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I am submitting herewith a thesis written by Kathryn Merritt Flynn entitled "Monoclonal Antibodies for Characterization of a Tumor-Associated Antigen from a Mouse Lung Carcinoma." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biomedical Sciences.

Stephen J. Kennel

Stephen J. Kennel, Major Professor

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The Graduate School

MONOCLONAL ANTIBODIES FOR CHARACTERIZATION OF A TUMOR-ASSOCIATED ANTIGEN FROM A MOUSE LUNG CARCINOMA

A Thesis

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Degree

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ABSTRACT

Binding specifities of putative tumor-specific antibodies intended for use in radioimaging of tumors are routinely characterized by *in vitro* binding assays to tissue cultured cells, frozen tissue sections and tissue lysates. 135-13C MoAb was characterized *in vitro* as specific to line 1 alveolar carcinoma cells and other lung carcinomas from mice. 135-13C MoAb precipitates a protein of 180,000 M.W., tumor-surface protein-180, from solubilized surface-labeled line 1 cells. I analyzed the distribution of radioiodinated 135-13C MoAb and 135-14 MoAb in tumor-bearing mice. Localization indexes for lung, spleen and kidney indicated that 135-13C MoAb might be binding to normal cells of these organs. Results from the analysis of clearance of radiolabeled 135-13C MoAb and control MoAb, 135-14, from the blood of normal and tumor-bearing mice are consistent with this hypothesis since 135-13C MoAb is cleared from the blood faster than the control MoAb, 135-14, in normal as well as tumor-bearing mice. Results show that putative imaging antibodies should be analyzed for clearance kinetics *in vivo* in normal subjects prior to imaging trials to determine if there is significant binding to normal cell sites.

135-13C MoAb was not expected to bind to normal cell sites. Characterization of TSP-180-like antigens from normal cells requires a more sensitive assay for the detection and isolation of proteins than the assays used in the characterization of 135-13C MoAb. A MoAb (346-11) to a second site on TSP-180 was developed for future use in a two-site immunoassay. Two site assays increase the sensitivity of

detection by concentrating the antigen with one MoAb prior to detection of antigen with a second MoAb. 346-11 binds to the surface of line 1 cells. It specifically precipitates TSP-180 from surface-labeled line 1 cells as identified by SDS-PAGE of immunoprecipitates of solubilized line 1 cells. Reciprocal competition binding analysis of 135-13C MoAb and 346-11 MoAb demonstrated that 346-11 binds to a different site than 135-13C MoAb on the TSP-180 molecule.

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CHAPTER I

INTRODUCTION

Review of Radioimaging

The idea of using antibodies to tumor-specific antigens for *in vivo* radioimaging of tumors is not new (e.g. see Pressman and Korngold, 1953; Bale et al., 1955). Initially, progress in this field was limited by the heterogeneity of the key reagent, antibody. Antisera contain complex mixtures of immunoglobulins which can bind to many different sites or determinants on target cells. Different preparations of antisera are unique and each must be characterized extensively to ensure a similar operational specificity from batch to batch (Rasche, 1982; Kennel et al., 1984). Purification of antibody from antisera requires extensive absorption onto normal cells to remove nonspecific components. This multistep purification process is rarely successful at removing all nonspecific components (Raschke, 1982; Damjanov and Knowles, 1983). Furthermore, preparations obtained from conventional antisera vary in quality, biochemical properties and specificity (Raschke, 1982; Damjanov and Knowles, 1983). Even when antisera were determined to be tumor specific by one test or another, they were subsequently found to react with normal cell components if different criteria were employed (Iwaki et al., 1982; Brown et al., 1981b; Damjanov and Knowles, 1983; Kennel et al., 1984).

A technique for monoclonal antibody production introduced by Kohler and Milstein (1975, 1976) has increased the possibilities for the successful use of anti-tumor antibodies in radioimaging of tumors. Monoclonal antibodies are specific for a single epitope on an antigen. Problems associated with the heterogeneity in binding capacity are eliminated. Theoretically, one could expect to find antibodies that are directed to truly tumor specific antigens. Monoclonal antibodies are homogeneous and can be purified easily in virtually unlimited quantities from ascites fluid or from cell supernatants. Furthermore, these purified reagents can be produced reproducibly and indefinitely since hybridoma cell lines can be cryopreserved.

In recent years there have been several researchers who have successfully employed both monoclonal and polyclonal antibodies for the _in vivo_ radioimaging of tumors (e.g. Houston et al., 1980; Colcher et al., 1983; Key et al., 1983; Scheinberg et al., 1982; Weinstein et al., 1983; Bernhard et al., 1983; Mach et al., 1981; van Nagell et al., 1980). Generally, antibodies to tumor-specific or tumor-associated antigens were labeled with a radioisotope such as iodine-131 or indium-111 and injected into a tumor-bearing experimental animal. The distribution of the antibody _in vivo_ is determined by excising organs and tissues at various time points and analyzing these for radioactivity. The results are computed as localization indexes.

Radioimages of the distribution of the isotope-labeled antibody in the whole animal can be obtained with a gamma camera. This technique generally utilizes blood pool subtraction techniques to obtain a clear image of the tumor (DeLand et al., 1980; Pimm et al., 1982). Although progress in the field of tumor imaging has been fairly dramatic in animal model systems, there are only a few human systems where radioimaging has been attempted and it has met with somewhat limited success (e.g. Mach et al., 1981; van Nagell et al., 1980; Larson et al., 1983; Goldenberg et al., 1980). For example, monoclonal and polyclonal anti-CEA (carcinoembryonic antigen) antibodies have been successfully employed in the imaging of human carcinomas and ovarian cancers (van Nagell et al., 1980; Mach et al., 1981); anti-p97 monoclonal antibodies have been used by Larson et al. (1983) to image melanomas; and hepatocellular and germ cell carcinomas have been imaged with polyclonal antibodies to alpha-fetoprotein (Goldenberg et al., 1980).

Some of the problems encountered in imaging have been the high background from radiolabeled antibody in the blood (Rollo et al., 1980; Levine et al., 1980) and a high nonspecific accumulation of the radiolabeled antibody in organs such as liver (Deland et al., 1980; Rollo et al., 1980). Background subtraction techniques were designed to correct for high background in the blood and nonspecific accumulation in organs. This has increased the sensitivity of imaging but nonspecific accumulation still remains a problem (Larson et al., 1983).

Another problem in imaging is that of resolution. In the anti-CEA system, tumors that are less than 2 cm in diameter cannot be detected (van Nagell et al., 1980) and in the anti-p97 system tumors must be larger than 1.5 cm for detection (Larson et al., 1983). Thus the utility of radioimaging in detecting metastases may be limited by the size of the lesions.

The location of the tumor in vivo also effects whether imaging techniques will be sufficient for detection. Tumors of sufficient size for detection in one location can go undetected if located in areas with poor object contrast, such as the liver (Rollo and Patton, 1980). False positive images are also fairly frequent, indicating problems with specificity in imaging systems. The production of false positives can be partially corrected by using different background subtraction techniques (Mach et al., 1981). Nevertheless, the general conclusion from clinical studies is that radioimmunodetection techniques are not perfected and further study in animal model systems is necessary to make this technique clinically useful (Mach et al., 1981; Larson et al., 1983).

All of the radioimaging studies performed to date in a clinical situation have been attempted with antibodies that are directed against tumor-associated, not tumor-specific, antigens. There is some question whether truly tumor-specific antigens exist at all since most of the putative tumor-specific antigens that aren't tumor-virus associated have been found to be present on normal cells (Damjanov and Knowles, 1983). For example, p97 was thought to be

specific to melanomas (Woodbury et al., 1980) until more sensitive assays were used which were able to detect p97 in normal tissues (Brown et al., 1981b). In subsequent experiments it was shown that p97 is similar to transferrin, which is known to be present in normal cells (Brown et al., 1982). However, p97 is present at much higher levels in melanomas (100 fold greater concentration) than in normal adult tissues (Brown et al., 1981b) and operationally it can act as a tumor specific antigen (Larson et al., 1983). Although exact specificity for tumor cells is a desirable characteristic for an imaging antibody, it is clear from the initial clinical studies it is not an absolute requirement. Potentially, an antigen can be an adequate target for radiolabeled antibodies if it is present on the tumor cells at a much greater density or in much larger amounts than on normal cells. Even in the anti-CEA system, where large amounts of circulating antigen are known to be present in the form of immune complexes, moderately successful radioimaging of tumors with polyclonal and monoclonal anti-CEA antibodies has been accomplished (Mach et al., 1981; van Nagell et al., 1980). Nevertheless, as discussed above, clinical trials have not been completely successful due to background problems, nonspecific accumulation of radiolabeled antibody in certain organs, poor object contrast, and false positive results. More research is needed in animal model systems to correct these problems and thus improve imaging systems. For example, one could test whether the use of well-defined anti-tumor monoclonal antibodies, rather than antisera or monoclonal antibodies known to

have normal cell specificity, significantly improves imaging. These antibodies could be characterized for their *in vivo* specificity using the double label method of Pressman et al. (1957) where the distribution of a I-125 labeled tumor specific monoclonal antibody (MoAb) is compared to the distribution of a I-131 labeled control MoAb. One could also test whether the combined use of two radiolabeled antibodies to different determinants on the target antigen decreases false positives or improves resolution by increasing the levels of radioactivity on tumor cells (Goldenberg, 1983). Furthermore, animal models can elucidate whether there are problems with the imaging antibody prior to clinical application.

Aims of Research

The original intent of the thesis research was to image a lung carcinoma of the BALB/c mouse with a radiolabeled monoclonal antibody directed to a surface protein on the lung carcinoma. Data for radiolocalization and blood clearance kinetics of a putative tumor-specific monoclonal antibody are presented in this thesis. Tumor imaging attempts, as defined by localization indexes, were unsuccessful. A problem was encountered during these studies that is generally applicable to radioimaging studies. Although the monoclonal antibody was extensively characterized *in vitro* for its binding specificity and was determined to be tumor-specific, these *in vivo* experiments indicate that there is extensive binding to normal cell sites. Thus, *in vitro* characterization of an imaging antibody may not be sufficient for determining *in vivo* binding specificity. It

may be necessary to characterize putative imaging antibodies *in vivo* in normal subjects prior to imaging trials (Kennel et al., 1984 and this thesis).

Experimental Model

The tumor system used in this research was a spontaneous alveolar carcinoma from a BALB/c mouse, line 1. The tumor cells injected intra- muscularly (i.m.) are weakly antigenic and highly metastatic in the syngeneic host (Yuhas et al., 1974a, 1975). Kennel et al. (1979, 1980) characterized a tumor cell surface protein on line 1 cells using heterologous antisera. They found a 180,000 M.W. protein they called tumor surface protein-180 (TSP-180) that could be immunoprecipitated from solubolized line 1 cells with absorbed goat and rat antisera prepared against line 1 cells. Antisera to large external transformation sensitive protein (fibronectin), CEA, viral proteins and filamen did not precipitate this protein. Furthermore, in competition studies with radiolabeled line 1 cells, solubolized Moloney sarcoma cells (MSC), normal lung cells, BALB/c fibroblasts, mouse mammary tumors, BALB/c embryonic tissue, rat tracheal tumor cells and human alveolar tumor cells did not compete for precipitation of radiolabeled TSP-180 against *in vivo* absorbed rat anti-line 1 serum. Line 1 cells from tissue culture or excised from tumors competed in this reaction. Kennel et al. (1980) also found that other mouse lung carcinomas could compete in this assay, as well as adenomas, to a more limited extent. They isolated TSP-180 from these carcinomas, compared the proteins by limited protease digestion and

found a highly conserved sequence homology indicating that TSP-180 represents a common cell surface protein on mouse lung carcinomas. This indicates that TSP-180 is potentially a good candidate for a target antigen in radioimaging of lung carcinomas in the mouse.

