Cloning of the Murine Counterpart of the Tumor-Associated Antigen H-L6: Epitope Mapping of the Human and Murine L6 Antigens†

Caroline P. Edwards,‡,§ Andrew G. Farr,‡,|| John S. Marken,⊥ Andrew Nelson,‡ Jürgen Bajorath,⊥ Karl Erik Hellström,⊥ Ingegerd Hellström,⊥ and Alejandro Aruffo*,⊥

Department of Biological Structure and Department of Immunology, University of Washington School of Medicine, Seattle, Washington 98195, and The Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, Washington 98121

Received April 12, 1995; Revised Manuscript Received July 28, 1995⊗

ABSTRACT: The murine monoclonal antibody (mAb) L6 was raised against human lung carcinoma cells and found to recognize an antigen which is highly expressed on lung, breast, colon, and ovarian carcinomas. Promising results in phase 1 clinical studies with this antibody or its chimerized counterpart suggest the antigen recognized by mAb L6 (H-L6) is an attractive target for monoclonal antibody-based cancer therapy. Further development of L6 as an anti-tumor-targeting agent would benefit from the development of a murine model. However, initial attempts to develop such a model were hampered by our inability to generate antibodies against the murine homologue of the L6 antigen, M-L6. Here we describe the preparation of the mAb 12A8, which was raised against murine thymic epithelial cells, the tissue distribution of the murine antigen recognized by 12A8, the cloning of a cDNA encoding the 12A8 target antigen, and the demonstration that this antigen is M-L6. Using H-L6/M-L6 chimeric proteins, we show that the region of the M-L6 protein recognized by mAb 12A8 corresponds to the region of H-L6 recognized by mAb L6. There are five amino acid differences in the regions of the H-L6 and M-L6 proteins recognized by L6 and 12A8, respectively. We further mapped the protein epitope recognized by L6 by individually exchanging each of these residues in H-L6 with the corresponding residue found in M-L6. Substitution of the single H-L6 residue Leu122 with Ser resulted in the H-L6 mutant HL6-L122S which failed to bind L6. The HL6-L122S mutant also failed to bind 12A8. Substituting residue Ser122 in M-L6 with Leu did not prevent 12A8 binding and did not result in L6 binding. The availability of mAb 12A8 and the finding that it recognizes the same region of M-L6 that is recognized by L6 on H-L6 might allow the development of a murine tumor model in which the L6 antigen can be further evaluated as a therapeutic target.

An increasing body of evidence suggests that monoclonal antibodies (mAbs)[1] which recognize antigens preferentially expressed on tumor cells can be used therapeutically to treat cancer. These antibodies can mediate host-directed killing of antigen-positive tumor cells, bind antigen on the tumor cells causing a decrease in proliferation, and/or be used to selectively deliver high doses of cytotoxic agents to tumors [for reviews, see Emery and Adair (1994)]. For example, very encouraging therapeutic findings have been obtained with radiolabeled anti-CD20 mAb to treat patients with advanced B cell leukemia (Press et al., 1993).

We have been interested in identifying mAbs that cross-react with antigens overexpressed on carcinomas and developing these antibodies for use clinically in the treatment of cancer. One such antibody, L6, has been shown to recognize an epitope preferentially expressed on a number of human tumors including breast, ovarian, lung, and colon carcinomas (Hellström et al., 1985). L6 is able to fix human complement and mediate antibody-dependent cellular cytotoxicity with human mononuclear cells. In nude mice, it was found to localize to antigen-positive tumor cells and suppress their outgrowth (Hellström et al., 1986; Lavie et al., 1989). In a phase I clinical trial with patients with recurrent breast, colon, lung, and ovary cancers that had failed conventional therapy, L6 was found to be well tolerated and shown to preferentially localize to the tumors (Goodman et al., 1990). One patient in this study, with recurrent breast cancer, underwent complete, albeit temporary, remission following L6 treatment (Goodman et al., 1990). More recently, partial tumor regressions were observed in a few patients receiving therapeutic doses of a radiolabeled chimerized L6 in a phase I study (DeNardo et al., 1991).

Recently we isolated a cDNA clone encoding H-L6 (Marken et al., 1992), partially determined the membrane topology of H-L6, and located the epitope recognized by L6 (Marken et al., 1994). The H-L6 antigen is an integral membrane protein with a predicted molecular mass of ~22 kDa and a member of the "tetraspan" family of proteins (Wright & Tomlinson, 1994). Other members of this family include the leukocyte antigens CD9 (Boucheix et al., 1991), CD37 (Classon et al., 1989), CD53 (Amiot, 1990), CD63

† This work was supported in part by U.S. Department of Energy Grant DE-F006-86-ER60409 (to C.P.E., A.G.F., and A.A.) and the Bristol-Myers Squibb Pharmaceutical Research Institute.

* Address correspondence to this author at BMS-PRI, 3005 First Ave., Seattle, WA 98121. Telephone: (206) 727-3695. Fax: (206) 727-3602.

‡ Department of Biological Structure, University of Washington School of Medicine.

§ Present address: Genentech Inc., 460 Point San Bruno Blvd., South San Francisco, CA 94080.

|| Department of Immunology, University of Washington School of Medicine.

⊥ The Bristol-Myers Squibb Pharmaceutical Research Institute.

⊗ Abstract published in *Advance ACS Abstracts,* September 15, 1995.

[1] Abbreviations: mAb, monoclonal antibody; H-L6, human L6 antigen; HBSS, Hank's balanced salt solution; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

0006-2960/95/0434-12653$09.00/0

(Hotta et al., 1988; Metzelaar et al., 1991), TAPA-1 (Oren et al., 1990); the carcinoma-associated antigen CO-029 (Szala et al., 1990); and the *Schitosoma mansoni* worm antigen SM23 (Wright et al., 1990). Several of these antigens have been implicated in cellular growth control. Like H-L6, CD63 and CD9 are overexpressed in certain cancers (Boucheix et al., 1991; Hotta et al., 1988; Metzelaar et al., 1991; Hellström et al., 1985). Interestingly, antibodies against TAPA-1 have been shown to have an antiproliferative effect on human lymphoid lines, particularly those derived from large cell lymphomas (Vaickus & Levy, 1985).

