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Human Tumor Rejection Antigens MAGE[1]

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Characterization of the MAGE genes has facilitated a molecular approach to identification of the genes encoding tumor-rejection antigens expressed on human cancer cells. MAGE proteins are normal tissue antigens compartmentalized in testicular cells that play an important role in the early phase of spermatogenesis. MAGE-1, -2, -3, -4, and -6 genes are preferentially expressed in many different cancers at both the mRNA and protein levels. More than half of human cancers of various histologic type express at least one of these MAGE genes. Demethylation induces MAGE antigens in cells, suggesting that MAGE genes are important developmentally regulated genes under methylating control. Thus, genetic instability in cells causing loss of this methylating control could result in the preferential expression of MAGE genes in cancer cells. Therefore, MAGE gene products may be appropriate target molecules for development of new cancer vaccine.

Key words: human cancer, MAGE, peptide antigen, specific immunity, tumor-rejection antigen.

A body of data spanning two decades has shown that human cancer cells express tumor-rejection antigens recognized by cytotoxic T lymphocytes (CTL) on the appropriate MHC class I antigens (*1-3*). van der Bruggen *et al.* reported the identification of human gene MAGE-1 which directs the expression of a tumor-rejection antigen (*3*). The gene MAGE-1, encoding an antigen MZ2-E, was identified by a procedure based on gene transfection. The molecular techniques used for these studies allowed the cloning of several new genes encoding melanoma-related antigens recognized by CTL (*4-7*). These tumor-rejection antigens are not truly foreign, but rather are differentiation antigens expressed by compartmentalized normal tissues. Although the known universe of the identified genes encoding tumor-rejection antigens is small at present, it is expanding rapidly.

The MAGE-1 gene is comprised of three exons spread over 4.5 kilobases (kb) and shows no homology to any reported gene (*3*). The MAGE gene family consists of at least 12 closely related genes located on the long arm of chromosome X (*8*). Among them, the MAGE-1 or -3 gene codes for tumor antigens on HLA-A1 and -Cw1601 or -A1 and -A2 recognized by CTL, respectively (*9-12*). The other MAGE genes (MAGE-4a, -4b, and -6), with the exception of MAGE-2 and -12, also encode a peptide antigen capable of binding HLA-A1 (*9*). The MAGE-1, -2, -3, -4 (-4a/-4b), -6, and -12 genes are preferentially expressed at the mRNA level in many different cancers, including melanomas, lung cancers, head-and-neck cancers, esophageal cancers, ovarian cancers, and breast cancers (*8-22*). These seven genes code for proteins of approximately 300 amino acids and the sequences of these proteins display 80 to 98% homology at the nucleotide level and 65 to 97% homology at the protein level (*8, 23*). Structure of MAGE-1, -2, and -3 genes and position of antigenic peptide for HLA-A1 are shown in Fig. 1. In contrast to preferential expression in cancer cells, no normal cells other than testicular cells express the MAGE gene (*24*). These results suggest that MAGE gene products are appropriate target molecules for specific immunotherapy of cancer. This manuscript updates information of MAGE genes and proteins in normal and malignant cells, and discusses their potential as a cancer vaccine.

MAGE gene expression in normal cells; expression in spermatogonia and primary spermatocytes of testis

A panel of normal adult tissues and some tissues from fetuses over 20 weeks old were negative for MAGE gene at the mRNA level, with the exception of testis and placenta (*8, 24*). The testis expressed all the MAGE genes except the MAGE-7 gene, while the placenta expressed MAGE-3, -4 and MAGE-8 to -11 genes (*8*). Immunoblot analysis was used for detection of the MAGE-1 and MAGE-4 proteins in the testis using anti-MAGE-1 and -4 antibodies (Ab) (*24*). Polyclonal anti-MAGE-1 Ab was reactive to the testes from two donors, but failed to react to the negative control (PBMC) (Fig. 2). This Ab recognized a 38 kDa protein as a major band and the additional 42 and 45 kDa proteins in the testes. The molecular weight of cellular MAGE-1 protein in melanomas was reported as a 46 kDa (*24-26*) that was expressed in the HEL leukemia cell line taken as a positive control (Fig. 2). The other positive cell line (K562) possessed 38 and 45 kDa MAGE-1 proteins. In contrast, this Ab failed to react to PBMC from a healthy donor (Fig. 2).