Kennel et al. (1981a) developed monoclonal antibodies to TSP-180 by fusing the spleen cells of a rat immunized with line 1 cells with the parent myeloma P3-X63-Ag8. The binding characteristics of these antibodies to surface proteins of the line 1 carcinoma were determined and it was found that the anti-TSP-180 monoclonal antibodies had binding constants that ranged from 1.2×10^7 to 1.5×10^8 liters/mol. Direct binding studies using radiolabeled monoclonal antibodies on line 1 and other mouse lung carcinomas verified that TSP-180 is expressed on the cell surface. This suggests that anti-TSP-180 monoclonal antibodies could be appropriate reagents for radioimaging of lung carcinomas. One of these anti-TSP-180 monoclonal antibodies, 135-13C, was chosen for studies on in vivo distribution and blood clearance kinetics in tumor-bearing and normal BALB/c mice.

It is important that a target antigen for radioimaging be present in significant amounts only on the target tumor cells' surface and be absent or present only at low levels on the surface of normal cells. Although the results of Kennel et al. (1979, 1980) indicated that TSP-180 is not present on normal cells, the assays used were not sensitive enough to be capable of detecting TSP-180 present at high densities on a few normal cells or at low levels on

normal cells. These levels could be high enough to prevent adequate accumulation of the monoclonal antibody in the tumor site. Studies of the clearance of the monoclonal antibody with time from the circulation of normal mice can indicate whether there is significant binding by normal cells (Kennel et al., 1984 and this thesis). Different classes and subclasses of immunoglobulin have different circulation times but the normal circulation time (t 1/2) of immunoglobulins is on the order of 4-10 days (Spiegelberg and Weigel, 1965). Rapid clearance of antibody (t 1/2 on the order of hours) indicates binding. It would be preferable to determine binding by normal cells in vitro, however. More sensitive assays than those used by Kennel et al. (1979, 1980) must be employed for this purpose.

A two-site immunoassay would increase sensitivity in the detection of TSP-180 in tissue lysates (Brown et al., 1981a, Hedin et al., 1983). In this assay, one monoclonal antibody to TSP-180 is covalently attached to a solid support, such as sepharose beads, and is used to concentrate TSP-180 from solubolized tissues. A second monoclonal antibody directed to a different epitope on TSP-180 is then radiolabeled and used for detection of TSP-180 attached on the solid support.

In order to develop a two-site assay, a monoclonal antibody to a second epitope on TSP-180 must be produced. Potentially, this second monoclonal antibody could also be used to enhance imaging. Used in combination with the first MoAb, it could increase the accumulation of radioactivity in target tumors (Goldenberg, 1983). Furthermore,

both antibodies could be used to purify TSP-180 antigens from tumor and normal cells by immunoaffinity chromatography facilitating comparative analysis of these antigens (August and Murphy, 1982). Comparative characterization could determine whether there are normal proteins related to TSP-180 or a family of TSP-180 proteins associated with different epitopes.

To facilitate the development of these studies, a monoclonal antibody to a second epitope on TSP-180 was developed. The intentional development of a monoclonal antibody to a second epitope on a target antigen is not common since it could be difficult to generate an adequate immune response to other epitopes on this molecule. Epitopes on TSP-180 other than the one recognized by 135-13C might be less antigenic. Kennel et al. (1981a) did not produce any monoclonal antibodies to different epitopes on TSP-180 using immunization with tumor tissue culture cells. However, it could be that the immunization and screening procedures used discriminated against antibodies to other epitopes. Foote and Kennel (unpublished observations) found that the epitope recognized by 135-13C could be destroyed by high salt treatment, such as treatment with 3M KSCN. This property was exploited in the production of the monoclonal antibody to a second epitope on TSP-180. The production and characterization of this monoclonal antibody (346-11) is described in this thesis.

CHAPTER II

MATERIALS AND METHODS

Cells

Line 1 cells were from a spontaneous alveolar cell carcinoma of a BALB/c AnNBdf mouse adapted to tissue culture after 12 *in vivo* passages (Yuhas, 1974a). Cells used in this study were from short term *in vitro* passages (11-48). A31 is a clonal line of BALB/c 3T3 fibroblasts (Aaronson and Todaro, 1968). A31 was obtained from Dr. R. W. Tennant (National Institutes of Health-Environmental Sciences) at passage 25 (Kennel, 1979). Line 1 and A31 cells were maintained as monolayers in McCoy's medium 5A (Grand Island Biological Company, Grand Island, New York) supplemented with 10% fetal bovine serum (Grand Island Biological Company), 2 mM glutamine, 100 units penicillin per ml, and 100 ug streptomycin per ml.

P3-X63-Ag8 myeloma was obtained from Dr. F. Fitch (University of Chicago) with the permission of C. Milstein and was maintained in Dulbecco's modified minimum essential medium with 20% fetal bovine serum (DME-20), 2 mM glutamine, 100 units penicillin per ml, and 100 ug streptomycin per ml as described (Kennel et al., 1981b).

Monoclonal Antibodies

135-13C, 133-13A, 133-13D and 135-14 were obtained from fusions with spleens from line 1 cell-immunized Fischer 344 rats and the parent myeloma P3-X63-Ag8 (Kennel et al., 1981a). 135-13C binds to a 180,000 M.W. protein called tumor surface protein-180 (TSP-180), as

determined by sodium-dodecyl sulfate polyacrylimide gels of immunoprecipitates, on the surface of line 1 alveolar carcinoma cells and other lung carcinomas from mice (Kennel et al., 1981a). It does not bind to other tumors (sarcomas) or to normal cells (Kennel, 1979). 133-13A and 133-13D bind to a surface protein with a molecular weight of 100,000 (P100) as determined by immunoprecipitation (Kennel et al., 1981a). P100 is present at high levels on all murine cells tested, including line 1 (Kennel et al., 1981a). 135-14 is used as a control monoclonal antibody since it does not bind to any of the cell lines tested (Kennel et al., 1981a and 1984).

346-11 was obtained from a fusion with a TSP-180-immunized Fischer 344 rat's spleen and the parent myeloma P3-X63-Ag8 (this thesis). It binds to TSP-180 on the line 1 cell surface, as determined by immunoprecipitation and direct binding assays. It binds to a different epitope on TSP-180 than 135-13C (see Results section).

Antibody from cell supernatants was precipitated by adding an equal volume of saturated ammonium sulfate in PBS, collected by centrifugation and resuspended in 1/50 the original medium volume. It was dialyzed against PBS and stored frozen at -20^{o}C (Kennel et al., 1981a). Monoclonal antibodies were purified from the concentrated culture supernatants with an affinity column of goat antiserum to rat immunoglobulin G (IgG) as described (Kennel et al., 1981a). Alternatively, hybridoma cells were injected intraperitoneally (i.p.) into pristane treated nu/nu mice to produce ascites fluid. IgG was

purified from ascites fluid by ammonium sulfate precipitation and ion exchange chromatography as described previously (Kennel, 1982).

In Vivo Distribution of TSP-180

Sixteen female mice, six weeks of age, were injected with 1 x 10^6 line 1 cells intramuscularly (i.m.) eight days prior to the distribution experiment. Three days prior to the experiment these mice and sixteen normal mice were given Lugol's solution in drinking water. 135-13C, the specific monoclonal antibody, was labeled to a specific activity of about 800 counts per minute (cpm) per ng with ^{125}I and the control monoclonal antibody, 135-14, was labeled with ^{131}I to a specific activity of about 70 cpm/ng by the chloramine-T method (McConahey and Dixon, 1966). ^{131}I labeled monoclonal antibodies were deliberately labeled to a lower specific activity than ^{125}I labeled monoclonal antibodies to minimize problems in the detection of ^{125}I labeled monoclonal antibodies from crossover of ^{131}I counts into the ^{125}I detection channel. Labeled immunoglobulins were separated from aggregrates and free iodine by gel filtration through LKB AcA 34 resin (LKB Instruments, Inc., Rockville, MD) equilibrated in bovine serum albumin (5 mg per ml in Phosphate Buffered Saline). Labeled 135-13C MoAb was tested for binding to line 1 cells in a direct binding assay to assure that the antibody activity was not destroyed in the labeling procedure as described (Kennel et al., 1981a).

One-hundred ug of ^{125}I 135-13C MoAb and 100 ug of ^{131}I 135-14 MoAb mixed together in a 200 ul volume was injected intravascularly

(i.v.) into all mice (16 tumor-bearing and 16 normal). At each time point after injection of the antibodies (0 hr, 2 hr, 5 hr, 8 hr, and 30 hr) two normal mice and two tumor-bearing mice were weighed, anaesthesized, bled from the femoral artery (fore leg), sacrificed and perfused through the heart with 10 ml of phosphate buffered saline (PBS) pushed through a 27 guage needle on a 10 ml syringe. The volume of blood recovered was recorded for each mouse. The total volume of blood in a mouse was assumed to be 8% of the body weight with 1 ul of blood equal to 1 mg (Kennel et al., 1984). The following organs and tissues were excised and weighed: heart, lung, liver, kidney, spleen, skin, brain, normal leg and tumor leg. The organs, tissues and a blood sample were analyzed for ^{125}I and ^{131}I in a Searle automatic gamma spectrometer.

Calculations were performed to determine the amount of ^{125}I 135-13C MoAb and ^{131}I 135-14 MoAb in the blood, organs, and tissues at every time point. All values were corrected for channel background and for crossover between the ^{125}I and ^{131}I channels.

In order to determine the total recovery of labeled antibody in the whole mouse at each time point, the total recovery of counts in skin and blood had to be estimated. Recovery in the blood was estimated by determining the fraction of blood recovered relative to the total volume of blood in the mouse (ul of blood recovered/mg of blood in mouse X 100 = % recovery). Radioactivity recovered in the blood was corrected to % recovery to obtain an estimate of total label in the blood (radioactivity recovered/% recovery X 100 = total

radioactivity in the blood). Radioactivity recovered in the skin had to be estimated since skin in the head and leg regions of the mouse is not recovered. An estimate of 75% recovery of skin is used to calculate radioactivity recovered in the skin.

Radioactivity from organs, tissues, and blood was totaled to obtain a value for the total radioactivity retained per mouse and was divided by total radioactivity injected and multiplied by 100 to determine the fraction of label retained. A similar calculation was performed to determine the fraction of label recovered per organ in relation to the total amount of label recovered per mouse. This calculation was performed for blood and all organs and tissues for both ^{125}I 135-13C MoAb and ^{131}I 135-14 MoAb. A localization index (L.I.) was then calculated using the % ^{125}I and % ^{131}I values obtained above for each organ and tissue as follows:

$$\text{L.I.} = \frac{\%\ ^{125}\text{I in organ}}{\%\ ^{131}\text{I in organ}}$$

Localization indexes indicate whether there is a specific distribution of 135-13C MoAb in any given organ. A localization index greater than 1.0 indicates that ^{125}I 135-13C specifically accumulates in the area relative to the control MoAb ^{131}I 135-14 and suggests the presence of TSP-180 or a protein bearing the epitope recognized by 135-13C MoAb in that area.

Clearance Kinetics

An experiment was designed to assess the clearance of ^{125}I 135-13C MoAb and ^{131}I 135-14 MoAb from normal and line 1 tumor-bearing mice. Twelve BALB/c females, six weeks of age, were injected i.m. with 1 X 10^6 line 1 cells nine days prior to the clearance experiment. These 12 mice and 12 normal mice were given Lugol's solution in drinking water for four days preceding the experiment. 135-13C MoAb was labeled with ^{125}I to a specific activity of about 700 cpm/ng and 135-14 was labeled with ^{131}I to a specific activity of about 40 cpm/ng by the chloramine-T method. Labeled antibodies were separated from aggregates and free iodine as before.

One-hundred ug of ^{125}I 135-13C MoAb and 100 ug of ^{131}I 135-14 MoAb mixed in a 200 ul volume of BSA/PBS per dose was injected i.v. into the tail vein of 12 tumor-bearing and 12 normal mice. Two tumor-bearing and two normal mice were bled and sacrificed immediately after antibody injection and at 1, 2, 5, 8, and 24 hours after injection. The volume of blood recovered from each mouse was recorded. Blood was clotted and serum collected. Calculations of the fraction of label recovered in the blood in relation to total radioactivity injected were performed as before.

