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Immunology

Identification of the *MAGE-1* gene product by monoclonal and polyclonal antibodies

(MAGE-1/tumor-rejection antigen/melanoma/testis)

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ABSTRACT **The human *MAGE-1* gene encodes a melanoma peptide antigen recognized by autologous cytotoxic T lymphocytes. To produce antibodies against the MAGE-1 gene product, several approaches were taken. Three oligopeptides were synthesized based on predicted MAGE-1 amino acid sequences and were used to generate rabbit anti-peptide antisera. In addition, a truncated *MAGE-1* cDNA was cloned into an *Escherichia coli* expression vector, and recombinant protein was produced and purified. All three rabbit anti-peptide antisera showed reactivity against the immunizing peptide, and one reacted with the recombinant MAGE-1 protein by immunoblotting, but none reacted with cell lysates from MAGE-1 mRNA-positive cells. The recombinant MAGE-1 protein was then used for the generation of mouse monoclonal and rabbit polyclonal antibodies. One IgG1 monoclonal antibody, MA454, as well as rabbit polyclonal antisera recognized a 46-kDa protein in extracts of MAGE-1 mRNA-positive melanoma cell lines. The antibodies showed no apparent cross-reactivity with products of the closely related *MAGE-2* and *MAGE-3* genes. Serological typing of normal and tumor cell lysates was in full agreement with mRNA analysis, showing expression of MAGE-1 protein in MAGE-1 mRNA-positive testis and a subset of melanomas but not in MAGE-1 mRNA-negative normal or tumor tissues. Transfection of the *MAGE-1* gene into a MAGE-1 mRNA-negative melanoma cell line resulted in the expression of the 46-kDa protein, confirming the identity of this protein as the *MAGE-1* gene product.**

The *MAGE-1* gene, cloned from human melanoma cell line MZ2-MEL, encodes the tumor-rejection antigen MZ2-E, which is recognized by autologous $CD8^+$ cytolytic T cells (CTLs) (1–3). *MAGE-1* consists of three exons, with the putative coding region residing entirely in the third exon. Further cloning work revealed that this gene belongs to a multigene family (2, 3). MZ2-MEL tumor cells, in addition to MAGE-1, also express *MAGE-2*, *MAGE-3*, and other closely related genes. The target for CTL recognition of MAGE-1 antigen was defined as a nonapeptide presented by HLA-A1 (4). *MAGE-2* and *MAGE-3* gene products, although structurally similar to *MAGE-1*, do not contain this nonapeptide and are not recognized by CTLs with specificity for MZ2-E.

The expression of MAGE-1 mRNA is detected in ≈40% of melanoma cell lines and melanoma tumors tested as well as in some breast tumors (5) and other tumor types (6). In the melanoma cell lines and tumor specimens, MAGE-2 and MAGE-3 mRNA expression does not coincide with MAGE-1 expression and appears to have a broader distribution (1, 7). In contrast to expression in malignant cells, initial analysis of mRNA expression of the *MAGE* gene family showed no evidence of expression in a wide range of normal tissues (2). Testicular tissue, however, was subsequently shown to express significant levels of several MAGE mRNA species, including MAGE-1 (7).

The protein product of *MAGE-1* and the other *MAGE* family members has not been identified. The present study was designed to develop a series of serological reagents against the *MAGE-1* gene product.

MATERIALS AND METHODS

Cell Lines and Tissues. Melanoma cell lines were previously described (1, 8). Normal and tumor tissues were obtained from the Departments of Pathology at The New York Hospital–Cornell Medical Center and Memorial Sloan–Kettering Cancer Center.

Detection of MAGE mRNA Expression. Expression of MAGE-1, MAGE-2, and MAGE-3 mRNA was determined by reverse transcription and subsequent polymerase chain reaction (RT-PCR) as described (2, 5). Briefly, total RNA was prepared from tissue culture cells or fresh-frozen tissue specimens. RT-PCR was then performed using several primer pairs. For MAGE-1 expression, primers CHO12 and CHO14 were used (5). MAGE-2 and MAGE-3 expression was analyzed in parallel using primers CHO8 and CHO9 (2). The latter two primers are derived from consensus sequences shared by *MAGE-1*, *MAGE-2*, and *MAGE-3* genes and allow amplification of cDNA from any of the three MAGE transcripts. PCR amplification was performed in a thermal cycler (Perkin–Elmer/Cetus), following conditions outlined previously (2, 5).

