Expression of Melanoma Antigen Gene by Cells from Inflamed Joints in Juvenile Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** To determine if synovial fluid (SF) cells from inflamed joints of patients with juvenile rheumatoid arthritis (JRA) express melanoma antigen gene (MAGE) RNA and protein.

Methods. The pattern of MAGE-A1 expression was analyzed in inflammatory synovial tissue and peripheral blood mononuclear cells (PBMC) from patients with JRA by immunocytochemistry, reverse transcription-polymerase chain reaction (RT-PCR), and flow cytometry.

Results. MAGE-A1, previously detected only in tumor cells, is strongly expressed in SF cells from patients with JRA. Immunocytochemistry revealed strong staining of SF cells in all of 22 specimens tested. PBMC from patients (7 of 7) also expressed MAGE-A1, but not as strongly as SF cells. Two-color immunofluorescence showed colocalization with CD4 and CD14. Flow cytometry on 3 samples of SF cells confirmed the presence of MAGE-A1 on the cell surface and intracellularly. Five of 5 SF cell samples were positive for MAGE-A1 by RT-PCR.

Conclusion. Mononuclear cells from inflamed joints and blood from patients with JRA express MAGE-A1. MAGE family proteins were previously thought to be expressed only by certain tumors and presented by HLA Class I, resulting in tumor cell lysis by cytotoxic T cells. The observation of MAGE-A1 expression in JRA suggests an association with autoimmune disease and a possible causal role for MAGE antigen in the chronic inflammation of JRA. (J Rheumatol 2002;29:2219–24)

Key Indexing Terms:
AUTOIMMUNITY RHEUMATOID ARTHRITIS CHRONIC INFLAMMATION
ANTIGEN MAGE ANTIGEN PRESENTATION

Juvenile rheumatoid arthritis (JRA), a chronic inflammatory disease of the synovial membrane leading to cartilage and bone erosions, is characterized by activation and proliferation of synoviocytes and infiltration of inflammatory cells[1,2]. The prevailing hypothesis for the etiology of arthritis is that an initiating environmental event or events (viral or bacterial infection, toxins, and/or others) results in a state of chronic inflammation in certain genetically predisposed individuals. Despite extensive research, the initiating factors that lead to chronic inflammation are not well defined.

Melanoma antigen genes (MAGE) are primarily expressed in cancer cells. The synovial hyperplasia and pannus formation in patients with arthritis has several features suggestive of malignancy. The pannus formation resembles that of a localized neoplasm[2,3]. Features such as angiogenesis and pleomorphic fibroblast-like cells with large pale nuclei and prominent nucleoli are suggestive of the dysregulation seen in malignant cells. Further, cultured synoviocytes from rheumatoid joints have properties of transformed cells, i.e., they grow rapidly, lose contact inhibition, tend to form foci, and grow under anchorage independent conditions.

This synovial transformation has been associated with the activation of protooncogenes that regulate the cell cycle and intracellular signaling cascades. In RA, increased expression of protooncogenes, such as early growth response gene-1 (*egr*-1), c-*fos*, c-*jun*, and c-*myc*, has been implicated in synovial cell proliferation and invasion[3]. Such genes may be normally quiescent genes that are re-expressed in times of rapid proliferation or activation. One of the MAGE genes, was isolated from an expression library using sera from pediatric patients with systemic lupus erythematosus (SLE)[4]. This finding suggests an immune response to MAGE proteins in diseases of immune dysregulation, and prompted our investigation on the role of MAGE proteins in chronic inflammatory diseases. This report shows the expression of MAGE-A1 at the site of inflammation in patients with JRA. Synovial fluid (SF) cells from patients with JRA were strongly positive for MAGE-A1 using immunocytochemistry, reverse transcription-polymerase chain reaction (RT-PCR), and flow cytometry.

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Supported by grants from the Arthritis Foundation, So. California Chapter, Children's Hospital of Orange County Padrinos Foundation, and Pediatric & Adolescent Diabetes Research and Education (PADRE) Foundation.