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Both the polyclonal anti-MAGE-4 Ab and anti-MAGE-4 mouse monoclonal Ab (R5) were reactive to the testes, but failed to react to the negative control (PBMC) (Fig. 2). Both the Ab recognized a 45 kDa protein as a major band and the additional 28, 35, and 40 kDa proteins in the testes, and a 45 kDa protein in the RPMI-1788 B cell line (positive control) (Fig. 2).

Although detailed studies are under investigation, these 38 to 46 kDa proteins or 28 to 45 kDa proteins might be either the MAGE-1 or MAGE-4 protein by itself, respectively, with different levels of glycosylation. Alternatively, the 38 to 45 kDa MAGE-1 or 28 to 40 kDa MAGE-4 might be a part of the MAGE-1 or -4 protein, respectively, processed by low molecular weight proteins in the cytoplasm possessing proteasome activity. Otherwise, these minor differences at the molecules would be due to a proteolytic degradation when samples were solubilized.

Immunohistochemical studies were also used to identify the cells expressing the MAGE-1 and -4 proteins in the testes using these Ab. Both the MAGE-1 (data not shown) and -4 proteins (Fig. 3) were observed in the nucleus and the cytoplasm of spermatogonia, but undetectable in spermatids and Sertoli's cells. MAGE-1 and -4 proteins were also detectable in the primary spermatocytes adjacent to the basement membrane with relatively large nuclei, but not in the primary spermatocytes far from the basement membrane with relatively small nuclei (Fig. 3). Therefore, MAGE proteins are normal tissue antigens compartmentalized in testicular cells probably playing an important role in the early phase of the spermatogenesis.

Spermatogenesis is a complex process (*27*), which may be divided into three phases based on functional considerations: (i) the proliferative phase (spermatogonia) in which

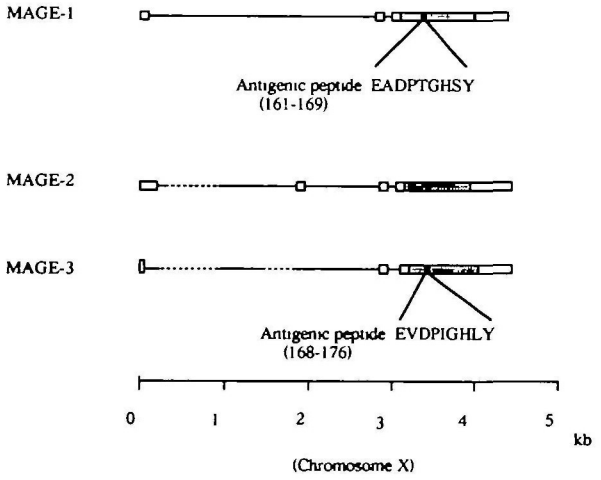

Fig. 1. **Structure of MAGE-1, -2, and -3 genes.** The lines show the genes that have been sequenced (*8*). Deletions are shown as dashed lines. Exons are shown as open boxes, with the open reading frame as a gray box. The region encoding the antigenic peptide on HLA-A1 recognized by CTL is marked in black

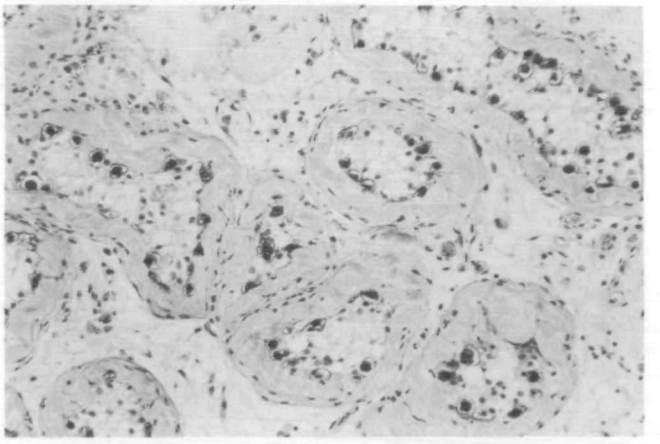

Fig 3 **MAGE-4 protein in the testicular cells.** Immunohistochemical studies were performed to identify the cells expressing MAGE-4 protein among the testicular cells with IgG of anti-MAGE-4b polyclonal Ab [×100]. Spermatogonia reactive to the Ab were adjacent to the basement membrane of the seminiferous tubules and smaller than the primary spermatocytes. Primary spermatocytes also reactive to the Ab were adjacent to the basement membrane and larger than the other cells with large nuclei Primary spermatocytes not reactive to the Ab were located far from the basement membrane with relatively small nuclei Spermatids were not reactive to the Ab, and were located far from the basement membrane and smaller than the primary spermatocytes or spermatogonia with mostly irregular shaped nuclei. Sertoli's cells were also not reactive, and were located far from the basement membrane with oval- or spindle-shaped nuclei.