Prokaryotic Expression Cloning in *Escherichia coli*. For expression cloning, RT-PCR products were directly cloned into plasmid pT7Blue (Novagen). Clones were analyzed by restriction mapping and subcloned into expression plasmid vectors with six-histidine tag. Plasmids pQE9, pQE10, and pQE11 (Qiagen, Chatsworth, CA) were used. Induction of recombinant protein expression by isopropyl β-D-thiogalactoside and purification of the fusion protein by Ni^{2+} ion affinity chromatography were performed following procedures recommended by the manufacturer. Protein synthesis was monitored by $NaDodSO_4$/polyacrylamide gel electrophoresis and silver staining. The protein yield was determined by colorimetric protein quantitation assay (Bio-Rad). In addition to MAGE-1 recombinant protein, mouse p53 recombinant protein was similarly produced and was used as a control target in various serological assays.

Peptide Synthesis and Production of Rabbit Antisera. The oligopeptides were synthesized by Research Genetics (Huntsville, AL) using the multiple antigenic peptide resin

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Abbreviations: mAb, monoclonal antibody; RT-PCR, reverse transcription polymerase chain reaction; CTL, cytotoxic T lymphocyte.

strategy (9). Rabbit immunizations with peptides and fusion proteins were by HRP (Denver, PA). Lysate of *Escherichia coli* was used to absorb contaminating antibodies in the rabbit antiserum.

Mouse Hybridoma Production. Purified recombinant protein was used to immunize BALB/c mice, and hybridomas were generated and cloned as described (8). The hybridoma supernatants were screened by solid-phase ELISA on microtiter plates, using the immunizing fusion protein as the target antigen. Mouse p53 recombinant protein expressed in the same vector system was used as the negative control.

Immunoblotting. For immunoblotting, tissue culture cells or fresh-frozen tissue samples were homogenized in Nonidet P-40 buffer (1% Nonidet P-40/50 mM Tris·HCl, pH 8.0/150 mM NaCl). Immunoblotting of 2-mercaptoethanol-reduced lysates was performed using a chemiluminescent detection system (Amersham).

RESULTS

Production of Recombinant MAGE-1 Protein. Total RNA extracted from MZ2-MEL3.1 cells was used for RT-PCR with CHO8/CHO9 primer pairs. The PCR products were cloned into the pT7Blue plasmid, and restriction maps were generated to identify clones containing *MAGE-1* cDNA in the correct orientation for expression. The selected cDNA insert was subcloned unidirectionally into pQE9, pQE10, and pQE11 using *Bam*HI and *Hin*dIII cloning sites in pT7Blue. Recombinant protein production was induced by isopropyl β-D-thiogalactoside and screened by $NaDodSO_4$/polyacrylamide gel electrophoresis. This recombinant *MAGE-1* cDNA construct encodes 163 amino acids corresponding to codons 57–219 of the predicted *MAGE-1* gene product, as well as 30 residues encoded by the vector sequences (Fig. 1). Accordingly, the expected fusion protein has 193 amino acids, with an expected molecular mass of ≈20–22 kDa. Recombinant clones in pQE10 vector were found to carry the cDNA insert in the correct open reading frame and produced an ≈20-kDa recombinant protein upon isopropyl β-D-thiogalactoside induction. This protein was partially purified by Ni^{2+} metal affinity column chromatography (Fig. 2*A*). In addition to the 20-kDa species, minor protein species of 70 kDa, 43 kDa, 17 kDa, and 15 kDa were copurified (see below).