The fraction of label in the serum associated with active 135-13C MoAb was determined by a direct binding assay on line 1 cells (Kennel et al., 1981a). Sera recovered from normal mice sacrificed immediately after antibody injection (time 0) were incubated with line 1 cells. The fraction of label bound with 50 ng (as determined

by specific activity) of ^{125}I 135-13C MoAb added was used as a reference. The fraction of label bound with the addition of 50 ng of ^{125}I 135-13C from sera taken at 1, 2, 5, 8, and 24 hours after antibody injection was normalized to the fraction bound with sera from time zero (% active antibody). The fraction of active antibody was multiplied by the fraction of radioactivity recovered in the blood per mouse to determine the fraction of antibody activity (% antibody activity) remaining in the circulation at each time point. The t1/2 of clearance of active antibody from normal and tumor-bearing mice was determined from a plot of % antibody activity versus time.

The fraction of intact control immunoglobulin recovered from the circulation with time was determined by immunoprecipitation. Fifty ul of 25 mg/ml BSA, 100 ul of goat antiserum to rat IgG, 5 ul of rat sera and 5 ul of test samples were incubated for one hour at 37°C in 12 X 75 mm glass test tubes. Test samples included the antibody mixture and sera samples from all time points. Precipitates were collected by centrifugation, washed twice in PBS, and analyzed for ^{131}I. Cpm precipitated were compared to cpm added to determine the fraction of cpm associated with intact (precipitable) immunoglobulin.

Purification of TSP-180

TSP-180 was purified from line 1 tissue culture cells by immunoaffinity chromatography. 135-13C MoAb was coupled to epichlorhydrin-crosslinked, desulfonated, Sepharose 4B activated with cyanogen bromide (Kennel, 1976) to a density of 10 mg protein per ml of beads. 133-13A MoAb was also coupled to Sepharose 4B at the same

density. Approximately 2 X 10^9 line 1 cells were washed two times with PBS, scraped, pelleted by centrifugation and resuspended in a 10% suspension (1g/10 ml) of PBS, 1% Nonidet-P-40 (NP40), phenylmethylsulfonylflouride (PMSF, 10 ug/ml), 1 mM NaN_3, DNase II (10 ug/ml) and 2-3 mM $MgCl_2$. The cells were homogenized in a Polytron (Brinkman Instruments) at a setting of 6 for 15-20 seconds, three times. One 100 mm plate of line 1 cells was radiolabeled with lactoperoxidase, H_2O_2, and 2 mCi of carrier-free ^{125}I as previously described (Kennel, 1979). Labeled cells were solubolized by sonication in a Bronson Sonifier and added to the cell homogenate. The homogenate was centrifuged and the pellet discarded.

Sepharose beads coupled with MoAb were pre-treated with 1 M NaCl and washed three times with PBS prior to use. Fifty ml of line 1 cell homogenate was incubated with 0.5 ml of 133-13A beads overnight at 4^oC to bind P100, a surface protein on line 1 cells that is present at much higher levels than TSP-180 (Kennel et al., 1981a). The homogenate was then incubated with 0.5 ml of 135-13C beads overnight at 4^oC. The 135-13C beads were washed one time with PBS, loaded in a 1 ml column, and washed with 60-65 ml of PBS containing 5% NP40. Bound protein was eluted (TSP-180-AC) with 3M KSCN containing 5% NP40 and collected in 1 ml fractions. Fractions were analyzed for ^{125}I in a Searle automatic gamma spectrometer and the fraction containing the majority of the counts was dialyzed against PBS and concentrated to about 0.5 ml by vacuum dialysis. Protein yields were

measured on 50 ul test samples extracted with 1 ml of ether to remove interfering detergent (Lowrey et al., 1951).

For the characterization of TSP-180-AC, sodium dodecyl sulfate polyacrylimide gel electrophoresis (SDS-PAGE), immunoprecipitation, and autoradiography were performed as described (Kennel, 1979).

Production of 346-11 Monoclonal Antibody

A Fischer 344 rat, 4 weeks of age, was immunized with TSP-180-AC according to the following schedule: Day 1, injected with 3.5 ug TSP-180-AC emulsified in complete Freunds adjuvant intradermally (i.d.); Day 13, injected 3.5 ug TSP-180-AC emulsified in incomplete Frends adjuvant (IFA) i.d.; Day 23, injected 11 ug TSP-180-AC emulsified in IFA i.d.; Day 48 and 49, injected 11 ug TSP-180-AC in PBS i.p. The rat was bled on Day 16 through the orbital sinus and sera tested for precipitation of radiolabeled TSP-180-AC. Precipitation confirmed that the rat was immune. On Day 50, the rat was sacrificed, bled for a source of immune serum, and spleen cells (3×10^7) were fused with 5×10^7 P3-X63-Ag8 myeloma cells with 45% polyethyleneglycol as described by Fazekas de St. Groth et al. (1980). Cells were suspended in DME-20 medium containing hypozathine, aminopterin, and thymidine (DME-HAT) to select against parent myeloma cells (Kohler and Milstein, 1975). Cells were plated into twelve, 96-well microtiter plates (Costar) containing a feeder layer of peritoneal exudate cells (PECS; Fazekas de St. Groth et al., 1980).

PECS were obtained from a BALB/c mouse by injection of 5 ml of sterile 11.6% sucrose into the peritoneal cavity, massage of the area and drainage of the cavity with the syringe. PECS were pelleted, resuspended in DME-HAT and plated at 1 drop per well (about 1-2 X 10^3 PECS in 50-75 ul/well). Cultures were fed on Day 4 after fusion with 1 drop DME-HAT, on Day 7 with medium lacking aminopterin (HT) and on Day 11 with DME-20. Hybridoma cultures were screened for antibody production to line 1 cells on Day 12. Screening of culture fluids for antibody production to line 1 cells was performed with gluteraldehyde-fixed line 1 cells grown to 70-80% confluence in 96-well microtiter plates with a double antibody binding assay as described (Kennel et al., 1981a). Cultures positive for antibody production were subcultured into 24-well plates and then to 25 ml flasks (Corning Glass Works, Corning, New York). Hybridomas positive for antibody production on the third screen were frozen at approximately -10°/minute in nitrogen vapor using 1 X 10^7 cells/ml DME-20 containing 5% dimethyl sulfoxide.

Competition Assays

Purified monoclonal antibodies were radiolabeled with carrier-free ^{125}I by the chloramine-T method (McConahey and Dixon, 1966). Binding assays were conducted in 6-well plates of 80-90% confluent viable line 1 cells with 1 ug of radiolabeled 346-11 or 135-13C MoAb added per well and six 3-fold dilutions (1/100-1/24,300) of competitor (346-11, 133-13A or 135-13C MoAb) added to each well in duplicate. The amount of radiolabeled MoAb bound in the presence of

competitor was compared to the amount bound in the absence of competitor and the percent of competition was calculated as follows: 1-(ng MoAb bound with competitor/ng MoAb bound without competitor) X 100 = % competition.

Ascites fluids used in the competition assays were titered by a double antibody binding assay (Kennel et al., 1981b) to calibrate the amount of competitor used. The dilution needed to reach 50% of the maximum binding was used to calculate antigen binding capacity (ABC). The ABC of each ascites fluid containing 133-13A, 135-13C or 346-11 was calculated from the reciprocal of the volume of the reaction in ml, and the amount bound at 1/2 maximum binding (1/dilution X 1/volume X ng bound = ng/ml = ABC). All dilutions of ascites fluids used in the competition assays were expressed in terms of the ABC added.

CHAPTER III

RESULTS

Distribution of Iodinated MoAb in Normal and Tumor-Bearing Animals

The distribution of ^{125}I 135-13C in BALB/c mice bearing line 1 tumors approximately 1 cm in diameter was determined at various time points after i.v. injection. Figure 1 illustrates the distribution of ^{125}I 135-13C in the tumor leg and the contralateral normal leg with time. The $\%^{125}I$ recovered per gram of tissue weight relative to the total cpm recovered per mouse was 3-fold higher in the tumor leg than in the normal leg.

In control studies ^{131}I 135-14 and ^{125}I 135-13C were injected simultaneously into tumor-bearing mice and localization indexes were calculated. Lung, liver, spleen, kidney, tumor-bearing leg, normal leg and blood were sampled at various time points (Figure 2). A localization index of 1.0 indicates that the distribution of ^{125}I 135-13C is nonspecific since it is identical to the distribution of the control MoAb, 135-14. The apparent accumulation of 135-13C in the tumor leg is nonspecific with localization indexes falling consistently around 1.0. The blood, normal leg and liver also have localization indexes close to 1.0 at all time points, indicating nonspecific distribution of the anti-TSP-180 MoAb in these organs. In contrast, localization indexes for lung, kidney and spleen are above 2.0 at several time points after injection of antibody indicating specific accumulation of 135-13C MoAb in these organs. Statistical

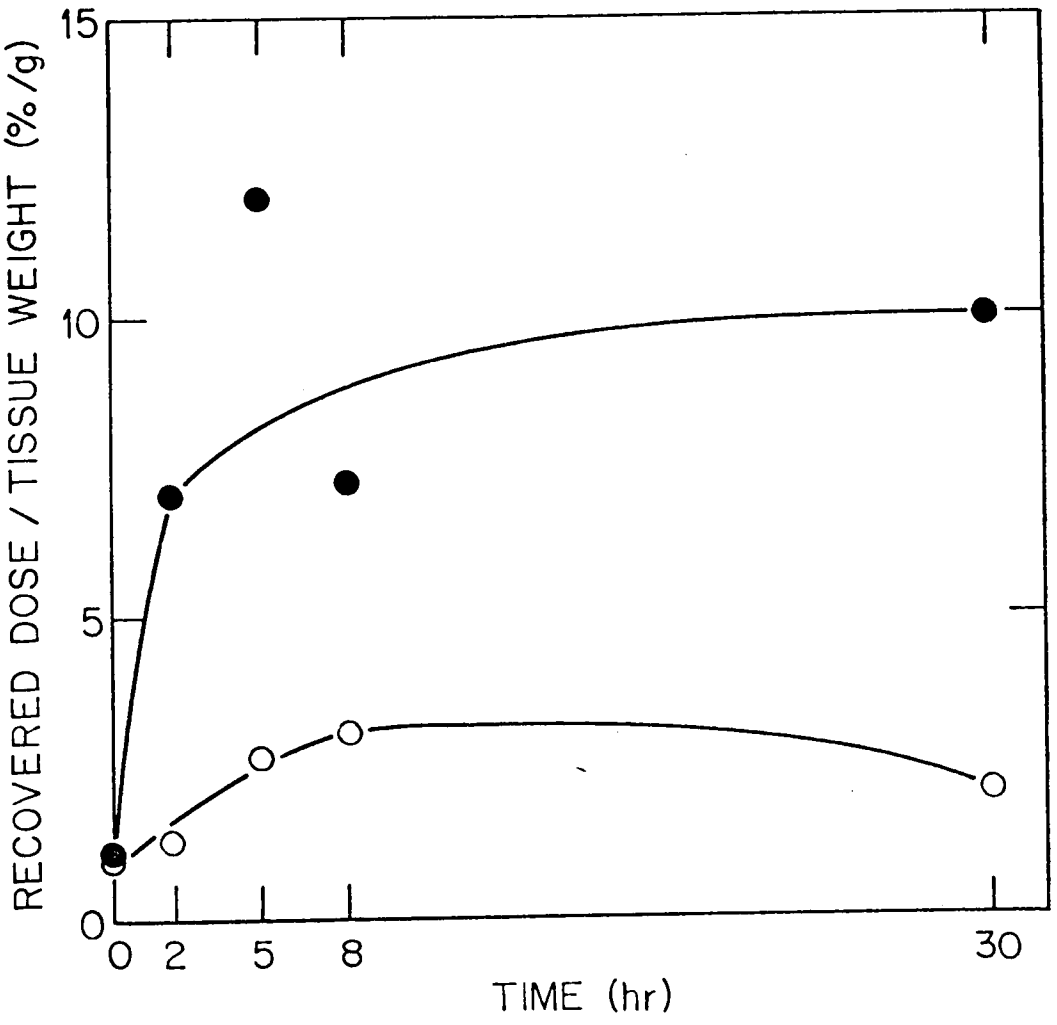

Figure 1. Distribution of I-125 135-13C in the tumor-bearing leg (●-●) and the normal leg (O-O) of BALB/c mice bearing approximately 1 cm in diameter line 1 tumors. Data normalized to the total radioactivity recovered per mouse at each time point.