Hydrophobicity profiles have shown that these proteins contain short amino- and carboxy-terminal hydrophilic domains and four hydrophobic domains. Each of these four hydrophobic domains is long enough to traverse the plasma membrane once and are separated by three hydrophilic regions (numbered I, II, and III) of varying length. Early studies on the membrane topology of TAPA-1 suggested that the amino- and carboxy-terminal hydrophilic domains, as well as hydrophilic region II, are located intracellularly (Levy et al., 1991). Conversely, the first and third hydrophilic regions are presumed to be located extracellularly. In agreement with this predicted membrane topology, we recently reported that the hydrophilic region III of H-L6 is located extracellularly and that the protein epitope recognized by L6 maps to the amino terminus of this region (Marken et al., 1994).

To further develop L6 for use clinically, a murine model of L6 immunotherapy needs to be established. This model would allow the detailed study of the consequences of active and passive immunity to this self-antigen following antibody treatment. In an effort to establish such a murine model, we isolated a cDNA clone encoding M-L6 and prepared two peptides based on the predicted amino acid sequence of the third hydrophilic region of M-L6. These peptides were used to generate antibodies which we hoped would recognized M-L6. However, the anti-peptide antibodies were unable to recognize native M-L6.

As part of a separate program on thymocyte differentiation and the role of thymic epithelial cells in this process, we made mAbs that recognize proteins expressed by murine thymic epithelium using the murine thymic epithelial derived cell line TEC (Glimcher et al., 1983) as an immunogen and isolated cDNA clones encoding the antigens recognized by these antibodies. Here we describe the preparation of mAb 12A8 and the results of experiments to determine the tissue distribution of the antigen recognized by this antibody as well as the isolation of a cDNA clone encoding this antigen. Characterization of this cDNA clone led to the surprising discovery that it encoded M-L6. H-L6/M-L6 chimeras were made and employed to map the location of the protein epitope recognized by 12A8. H-L6 and M-L6 point mutants were prepared for fine-mapping of the protein epitope recognized by L6.

MATERIALS AND METHODS

Cells Lines. COS cells were grown and maintained in DMEM (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and 100 units/mL penicillin and 100 μg/mL streptomycin. Other cells used in this study included the murine keratinocyte cell line PAM-212 (Yuspa et al., 1980), and the thymic stromal cell lines TEC (Glimcher et al., 1983), TE-71 (Farr et al., 1989), and Z210R.1 (Friend et al., 1994).

Production of mAb 12A8. A rat received four intraperitoneal injections of 5×10^6 TEC cells in Hank's balanced salt solution (HBSS) at weekly intervals. Four days after the last immunization, the rat was sacrificed, and rat spleen cells were used to prepare hybridomas. The hybridoma supernatants were screened by flow cytometry for reactivity with TEC cells. Hybridomas were cloned 3 times by limiting dilution. One of the hybridomas resulting from this fusion produced mAb 12A8, an IgG2a rat mAb.

Immunohistochemistry. MAb 12A8 was used for immunohistochemistry studies as previously described (Farr et al., 1992). In brief, freshly excised tissues were immersed in OCT (Miles Diagnostics, Elkhart, IN) and frozen in a dry ice/methanol slurry. Cryostat sections (6 μm thick) were mounted on aminoalkylsilane-treated slides (Rentrop et al., 1986) and air-dried for at least 2 h before fixation (15 min in acetone at −20 °C) before further processing. Slides were rinsed in phosphate-buffered saline (PBS) and then incubated for 1 h with primary antibodies in the form of exhaustively grown hybridoma supernatants. After several washes in PBS, the sections were incubated with anti-rat IgG antibodies (Pierce Chemical Co., Rockford, IL) previously derivatized with digoxigenin 3-*O*-methylcarbonyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester (Boehringer Mannheim, Indianapolis, IN) following the manufacturer's protocol. After additional washing in PBS, sections were incubated with peroxidase-conjugated Fab fragments of goat anti-digoxigenin antibodies (Boehringer Mannheim). Sections were again washed, and peroxidase activity was demonstrated with the use of hydrogen peroxide and 3,3-diaminobenzidine in 50 mM Tris buffer, pH 7.6. Sections were photographed without additional counterstaining.

Expression Cloning of the Antigen Recognized by mAb 12A8. The 12A8 antigen was cloned using the expression cloning techniques described elsewhere (Aruffo & Seed, 1987; Seed & Aruffo, 1987). Briefly, 10 10-cm plates of approximately 70% confluent COS cells were transfected with a cDNA library generated from the murine epithelial cell line TE-71 (Marken et al., 1992). Seventy-two hours post-transfection, the cells were harvested, centrifuged, resuspended in 0.5 mM EDTA/PBS/5% FBS/0.02% NaN_3 containing 10 μg/mL mAb 12A8, and incubated on ice for 60 min. The unbound antibodies were removed from the incubation mixture by centrifuging the cells through a 2% ficoll gradient. The cells were resuspended in 0.5 mM EDTA/PBS/5% FBS/0.02% NaN_3, divided equally onto six 60 mm anti-rat Ig antibody-coated plates, and incubated for 3 h at room temperature. The nonadherent cells were removed by gently washing the plates with 0.5 mM EDTA/PBS/5% FBS/0.02% NaN_3, and the plasmid DNA from the adherent cells was recovered by the method of Hirt (1967). The recovered plasmid DNA was transformed into MC1061/P3 cells and amplified in liquid culture, and the transformed *Escherichia coli* were used to initiate a second round of COS cell transfection by protoplast fusion (Seed & Aruffo, 1987). After the five rounds of transfection, enrichment, and plasmid recovery, the enriched plasmid DNA was transformed into MC1061/P3 cells. Ten pools, containing 10 transformants each, were amplified in liquid culture, and the purified plasmid DNA was transfected into COS cells. The COS cells were analyzed for 12A8 antigen expression by

immunofluorescence. A single transfectant bound 12A8 and not a control rat antibody. The DNA from that pool was again transformed into MC1061/P3 cells. Thirty-six individual colonies were picked and amplified in liquid culture. The plasmid DNA was transfected into COS cells and assayed for 12A8 antigen expression by immunofluorescence. A single transfectant bound 12A8 and not a control rat antibody.