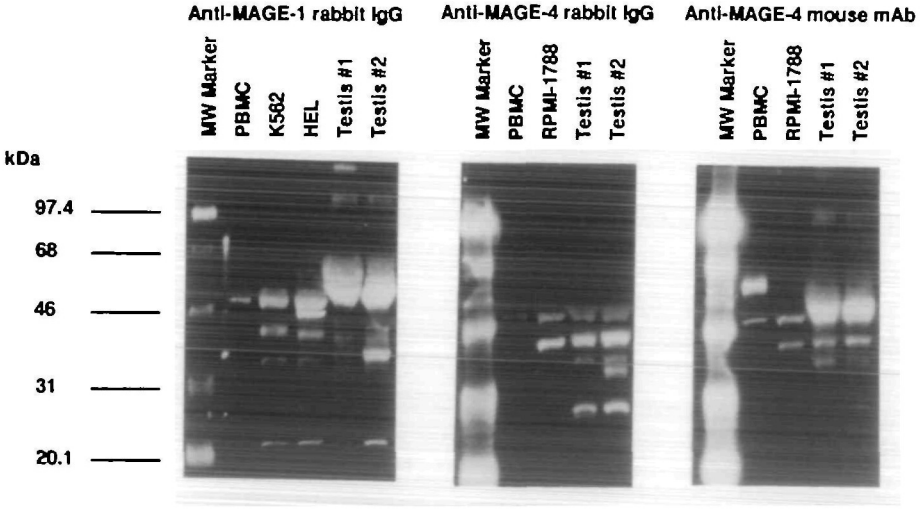

Fig. 2 **Immunoblot analysis of the MAGE-1 and -4 proteins.** The polyclonal anti-MAGE-1 and -MAGE-4 Ab and monoclonal anti-MAGE-4 Ab (R5 mAb) were tested for their reactivity to the positive control (K562 and HEL for MAGE-1 and RPMI-1788 for MAGE-4), the negative control (PBMC), and the testes of two donors by the immunoblot analysis.

cells undergo rapid divisions, (ii) the meiotic phase (spermatocytes) in which genetic material is recombined and segregated, and (iii) the differentiation phase (spermatids) in which spermatids transform into cells equipped to reach and fertilize the egg (*27*). The young spermatocytes, especially preleptotene spermatocytes, seem positive for the MAGE proteins based on their location and morphology. In contrast, the spermatocytes at the late stages seem negative. Other approaches shall be performed to identify the specific types of MAGE positive cells among the various stages of spermatocytes (*27*).

The expression of MAGE-4, but not MAGE-1 gene was observed at the mRNA level in the two placentas, in agreement with a previous report (*8*). However, neither MAGE-1 nor -4 protein was detectable in any of these placentas by immunoblot analysis (data not shown). Furthermore, none of these Ab immunohistochemically showed the apparent reactivity to any of the cells in the placentas (data not shown). Detailed studies using different placental specimens are under investigation.

Recent studies have demonstrated that the human tumor antigens recognized by CTL are not truly foreign, but rather are normal differentiation antigens expressed in the compartmentalized tissues. MAGE-1 gene is expressed in normal skin during wound healing (*28*). Our study has identified the MAGE protein positive testicular cells, and confirmed that MAGE proteins are one of the normal tissue antigens recognized by the host CTL. MAGE proteins would play an important role in the early phase of the spermatogenesis. These results are important findings relative to the biological functions of MAGE proteins and also important to understanding the nature of cancer antigens recognized by immune system.