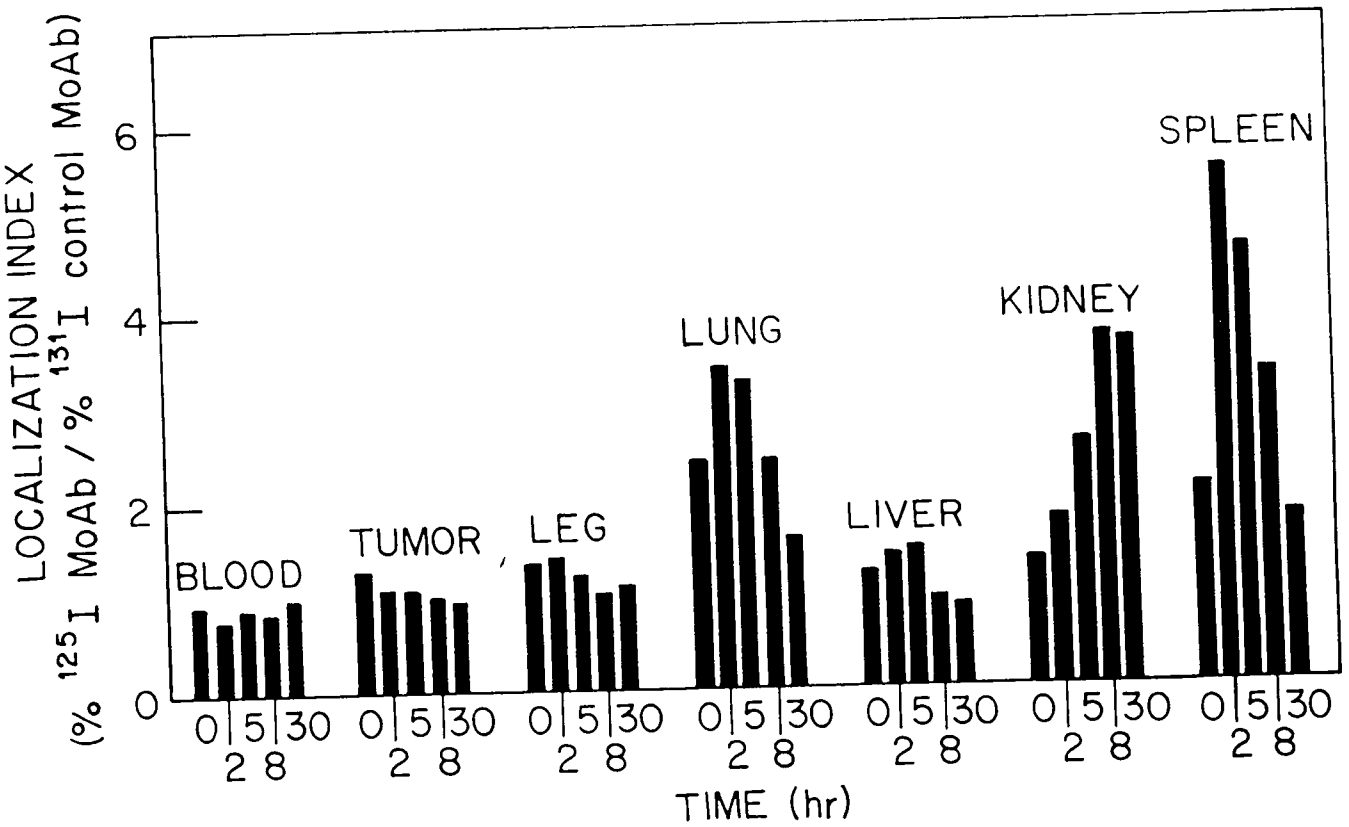

Figure 2. Localization indexes of I-125 labeled 135-13C MoAb to TSP-180/ I-131 labeled 135-14 control MoAb in BALB/c mice bearing approximately 1 cm in diameter line 1 tumors growing intramuscularly in the right rear leg. 100 ug of each antibody was injected i.v. per mouse. Duplicate animals were sacrificed for each time point and organs and tissues excised. Localization indexes were calculated as in Materials and Methods.

analysis was performed to determine if localization indexes for each organ where significantly different than the localization indexes for blood at each time point (data not shown). Results were clearly at odds with reason. There are not enough samples at each time point to yield an accurate reflection of the statistical significance of this data.

The patterns of localization indexes with time in the organs that show specific accumulation are not identical. Localization indexes in lung and spleen follow similar patterns increasing from immediately after antibody injection to two hours and decreasing thereafter. In contrast, a steady increase in the localization index is seen in the kidney up to thirty hours. Localization indexes cannot be accurately calculated for time points later than 30 hours since the detection of isotope remaining in the mouse is close to background levels.

Blood Clearance Kinetics

To test the contention that 135-13C binds to sites in normal mice, clearance of ^{125}I 135-13C MoAb and ^{131}I 135-14 control MoAb from the circulating blood of line 1 tumor-bearing and normal mice was studied. Figure 3 (A and B) illustrates the clearance of radioactivity of ^{125}I 135-13C and ^{131}I 135-14 from the circulation of normal and tumor-bearing mice with time. Values for isotope recovered are corrected for the total blood volume as described in Materials and Methods. Clearance of radiolabeled antibody occurs in two phases, with rapid clearance between zero and 8 hours after

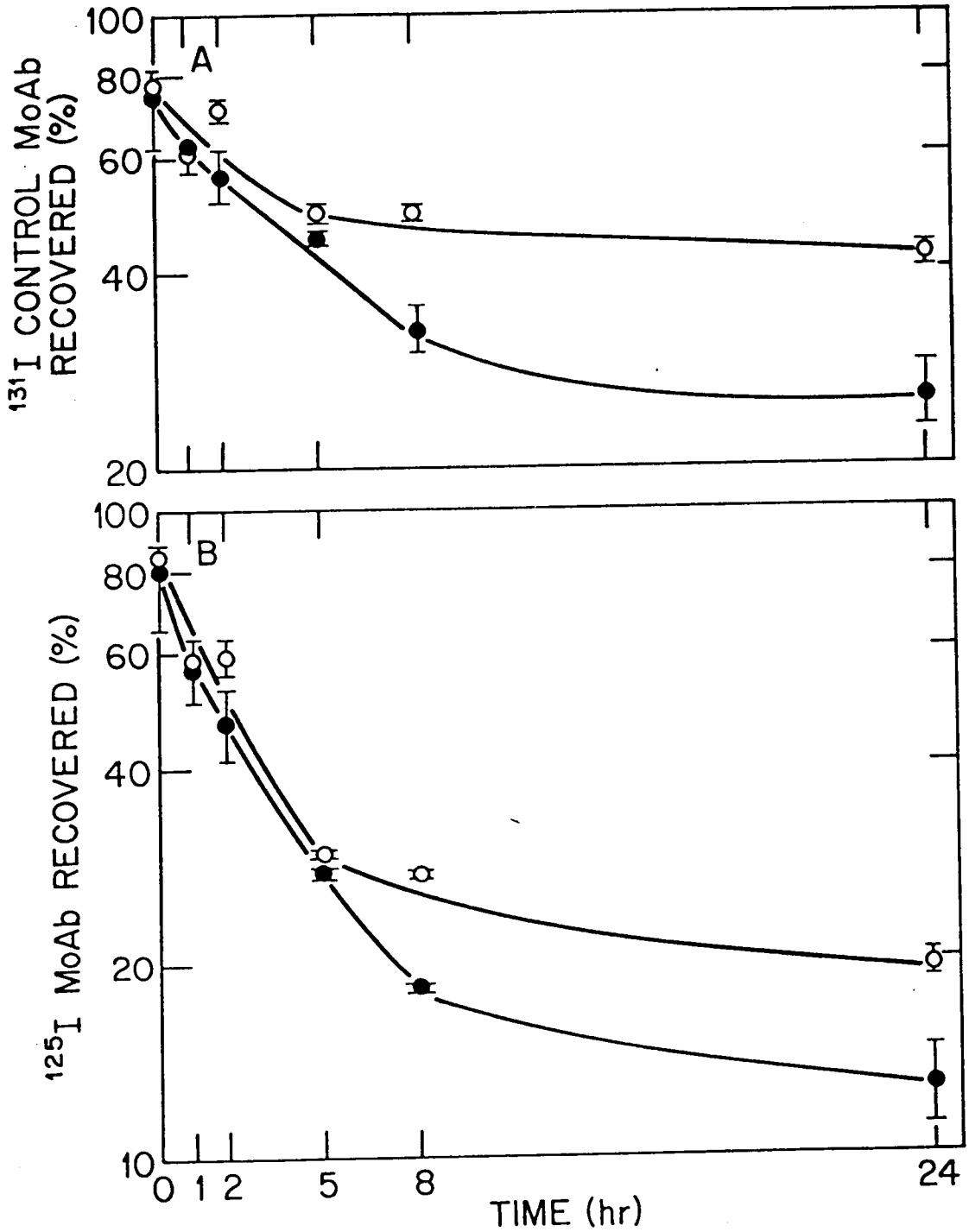

Figure 3. Recovery of radioactivity injected i.v. into tumor-bearing (●-●) and normal (O-O) mice. A) Recovery of the I-131 labeled control MoAb 135-14. B) Recovery of the I-125 labeled specific MoAb 135-13C. Duplicate animals were injected with 100 µg of each MoAb, anaesthesized and bled at each time point.

antibody injection and slower clearance between 8 and 24 hours. Control MoAb is cleared in a qualitatively identical manner by normal and tumor-bearing mice, as is specific MoAb. However, clearance of ^{131}I 135-14 cpm (t1/2 = 5.5 hours) is slower than clearance of ^{125}I 135-13C cpm (t1/2 = 2.5 hours) in the first phase of clearance (Figure 3A and 3B). T1/2's cannot be calculated accurately for the second phase of clearance with this data but a rough estimate of t1/2 = 1 day for both ^{125}I 135-13C and ^{131}I 135-14 can be determined (Figure 3, A and B).