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Submitted November 21, 2001; revision accepted April 1, 2002.

MATERIALS AND METHODS

Source of SF cells. SF aspirates were withdrawn for therapeutic reasons from patients attending the rheumatology clinic at Children's Hospital of Orange County (CHOC). Twenty-two pediatric patients with JRA who fulfilled the American College of Rheumatology 1986 revised criteria[5] were included. There were 18 females and 4 males ranging in age from 2 to 16 years (mean 10 yrs). The study included 9 pauciarticular, 3 pauciarticular to polyarticular, 6 polyarticular, and 4 systemic onset JRA. The Institutional Review Board at CHOC approved a protocol to collect blood and SF at the time of an essential test or during treatment. SF cells were spun down and washed in 1× Hanks' balanced salt solution (HBSS; Irvine Scientific, Irvine, CA, USA) within 1 h of obtaining the sample and resuspended in HBSS with 5% fetal calf serum for cytospins. Cytospins onto glass slides were performed using a cytocentrifuge (Thermo Shandon, Pittsburgh, PA, USA). Peripheral blood mononuclear cells (PBMC) were isolated from blood using Ficoll-Paque centrifugation (Amersham-Pharmacia, Piscataway, NJ, USA). Cytospin slides were stored at –20°C until use.

Immunocytochemistry. After fixation (acetone/methanol 1:1 for 2 min), slides were incubated at room temperature with murine monoclonal antibody to MAGE-A1 1:50 (MAGE-1 Ab-4; Neomarkers, Inc., Fremont, CA, USA), CD3, CD4, CD8 at 1:50, CD14 1:20 (Dako, Carpenteria, CA, USA) or control isotype IgG1 (MOPC 21; Sigma, St. Louis. MO, USA) for 3 h. Staining was performed using the Dako LSAB2 System, Peroxidase/AEC in the experiments shown in Figure 1. Slides were counterstained with Mayer's hematoxylin. Staining in the experiments shown in Figure 2 was performed using the Dako LSAB2 System, Peroxidase/DAB. Slides were counterstained with Harris hematoxylin.

For double labeling, slides were first incubated with anti-MAGE-A1 Mab (1 h), followed by phycoerythrin (PE) conjugated sheep anti-mouse IgG (Sigma) at 1:50 for 1 h. FITC labeled antibodies (1:100) (BD Biosciences; www.bdfacs.com) to CD3, CD4, or CD14 were then added for 1 h. Slides were analyzed by fluorescence microscopy.

Flow cytometry. Cells were centrifuged, resuspended in phosphate buffered saline (PBS) at 1×10^6 cells in 100 μl/tube, and incubated with anti-MAGE-A1 1:2 (MAGE-1 Ab-4; Neomarkers) or control isotype IgG1 (MOPC 21; Sigma) for 30 min at 4°C. The cells were centrifuged and incubated with sheep anti-mouse IgG-PE 1:10 (Sigma) for 30 min at 4°C. After centrifugation, the cells were resuspended in PBS for flow cytometric analysis. Intracellular staining was performed as above using the