Expression of the MAGE genes in various cancers at the mRNA level

The MAGE-1, -2, -3, -4 (-4a/-4b), and -6 genes are preferentially expressed in many different cancers at the mRNA level as evaluated by reverse transcriptase-polymerase chain reaction (RT-PCR) (*8–22*). More than half of

TABLE I. **Expression of MAGE genes in human cancers.**

	MAGE-1	MAGE-2	MAGE-3	MAGE-4
	(%)			
Melanoma	36		65	
Head and neck squamous cell carcinoma	33	41	43	27
Lung cancer	34	40	38	25
Gastric Cancer	44	56	56	
Serous adenocarcinoma (ovarian)	47	21	26	16
T cell leukemia	52	13	9	0

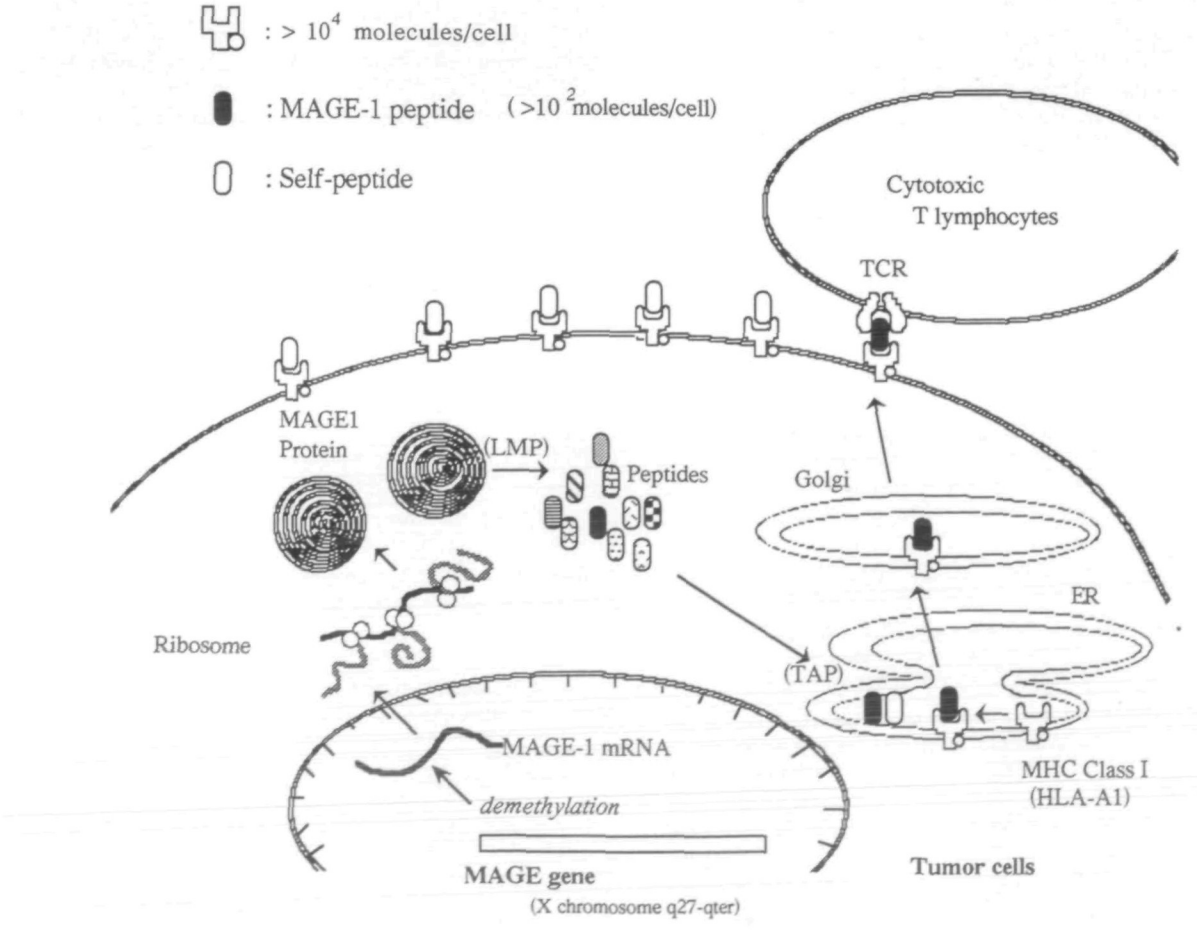

Fig. 4. **Expected scheme for expression of MAGE genes in cancer cells and T cell recognition of the peptide antigen on HLA-A1 molecule.**

human cancers of various types of histologies, except for renal cell carcinoma and myeloid leukemia, express at least one of these MAGE genes. A summary of MAGE-1, -2, -3, and -4 gene expression is shown in Table I. These cancers include metastatic melanoma, head and neck cancer, lung cancer, gastric cancer, ovarian cancer, and T cell leukemias (Table I). The MAGE-6 gene that possesses the highest homology with MAGE-3 gene is also highly expressed in various cancers with different histologies. A substantial number of breast cancers, esophageal cancers, bladder cancers also expressed MAGE genes (*28*). In contrast, myeloid-monocytic leukemia rarely expressed the MAGE-1 or -3 gene (*12, 24*).