Generation of Human/Murine L6 Chimeras. Mutants were generated by the overlap extension polymerase chain reaction (PCR) method as previously described (Ho et al., 1989). To reduce the chance of unwanted amino acid substitutions during amplification, pfu polymerase (Stratagene, La Jolla, CA) was used. The 5′ and 3′ halves of human−murine L6 antigen chimeras were generated by PCR using internal oligonucleotides with sequences encoding the mutations (L[120]K, L[122]S, S[124]A, L[125]H, Q[127]V, LDSLGQ-[122−127]SDAHGV). The halves were joined by overlap−extension PCR to generate the full-length chimeras. The FLAG epitope tag, DYKDDDDK (IBI, New Haven, CT), was added by PCR to the full-length H-L6 antigen or the HL6-L122S chimera at the carboxy terminus between the final L6 cysteine residue and the stop codon. The outside 5′ primers used for all the constructs contained sequences for the *Hin*dIII restriction endonuclease site and the 3′ reverse primers contained sequences for the *Xba*I restriction endonuclease site. The resulting chimeras were subcloned into the CDM8 vector (Seed, 1987) at the *Hin*dIII/*Xba*I sites. All the clones were sequenced in the areas of the mutations to ensure that the proper substitutions had been generated.

Immunofluorescence and Flow Cytometry. One day prior to transfection COS cells were freshly plated onto 35 mm plates such that they would be approximately 70% confluent at the time of transfection. Using the DEAE-dextran method (Aruffo & Seed, 1987), the COS cells were either mock-transfected or transfected with ~1 μg/mL purified DNA obtained from single *E. coli* colonies. After 48−72 h, the cells were washed 2 times with PBS and incubated for 1 h on ice with 10 μg/mL 12A8 or mAb L6 in 10% FBS containing media. The cells were then washed 2 times with PBS and incubated for 1 h on ice with 10 μg/mL the appropriate fluorescein isothiocyanate-conjugated secondary antibody [goat anti-rat (Cappell, Durham, NC) and goat anti-mouse (Cappell), respectively]. After two washes with PBS, the cells were examined by fluorescence microscopy (Nikon, Melville, NY).

Adherent stromal cells were recovered by first washing with Ca^{2+},Mg^{2+}-free HBSS, followed by a brief treatment with Ca^{2+},Mg^{2+}-free HBSS containing 0.5 mM EDTA. Stromal cells were suspended in HBSS containing 1% FBS and 0.1% NaN_3 to a concentration of 5 × 10^6 cells/mL and incubated with 100 μL of hybridoma supernatant for 1 h at 4 °C. After the cells were washed twice, phycoerythrin-conjugated goat anti-rat Ig antibodies (Caltag, San Francisco, CA) (2 μg/mL) were added to each cell sample. After an additional hour incubation, the cells were washed 2×, the second time in PBS containing FBS and sodium azide. Cells were resuspended in 250 μL of the PBS wash solution, and propidium iodide was added as a viability marker. Flow cytometry was performed with a FACScan unit (Becton Dickinson, San Jose, CA), and resulting data were analyzed with Reproman software (True Facts, Seattle, WA). At least 10 000 live cells were analyzed in each sample.

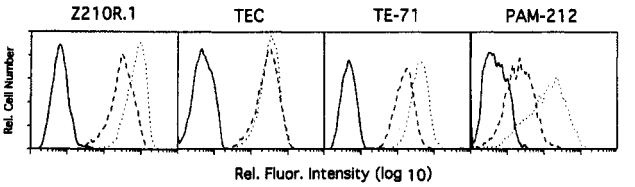


FIGURE 1: Reactivity of 12A8 with murine cell lines. Flow cytometry analysis of murine cell lines stained with the anti-M-L6 mAb 12A8 (dotted line), the anti-murine CD44 mAb D12 (dashed line), or secondary antibody alone (solid line). The name of the murine cell line used is shown above each plot.

COS cell transfectants were analyzed by flow cytometry in a similar manner. Seventy-two hours post-transfection, the cells were lifted from the plate in 0.7 mM EDTA/PBS/dextrose solution, washed, and resuspended in 10% FBS/DMEM; 1 × 10^6 cells were incubated for 1 h on ice in the presence of 10 μg/mL of the specified antibodies. The cells were washed twice with 10% FBS/DMEM and incubated with 10 μg/mL of the appropriate secondary fluorescein-conjugated antibody for 1 hour on ice. After two washes, the cells were resuspended in 2% paraformaldehyde/PBS and analyzed by FACScan (Becton Dickinson) using the PC LYSYS software.

Preparation of Anti-M-L6 Polyclonal Sera. Peptides encompassing residues A116−G137 (P678) and S147−T161 (P679) from the third hydrophilic domain of M-L6 were generated using Boc amino acids on an ABI 430 synthesizer. A portion of the preparations was conjugated by the sulfo-ssmcc method to ovalbumin. Two rabbits were immunized with a cocktail of the ova-P678 and ova-P679 peptides in 2 mL of incomplete Freunds adjuvant at four sites twice at 4 week intervals. Sera were collected prior to immunization and at 4 and 8 weeks after immunization. The 8 week serum (R001) was used in the experiments described here.

ELISA Assays. Unconjugated peptides, P678 and P679, were bound to Immulon II 96 well plates (Dynatech, Chantilly, VA) overnight at room temperature in 50 mM sodium bicarbonate buffer (pH 9.6) at a concentration of 10 μg/mL. BSA was also bound at 10 μg/mL as a negative control. The plates were then blocked with 1× specimen diluent (Genetic Systems, Seattle, WA) for 1 h at room temperature. After being washed 5 times, the plates were incubated with serially diluted anti-peptide rabbit sera from 1:500 to 1:4000 in 2% FBS/PBS or serially diluted 12A8 from 2 to 0.25 μg/mL in duplicate wells for 1 h at room temperature. After the plates were washed, they were incubated with either HRP-conjugated goat anti-rat Ig (1:3000; TAGO, Burlingame, CA) or HRP-conjugated goat anti-rabbit Ig (1:3000; Jackson Laboratories, Bar Harbor, ME) for 1 h at room temperature. After being washed, bound HRP-conjugated antibody was assayed using chromagen, H_2O_2/citrate-buffered, and sulfuric acid according to the manufacturer's instructions (Genetic Systems).