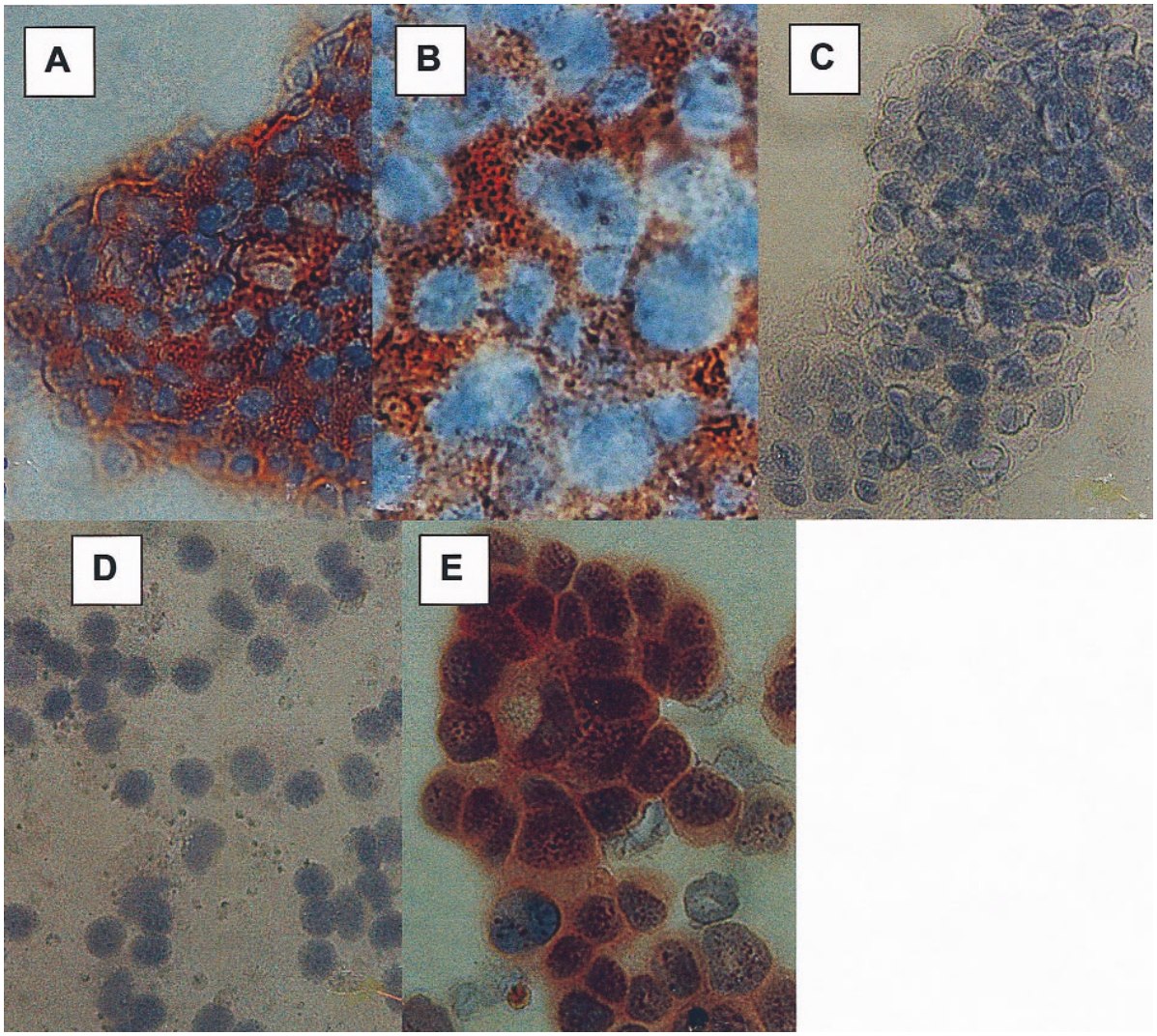

Figure 1. Immunocytochemistry with anti-MAGE-A1 Mab. Cytospin slides were stained with Mab to MAGE-A1 or control isotype antibody and anti-mouse Ig/HRP/AEC (Dako). A. SF cells with anti-MAGE-A1 (original magnification ×400). B. SF cells with anti-MAGE-A1 (original magnification ×1000). C. SF cells with isotype IgG control. D. Normal PBMC with anti-MAGE-A1. E. HEp-2 epithelial carcinoma line stained with anti-MAGE-A1.