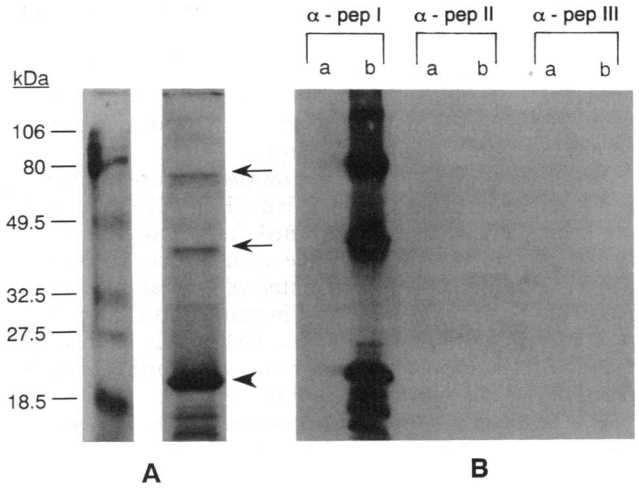
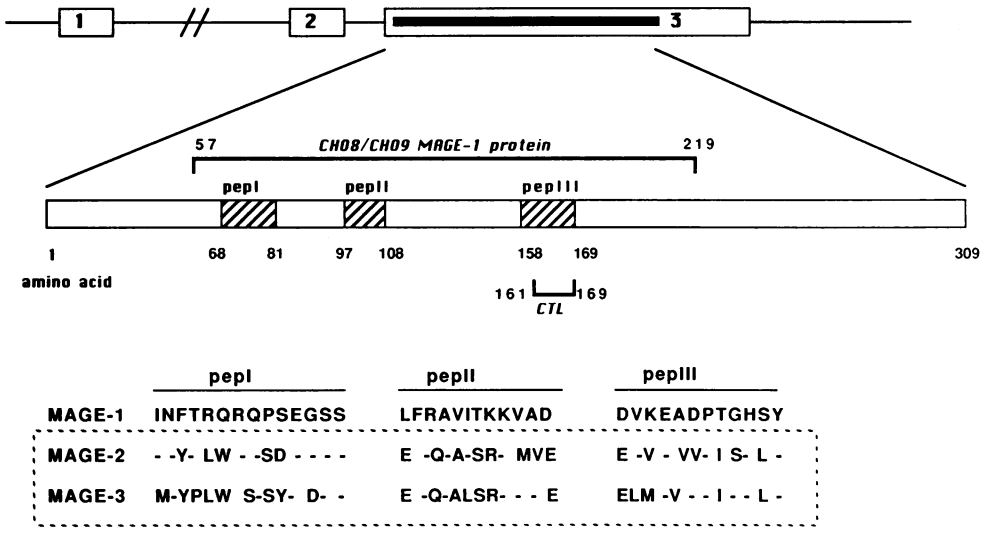


FIG. 2. (*A*) Silver-stained gel of affinity-purified CHO8/CHO9 MAGE-1 recombinant protein. The major species is at ≈20 kDa (arrowhead), and several minor components (e.g., ≈43 kDa and ≈70 kDa, arrows) are also present. (*B*) Immunoblotting of recombinant MAGE-1 protein (lanes b) against three rabbit anti-peptide antisera (α-pepI, α-pepII, and α-pepIII) at 1:1000 dilution. Only anti-pepI antiserum showed strong reactivity. In addition to the 20-kDa species, all other minor bands in silver-stained gel also showed positive reactions, indicating that they are related proteins. Recombinant mouse p53 protein (molecular mass, 45 kDa) expressed in the same vector system was nonreactive (lanes a).

Rabbit Anti-Peptide Antisera. Three oligopeptides (pepI, pepII, and pepIII) were synthesized based on the predicted MAGE-1 amino acid sequence and used for rabbit immunization (Fig. 1). pepIII, a 12-mer, contains the epitope recognized by CTLs (amino acids 161–169). By ELISA, all hyperimmune rabbit sera showed high-titer reactivity toward the immunizing peptides. However, only anti-pepI rabbit serum reacted strongly with the CHO8/CHO9 MAGE-1 recombinant protein by immunoblotting (Fig. 2*B*). The additional protein species copurified with the major 20-kDa fusion protein also showed reactivity with the antibody, suggesting that they represent aggregates of the 20-kDa fusion protein. All antibody reactivity could be absorbed by pepI but not by an unrelated peptide. The pepI sequence corresponds to



	pepI	pepII	pepIII
MAGE-1	INFTRQRQPSEGSS	LFRAVITKKVAD	DVKEADPTGHSY
MAGE-2	- -Y- LW - -SD - - - -	E -Q-A-SR- MVE	E -V - VV- I S- L -
MAGE-3	M-YPLW S-SY- D- -	E -Q-ALSR- - - E	ELM -V - - I - - L -