RESULTS AND DISCUSSION

Preparation of mAb 12A8 and Immunoreactivity with Normal Murine Tissues. The 12A8 mAb was generated by immunizing rats with the SV40-transformed murine thymic epithelial cell line TEC (Glimcher et al., 1983). Three murine epithelial cell lines (TEC, TE-71, and Z210R.1) and a murine keratinocyte cell line (PAM-212) were stained with 12A8 or D12, a rat mAb reacting with murine CD44, and

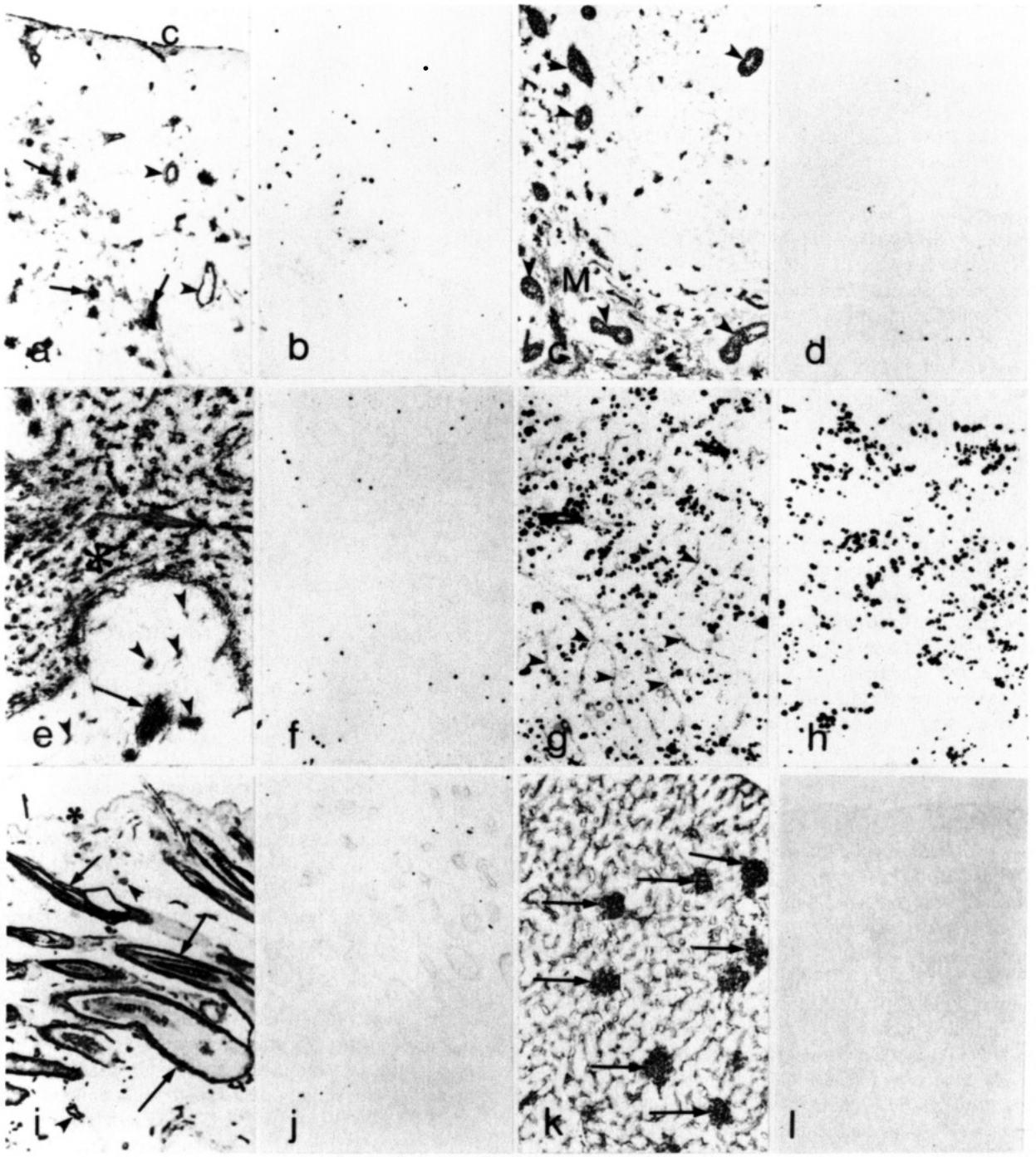


FIGURE 2: Reactivity of 12A8 with selected murine tissues. Panels a, c, e, g, i, and k depict labeling observed with the 12A8 mAb, while panels b, d, f, g, j, and l demonstrate endogenous peroxidase activity and background staining of the tissue. Panels a and b, thymus: arrowheads = blood vessels, arrows = medullary epithelial cells, c = capsule. Panels c and d, lymph node: arrowheads = postcapillary venules, m = medulla. Panels e and f, spleen: arrowheads = arterioles in white pulp, arrow = central artery, asterisk = red pulp. Panels g and h, bone marrow: arrowheads = venous sinusoids. Panels i and j, skin: arrowheads = dermal blood vessels, arrows = hair follicles, asterisk=epidermal epithelium. Panels k and l, kidney: arrows =glomuli. a−1 = 90×.