135-13C MoAb recovered in the serum of normal and tumor-bearing mice was tested for antigen binding capacity at each time point by a direct binding assay on line 1 cells to determine if the differences in clearance kinetics in the first phase of clearance between 135-13C and 135-14 were due to specific binding of ^{125}I 135-13C. The percent of ^{125}I MoAb binding relative to total ^{125}I from recovered serum was determined. Results were normalized to the percent of binding with antibody from the serum of normal mice bled directly after injection (zero time) as described in Materials and Methods. Figure 4 illustrates the clearance of active antibody from the blood of normal and tumor-bearing mice with time. The first phase of clearance of antibody activity (t1/2 = 1.25 hours) is more rapid than the first phase of clearance of cpm (t1/2 = 2.5 hours). Faster clearance of antibody activity relative to the clearance of isotope indicates a preferential depletion of specific antibody IgG from the circulation of both normal and tumor-bearing mice. By 24 hours, the fraction of active

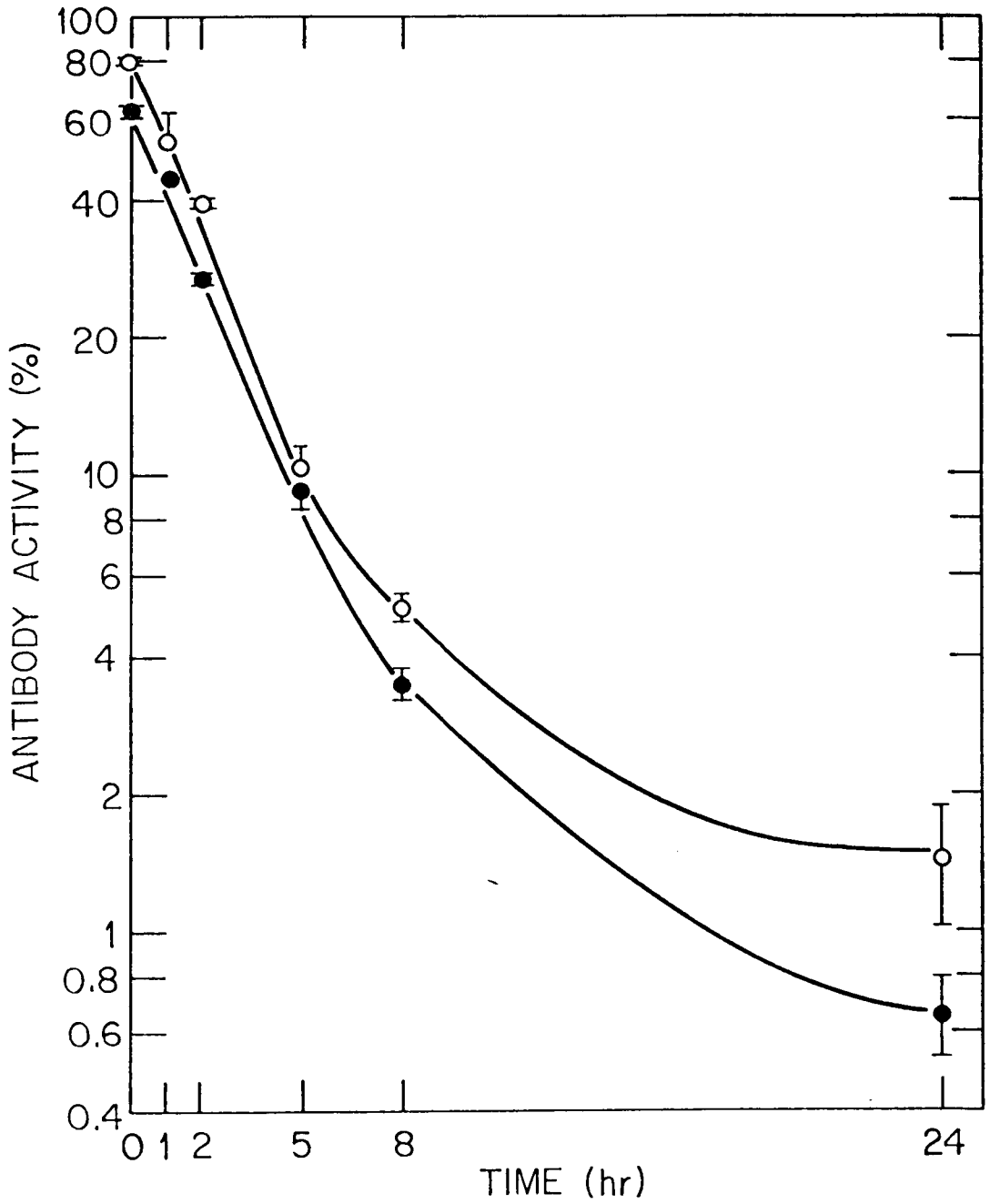

Figure 4. Active antibody recovered after in vivo passage in tumor-bearing (●-●) and normal (O-O) mice. Normalized to the direct binding of the antibody recovered immediately after antibody injection (0 time) from normal mice as shown in Materials and Methods.

antibody remaining is less than 2% while the fraction of ^{125}I isotope remaining is between 13 and 19% in tumor-bearing and normal mice (Figure 4 vs. Figure 3B).

The fraction of active ^{131}I 135-14 control MoAb cannot be measured by a direct binding assay since 135-14 MoAb does not have a known binding specificity. However, the fraction of control MoAb associated with intact IgG in the recovered serum was determined by immunoprecipitation of ^{131}I with goat antiserum to rat IgG. Eighty-five to 95% of the control MoAb was associated with intact IgG at all time points (data not shown). Normalizing to the fraction of intact antibody recovered in the serum of mice sacrificed immediately after antibody injection (0 time), as above, showed that all of the ^{131}I MoAb is associated with intact IgG at every time point.

The fraction of control MoAb remaining in the serum at 24 hours was measured as the fraction of ^{131}I counts relative to counts injected remaining in the sera as described in Materials and Methods. Twenty-five to 44% of the control MoAb remains in the sera at 24 hours (Figure 3A). In contrast, only 0.65-1.4% of active 135-13C MoAb remains in the serum at 24 hours (Figure 4). Active antibody is, therefore, cleared much more completely by 24 hours than control antibody by both normal and tumor- bearing mice.

The first phase of clearance of active antibody is considerably faster (t1/2 = 1.25 hours) than the first phase of clearance of control MoAb (t1/2 = 5.5 hours). Furthermore, clearance kinetics of active antibody are identical in normal and tumor-bearing mice

(Figure 4). Preferential depletion of active antibody relative to the control MoAb does not, therefore, result from the binding of specific antibody to the line 1 tumor. Preferential depletion of active antibody in normal mice supports the contention that 135-13C is binding to sites in normal mice.

Assay for TSP-180 in Tissue Lysates

In an effort to develop an *in vitro* assay for the detection and quantitation of TSP-180 in BALB/c organs, an indirect binding assay on crude membrane fractions from line 1 tumors and organs was designed. Crude membrane preparations of excised tumors, lungs, livers, spleens, and kidneys were prepared by differential centrifugation of organ homogenates (Brown et al., 1981a). Membrane fractions were attached to 96-well plates using poly-l-lysine (Kennel, 1982). 135-13C MoAb was used as the primary antibody and radiolabeled goat or mouse antibody to rat IgG was used as the secondary antibody. After numerous modifications and trials, varying both the concentration of membrane added and the concentration of primary antibody used for detection, this assay proved to be unreliable (data not shown).

It was noted that Brown et al. (1981) in the human melanoma system successfully quantitated p97 in detergent solubilized membrane preparations of normal tissues and tumors using a double- determinant immunoassay. This type of assay requires an antibody to a second epitope on the target antigen. One antibody is used to immobilize the antigen and a second antibody is used for detection. All the

monoclonal antibodies to TSP-180 developed by Kennel et al. (1981a) were directed to the same epitope on TSP-180. Therefore, it would not be possible to develop a double-determinant (two-site) immunoassay unless an antibody to a second epitope was produced. The remaining sections in this chapter describe the development and characterization of a second monoclonal antibody to TSP-180 that could be suitable for use in a two-site immunoassay.

Purification of TSP-180

An appropriate immunogen is required for the development of a monoclonal antibody to a second epitope on TSP-180. An attempt to find a MoAb to a second site using the immunization protocol that was employed in the production of 135-13C MoAb (Kennel et al., 1981a) was unsuccessful. The probability for the successful production of a hybridoma secreting antibody to a second site would be vastly increased if the first site was masked or destroyed on the immunogen prior to immunization. Foote and Kennel (unpublished observations) discovered that the epitope recognized by 135-13C was destroyed by 3M KSCN treatment if they used 3M KSCN to elute TSP-180 from an immunoaffinity column prepared with 135-13C MoAb. This property was exploited in the purification of TSP-180 for use as an immunogen. TSP-180 was purified by immunoaffinity chromatography from line 1 tissue culture cell lysates. Two hundred plates of cells containing one plate of ^{125}I lactoperoxidase-labeled cells as a marker were solubilized and TSP-180 purified on a 135-13C immunoaffinity column. Radioactivity is associated with surface proteins. TSP-180 was

eluted from the column with 3M KSCN. The migration in a 4 to 10% gradient polyacrylimide gel of the protein(s) recovered from the affinity column is shown in an autoradiogram (Figure 5). There is one major band at approximately 150,000 M.W. and two minor bands at 175,000 and 135,000 M.W. (Figure 5). Protein purified from this 135-13C MoAb affinity column does not migrate at 180,000 M.W. Nevertheless, since the protein was purified from a highly specific column, it was called TSP-180-AC.

Characterization of Purified TSP-180-AC

Immunoprecipitates (IP) of chloramine-T labeled ^{125}I TSP-180-AC were set up to test whether TSP-180-AC could be precipitated specifically by selected antisera. Autoradiograms of SDS-PAGE profiles of these IPs are shown in Figure 6. It was found that specific protein bands (Figure 6, slot 1) were precipitated by rat antisera to line 1 cells (Figure 6, slot 3) and by rat antisera to TSP-180-AC (Figure 6, slot 4) but were not precipitated by the normal rat sera control (slot 2) or 135-13C MoAb (slot 5). This indicates that TSP-180-AC, purified by its interaction with 135-13C bound to an affinity column, is recognized as specific to line 1 cells and as TSP-180-AC but is no longer capable of binding to the 135-13C MoAb that was used to purify it. The implication is that the determinant recognized by 135-13C was destroyed or lost when TSP-180-AC was eluted from the affinity column with 3M KSCN. Since an antibody to a different determinant on TSP-180 was desired, this preparation of TSP-180-AC was used as an

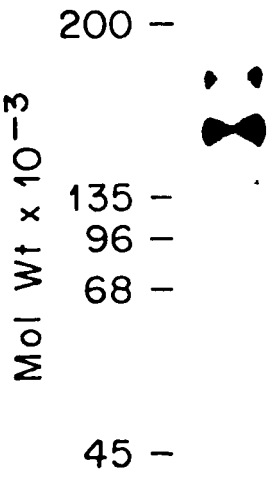

Figure 5. Autoradiogram of a 4-10% gradient SDS-PAGE of 25 µl (1.8 ug Lowrey's protein) of eluate from a 135-13C immuno-affinity column incubated with line 1 cell lysate containing one plate of I-125 lactoperoxidase surface labeled line 1 cells.

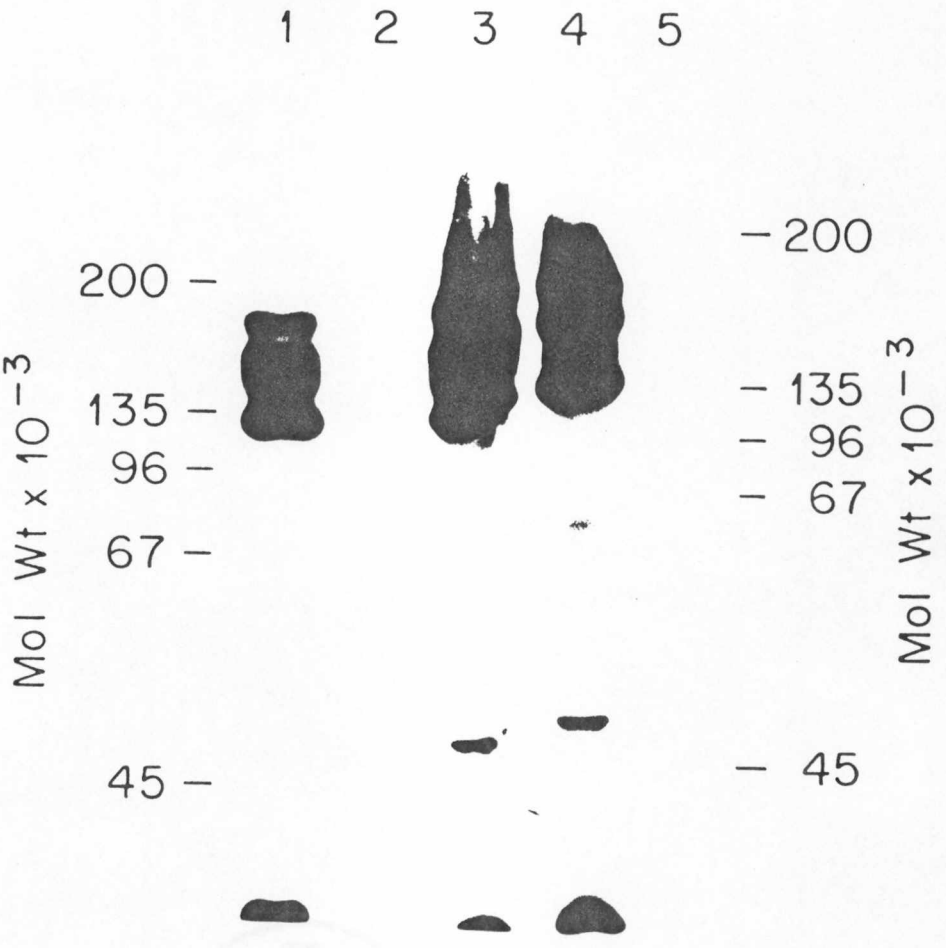

Figure 6. SDS-PAGE autoradiogram of immunoprecipitates of I-125 labeled TSP-180-AC (slot 1) with normal rat sera (slot 2); rat antiserum to line 1 cells (slot 3); rat antiserum to TSP-180-AC (slot 4); and 135-13C MoAb with normal rat serum added (slot 5).

immunogen in the production of a monoclonal antibody to a second site on the TSP-180 molecule.