FIG. 1. MAGE-1 oligopeptides and recombinant protein. The *MAGE-1* gene (*Top*) consists of three exons (open boxes), with the coding sequence (solid bar) residing entirely within exon 3. (*Middle*) Detailed amino acid boundaries of the oligopeptides (pepI, pepII, and pepIII) and the CHO8/CHO9 MAGE-1 recombinant protein. The nonapeptide composing the CTL recognition site is also indicated (amino acid 161–169, *CTL*). (*Bottom*) Amino acid sequences of MAGE-1 pepI, pepII, and pepIII. The corresponding sequences from putative MAGE-2 and MAGE-3 proteins were compared, and dashes in the MAGE-2 and MAGE-3 sequences indicate sequence identity to MAGE-1.

amino acids 68–81 of the predicted MAGE-1 protein, and it shows 64% identity to MAGE-2 and 36% identity to MAGE-3 (Fig. 1). Despite the strong reactivity of anti-pepI against MAGE-1 recombinant protein, immunoblots with the cell lysate of MZ2-MEL3.1, a MAGE-1 mRNA-positive cell line, showed no detectable MAGE protein.

Mouse Monoclonal Antibodies (mAbs). Mouse mAbs were generated against partially purified CHO8/CHO9 MAGE-1 recombinant protein and screened for reactivity with the fusion protein by ELISA. Five clones reactive with the MAGE-1 fusion protein were obtained, and all five showed moderate to strong reactivity in immunoblots (Table 1). No reactivity was seen toward mouse p53 protein expressed in the same plasmid vector. These mAbs were further tested for reactivity against melanoma cell line lysates.

MA454 Detects a MAGE-1-Specific 46-kDa Protein. For testing mAb reactivity, Nonidet P-40 cell lysates of three cell lines were used as the screening panel—namely, MZ2-MEL3.1, MZ2-MEL2.2, and SK-MEL-187. MZ2-MEL2.2 is a *MAGE-1* loss variant derived from the *MAGE-1*-positive parental MZ2-MEL3.1 line by CTL selection (2). By RT-PCR, the MAGE mRNA expression profiles of these three cell lines were MAGE-$1^+2^+3^+$ (MZ2-MEL3.1), MAGE-$1^-2^+3^+$ (MZ2-MEL2.2), and MAGE-$1^-2^-3^-$ (SK-MEL-187).

Of the five fusion protein-reactive mAbs, one, MA454, reacted with a 46-kDa protein present in the MZ2-MEL3.1 lysate but not in MZ2-MEL2.2 or SK-MEL-187 lysates. Three additional melanoma cell lines were tested, and the results showed the presence of the 46-kDa protein only in cell lines positive for MAGE-1 mRNA, irrespective of MAGE-2 or MAGE-3 expression status (Fig. 3*a*).

Normal tissue lysates were prepared from liver, kidney, and testis. Tumor tissue lysates were obtained from four melanomas, including one MAGE-$1^+2^+3^+$, two MAGE-$1^-2^+3^+$, and one MAGE-$1^-2^-3^-$ by RT-PCR. The 46-kDa protein was identified in testis and in the MAGE-1^+ melanoma sample but not in the other tissues. This pattern was in complete agreement with the MAGE-1 mRNA expression data (Fig. 4).