analyzed by flow cytometry. All cell lines examined displayed cell-surface staining with the 12A8 mAb and D12. The level of staining with 12A8 varied among the four cell lines, with Z210R.1 > TE-71 > TEC > PAM-212 (Figure 1). To examine the tissue distribution of the antigen recognized by this antibody, sections of murine thymus, lymph node, spleen, bone marrow, skin, and kidney tissue were stained with mAb 12A8. As shown in Figure 2, there was widespread reactivity of 12A8 with murine tissue. Reactivity with endothelial cells was a common feature. In the thymus, labeling of the capsule and scattered stromal cells was observed (panel a). There was strong labeling of high endothelial venules in lymph node as well as scattered stromal cells in medullary areas (panel c). In the spleen (panel e), there was extensive reactivity with sinusoidal endothelium in red pulp, central artery in white pulp, and small arterioles in the white pulp. Reactivity of 12A8 with bone marrow was restricted to the delicate venous sinusoidal endothelium (panel g). In the skin, 12A8 reacted strongly with small dermal vessels and epithelial cells associated with the hair follicles but exhibited little if any reactivity with stratified keratinizing epithelium (panel i). Glomeruli and

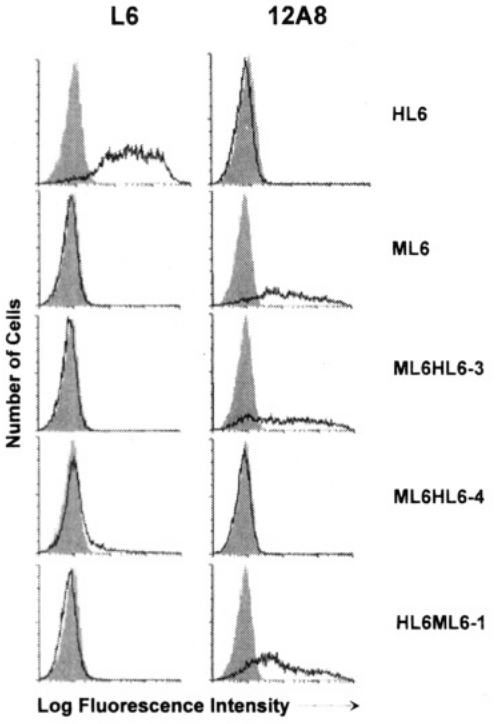


FIGURE 3: Binding of L6 and 12A8 to H-L6, M-L6, and H-L6/M-L6 chimeras. Flow cytometry profiles of COS cells expressing H-L6, M-L6, or the ML6HL6-3, ML6HL6-4, and HL6ML6−1 chimeras stained with either L6 of 12A8 (unshaded profiles) or the appropriate secondary antibody alone (shaded profiles).

interstitial tissue in the kidney were labeled extensively with 12A8 (panel k).

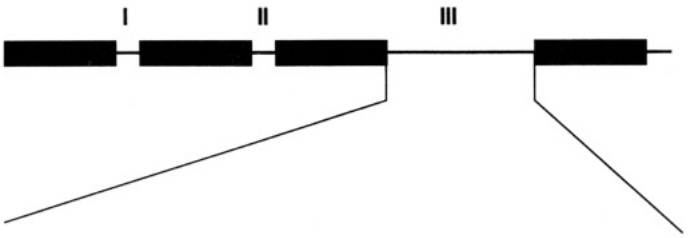
Isolation and Characterization of a cDNA Clone Encoding the Antigen Recognized by 12A8. A cDNA library prepared from mRNA isolated from the murine thymic epithelial cell line TE71 (Marken et al., 1992) was screened with mAb 12A8 using a COS cell-based transient expression−antibody selection cloning system (Marken et al., 1992; Seed & Aruffo, 1987). This procedure led to the isolation of a cDNA clone that could direct the expression of a molecule on transfected COS cells which bound mAb 12A8 (Figure 3). DNA sequencing of this clone revealed that it encoded M-L6, the murine homologue of H-L6 (Marken et al., 1994). Mock-transfected COS cells or COS cells transfected with the cDNA encoding H-L6 or M-L6 were tested for their ability to bind to 12A8 or L6. MAb 12A8 only bound to COS cells transfected with M-L6, whereas L6 only recognized COS cells transfected with H-L6 (Figure 3). Neither mAb recognized mock-transfected COS cells. This lack of cross species reactivity was exploited to map the location of the protein epitope recognized by 12A8.

Identification of the Protein Epitope Recognized by 12A8. Prior to the availability of 12A8, we attempted to generate anti-M-L6 antibodies by immunizing rabbits with 2 peptides with amino acid sequences corresponding to the first 22 amino acids of the third hydrophilic domain of M-L6 (P-678, Figure 4) and the last 15 amino acids in this same domain (P-679, Figure 4). These M-L6 peptides were designed on the basis of our previous results which showed that the third hydrophilic region of H-L6 is located extracellularly and contains the protein epitope recognized by L6 (Marken et al., 1994). To examine the feasibility of using these peptides to generate mAbs against M-L6, we prepared polyclonal anti-peptide sera in rabbits and tested for antibodies that recognized the immunogens and native M-L6 expressed by transfected COS cells (Figure 5 and data not shown). Although we were able to generate polyclonal sera that recognized the unconjugated immunizing peptides (data not shown), these sera did not recognize native M-L6 (Figure 5).

To evaluate whether mAb 12A8 could be used to develop a murine model of anti-L6 antigen immunotherapy, we set out to map the location of the protein epitope recognized by 12A8. Using the two peptides described above, we found that 12A8 weakly bound to P-678 but did not bind to P-679 (data not shown). This result suggested that 12A8 recognizes a protein epitope located at the amino terminus of hydrophilic region III of M-L6 which corresponds to the region of H-L6 recognized by L6 (Marken et al., 1994).

