.

**Recognition of the 84 kD protein by human sera**

The 84 kD protein preparation was transferred onto Millipore nitrocellulose and used to screen for the presence of antibodies against the protein in the sera of cancer patients or healthy individuals. Among the 212 sera tested 21 recognized the 84 kD protein in this screening test. Some examples are shown in Figure 5. In order to confirm the validity of the test, Western blot analysis was carried out using extracts of dimethylsulfoxide treated or untreated HL-60 cells. Despite the complexity of bands due to the homologous system, a band of 60 kD with a positive patient was seen only in extracts of non-differentiated HL-60 (Figure 6); which indicated recognition of the c-myc protein by the positive serum. The c-myc protein being found in nuclei, it was determined whether the 60 kD protein recognized by the positive serum was present in nuclear extracts. Figure 7 clearly shows that the positive serum reacted with a**nuclear** 60 kD protein.

None of the 41 sera screened contained antibodies to the 84 kD protein. Positive sera were from patients with colorectal cancer (4 out of 6), breast cancer (12 out of 125) osteosarcoma (1 out of 2), cancers of unknown origin (3 out of 3), ovarian cancer (1 out of 9) (Table I). Four sera from

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**Figure 2** HL-60 cell protein pattern recognized by anti-human c-myc antiserum using Western blot analysis: (a) 60 μg dimethylsulfoxide treated HL-60 proteins; (b) 60 μg HL-60 proteins. The human c-myc protein is indicated by a triangle.

**Figure 3** Western blot analysis of dimethylsulfoxide treated and untreated HL-60 nuclei: HL-60 was cultivated with (a) or without (b, c) dimethylsulfoxide (1.25%) for 48 h: (a, b) anti-84 kD protein antiserum (1/100 dilution); (c) anti-human c-myc antiserum (1/30 dilution). The human c-myc protein is indicated by a triangle.

**Figure 4** Protein patterns from various cell lines recognized by anti-human c-myc antiserum: (a) 60 μg dimethylsulfoxide treated HL-60 proteins; (b) 60 μg HL-60 proteins; (c) 60 μg OD262 proteins; (d) 60 μg MCF-7 proteins. The human c-myc protein is indicated by a triangle.
A 68-60-68K-60K-45K-25K

Figure 5 Screening of human sera: Samples from cancer patients were tested by Western blot analysis using 15 μg partially purified (~5% of total protein) 84 kD protein as antigen: 1, 2, 4, negative sera (1/20 dilution); 3, 5 positive sera (1/20 dilution). The 84 kD protein is indicated by a triangle.

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Table I Classification of responses according to cancer type

| Cancer type                  | No. positive | No. negative |
|------------------------------|--------------|--------------|
| Breast (adenocarcinoma)      | 12           | 113          |
| Melanoma                     | 0            | 5            |
| Colon                        | 4            | 2            |
| ORL                          | 0            | 3            |
| Ovary                        | 1            | 8            |
| Hodgkin's                    | 0            | 8            |
| Liver                        | 0            | 1            |
| Osteosarcoma                 | 1            | 1            |
| Uterus                       | 0            | 2            |
| Teratocarcinoma              | 0            | 1            |
| Neuroblastoma                | 0            | 2            |
| Cancers of unknown origin    | 3            | 0            |

Table I: Classification of responses according to cancer type

patients with autoimmune disease (lupus erythematosus) did not react with the 84 kD protein.

Competition between the 84 kD protein and synthetic c-myc peptides

Further evidence that the positive sera contain antibodies against epitopes of the c-myc protein was obtained when positive sera were pre-incubated with either peptide E corresponding to the amino acid sequence of the c-myc protein (305–318) or the peptide F(421–439). Recognition of the 84 kD protein by a positive serum was diminished after pre-incubation with either of the two synthetic peptides (Figure 8A).

Interestingly, another positive serum reacted only with peptide F and not with peptide E (Figure 8B).

These results demonstrate that the positive sera contain antibodies against epitopes contained in the amino acid sequences E and F of the c-myc protein.
anti-human c-myc antiserum. However, in common with other authors (Faletto et al. 1985; Persson et al. 1984a) this antiserum was found to recognize not only the c-myc protein but also another peptide of 42 kD. The latter protein disappears more slowly than the c-myc gene product when HL-60 cells are treated with dimethylsulfoxide (Faletto et al., 1985).

In the absence of absolute specificity of the antiserum the question was raised whether the 84 kD protein recognized by the c-myc antiserum was similar to the human 42 kD protein rather than to the human c-myc product. To answer the question, polyclonal antibody against the 84 kD protein was prepared which recognizes the nuclear 60 kD protein of HL-60. It was concluded that the 84 kD protein of H. halobium has similar epitopes to the human c-myc protein. Our results are consistent with the observed homologies between a part of the genome of H. halobium and the v-myc oncogene (Ben-Mahrez et al., 1988a).

Using the archaebacterial 84 kD protein (Figure 5) or HL-60 extracts (Figures 6 & 7) as antigen, it was found that 21 out of 212 cancer patient sera contained circulating antibodies against the c-myc protein. The results obtained with either of the two antigens (84 and 60 kD proteins) were the same. However, it is clear that the 84 kD protein preparation has two advantages over HL-60 extracts viz. the simplicity of the response patterns and the relative ease of preparation of the 84 kD protein.

Besides, it was shown by competition experiments that the positive sera reacted in a different manner with two peptides corresponding to two regions of the c-myc protein, probably indicating differences in the antibodies produced by the patients.

Although we have not studied a large panel of various cancer types statistically it appears that circulating antibodies to c-myc are not restricted to a particular type of cancer but are more likely to be present in patients with various types. The fact that many sera do not react with the 84 kD protein may be due to the absence of antibodies cross-reacting with the 84 kD protein (independently of the fact that anti c-myc antibodies may be present) or to an inhibitory effect of idiotypic antibodies in these sera.

As the myc gene product is a nuclear protein (Persson & Leder, 1984b), these antibodies could be classified among the antibodies to nuclear antigens (ANA), which have been clinically associated with numerous autoimmune diseases. In some malignant diseases ANAs may be important for diagnosis (Tan, 1982; Klein et al., 1974). On the other hand, an immunization process may occur when the c-myc product is exposed to immunocompetent cells due to tumour lysis provoked by a therapeutically induced or natural tumour necrosis. This immunization could be more frequent when the tumour is infiltrated by mononuclear cells, or when the tumour is close to a lymphoid organ such as Peyer's patches in colon tumours (Martin et al., 1986). Whether antibodies to the human c-myc protein reflect an autoimmune process or one induced by sensitisation during tumour necrotic or inflammatory processes remains to be studied.

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Discussion

Archaeabacteria possess several eukaryotic characteristics (Zillig et al., 1985). This observation has been supported by immunological techniques which have shown that the DNA polymerase or the alpha type DNA polymerase from archaeobacteria share common epitopes with their eukaryotic counterparts (Huet et al., 1983; Kohiyama et al., 1986). In the same way we have identified an 84 kD protein from H. halobium having similar epitopes to the human c-myc protein and developed a serological screening test to detect antibodies against the c-myc product.

The human c-myc protein was identified in the present paper as a nuclear protein from HL-60 cells of approximately 60 kD by Western blot analysis using a commercial anti-human c-myc antiserum. However, in common with other authors (Faletto et al. 1985; Persson et al. 1984a) this antiserum was found to recognize not only the c-myc protein but also another peptide of 42 kD. The latter protein disappears more slowly than the c-myc gene product when HL-60 cells are treated with dimethylsulfoxide (Faletto et al., 1985).

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