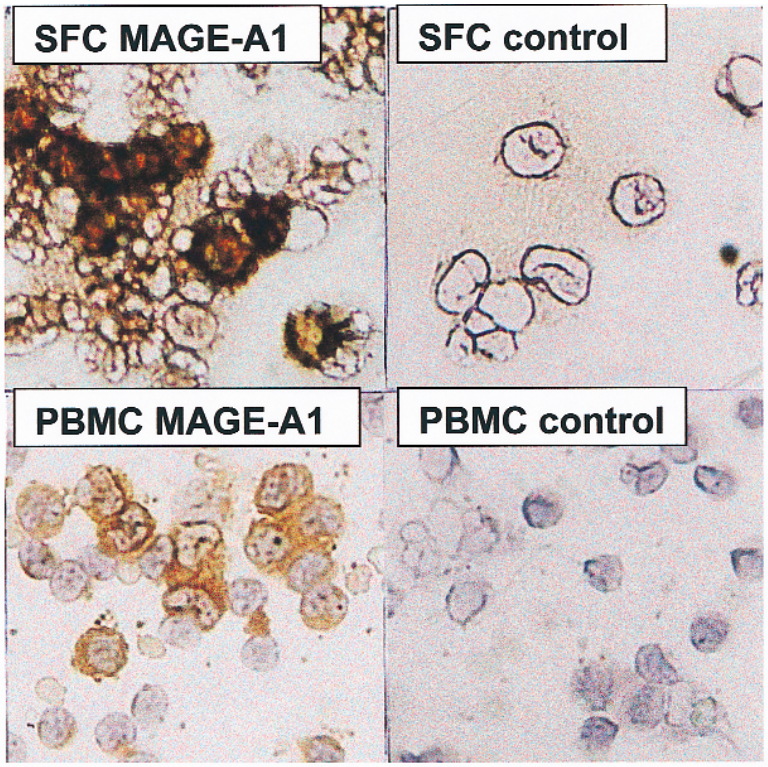

Figure 2. Immunocytochemistry with anti-MAGE-A1 Mab on SF cells and PBMC from a patient with polyarticular JRA. Cytospin slides were stained with Mab to MAGE-A1 or control isotype (IgG1) antibody (DAB). Top left: SF cells with anti-MAGE-A1. Top right: SF cells with isotype control antibody. Lower left: PBMC with anti-MAGE-A1. Lower right: PBMC with isotype (IgG1) control antibody. (All original magnification ×400.)

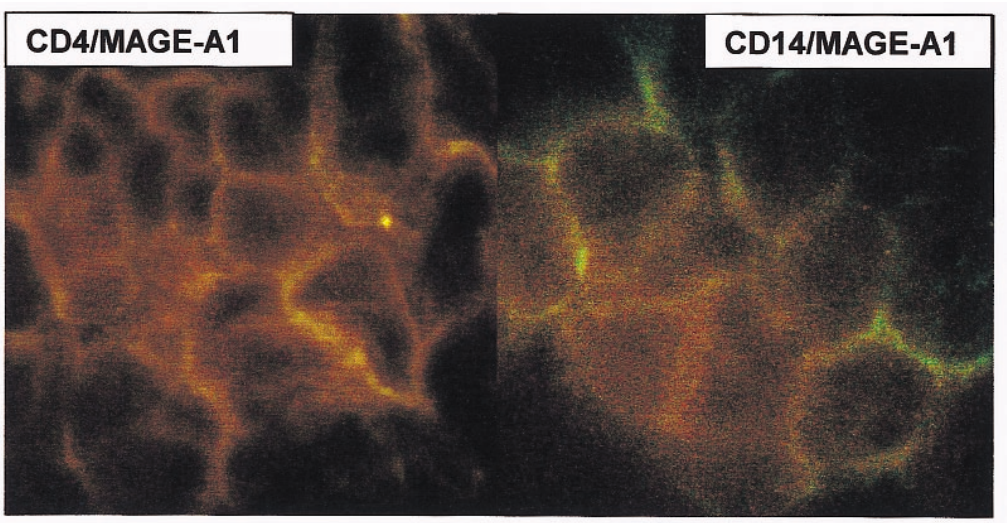

Figure 3. Immunofluorescence of SF cells. Cytospin slides of SF cells were double labeled with anti-MAGE-A1/PE (red) and FITC conjugated (green) anti-CD4 or CD14. Left: SF cells labeled with anti-MAGE-A1 and anti-CD4. Right: SF cells labeled with anti-MAGE-A1 and anti-CD14.