Expression of the MAGE genes in cancer at the protein level

The investigation of MAGE gene products in cancer cells at the protein level has not been fully investigated. Therefore we studied the expression of the MAGE-4 protein in lung cancers with the polyclonal Ab and monoclonal Ab (R5 mAb) against recombinant MAGE-4b protein. Forty-four lung cancer tissue specimens and 12 established lung-cancer cell lines were investigated for expression of MAGE-4 protein by ELISA with these Ab. The limit of sensitivity was 10 pg/mg tissue for lung cancer tissues or 10 pg/10^4 cells for cell lines (*18, 29*). Thirteen of 44 (30%) cancer tissues possessed detectable levels of MAGE-4 protein, ranging from 18 to 55,989 pg/mg tissue. These 13 cancers were 4 of 30 (13%) adenocarcinomas, 7 of 10 (70%) squamous-cell carcinomas, and 2 of 3 small-cell lung carcinomas. MAGE-4 gene at the mRNA level was positive in 6 of 13 samples in which the levels of cellular MAGE-4 protein were high (570 to 55,989 pg/mg). In contrast, the MAGE-4 gene at the mRNA level was negative in the other 7 samples in which the levels of MAGE-4 protein were very low (18 to 362 pg/mg). None of the other 31 cancer tissues had >10 pg/mg of MAGE-4 protein or detectable levels of MAGE-4 gene expression at the mRNA level. None of nontumorous lung tissues, peripheral blood lymphocytes, cells of fibroblast cell lines or keratinocyte cell lines was positive for MAGE-4 protein (data not shown). These results suggest that MAGE antigens are consistently expressed in cancer cells and tissues at the protein level, and an ELISA with anti-MAGE-1 Ab is more sensitive and quantitatively accurate than the RT-PCR method for measuring MAGE gene products in cancers.

Induction of MAGE genes

We have recently found that the demethylating agent 5′-Aza-deoxycytocytididine (DAC) induces MAGE-1 antigen in normal and malignant lymphoid cells and also in myeloid-monocyte cell lines in most cases (Shichijo *et al.*, unpublished results). The other MAGE genes were also induced in these cells with few exceptions. These results suggest that demethylation alone is a sufficient stimulus to induce MAGE tumor-rejection antigens in both normal and malignant lymphoid cells in most cases. The eukaryotic genome is methylated at the 5 carbon of cytosines that occur in 5′-CpG-3′ dinucleotides (*30, 31*). Demethylation seems to up-regulate accessibility of DNA to transcription factors (*32*). Incubation of cells with DAC may make sufficient unmethylated DNA in the cells to allow binding of transcription factors to induce MAGE gene expression.

TABLE II. **MAGE antigen peptides and HLA-class I restriction.**

MAGE	Peptide	HLA-A locus
MAGE-1	EADPTGHSY	HLA-A1
MAGE-1	SAYGEPRKL	HLA Cw1601
MAGE-3	EVDPIGHLY	HLA-A1
MAGE-3	FLWGPRALY	HLA-A2
MAGE-4a	EVDPASNTY	HLA-A1
MAGE-4b	EVDPTSNTY	HLA-A1
MAGE-6	EVDPIGHVY	HLA-A1

Many developmentally-regulated genes are under methylation control. Demethylation caused by gene targeting of methyltransferase in mice results in severe developmental abnormalities and early death (*31*). MAGE tumor antigens are constantly observed in spermatogonia and a part of primary spermatocytes and seems to play an important role in early phase of spermatogenesis (*24*). All these results suggest that MAGE genes are important developmentally-regulated genes under methylating control. Genetic instability in cells may induce loss of this methylating control, resulting in the preferential expression of MAGE genes in cancer cells. Expected scheme for expression of MAGE genes in cancer cells and T cell recognition of the peptide antigen on HLA-A1 is shown in Fig. 4.

