

Detection of Tumor-associated Antigen Gene Expression in Peripheral Blood by RT-PCR in Combination with the mRNA Isolation Kit for Blood/Bone Marrow

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Abstract

A poly(A)-mRNA purification procedure, applying Boehringer Mannheim's new RNA/DNA Stabilization Reagent for Blood/Bone Marrow and the new mRNA Isolation Kit for Blood/Bone Marrow, was tested to prove its applicability for the detection of disseminated tumor cells from peripheral blood, by use of the poly(A)-mRNA fraction in combination with a highly sensitive RT-PCR procedure. In order to establish the sensitivity of the technique, peripheral blood samples from healthy donors were spiked with defined numbers of melanoma cells from an established tumor cell line.

Introduction

The incidence of malignant melanoma of the skin is increasing at rates ranging from 3 and 7% per year in many European countries (1,10). To date, no chemotherapeutic regimens appear to prolong median survival of patients with metastatic disease in AJCC stages III and IV (2).

The possibility of generating clinically significant immune responses directed against tumor associated antigens (TAA), has long been suspected and the cloning of a number of melanoma TAAs (e.g., MAGE-1 or MAGE-3) led to the identification of several classes of HLA-class I restricted TAA epitopes, serving as targets to cytotoxic T-lymphocytes (CTL) (6,13,14). TAA of the MAGE family are of considerable interest in light of their specificity, albeit they are only expressed in a proportion of melanomas ranging from 30 to 60% of cases. Differentiation antigens, including MART-1/Melan-A (4,8), gp100 (5) and Tyrosinase (3,15) are expressed by tumor cells and by non-transformed melanocytes. Defined synthetic epitopes were sufficient to expand specific CTLs *in vivo* in melanoma patients (7). Almost all melanomas express these genes, which are detectable in the majority of melanoma cells, as shown by immunohistochemical studies.

Selection of patients for antigen-targeted immunotherapy relies on the analysis of antigen expression, as assessed by RT-PCR on mRNA isolated from tumor tissue. The possible presence of disseminated tumor cells in peripheral blood of patients undergoing treatment could serve as an independent prognostic criterion.

In order to obtain a high sensitivity in RT-PCR, inhibitory components that occur naturally in the blood, and high concentrations of total RNA should be avoided. Given these basic considerations, we tested a poly(A)-mRNA purification procedure developed by Boehringer Mannheim, consisting of a RNA/DNA Stabilization Reagent for Blood/Bone Marrow and a mRNA Isolation Kit for Blood/Bone Marrow. To prove applicability of the procedure for the detection of disseminated tumor cells from peripheral blood, we used the poly(A)-mRNA fraction in combination with a highly sensitive RT-PCR procedure (9,11). In order to establish the sensitivity of the technique, peripheral blood samples from healthy donors were spiked with defined numbers of melanoma cells from an established tumor cell line.

Material and Methods

In order to achieve semi-quantitative RT-PCR results, five 1 ml aliquots of peripheral blood from healthy donors were spiked with 1×10^4 , 1×10^3 , 1×10^2 , 1×10^1 , and 1 melanoma cells (HBL cell line, kindly provided by Dr. Ghanem, Free University of Brussels, Belgium). The poly(A)-mRNA was isolated by using the RNA/DNA Stabilization Reagent for Blood/Bone Marrow in combination with the mRNA Isolation Kit for Blood/Bone Marrow. One milliliter of blood, containing the spiked melanoma cells was lysed by addition of 5 ml of the RNA/DNA Stabilization Reagent for Blood/Bone Marrow, resulting in simultaneous lysis of cells and immediate stabilization of the nucleic acids, contained therein. Stabilized blood samples were stored at -20°C or immediately processed for poly(A)-mRNA purification, using the mRNA Isolation Kit for Blood/Bone Marrow as described in the pack insert. Total nucleic acids are prepared by taking advantage of their ability to non-specifically adsorb to silica surfaces (e.g., glass magnetic particles). Upon magnetic separation, total nucleic acids are eluted from the particles. The mRNA fraction is purified by hybridization to biotin-labeled oligo-dT and subsequent capturing by streptavidin-coated magnetic particles. The entire mRNA purified from each 1 ml aliquot was directly converted to cDNA according to standard procedures. Subsequently, the entire cDNA contained in each sample, corresponding to the templates of approximately 2×10^6 peripheral blood mononuclear cells (PBMCs) plus 1×10^4 , 1×10^3 , 1×10^2 , 1×10^1 , or 1 melanoma cells was used for one PCR reaction (see Figure 1).