Monoclonal Antibody Production

A rat was immunized with TSP-180-AC and was bled for a source of immune sera. The rat was sacrificed for hybridoma production. Spleen cells were fused with the parent myeloma P3-X63-Ag8 with polyethyleneglycol and then plated onto feeder layers of peritoneal exudate cells (PECS) in twelve 96-well plates (Fazekas de St. Groth et al., 1980). Two-thirds of the wells were positive for hybrid growth after selection with HAT medium. A primary screen of supernatants from all wells on gluteraldehyde-fixed monolayers of line 1 cells showed that about 3% (37 wells) were positive for antibody to line 1 cells. These cultures were transferred to 24-well plates and tested for binding to gluteraldehyde-fixed line 1 and A31 (TSP-180 negative) cells. Thirteen tested positive on line 1 cells and negative on A31 cells. However, only one hybrid, 346-11, consistently tested positive on line 1 cells in subsequent screens. 346-11 was expanded in culture, cells cryopreserved, and Ig from culture supernatants was concentrated by ammonium sulfate precipitation for future use and characterization.

Binding Specificity of 346-11 Monoclonal Antibody

Specificity of 346-11 MoAb for TSP-180 was determined by its ability to immunoprecipitate TSP-180 from surface-labeled proteins of line 1 cells. Figure 7 shows the autoradiogram of SDS-PAGE of IPs.

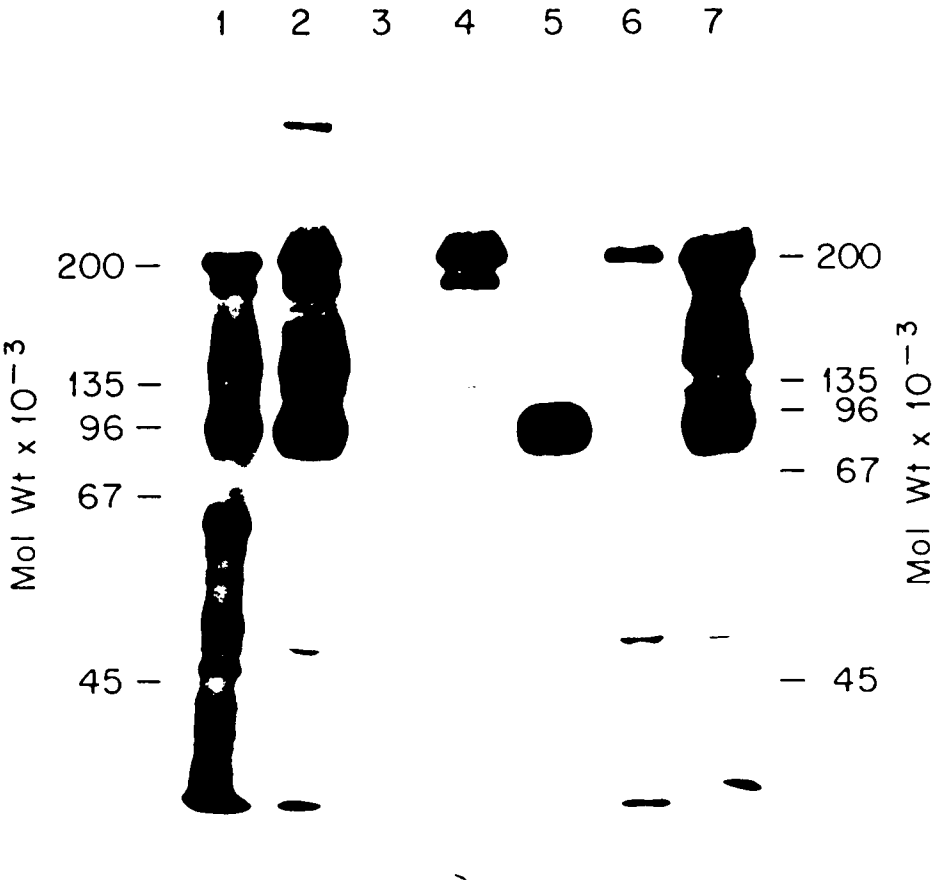

Figure 7. Autoradiogram of immunoprecipitates with I-125 surface labeled-line 1 proteins. Labeled cell lysate is shown in slot 1. Immunoprecipitates are with rat antiserum to line 1 cells (slots 2 and 7); normal rat sera (slot 3); 135-13C MoAb (slot 4); 133-13D MoAb (slot 5) and 346-11 MoAb (slot 6). 4-10% gradient SDS-PAGE gel.

Positive controls were run with 135-13C MoAb, 133-13D MoAb (directed to P100), and rat antisera to line 1 cells. A negative control was run with normal rat sera. Rat antisera to line 1 cells (slot 2 and 7) precipitates several proteins with molecular weights ranging from 200,000 to about 70,000 and with one band at 50,000 M.W. 135-13C (slot 4) predominately precipitates a protein of 180,000 M.W., TSP-180. Minor bands are probably contaminants or degradation products since this MoAb was previously determined to be monospecific (Kennel et al., 1981a). 133-13D (slot 5) precipitates a protein of around 100,000 M.W. The negative control, normal rat sera (slot 3), does not precipitate any labeled proteins from line 1 cell lysates.

346-11 (slot 6) precipitates a protein of about 180,000 M.W., with minor bands at about 170,000 M.W. and 50,000 M.W. 346-11 MoAb does not precipitate as much protein at 180,000 M.W. as 135-13C MoAb. These results show that 346-11 MoAb precipitates a TSP-180 like protein from line 1 cell surface proteins.

Competition Analysis

To prove that 346-11 MoAb and 135-13C MoAb bind to different epitopes on TSP-180 on line 1 cells, competition assays were performed (Stahli et al., 1983). Ascites fluid from nu/nu BALB/c mice containing MoAb from 346-11 cells was tested for its ability to compete with 1 ug of ^{125}I-labeled 135-13C for binding to 80% confluent monolayers of line 1 cells in 60 mm, six-well plates. Ascites fluid containing 135-13C MoAb was used as a positive control and ascites fluid containing 133-13A MoAb (directed to P100) was used as a

negative control. All dilutions were adjusted for antigen binding capacity (ABC = ng/ml needed to give half maximum binding on line 1 cells) and expressed as ABC units in ng/ml for each ascites so they can be directly compared in the competition assays. Figure 8 illustrates that 346-11 ascites fluid does not compete for the binding of TSP-180 with ^{125}I 135-13C at all dilutions tested, nor does the negative control 133-13A ascites fluid. 135-13C ascites fluid competes well with purified 135-13C in this same range of dilutions, reaching a level of about 91% competition at the highest concentration of competitor added. This verifies that 346-11 MoAb does not bind to the same epitope as 135-13C MoAb on TSP-180.

The reciprocal experiment was performed to directly confirm this result. 135-13C ascites fluid, 133-13A ascites fluid (negative control) and 346-11 ascites fluid (positive control) were tested for their ability to compete with 1 ug of ^{125}I-labeled 346-11 MoAb for binding to line 1 cells. Figure 9 illustrates the results of this experiment. 346-11, as expected, competes well with itself reaching a level of 95% competition for binding sites at the highest concentration of competitor added. 135-13C ascites fluid and 133-13A ascites fluid do not perform well as competitors although there is a very low level of competition at the highest concentrations of competitor added. This is probably a nonspecific effect since both the experimental 135-13C ascites fluid and the control 133-13A ascites fluid show these very low levels of competition.

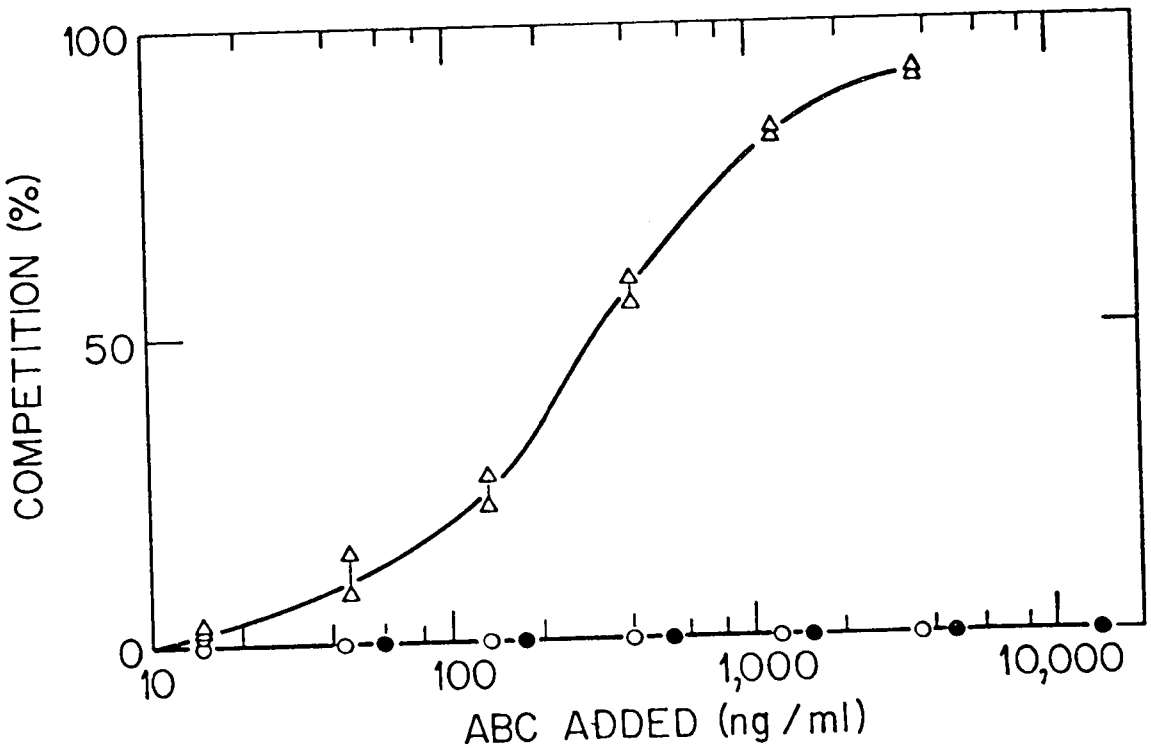

Figure 8. Competition against 1 µg of I-125 135-13C for binding to line 1 cells with 135-13C ascites fluid (△-△), 346-11 ascites fluid (O-O) and 133-13A ascites fluid (●-●). All dilutions of ascites fluids in duplicate.