Cytofix/Cytoperm Plus Kit (Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. The data were acquired on a FACS Vantage (Becton Dickinson, San Jose, CA, USA) and analyzed by CELLQuest software (Becton Dickinson). The FACS Vantage was aligned and optimized for use according to the manufacturer's recommendations. Fixed and permeabilized SF cells and large viable low side-scatter, surface stained SF cells were gated and analyzed for FL-2/PE fluorescence. 7-Amino actinomycin-D nuclear stain was used to assess viability in the nonpermeabilized, surface stained population.

RNA preparation and RT-PCR. Total RNA was extracted from SF cells using an RNeasy kit (Qiagen, Valencia, CA, USA). For cDNA synthesis, total RNA was pretreated with DNase I (BRL/Life Technologies, Gaithersburg, MD, USA) and reverse transcribed: 1–2 μg total RNA was incubated at 37°C for 1 h using a First Strand cDNA Synthesis kit

(Amersham-Pharmacia). After cDNA synthesis, PCR was performed using the same conditions as described[4]. The primers used were (5′–3′) MAGE-A1F: TCA GAT CAT GCC CAA GAC AGG; and MAGE-A1 R: ACT CAG CTC CTC CCA GAT TTC (annealing temperature 58°C). ß$_2$-microglobulin: ß$_2$m-A: ACC CCC ACT GAA AAA GAT GA; and ß$_2$m-B: ATC TTC AAA CCT CCA TCA TG (annealing temperature 55°C).

RESULTS

MAGE-A1 protein expression. Immunocytochemistry with Mab to MAGE-A1 was performed on fresh cytospin preparations of cells from joint aspirates obtained from 22 patients. Strong cytoplasmic staining was consistently seen in large and small aggregates of cells (Figure 1A, 1B). Single mononuclear cells in these preparations were usually positive, but the staining was not as strong. Polymorphonuclear cells were negative for MAGE-A1. Isotype control slides were consistently negative (Figure 1C). PBMC from healthy donors were used as negative controls (Figure 1D). In contrast, the positive control, an epithelial carcinoma cell line (HEp-2), showed > 80% strongly positive cells (Figure 1E). Immunocytochemistry with Mab to CD3, CD4, CD8, and CD14 was also done on SF cells. CD4 and in some cases CD14 were found staining similar populations of cells. CD3 was positive on smaller cells and CD8 was positive on only a few cells (< 1 in 200; data not shown).

Three SF cell samples from patients with septic arthritis were analyzed. MAGE staining was difficult to evaluate because of the cellular composition of the aspirates (primarily polymorphonuclear cells) and poor cellular viability. One of the samples was clearly negative, but the other 2 were inconclusive, with nonspecific background staining (data not shown).

PBMC from 7 patients with JRA (4 pauciarticular, 3 polyarticular) were examined by immunocytochemistry with MAGE-A1 and were also found to contain a portion of cells positive for MAGE-A1. Figure 2 shows staining for MAGE-A1 in SF cells and PBMC from an individual with polyarticular JRA.

To characterize MAGE-A1 positive SF cells, 2-color immunofluorescence analysis with Mab to MAGE-A1 (PE, red color) and FITC labeled (green) CD3 (T cell receptor complex), CD4 (T helper cell/monocyte marker), or CD14 (monocyte marker) antibody was performed. Figure 3 shows colocalization (yellow/orange) of MAGE-A1 and CD4. Small areas of colocalization are apparent with CD14; however, some cells or areas of cells stain only for CD14. No colocalization was seen with CD3 (data not shown).