The following primers were used for the PCR-reaction: β -actin sense, 5'-CACCCA-CACTGTGCCATC; β -actin anti-sense, 5'-CTAGAAGCATTGCGGTGAC, amplifying a 650-bp gene fragment; gp100 sense, 5'-CTGTGCCAGCCTGTCTAC; gp100 anti-sense, 5'-CACCAATGGGACAAGAGCAG, amplifying a 334-bp fragment; MART-1/Melan-A sense, 5'-AGATGCCAAGAGAA-GATCTC; MART/Melan-A anti-sense, 5'-GCTCTTAAGGTGAATAAGGTGG, amplifying a 364-bp gene fragment; Tyrosinase sense, 5'-TTGGCAGATTGTCTGTAGCC; Tyrosinase anti-sense, 5'-AGGCATTGTGC-ATGCTGCTT, amplifying a 284-bp gene

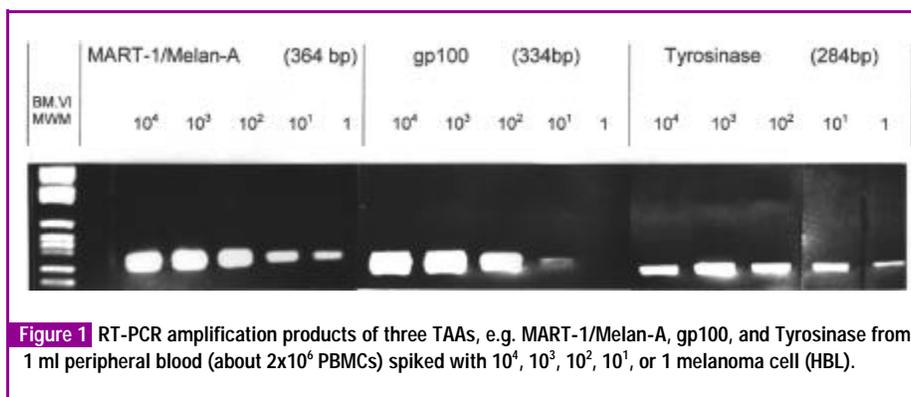


Figure 1 RT-PCR amplification products of three TAAs, e.g. MART-1/Melan-A, gp100, and Tyrosinase from 1 ml peripheral blood (about 2×10^6 PBMCs) spiked with 10^4 , 10^3 , 10^2 , 10^1 , or 1 melanoma cell (HBL).

fragment. All primer pairs were derived from different exons in order to allow discriminative detection of contaminant PCR products derived from genomic DNA. In addition, the absence of residual genomic DNA in the poly(A)-mRNA preparation was tested by performing PCR reactions with the mRNA preparations prior to cDNA generation.

PCR was performed using the following profile: 20 s of denaturation at 94°C , 20 s of annealing at 58°C , and 40 s of extension at 72°C using "hot-start" technique (5 min at 94°C). For the detection of Tyrosinase, gp100, and MART-1/Melan-A, the PCR was cycled 60 times, for the control β -actin, 25 times.

Results and Discussion

In order to validate use of the poly(A)-mRNA isolation procedure, using the RNA/DNA Stabilization Reagent for Blood/Bone Marrow and the mRNA Isolation Kit for Blood/Bone Marrow and to determine the sensitivity of the assay, we added varying numbers of cells from the melanoma cell line HBL to aliquots of peripheral blood. Previous expression studies had revealed a relatively high expression of these markers (MART-1/Melan-A, gp100, and Tyrosinase) in the cell line HBL as compared to other melanoma cell lines (data not shown). The purification procedure coupled to our RT-PCR protocol was sensitive enough to detect the expression of the TAA MART-1/Melan-A in as little as 1 cell per 1 ml blood within the background of the mRNAs of 2×10^6 PBMCs (Figure 1). For gp100, the detection limit was 10 cells/ml blood. Tyrosinase gene expression was detected down to 1 cell/ml blood. The results were obtained with a single PCR (60 cycles), which we preferred to nested PCR approaches because of the shorter handling

time. The qualities of the different RNA preparations were shown to be positive for β -actin (25 cycles/data not shown). All primer pairs were derived from different exons in order to allow discriminative detection of PCR products eventually derived from genomic DNA-contaminating cDNA preparations. The possibility of amplification of residual chromosomal DNA in the poly(A) mRNA preparation was ruled out by performing PCR reactions with the mRNA preparations prior to cDNA generation that gave no positive results.

We preferentially use poly(A)-mRNA instead of total RNA as templates for RT-PCRs. Comparing the same amounts of these two different types of RNA-templates, the target mRNA is clearly enriched in a poly(A)-mRNA. The absence of inhibitory factors, high concentrations of total-RNAs and chromosomal DNA in the poly(A)-mRNA preparations, additionally increased the sensitivity and specificity of the reactions (data not shown).

The use of the RNA/DNA Stabilization Reagent for Blood/Bone Marrow in this protocol both simplified the handling of the peripheral blood samples and enhanced reliability of the procedure. Ficoll[®] gradient centrifugations were not necessary and the risk of losing tumor cells during this step was avoided. Another advantage is the possibility to freeze and store the peripheral blood lysate right after the addition of the RNA/DNA Stabilization Reagent for Blood/Bone Marrow at -20°C , thus immediately preventing nucleic acids from degradation.

The products described in this article are for research purposes only.