Rabbit Antisera Against Recombinant MAGE-1 Protein. Two rabbits were immunized with the CHO8/CHO9 MAGE-1 fusion protein. The resulting antisera reacted with the fusion protein at a titer of 1:200,000 by ELISA. Immunoblotting with MZ2-MEL3.1, MZ2-MEL2.2, and SK-MEL-187 cell lysates revealed a 46-kDa protein in the MZ2-MEL3.1 cells, which comigrated with the protein recognized by MA454 (Fig. 3*b*). Results of tissue lysates were identical to those obtained with MA454 (Fig. 4*c*).

Reactivity of *MAGE-1* Transfectant with MAGE-1 Antibodies. To confirm that the 46-kDa protein recognized by MA454 and rabbit anti-MAGE-1 antisera is encoded by *MAGE-1*, we analyzed MZ2-MEL2.2-ET1, a MZ2-MEL2.2 subclone that had been transfected with the *MAGE-1* gene. This transfectant expresses the MAGE-1 protein and is recognized by MZ2-E-specific CTLs (2). Immunoblot analysis of this cell line identified the 46-kDa protein, as detected by MA454 and rabbit anti-MAGE-1. In contrast, the parental MZ2-MEL2.2 cell line was negative for the 46-kDa protein (Fig. 3*c*).

Table 1. Reactivity of mouse anti-recombinant MAGE-1 mAbs toward recombinant MAGE-1 protein and control p53 protein

	ELISA*		Immunoblot†	
mAb	MAGE-1	p53	MAGE-1	p53
MA32	++	−	++	−
MA231	+	−	++	−
MA399	++	−	++	−
MA430	++	−	+++	−
MA454	++	−	+++	−

*ELISA titer using hybridoma supernatants: −, <1:16; +, 1:64; ++, 1:256.

†Immunoblot signal intensity: −, negative; +, weak; ++, moderate; +++, strong.

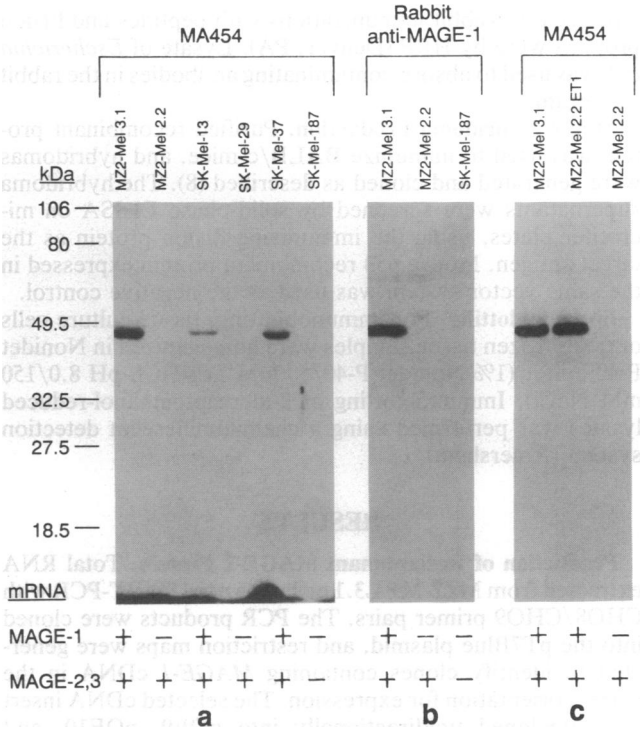


FIG. 3. Immunoblot analysis of anti-MAGE-1 antibodies against melanoma cell line lysates. The presence or absence of MAGE-1, MAGE-2, and MAGE-3 mRNA expression in these cell lines is indicated at the bottom. (*a*) Mouse mAb MA454 against six melanoma lines. Three cell lines—i.e., MZ2-MEL3.1, SK-MEL-13, and SK-MEL-37—were positive for MAGE-1 mRNA expression, and they were also positive for the 46-kDa species. (*b*) Rabbit anti-MAGE-1 polyclonal antiserum reacted with the identical 46-kDa protein in the MZ2-MEL3.1 lysate. (*c*) MZ2-MEL2.2-ET.1, a MAGE-1 transfected cell line, was positive for the 46-kDa protein, as detected by MA454. In contrast, the parental line (MZ2-MEL2.2) was negative.