Flow cytometry of synovial aspirate cells. To characterize cell surface and intracellular expression of MAGE-A1, flow cytometry was performed on 3 SF cell samples (2 patients with polyarticular and one with pauciarticular JRA). Cell aggregates were disrupted into a single cell suspension and stained for surface or intracellular MAGE-A1 by flow cytometry, as described in Materials and Methods. In these studies, the cells analyzed included all SF cells (excluding only cellular debris). Six percent of the SF cell population was positive for surface expression of MAGE-A1 compared with isotype control (Figure 4A). Intracellular expression (Figure 4B) on fixed permeabilized cells was positive in a larger portion of cells (60–65%) compared with isotype control.

MAGE-A1 mRNA in SF cells. To study the expression of MAGE-A1 in synovial cells from patients with JRA, RNA was isolated from SF cell samples from 5 patients with pauciarticular JRA. RT-PCR was performed with primers specific for MAGE-A1. All samples were positive, showing a 100 bp band (Figure 5) corresponding to the amplified MAGE-A1 gene segment. The HEp-2 cell line was used as a positive control.

DISCUSSION

The chronic inflammatory process in the joints of children with JRA is not well understood. Our finding of MAGE-A1 expression in SF cells of all patients with JRA we studied suggests that de novo expression may be causal to the inflammatory process in these patients. The data from immunocytochemistry, 2-color fluorescence, and flow

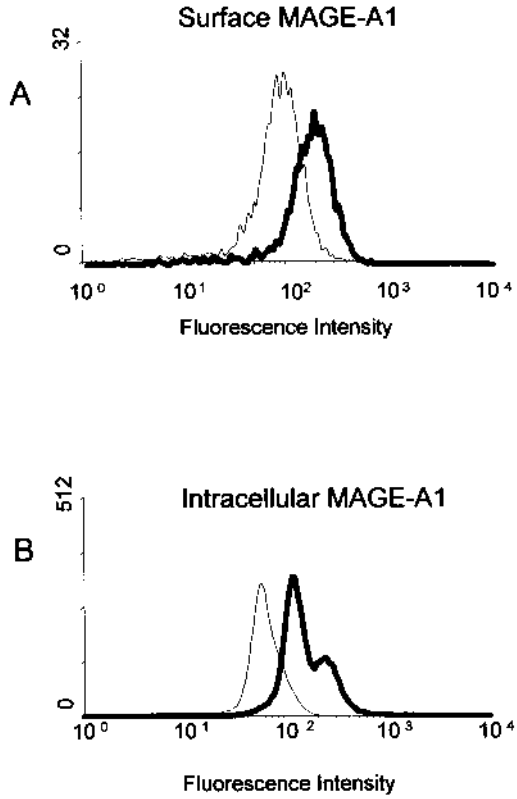

Figure 4. Representative flow cytometry data of SF cells. Pauciarticular JRA SF cells were incubated with anti-MAGE-A1 Mab (bold lines) or isotype antibody (fine lines) and labeled with sheep anti-mouse IgG-PE. A. Surface labeling. B. Intracellular labeling performed after permeabilization.

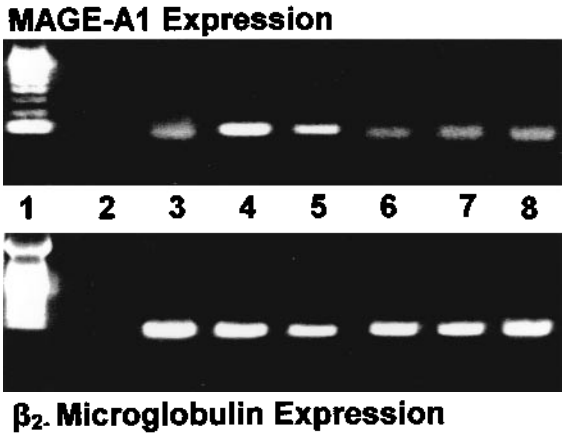

Figure 5. MAGE-A1 mRNA expression in SF cells. mRNA was isolated from JRA SF cells and converted to cDNA. PCR with primers for MAGE-A1 showed a 100 bp band in all patients studied (Lanes 3, 4, 6–8). Lane 1: markers. Lane 2: negative control. Lane 5: HEp-2 cell line. Lower panel: $ß_2$-microglobulin PCR products of each sample served as controls.

cytometry suggest that MAGE-A1 is expressed primarily in CD4+/CD14+ monocytes/macrophages. The finding of weaker MAGE-A1 expression in PBMC compared with SF cells may reflect increased activation of infiltrating mononuclear cells in the synovial milieu. The presence of MAGE-A1 in circulating mononuclear cells is evidence for the systemic aspect of JRA.