The protein epitope recognized by 12A8 was mapped in more detail by examining the reactivity of 12A8 and L6 to a series of H-L6/M-L6 chimeras. Initially, two hybrid proteins, ML6HL6-3 and ML6HL6-4, were used in which residues of M-L6 were replaced with the corresponding residues from H-L6 (Figure 4). These chimeras had been prepared previously to map the location of the protein epitope recognized by L6 (Marken et al., 1994). In ML6HL6-3, residues K120, N142, and K148 in M-L6 were replaced with the corresponding residues from H-L6: L, D, and E, respectively (Figure 4). In ML6HL6-4, residues K120, S122, A124, H125, and V127 in M-L6 were replaced with the corresponding residues from H-L6: L, L, S, L, and Q, respectively (Figure 4). COS cells transfected with ML6HL6-3 did not bind L6 (Figure 3), while transfectants expressing ML6HL6-4 did (Figure 3), indicating that residues L122, S124, L125, and Q127 in H-L6 are critical for L6 binding. However, ML6HL6-4 bound mAb L6 only weakly. This observation, which is reproducible (Marken et al., 1994) and can be detected by flow cytometry (Figure 3) and immunofluorescence microscopy (data not shown), suggests that additional amino acids in H-L6 are required for maximal L6 binding and/or that the conformation of this eight amino acid stretch in the context of M-L6 is different from H-L6. COS cells expressing ML6HL6-3 bound 12A8 (Figure 3) while COS cells expressing ML6HL6-4 did not. These observations are consistent with the peptide binding data and provide further evidence that the M-L6 protein epitope recognized by 12A8 is located in the amino terminus of the third hydrophilic region of M-L6. Furthermore, these data indicate that M-L6 residues K120, N142, and K148 are not critical for 12A8 binding. Therefore, of the five mutations present in ML6HL6-4, only four may be the cause of the lack of 12A8 binding because the presence of the K120L mutation in ML6HL6-3 does not affect binding of that chimera to 12A8.

To conclusively demonstrate that the M-L6 protein epitope recognized by 12A8 is located in the amino-terminal region of the third hydrophilic domain of M-L6, we prepared an additional H-L6/M-L6 chimera, HL6ML6-1, in which the four residues identified above were replaced in H-L6 with the corresponding residues from M-L6 (Figure 4). In HL6ML6-1, residues L122, S124, L125, and Q127 in H-L6 were replaced with the corresponding amino acids found in M-L6: S, A, H, and V, respectively (Figure 4). COS cell transfectants expressing HL6ML6-1 bound 12A8 but not L6 (Figure 3). Taken together, these results demonstrate that the M-L6 protein epitope recognized by 12A8 maps to the



G L A E G P L C L D S L G Q W N Y T F A S T E G Q Y L L D T S T W S E C T E P K H I V E W N V S L F S	HL6
G L A E G P K C S D A H G V W N Y T F A S T E G Q Y L L N S S M W S K C Y E P K H I V E W H V T L F S	ML6
A E G P K C S D A H G V W N Y T F A S T E G peptide 678 S K C Y E P K H I V E W H V T peptide 679	
G L A E G P L C S D A H G V W N Y T F A S T E G Q Y L L D S S M W S E C Y E P K H I V E W H V T L F S	ML6HL6-3
G L A E G P L C L D S L G Q W N Y T F A S T E G Q Y L L N S S M W S K C Y E P K H I V E W H V T L F S	ML6HL6-4
G L A E G P L C S D A H G V W N Y T F A S T E G Q Y L L D T S T W S E C T E P K H I V E W N V S L F S	HL6ML6-1
G L A E G P K C L D S L G Q W N Y T F A S T E G Q Y L L D T S T W S E C T E P K H I V E W N V S L F S	HL6-L120K
G L A E G P L C S D S L G Q W N Y T F A S T E G Q Y L L D T S T W S E C T E P K H I V E W N V S L F S	HL6-L122S
G L A E G P L C L D A L G Q W N Y T F A S T E G Q Y L L D T S T W S E C T E P K H I V E W N V S L F S	HL6-S124A
G L A E G P L C L D S H G Q W N Y T F A S T E G Q Y L L D T S T W S E C T E P K H I V E W N V S L F S	HL6-L125H
G L A E G P L C L D S L G V W N Y T F A S T E G Q Y L L D T S T W S E C T E P K H I V E W N V S L F S	HL6-Q127V
G L A E G P K C L D A H G V W N Y T F A S T E G Q Y L L N S S M W S K C Y E P K H I V E W H V T L F S	ML6-S122L

FIGURE 4: Line drawing of the L6 antigen and description of H-L6 and M-L6 chimeras and point mutants. The three hydrophilic regions in the L6 antigen are labeled I, II, and III and are shown above their predicted locations in a line-drawing representation of the L6 antigen. The four hydrophobic domains are represented by dark boxes. The amino acids in hydrophilic region III of the human and murine L6 antigen are shown. Residue differences between H-L6 and M-L6 in this region are underlined. Amino acid substitutions in the chimeras and point mutants are shown in boldface type and are underlined. The amino acid sequences of the two M-L6 peptides, P-678 and P-679, are shown.

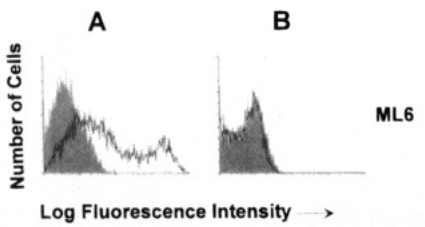


FIGURE 5: Binding of rabbit polyclonal sera to native M-L6. Flow cytometry profiles of COS cell transfectants expressing M-L6 were stained with 12A8 (A, unshaded profile) or the rabbit anti-M-L6 peptide antisera (B, unshaded profile) or the appropriate secondary antibody alone (A and B, shaded profiles).

equivalent position in M-L6 as the H-L6 protein epitope recognized by L6. These observations suggest that 12A8 might be a good choice for an anti-M-L6 antibody with which to establish a murine model of anti-L6 antigen immunotherapy. Using 12A8 would eliminate possible differences between the human and murine systems that might result from using mAbs which recognize different regions of the L6 protein in different species.

Identification of Residues Critical for L6 Binding. One of the initial objectives of this project was to gain understanding of the molecular features of the epitope recognized by L6 and to use this information to generate novel anti-H-L6 mAbs which recognize different H-L6 epitopes. For this reason, we extended our initial work on the mapping of the protein epitope recognized by the mAb L6 by examining the contribution of individual residues in the amino-terminal region of the third hydrophilic domain of H-L6 which are required for L6 binding. We focused our attention on the five residues which differ between H-L6 and M-L6 in this region. To investigate the individual contribution of these residues to L6 binding, each residue in H-L6 was replaced with the equivalent residue in M-L6. This resulted in a set of H-L6/M-L6 point mutant chimeras. The binding of each point mutant to L6 and 12A8 was examined. In particular, residues L120, L122, S124, L125, and Q127 in H-L6 were individually mutated to the corresponding amino acid of M-L6, giving rise to the point mutants HL6-L120K, HL6-L122S, HL6-S124A, HL6-L125H, and HL6-Q127V, respectively (Figure 4). These mutants were individually transfected into COS cells and tested for their ability to bind either L6 or 12A8. Mutants HL6-L120K, HL6-S124A, HL6-L125H, and HL6-Q127V bound L6 but not 12A8 (Figure 6). Mutant HL6-L122S bound neither mAb L6 nor 12A8 (Figure 6).