DISCUSSION

The *MAGE-1*-encoded MZ2-E antigenic peptide of human melanoma is a prototype for molecularly defined human tumor antigens recognized by autologous CTLs (1, 2). Detailed characterization of this antigenic system may provide important leads toward the goal of augmenting T-cell-mediated immunity in cancer patients. As part of these studies, serological probes are essential to gain information about the expression of MAGE protein in normal and tumor tissues, the biochemical properties of these proteins, their subcellular distribution, and their function. The present report describes mAbs and polyclonal antibodies generated against the MAGE-1 protein using several different approaches.

Since the *MAGE-1* DNA sequence and its open reading frame are known, synthetic peptides based on the predicted amino acid sequence of the encoded protein were employed as immunogens. A similar approach has been used to generate mAbs and polyclonal antibodies toward various viral (e.g., refs. 11 and 12), parasitic (13), and cellular proteins (e.g., refs. 14–18). However, several studies have shown that a significant proportion of the antibodies generated by this strategy react well with the immunizing peptides but are unreactive with the corresponding intact protein (e.g., refs.

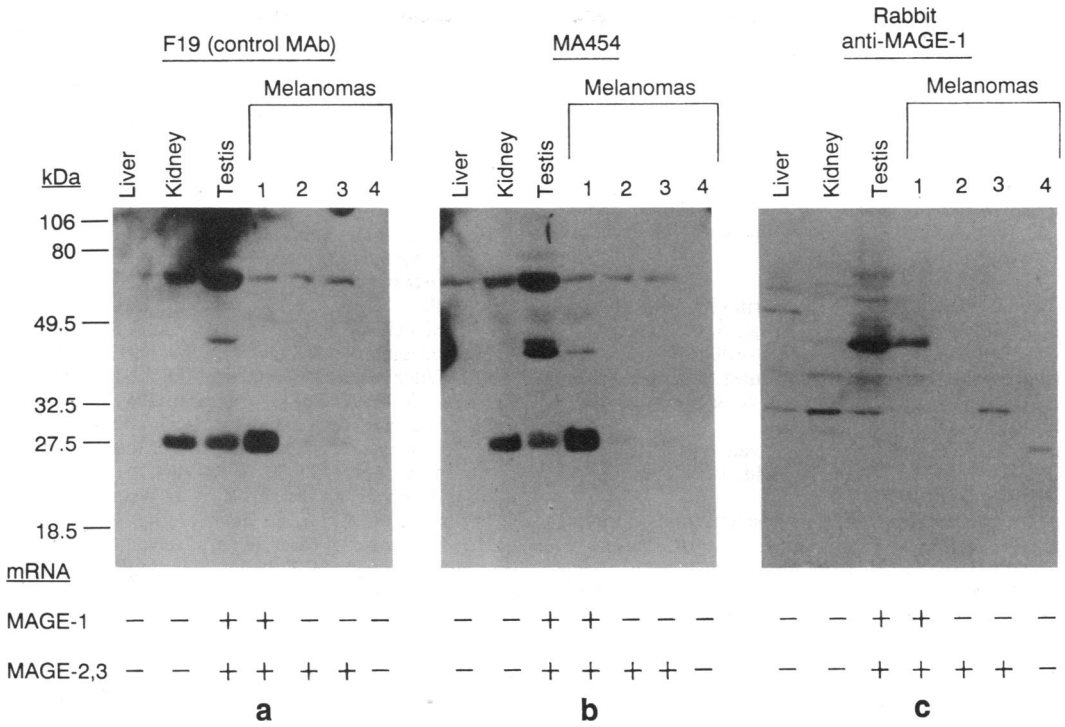


FIG. 4. Immunoblot analysis of anti-MAGE-1 antibodies against tissue lysates. Liver, kidney, testis, and four melanomas were tested, and the status of MAGE-1, -2, and -3 mRNA expression is shown at the bottom. An unrelated, nonreacting mAb (F19, ref. 10) was used as negative control mAb. The 46-kDa species was detected only in testis and one melanoma, in agreement with MAGE-1 mRNA expression.