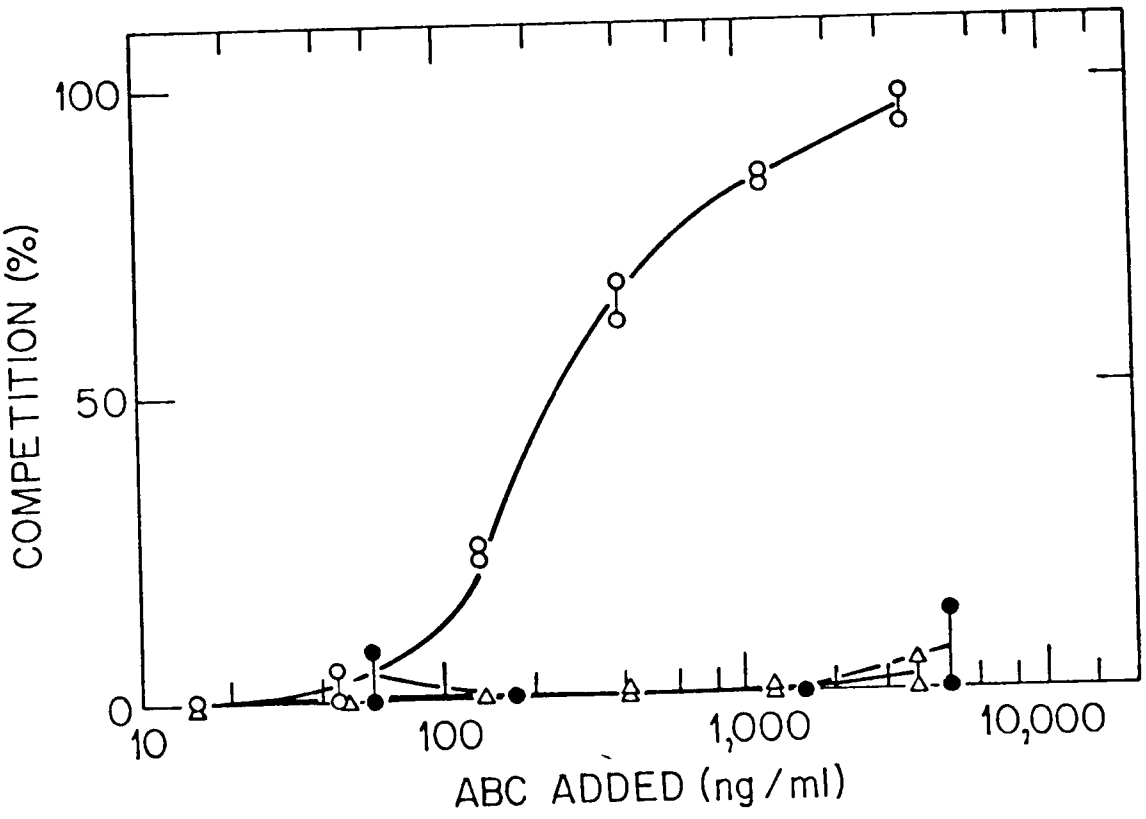

Figure 9. Competition against 1 µg of I-125 346-11 with 356-11 ascites fluid (O-O), 135-13C ascites fluid (Δ-Δ), and 133-13A ascites fluid (●-●). All dilutions of ascites fluids in duplicate.

Binding of radiolabeled 346-11 MoAb to the surface of line 1 cells indicates that the 346-11 epitope is expressed on the surface of viable cells.

One final experiment was performed to verify that 135-13C MoAb and 346-11 MoAb bind to different epitopes on TSP-180. Radiolabeled 135-13C MoAb (0.5 ug) and 346-11 (0.5 ug) were bound to line 1 cells separately or mixed together. The results (Table I) show that the binding of these two monoclonal antibodies was additive indicating that they do not interfere with each other for binding sites on TSP-180 (Stahli et al., 1983).

In summary, a monoclonal antibody (346-11) was developed to an epitope on TSP-180 that is distinct from the epitope recognized by 135-13C MoAb. Both 346-11 MoAb and 135-13C MoAb precipitate a 180,000 M.W. surface protein from solubilized line 1 cells. Neither can compete with the other for binding to line 1 cells.

Table I. Binding of I-125 Labeled MoAbs to Line 1 Cells

	CPM Bound	
MoAb added	1	2
346-11[a] (0.5 ug)	46853	433988
135-13C[b] (0.5 ug)	372895	366262
135-13C + 346-11 (0.5 ug + 0.5 ug)	864264	881163

[a]Specific activity = 7378 cpm/ng.

[b]Specific activity = 3898 cpm/ng.

CHAPTER IV

DISCUSSION

Distribution Studies and Blood Clearance Kinetics

A good imaging antibody binds preferentially to the tumor cell surface *in vivo* and binds to an antigen that is expressed at high densities on the tumor cell surface. The utility of an imaging antibody is influenced by the affinity of the antibody for the target antigen and by the purity of the imaging antibody. The affinity and purity of the antibody determine the fraction of antibody that can bind to tumor cells. Kennel et al. (1983a) determined that antibodies must have binding constants of 10^8 M^{-1} or greater to be useful for tumor imaging. They also found that the fraction of antibody that can bind to the cell surface is directly proportional to antibody purity.

The target tumor also plays a key role in the success of imaging with radiolabeled antibodies. The antibody must be able to reach the target tumor *in vivo* through the circulation. Tumors with poor vascularization would not be appropriate candidates for imaging since they are not readily accessable via the circulation.

Characterization of antibodies with potential for tumor radioimaging has routinely been performed by *in vitro* determination of the binding specificity of the antibodies to cells and tissue lysates (Steplewski and Koprowski, 1982; Raschke, 1982; Damjanov and Knowles, 1983). It is not possible to test all normal tissues and

cells for antigen expression *in vitro*. Furthermore, *in vitro* assays cannot measure the amount of antigen in normal tissues relative to that found in the tumor or assess the effect of antigen expression on normal cells to tumor imaging (Kennel et al., 1984). Successful imaging of tumors has been accomplished even when antigen is present on normal cells as demonstrated by the imaging of human carcinomas with anti-CEA antibodies (van Nagell et al., 1980; Mach et al., 1981), of melanomas with anti-p97 antibody (Larson et al., 1983) an of hepatocellular and germ cell carcinomas with anti-alpha-fetoprotein antibodies (Goldenberg et al., 1980). This thesis presents a means to test one aspect of an antibody's imaging potential by analysis of the antibody's clearance kinetics in normal animals. This method could be used to assess an antibody's imaging potential in normal human subjects prior to imaging trials.

The antibody used in this study, 135-13C, had been previously characterized as directed to a putative tumor-specific antigen, TSP-180, by *in vitro* binding criteria (Kennel et al., 1981a). *In vivo*, radiolabeled 135-13C accumulates in the tumor-bearing leg (Figure 1, page 23), however analysis of a distribution of ^{125}I 135-13C relative to the distribution of ^{131}I 135-14 control MoAb in tumor-bearing mice indicates that the accumulation of 135-13C MoAb is not specific (Figure 2, page 24). The control MoAb, 135-14, accumulates in the tumor-bearing leg to the same extent as 135-13C MoAb. Non-specific accumulation of immunoglobulins is probably due to poor or altered vascularization of the tumor which results in a fluid pool in the tumor

and subsequent accumulation of serum proteins, including immunoglobulins (Jewell and Aarons, 1979; Day, 1973; and Witz, 1977). In fact many solid tumors developed from transplanted cells, including line 1 cell tumors, develop serum pools in the extracellular space. Non-specific accumulation of immunoglobulins in the tumor could mask a small amount of specific binding of 135-13C MoAb to the tumor.

135-13C has relatively large (3-5) localization indexes in lung, kidney, and spleen of the BALB/c mouse (Figure 2, page 24), suggesting the presence of the epitope recognized by 135-13C MoAb on normal cells from these organs. It is not likely that localization of 135-13C in the lung is due to binding to metastases in the lung from the original line 1 cell inoculum since too few lung metastasis would be present at day 8. Yuhas et al. (1974b) only found one mouse out of eight with lung metastases 28 days after the mice received 4×10^7 line 1 cells subcutaneously in the dorsal cervical region.

Localization indexes in the spleen and lung are highest two hours after injection of antibody and decrease thereafter whereas localization indexes in the kidney continue to rise up to 30 hours. It is possible that increased localization of ^{125}I 135-13C in the kidney is due to an accumulation of degradation products of 135-13C. Blood clearance kinetics indicate that active ^{125}I 135-13C is cleared faster than ^{131}I 135-14 or inactive ^{125}I 135-13C. Binding to epitopes in the normal animal, such as on lung and spleen, and subsequent degradation of 135-13C could lead to an increase in labeled degradation products of 135-13C in the kidney at later time points.

Blood clearance kinetics of ^{125}I 135-13C and ^{131}I 135-14 control MoAb from normal and tumor-bearing mice was performed to determine if localization of 135-13C MoAb to BALB/c organs could be a result of binding to epitopes in normal mice. The clearance of ^{125}I 135-13C MoAb was compared to the clearance of ^{131}I 135-14 control MoAb. It was found that the clearance of both antibodies occurred in two phases with the initial phase of clearance more rapid than the second phase of clearance (Figure 3, A and B, page 26). The specific MoAb, 135-13C, was cleared more rapidly (t1/2 = 2.5 hrs) than the control MoAb (t1/2 = 5.5 hrs), 135-14, in the first phase of clearance in both normal and tumor-bearing mice. Shorter clearance times of 135-13C MoAb relative to the control MoAb could indicate that 135-13C MoAb is binding to antigen in the animal. Alternatively, it could indicate that 135-13C has a unique Fc interaction with host cells or that 135-13C MoAb is more extensively damaged than 135-14 MoAb during the labeling procedure. A unique Fc interaction would not have been detected by the competition assays used to characterize the binding specificity of 135-13C (Kennel et al., 1981a). However, clearance of 135-13C MoAb and 135-14 MoAb with the Fc portions removed (F(ab)'2) is qualitatively identical to the clearance of intact MoAbs (data not shown) so this alternative is not likely. The second alternative was tested by column chromatography of ^{125}I 135-13C MoAb from the serum of normal and tumor-bearing mice (data not shown). No low molecular weight fragments of 135-13C MoAb were detected. Therefore, differences in clearance probably do not result from extensive damage to

135-13C MoAb during the labeling procedure although minor structural alterations cannot be ruled out.

The second phase of clearance was roughly identical for both antibodies with a t1/2 of about 1 day. The two phases of clearance may result from differential clearance of intact and degraded immunoglobulin molecules, from differential clearance as a function of the degree of radioactive iodine substitution since iodination of immunoglobulin molecules is not uniform (McConahey and Dixon, 1966) or from dehalogonation of a fraction of the labeled antibodies with subsequent rapid elimination of free iodine. Radiolabeled 135-14 can be precipitated as intact immunoglobulin with antiserum with serum samples taken at every time point and, as discussed above, degradation products of 135-13C could not be detected by column chromatography suggesting that the first phase of clearance does not result from the faster clearance of degradation products. Differential substitution of radioactive iodine on antibody molecules, however, might account for the two phases of clearance of ^{131}I 135-14 and at least for part of the two phases of clearance observed with ^{125}I 135-13C. Furthermore, the clearance of immunoglobulins is influenced in part by the number of terminal sialic acids on the molecule (Spiegelberg and Weigle, 1965). Chloramine-T, used in the labeling procedure, is an oxidizing agent which can destroy terminal sialic acids (McConahey and Dixon, 1966). The two phases of clearance may result from clearance of antibodies with different degrees of destruction from the chloramine-T. These antibodies would still be recognized as intact

IgG by immunoprecipitation with antisera. Faster initial clearance of ^{125}I 135-13C could also be due to clearance of most of the active 135-13C MoAb in the first phase of clearance (less than 2% of active 135-13C MoAb was detected by specific binding at 24 hours after antibody injection).

The t1/2 of the second phase of clearance obtained in this study for 135-14 control MoAb (1 day) is not in agreement with values found in the literature. Spiegelberg and Weigle (1965) documented the circulation times of immunoglobulins and their fragments in several species. They found that different classes and subclasses of immunoglobulin had different circulation times but that most of the t1/2's of circulation are on the order of 4-10 days. Kennel et al (1984) measured the clearance of ^{131}I 135-14 and found a t1/2 of clearance of 5.8 days. This study was conducted with antibody injected i.p. whereas clearance studies in this thesis were conducted with antibody injected i.v. Repeat experiments confirmed that i.p. and i.v. injection routes give different results (data not shown). Relying on isotope as an accurate measure of active antibody is not adequate (Kennel et al., 1983a) since labeling is not homogeneous and since even the best antibody preparations contain some contaminants.