The observation that COS cells transfected with a cDNA encoding HL6-L122S failed to bind either L6 or 12A8 suggested that residue L122 in H-L6 is critical for L6 binding and antibody specificity. However, we could not exclude the possibility that this protein was not expressed by the COS cell transfectants. To address this possibility, a tag polypeptide (DYKDDDDK, FLAG) was appended to the carboxy terminus of HL6-L122S and to the wild-type H-L6, generating HL6-L122S$_{FLAG}$ and HL6$_{FLAG}$, respectively. Plasmids containing cDNAs encoding HL6-L122S$_{FLAG}$ and HL6$_{FLAG}$ were transfected into COS cells, and the transfectants were tested for their ability to bind mAb L6, 12A8, or an anti-FLAG antibody, M2 after fixation and permeabilization. Addition of the FLAG epitope to the carboxy terminus of the wild-type H-L6 conferred M2, antibody binding and did not disrupt the epitope recognized by L6 (data not shown). Neither 12A8 nor L6 bound to the COS cells expressing HL6-L122S$_{FLAG}$; however, these cells bound M2, indicating that the protein is expressed (data not shown). This suggests that the Leu at position 122 in H-L6 is critical for L6 binding

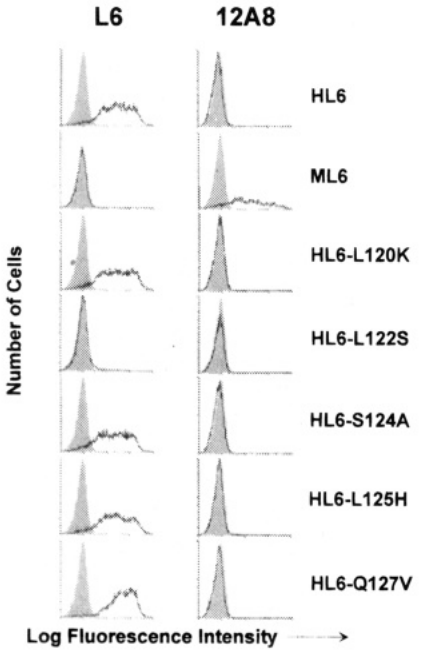


FIGURE 6: Binding of L6 and 12A8 to H-L6, M-L6, and H-L6 point mutants. Flow cytometry profiles of COS cells expressing H-L6, M-L6, and the H-L6 point mutants stained with either L6 or 12A8 (unshaded profiles) or the appropriate secondary antibody alone (shaded profiles).

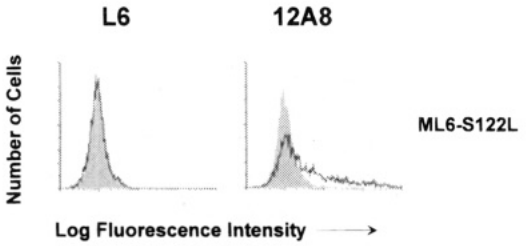


FIGURE 7: Binding of L6 and 12A8 to an M-L6 point mutant. Flow cytometry profiles of COS cells expressing the ML6-S122L point mutant binding to L6 and 12A8 (unshaded profile) or the appropriate secondary antibody alone (shaded profile).

and specificity and that only one residue difference between H-L6 and M-L6 may be sufficient for nonbinding of L6 to the murine antigen.

In addition to demonstrating that Leu122 in H-L6 is critical for L6 binding, these results show that the presence of residue S122 in the HL6-L122S mutant is not sufficient to confer the binding specificity required for 12A8. To examine whether the L122 was sufficient to confer mAb L6 binding in a M-L6 background, an additional point mutant chimera was generated, ML6-S122L, in which residue S122 in M-L6 was replaced by Leu (Figure 4). COS cell transfectants expressing ML6-S122L did not bind mAb L6 but did bind 12A8 (Figure 7). These results show that the presence of Leu122 is necessary but not sufficient for L6 binding and that Ser122 in M-L6 is not a critical residue of the protein epitope recognized by 12A8. Furthermore, the observation that ML6-S122L did not bind L6 indicates that one or more of the amino acid differences between H-L6 and M-L6 in this region play an important role in L6 binding and specificity. It is possible that the limited sequence variability of the epitope regions in M-L6 and H-L6 causes some defined, albeit subtle, changes in tertiary structure and that these changes lead to differences in recognition by 12A8 and L6, respectively. Taken together, these results suggest two possibilities. First, the replacement of Leu by Ser in the HL6-L122S point mutant results in the loss of a residue which is involved in forming an important molecular contact between H-L6 and a residue(s) in the L6 antigen binding site. Second, it is possible that this amino acid substitution perturbs the local structure of the L6 binding epitope, resulting in the inability of L6 to recognize HL6-L122S.

Conclusions. We have shown that 12A8, a rat mAb raised against murine thymic epithelial cells, recognizes M-L6, the murine homologue of the human tumor antigen recognized by the L6 mAb. In addition, we have shown that 12A8 recognizes an equivalent region on M-L6 as the region of H-L6 recognized by L6. Taken together, the data suggest that 12A8 is a good candidate mAb with which to establish a murine model of tumor immunotherapy to the L6 self-antigen which is overexpressed by a large number of human tumors. However, before such a model can be established, additional experiments need to be carried out. These experiments include examining the ability of 12A8 to fix complement and direct antibody-mediated cellular cytotoxicity, two properties of the L6 antibody which have been implicated in its ability to mediate tumor cell destruction (Hellström et al., 1986; Lavie et al., 1989). In addition, it will be important to examine whether the expression of this self-antigen is similar in mice and humans. If there are substantial differences, 12A8 might have a toxicity profile which is quite distinct from that of L6. Lastly, 12A8 can now be used to identify murine tumors which overexpress M-L6 to be used in this murine model.