16 and 17). In addition, anti-peptide antibodies may react only with denatured proteins but not with proteins in their native conformation (e.g., refs. 15 and 18). Several factors appear to be important in determining the reactivity of anti-peptide antibodies. These include the length of the immunizing peptide (19), the accessibility of the antigenic epitope, and the hydrophilicity of the peptide (20). Different carriers and the chemical linkage between carrier and peptides may also affect the serologic response (20, 21). In this study, three peptide segments were selected as immunogens for the production of MAGE-1-specific antibody. To enhance the probability of obtaining MAGE-1-specific reagents, peptide sequences with least sequence homology to the putative MAGE-2 and MAGE-3 proteins were chosen. However, this consideration significantly restricts the location and the size of the peptides synthesized. Only one of the three antisera reacted with the recombinant MAGE-1 protein in its denatured form, and none reacted with the intact protein from cell lysates by immunoblotting. Lack of reactivity of these rabbit anti-peptide antisera may be explained in part by the small size and/or the insufficient hydrophilicity of the immunizing peptides.

As an alternative approach, a prokaryotic expression system was used to generate MAGE-1 recombinant protein for the production of mouse mAbs and polyclonal rabbit antibody. Based on the high degree of overall sequence identity among *MAGE-1* and other *MAGE* genes, this approach appeared less likely to generate antibodies specific for the MAGE-1 gene product. However, MA454 and rabbit anti-recombinant MAGE-1 antisera show apparent MAGE-1 specificity, reacting only with 46-kDa products in MAGE-1 mRNA-positive samples. At least two explanations can be offered for this observation. (*i*) The antibodies are indeed MAGE-1 protein-specific, and selectivity may be due to the fact that MAGE-1 protein carries major immunogenic determinants not shared by the other MAGE proteins. (*ii*) The MAGE-1 protein may be expressed at much higher levels than other MAGE gene products, even in cells that express comparable levels of MAGE-1, -2, and -3 mRNA, and the antibodies may therefore not be MAGE-1 protein-specific.

Of five mAbs that reacted strongly with recombinant MAGE-1, only MA454 reacted with MAGE-1 protein product in cell lysates. The possibility that the other mAbs are directed against vector sequences in the fusion protein was ruled out by testing against recombinant mouse p53 molecule expressed in the same plasmid construct; it is therefore likely that the MAGE-1 epitopes recognized by these other mAbs represent antigenic determinants that are not accessible in the intact protein. Attempts to produce full-length MAGE-1 recombinant protein in the same plasmid have been unsuccessful (data not shown), and it is possible that full-length MAGE-1 protein may interfere with the growth of the host bacteria. Thus, prokaryotic expression systems, despite their relative simplicity, have certain drawbacks. First, not all eukaryotic genes can be expressed, and the level of protein expression is highly variable among genes that are expressed. Second, the antibodies generated using these proteins as immunogens, although having greater potential than anti-peptide antibodies, may still be suboptimal regarding their reactivity toward native cellular protein. Eukaryotic expression systems—e.g., the baculovirus vectors in insect cells (22)—may therefore provide an alternative in the study of some antigens. Despite the apparent MAGE-1 specificity of MA454 and polyclonal rabbit anti-MAGE-1 antibody, initial attempts to apply these reagents to immunohistochemical studies of tissue sections have been unsuccessful (data not shown). Although technical problems may play a role, it is also likely that these antibodies preferentially react with the denatured linearized molecule and have little affinity toward the native protein.

The apparent molecular mass of the MAGE-1 product defined in the present study, 46 kDa, is significantly higher than the 34.3-kDa protein product predicted on the basis of the primary *MAGE-1* sequence (7). A possible explanation for this discrepancy is that MAGE-1 proteins migrate in an anomalous fashion in $NaDodSO_4$/polyacrylamide gel. The

deduced MAGE-1 amino acid composition reveals a high content in acidic residues, which may reduce $NaDodSO_4$ binding and lower the polypeptide mobility during electrophoresis, as previously reported (23–26). Posttranslational modifications—e.g., glycosylation of the MAGE-1 protein—could also account for the increase in the molecular mass.

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