MAGE peptides recognized by CTL

Characterization of the MAGE genes has facilitated the molecular approach to identification of genes encoding tumor rejection antigens expressed on human cancer cells. The summarized scheme of expression of MAGE gene and T cell recognition of MAGE antigen on the groove of HLA-A1 molecule in cancer cells is shown in Fig. 4. A MAGE-1-encoded nonapeptide (EADPTGHSY) is presented to autologous CTL by an HLA-A1 molecule (*9*). MAGE antigen peptides presented by HLA-A molecules are shown in Table II. Like MAGE-1, a MAGE-3-encoded nonapeptide (EVDPIGHLY) is also presented to the CTL by an HLA-A1 molecule (*9, 12*). MAGE-4 and -6-encoded nonapeptides (EVDPASNTY and EVDPIGHVY, respectively), but not MAGE-2 encoded nonapeptide (EVVPISHLY) can competitively inhibit the binding of MAGE-1 or -3 nonapeptide to HLA-A1 molecule (*9*). Based on these results, the two amino acids asparatic acid [D] of the position 3 and tyrosine [Y] of the position 9 are suggested to be the agretopes that are needed for binding to the groove of HLA-A1 molecule (*9*). Epitope analysis using three CTL clones recognizing MAGE-1 nonapeptide on HLA-A1 have shown that modifications of residues at positions 5, 6, or 7 in the antigenic peptide affected recognition by the three CTL, while each of the modifications of residues at positions 1, 4, or 8 affected recognition by one CTL only (*33*). The sequences of T cell receptor α and β chains in these CTL clones are completely different from each other (*33*). These results suggest that the degree of diversity in terms of both epitope specificity and T cell receptor structure of tumor specific immunity is bigger than that of prediction.

The other MAGE-1-encoded nonapeptide (SAYGEPRKL) is also presented to CTL by an HLA-Cw1601 molecule (*10*). Furthermore, the MAGE-3 peptide (FLWGPRALV) seems to be presented to CTL by HLA-A2 (*11*). This MAGE-3 peptide may prove to be a useful target for specific anti-tumor immunization of HLA-A2+ cancer

patients if the peptide is truly recognized by CTL derived from cancer patients. The proportion of melanoma patients expressing this MAGE-3/HLA-A2 antigen would be larger than that expressing MAGE-1/HLA-A1 antigen because about half of individuals are HLA-A2 positive in Caucasian or other ethnic populations. The identification of additional MAGE-encoded tumor antigens presented by different HLA molecules will increase the number of patients eligible for immunization.

Development of cancer vaccine

Various non-specific immunotherapies has been energetically performed during the past two decades. These nonspecific immunotherapies are: (i) BCG, bacterial products (OK-432 *etc.*) and the other biological response modifies (BRM) all that could augment macrophage functions and natural killer (NK) cell activities; (ii) interferon, interleukin 2, or the other cytokines all that augment NK and macrophage functions and also induce lymphokine activated killer (LAK) cells; and (iii) adoptive transfer of LAK cells or tumor-infiltrating lymphocytes (TIL). However, almost all of these therapies failed to obtain consistent and observed clinical response even in melanomas and renal cell carcinomas that were relatively sensitive to immunotherapy. Monoclonal Ab against melanomas and lymphomas also failed to obtain consistent clinical response.

Several active immunization trials involving either allogeneic or autologous irradiated tumor cells have also been carried out over the past two decades. Other trials have involved irradiated autologous cells, such as renal carcinoma cells, colon carcinoma cells mixed with BCG or haptenized melanoma cells. In several of these trials, disease-free interval and patient survival showed improvement relative to controls. However, no clear conclusion has emerged as how to render these procedures more effective.

The identification of human tumor-rejection antigens and the following elucidation of molecular mechanisms of host-tumor interaction opens possibilities for development of new cancer vaccines. The patients likely to benefit from immunization with a defined antigen can be identified if a small tumor sample is available. The expression of genes encoding human tumor-rejection antigens can be ascertained readily with very small tumor sample. The patients can also be typed for HLA alleles. Thus, one should know whether a tumor expresses a peptide-HLA combination corresponding to a known tumor antigen recognized by CTL. For the immunization of these patients, many different forms of the defined antigens can be tried for optimal immunization. Finally, it should become possible to determine whether CTL directed against the relevant antigen are generated as a result of immunization. By proceeding in this way, it should be possible to systematically resolve some of the uncertainties of previous immunization attempts. Because of the preferential expression in different types of cancer cells, the MAGE gene products may be one of the most appropriate target molecules for development of a new cancer vaccine.

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