The function of the MAGE genes is not known; however, expression of certain MAGE genes has been reported in blastocytes and trophoblasts, as well as in the placenta, suggesting they may play a role in embryogenesis[6,7]. Supporting this, MAGE genes have been described in a variety of childhood tumors, frequently those of embryonal origin[8,9].

Once developmental maturation is completed, MAGE genes are normally only expressed in the spermatogonia of the testes, where there is usually no expression of HLA Class I and, thus, no presentation of MAGE[10]. MAGE gene products may thereby not be available for negative selection of T cells in the thymus. During the rapid proliferation of malignancy or during repair from cell injury, MAGE genes may again be expressed. In tumors, it is believed that this initiates an immune response. We hypothesize that if MAGE proteins were presented on nonmalignant activated cells, they could also initiate an autoimmune response.

Since 1991, when van der Bruggen, *et al* cloned the MAGE-1 (MAGE-A1) gene, interest in these tumor related antigens has led to the development of tumor vaccines with the potential to promote tumor rejection. MAGE-A1 was cloned from a human melanoma cell line, MZ2-MEL, and encodes the tumor rejection antigen MZ2-E, which is recognized by autologous CD8+ cytotoxic T cells[11]. The target for the cytotoxic T lymphocyte recognition of the MAGE-A1 antigen in the melanoma cell line MZ2-MEL is a nonapeptide presented by HLA-A1, combining to form MZ2-E[11]. Subsequent studies report that MAGE-A1 is expressed in tumors of various histological types such as melanomas, lung, breast carcinomas, hematologic malignancies, and others.

Following this first report, 22 additional human MAGE genes have been identified. MAGE-A, B, and C genes are located on the X chromosome and are not expressed in normal tissue, except the testis and placenta[6,12,13]. Recent data show that the expression of MAGE genes in the testis is restricted to the germ-line cells. Since the germ-line cells lack expression of major HLA molecules, they are not expected to present MAGE on the cell surface.

Studies provide evidence that an immune response to another MAGE protein, MAGE-B2, occurs in patients with SLE. The MAGE-B2 gene, localized to Xp21.3, was cloned from a HEp-2 expression library using sera from patients with SLE[4].

To explain the role of MAGE in chronic inflammation and autoimmune disease, we hypothesize that an event causing cellular injury results in reactivation of genes that control cell development and proliferation. Genes such as MAGE-A1 that are not normally expressed are induced and presented on the cell surface in the context of the HLA antigen. This activates T cells, and since there is no tolerance to this rarely expressed antigen, an immune response is activated[14,15]. Following cell activation, cytokines are upregulated, providing costimulation for an inflammatory response. With prolonged stimulation, epitope spreading and widespread activation of the immune system may occur. This process is then chronically fueled by the presentation of the MAGE proteins and other self-antigens that may be exposed during tissue destruction. An HLA restricted response would help to explain the different responses of different individuals. Depending on the HLA context within which the MAGE peptides are presented, T cell avidity and responses may vary, as well as differences in the number of molecules expressed on the cell surface. Understanding the expression of MAGE genes in JRA may elucidate a pivotal step in the development of an ongoing chronic immune response and autoimmune disease in general. Future studies will address the expression of other MAGE genes, including MAGE-B2, in JRA and other autoimmune diseases. In addition, the function of MAGE genes as regulators of inflammatory/immune responses and as targets of autoimmunity will be studied.

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