References

1. Armstrong, B.K. (1988) Epidemiology of malignant melanoma: intermittent or total accumulated exposure to the sun? *J. Dermatol. Surg. Oncol.* **14**: 835–849.
2. Balch, C.M., Houghton, A.N., and Peters, L.C. (1993) Cutaneous melanoma. In DeVita, V.T., Hellman, S., and Rosenberg, S.A. (eds): *Cancer: Principles & practice of oncology*. Philadelphia: J.B. Lippincott Co., pp. 1612–1661.
3. Brichard, V., Van Pel, A., Wölfel, T., Wölfel, C., DePlaen, E., Lethé, B., Coulie, P., and Boon, T. (1993) The Tyrosinase gene codes for an antigen recognized by autologous cytolytic T-lymphocytes on HLA-A2 melanomas. *J. Exp. Med.* **178**: 489–495.
4. Coulie, P.G., Brichard, V., Van Pel, A., Wölfel, T., Schneider, J., Traversari, C., Mattei, S., De Plaen, E., Lurquin, C., Szikora, J.P., Renaud, J.C., and Boon, T. (1994) A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J. Exp. Med.* **180**: 35–42.
5. Cox, A.L., Skipper, J., Chen, Y., Henderson, R.A., Darrow, T.L., Shabanowitz, J., Engelhard, V.H., Hunt, D.F., and Slingluff, C.L. (1994) Identification of a peptide recognized by five melanoma-specific human cytotoxic T-cell lines. *Science* **264**: 716–719.
6. Gaugler, B., Van den Eynde, B., van der Bruggen, P., Romero, P., Gaforio, J.J., De Plaen, E., Lethé, B., Brasseur, F., and Boon, T. (1994) Human gene MAGE-3 codes for an antigen recognized on a melanoma by autologous cytolytic T-lymphocytes. *J. Exp. Med.* **179**: 921–930.
7. Jaeger, E., Bernhard, H., Romero, P., Ringhoffer, M., Arand, M., Karbach, J., Ilsemann, C., Hagedorn, M., and Knuth, A. (1996a) Generation of cytotoxic T-cell responses with synthetic melanoma-associated peptides *in vivo*: implications for tumor vaccines with melanoma-associated antigens. *Int. J. Cancer* **66**: 162–169.
8. Kawakami, Y., Elyahu, S., Delgado, C.H., Robbins, P.F., Rivoltini, L., Topalian, S.L., Miki, T., and Rosenberg, S.A. (1994) Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T-cells infiltrating into tumor. *Proc. Natl. Acad. Sci. U.S.A.* **91**: 3515–3519.
9. Noppen, C., Spagnoli, G. C., and Schaefer, C. (1996) Isolation of multiple mRNAs from a few eukaryotic cells: A fast method to obtain templates for reverse transcription-PCR (RT-PCR). *BioTechniques* **21**, (3):394–396.
10. Osterlind, A. (1992) Epidemiology on malignant melanoma in Europe. *Acta Oncol.* **31**: 903–908.
11. Schaefer, C. (1995) Isolation of mRNA from a few eukaryotic cells; a fast method to obtain template for highly sensitive quantitative reverse transkription-PCR (RT-PCR). *Boehringer Mannheim PCR manual*: 21–23.
12. Van der Bruggen, P., Traversari, C., Chomez, P., Lurquin, C., De Plaen, E., Van den Eynde, B., Knuth, A., and Boon, T. (1991) A gene encoding an antigen recognized by cytolytic T-lymphocytes on a human melanoma. *Science* **254**: 1643–1647.
13. Van der Bruggen, P., Bastin, J., Gajewski, T., Coulie, P.G., Boel, P., De Smet, C., Traversari, C., Townsend, A., and Boon, T. (1994a) A peptide encoded by human gene MAGE-3 and presented by HLA-A2 induces cytolytic T-lymphocytes that recognize tumor cells expressing MAGE-3. *Eur. J. Immunol.* **24**: 3038–3043.
14. Van der Bruggen, P., Szikora, J.P., Boel, P., Wildmann, C., Somville, M., Sensi, M., and Boon, T. (1994b) Autologous cytolytic T-lymphocytes recognize a MAGE-1 nonapeptide on melanomas expressing HLA-Cw*1601. *Eur. J. Immunol.* **24**: 2134–2140.
15. Wölfel, T., Van Pel, A., Brichard, V., Schneider, J., Seliger, B., Meyer zum Büschenfeld, K.-H., and Boon, T. (1994) Two Tyrosinase nonapeptides recognized on HLA-A2 melanomas by autologous cytolytic T-lymphocytes. *Eur. J. Immunol.* **24**: 759–764.

Product	Cat. No.	Pack Size
RNA/DNA Stabilization Reagent for Blood/Bone Marrow	1 934 317	for 50 ml of sample material
mRNA Isolation Kit for Blood/Bone Marrow	1 934 333	100 (50, 30) isolations from 1.5 ml (3 ml, 5 ml) isolation material

Also Available	Cat. No.	Pack Size
mRNA Isolation Kit for White Blood Cell (WBC)	1 934 325	100 (50, 30) isolations from 1.5 ml (3 ml, 5 ml) of sample material

These products are sold for laboratory use.