Finally, an additional motivation behind this work was to develop new anti-HL6 mAbs for clinical use. In order to do this, we isolated a cDNA clone encoding the L6 target antigen, examined the topology of the protein in the cell membrane, and mapped in detail the protein epitope recognized by L6. We previously showed that hydrophilic region III of H-L6 is located extracellularly. We now demonstrate that polyclonal antibodies raised against two peptides with amino acid sequences corresponding to the amino- and carboxy-terminal regions of hydrophobic region III failed to bind the native M-L6 protein. These data, in conjunction with our previous results and data on the nature of the L6 target epitope, indicate that generating antibodies to novel H-L6 epitopes will not be a trivial undertaking. Strategies designed to generate such antibodies should rely on the use of cells overexpressing H-L6 in its native conformation.

ACKNOWLEDGMENT

We thank J. Blake and U. Garrigues for the preparation of the M-L6 peptides and rabbit polyclonal sera, respectively, M. Neubauer for sequencing the H-L6 and M-L6 point mutants and chimeras, D. Hollenbaugh and P. Senter for critical review of the manuscript, and D. Baxter for help in preparation of the manuscript.

REFERENCES

Amiot, M. (1990) *J. Immunol. 145*, 4322−4325.

Aruffo, A., & Seed, B. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8573−8577.

Boucheix, C., Benoit, P., Frachet, P., Billard, M., Worthington, R. E., Gagnon, J., & Uzan, G. (1991) *J. Biol. Chem. 266*, 117−122.

Classon, B. J., Williams, A. F., Willis, A. C., Seed, B., & Stamenkovic, I. (1989) *J. Exp. Med. 169*, 1497−1502.

DeNardo, S. J., Warhoe, K. A., O'Grady, L. F., DeNardo, G. L., Hellström, I., Hellström, K. E., & Mills, S. L. (1991) in *Radioimmunotherapy with I-131 chimeric L-6 in advanced breast cancer*, Plenum Press, New York.

Emery, S. C., & Adair, J. R. (1994) *Exp. Opin. Invest. Drugs 3*, 241−251.

Farr, A. G., Hosier, S., Braddy, S. C., Anderson, S. K., Eisenhardt, D. J., Yan, Z. J., & Robles, C. P. (1989) *Cell. Immunol. 119*, 427−444.

Farr, A., Nelson, A., & Hosier, S. (1992) *J. Histochem. 40*, 651−664.

Friend, S. L., Hosier, S., Nelson, A., Foxworthe, D., Williams, D. E., & Farr, A. (1994) *Exp. Hematol. 22*, 321−328.

Glimcher, L. H., Kruisbeek, A. M., Paul, W. E., & Green, I. (1983) *Scand. J. Immunol. 17*, 1−11.

Goodman, G. E., Hellström, I., Brodzinsky, L., Nicaise, C., Kulander, B., Hummel, D., & Hellström, K. E. (1990) *J. Clin. Oncol. 8*, 1083−1092.

Hellström, I., Hellström, K. E., Rollins, N., Lee, V. K., Hudkins, K. L., & Nepom, G. T. (1985) *Cancer Res. 45*, 2210−2218.

Hellström, I., Beaumier, P. L., & Hellström, K. E. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 7059−7063.

Hirt, B. (1967) *J. Mol. Biol. 26*, 365−369.

Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., & Pease, L. R. (1989) *Gene 77*, 51−59.

Hotta, H., Ross, A. H., Huebner, K., Isobe, M., Wendeborn, S., Chao, M. V., Ricciardi, R. P., Tsujimoto, Y., Croce, C. M., & Koprowski, H. (1988) *Cancer Res. 48*, 2955−2962.

Lavie, E., Beaumier, P. L., Brodzinsky, L., Hellström, K. E., & Hellström, I. (1989) *Radiother. Oncol. 15*, 295−305.

Levy, S., Nguyen, V. Q., Andria, M. L., & Takahashi, S. (1991) *J. Biol. Chem. 266*, 14597−14602.

Marken, J. S., Schieven, G. L., Hellström, I., Hellström, K. E., & Aruffo, A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 3503−3507.

Marken, J. S., Bajorath, J., Edwards, C. P., Farr, A. G., Schieven, G. L., Hellstrom, I., Hellstrom, K. E., & Aruffo, A. (1994) *J. Biol. Chem. 269*, 7397−7401.

Metzelaar, M. J., Wijngaard, P. L., Peters, P. J., Sixma, J. J., Nieuwenhuis, H. K., & Clevers, H. C. (1991) *J. Biol. Chem. 266*, 3239−3245.

Oren, R., Takahashi, S., Doss, C., Levy, R., & Levy, S. (1990) *Mol. Cell. Biol. 10*, 4007−4015.

Press, O. W., Eary, J. F., Appelbaum, F. R., Martin, P. J., Badger, C. C., Nelp, W. B., Glenn, S., Butchko, G., Fisher, D., Porter, B., et al. (1993) *N. Engl. J. Med. 329*, 1219−1224.

Rentrop, M., Knapp, B., Winter, H., & Schweizer, J. (1986) *Histochem. J. 18*, 271−276.

Seed, B. (1987) *Nature 329*, 840−842.

Seed, B., & Aruffo, A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 3365−3369.

Szala, S., Kasai, Y., Steplewski, Z., Rodeck, U., Koprowski, H., & Linnenbach, A. J. (1990) *Proc. Natl. Acad. Sci. USA 87*, 6833−6837.

Vaickus, L., & Levy, R. (1985) *J. Immunol. 135*, 1987−1997.

Wright, M. D., & Tomlinson, M. G. (1994) *Immunol. Today 15*, 588−594.

Wright, M. D., Henkle, K. J., & Mitchell, G. F. (1990) *J. Immunol. 144*, 3195−3200.

Yuspa, S. H., Hawley-Nelson, P., Koehler, B., & Stanley, J. R. (1980) *Cancer Res. 40*, 4694−4703.

BI950817I