The clearance of active ^{125}I 135-13C MoAb was tested by using binding assays to assess the remaining active antibody. It was found that the t1/2 of clearance for active antibody (1.25 hrs) was faster than the t1/2 of clearance for radioactivity associated with 135-13C MoAb (2.5 hrs; Figure 4, page 28 vs. Figure 3B, page 26). The faster

clearance of active antibody relative to the clearance of isotope indicates a preferential depletion of specific antibody immunoglobulin from the circulation relative to other labeled molecules in the 135-13C preparation (mostly Ig; Kennel et al., 1983a). 135-13C was produced from a fusion of immune spleen cells with the parent myeloma P3-X63-Ag8. P3-X63-Ag8 secretes its own gamma$_1$ heavy chains and kappa light chains so hybridomas produced with this parent myeloma, such as 135-13C, probably secrete P3 immunoglobulin and hybrid immunoglobulin molecules as well as specific antibody (Kennel et al., 1983a). Even though purification procedures discriminate against P3 parent and hybrid Igs, immunoglobulin purified from 135-13C ascites fluid is not 100% active (Kennel et al., 1983a). Gel filtration columns were used to analyze ^{125}I MoAb from serum samples from every time point to determine if the isotope remaining in the serum is associated with intact or degraded Ig. Column profiles for all serum samples were qualitatively identical (data not shown). The dissociation of radioactivity recovered (13-19%) from the amount of active antibody (less than 2%) remaining at 24 hours is not due to a major degradation of immunoglobulin.

Clearance of active antibody from the circulation is qualitatively identical in normal and tumor-bearing mice (Figure 4, page 28). The presence of a tumor with high levels of TSP-180 antigen does not appear to effect the clearance pattern of anti-TSP-180 antibody from the ciculation. It should be noted, however, that initially there is more active antibody in normal mice (about 80%) than

in tumor-bearing mice (about 60%) in serum samples (Figure 4, page 28; t = 0). Repeat experiments confirmed this result (Kennel et al., 1984). This initial difference in active antibody levels is possibly due to binding to TSP-180 in the serum since TSP-180 can be detected at low levels in the serum of tumor-bearing animals (Kennel et al., 1980). However, clearance times from normal animals are fast as well and no TSP-180 was detected in normal mouse serum. Blood clearance kinetics indicate that 135-13C is specifically removed from the circulation (t1/2 = 1.25 hrs) of both normal and tumor-bearing mice relative to the control MoAb (t1/2 = 5.5 hrs) or the clearance of radioactivity associated with 135-13C (t1/2 = 2.5 hrs). The implication is that active 135-13C MoAb is binding preferentially to sites in the normal mouse.

Data from the distribution studies support this conclusion. There is no specific localization of the 135-13C MoAb in the tumor leg (LI = 1, Figure 2, page 24) even though 18% of the label retained in the mice is found in the tumor leg 5 hours after injection of antibody. In contrast, spleen, lung and kidney retain 6%, 1.7% and 8% of the label with localization indexes of 4.5, 3 and 2.5 at 5 hours after injection of antibody. High levels of expression of TSP-180 in normal organs could preclude adequate accumulation of ^{125}I 135-13C in the tumor for tumor imaging if these organs contain large amounts of antigen or if the antigen is more easily accessed in these organs through better vascularization than in the tumor. A significant level of binding to normal cell sites was not expected since

normal BALB/c cells and tissue lysates did not compete with anti-line 1 cell antisera for binding to line 1 cells (Kennel et al., 1979, 1980 and unpublished observations). Even if low levels of competition had been found, it would not necessarily indicate that the antibody could not be used as an imaging agent. As demonstrated in the human melanoma system (Brown et al., 1981b and Larson et al., 1983), low levels of target epitope expression on normal cells does not preclude imaging of the tumor when the level of expression of antigen on tumor cells is much greater. Therefore, an empirical test of the in vivo clearance kinetics of a putative imaging antibody from the circulation of a normal animal or subject would be the most accurate way to determine its potential utility as an imaging agent. As demonstrated, rapid clearance of active antibody from the circulation of normal mice suggests significant binding to normal cell sites with the subsequent potential for interference from these sites in the imaging of tumors.

Although an in vivo clearance test is a useful test for determining whether an antibody might be suitable as an imaging reagent, it would be preferable to identify and quantitate sites of epitope expression in the normal subject in vitro prior to in vivo testing. There are limitations in using an in vivo blood clearance test in human systems since humans are not genetically identical. It is clear from Spiegelberg and Weigle's study (1965) that there are species differences in the clearance of immunoglobulins, although generally Igs are cleared in 4-10 days. It would be difficult to

extrapolate the clearance data from normal human subjects to the clearance of immunoglobulins in humans in general. Nevertheless, very rapid clearance of antibody (on the order of hours rather than days) would indicate binding of antibody to epitopes on normal cells. Blood clearance tests cannot, however, be used to screen an antibody for tumor specificity. An *in vitro* test is required for these purposes. An *in vitro* test would also allow for the elucidation of potential problems with the imaging antibody, such as a widespread distribution of the target epitope on normal cells or excessively high levels of target epitope in the normal animal or subject relative to antigen levels on the tumor cells. Furthermore, *in vitro* techniques could be used to isolate the proteins expressing the target epitope for comparative analysis. One could then determine the degree of homology between the normal and tumor-associated proteins thus elucidating any qualitative differences in protein expression between tumor and normal cell proteins bearing the target epitope. In conjunction with assays that quantitate the amount of epitope on normal and tumor cells, analysis of the isolated proteins would elucidate quantitative changes in protein expression between normal and tumor cells.

Monoclonal Antibody Development and Characterization

A two-site immunoassay could be used to quantitate antigen levels in normal tissue lysates (Brown et al., 1981a, 1981b). The development of this assay requires an antibody to a second epitope on TSP-180. A fusion with affinity chromatography purified tumor

surface protein-180 (TSP-180-AC) immunized rat spleen cells and the parent myeloma P3-X63-Ag8 produced a monoclonal antibody, 346-11, that is directed to a different site on TSP-180 than the site recognized by 135-13C MoAb.

The immunogen used to produce 346-11 MoAb, TSP-180-AC, was purified from detergent lysates of line 1 tissue culture cells by immunoaffinity chromatography with a 135-13C affinity column. TSP-180-AC was eluted from the column with 3M KSCN since it was known that the determinant recognized by 135-13C MoAb is destroyed or lost when 3M KSCN is used as the eluant (Foote and Kennel, unpublished observation). This eliminates the possibility of producing more antibodies to the epitope recognized by 135-13C. Purified TSP-180-AC does not migrate at 180,000 M.W., as determined by SDS-PAGE and autoradiography (Figure 5, page 33), but has one major radioactive band at 150,000 M.W. and two minor bands at about 175,000 and 135,000 M.W. It could be that the protein is degraded upon elution with 3M KSCN or during the concentration process. Unless TSP-180 is constructed in a very unusual manner, it is unlikely that elution with 3M KSCN degrades TSP-180 since high salt treatment does not disrupt peptide bonds. Degradation by proteases during the concentration process is more likely. During the solubilization of line 1 cells proteases may be released. PMSF is added to the cells prior to solubilization to inactivate proteases but inactivation may not be complete. KSCN unfolds proteins by disrupting polar interactions and unfolded proteins are more susceptible to protease attack so that TSP-180-AC

would be more prone to degradation after elution. Alternatively, it is possible that TSP-180-AC is not identical to TSP-180 but an antigenically related protein or that TSP-180 is composed of two subunits and TSP-180-AC lacks one of the subunits.

TSP-180-AC was identified by immunoprecipitation with immune sera but it was not recognized as TSP-180 by 135-13C (Figure 6, page 34). In light of the method of isolation, however, it seems likely that TSP-180-AC is TSP-180 or a degradation product of TSP-180. 135-13C could be directed to a conformational epitope on TSP-180 that is never restored after KSCN is removed. The antibody produced with TSP-180-AC, 346-11, binds to the surface of line 1 cell (Table I, page 42), precipitates authentic TSP-180 from solubilized surface-labeled line 1 cell (Figure 7, page 36) and reacts at a different epitope than 135-13C (Figures 8 and 9, pages 39 and 40).

Competition assays on line 1 cells were designed to directly demonstrate that 346-11 binds to a different site than 135-13C MoAb. Ascites fluids containing MoAbs were tested for their ability to compete with ^{125}I 135-13C MoAb for binding to line 1 cells (Figure 8, page 39). 346-11 MoAb and the negative control, 133-13A MoAb, did not compete for binding whereas 135-13C ascites fluid competed well with ^{125}I 135-13C. The failure of 346-11 ascites fluid to compete for binding indicates that 346-11 MoAb is binding to a different epitope on line 1 cells than 135-13C MoAb; however, it could be that the affinity constant (Ka) of 346-11 MoAb is much lower than the Ka of 135-13C MoAb and 346-11 is therefore unable to compete efficiently

for binding to line 1 cells. The Ka of 135-13C MoAb was previously determined as 1.2 X 10^9 liters/mole (Kennel et al., 1983a). The Ka of 346-11 MoAb was determined from direct binding data of ^{125}I 346-11 MoAb as about 1 X 10^8 liters/mole (data not shown). The affinity constant of 346-11 MoAb is ten times lower than the affinity constant of 135-13C MoAb. One would, however, still expect competition if 100 times more 346-11 MoAb than 135-13C MoAb was used if 346-11 was binding to the same site as 135-13C MoAb. Differences in the affinity of 346-11 MoAb for binding sites on line 1 cells were anticipated in the design of the competition experiments. Ascites fluids were titered and antibody dilutions expressed in terms of the antigen binding capacity (ABC in ng/ml), not in terms of concentration, so the values are directly comparable in terms of the potential for competition.

The reciprocal experiment, competition of ascites fluids containing MoAb with ^{125}I 346-11 for binding to line 1 cells was performed to confirm that the lack of competition with 346-11 MoAb for 135-13C binding sites was not due to the difference in affinity constants. 135-13C should compete for binding with ^{125}I 346-11 if it binds to the same site. Figure 9, page 40, illustrates that 135-13C MoAb and the negative control, 133-13A MoAb, could not compete against ^{125}I 346-11 for binding to line 1 cells. 346-11 MoAb competed significantly with iself. The lack of competition between 346-11 and 135-13C MoAbs for binding sites demonstrates that these MoAbs bind to different epitopes on the TSP-180 molecule (Stahli et al., 1983).

Direct confirmation that 346-11 and 135-13C bind to different epitopes was obtained by incubating ^{125}I 135-13C and ^{125}I 346-11 on viable line 1 cells separately and mixed together. The binding of these antibodies to the surface of line 1 cells was directly additive (Stahli et al., 1983) as demonstrated in Table I, page 42. These antibodies should be capable of increasing the sensitivity of detection of TSP-180 since their binding is additive.

The two-site assay proposed by Brown et al. (1981a) should be more sensitive than single site assays or competition radioimmunoassays (RIA) since the first antibody concentrates the antigen from the test sample. It is more specific since the antigen must have two epitopes to be detected. The limits of detection in the melanoma system were 1 fMol of antigen in a 1 ml sample (Brown et al., 1981a). In contrast, a competition RIA for CEA required an antigen concentration of 30 fMol/ml for 50% competition (Brown et al., 1981a).

Other investigators have employed two-site assays for the detection of antigen. Katus et al. (1982) utilized monoclonal antibodies that exhibit varying degress of cross-reactivity between cardiac and skeletal-muscle myosin light chains in a sandwich assay. They demonstrated increased specificity for cardiac myosin light chains when monoclonal antibodies to two epitopes on cardiac myosin light chains were employed. The range of this assay is only limited by the number of first-antibody binding sites immobilized on the column (Katus et al., 1982). Hedin et al. (1983) also demonstrated increased specificity for carcinomas in the detection of CEA in serum by a two-site

enzyme immunoassay. In this assay, the first antibody was immobilized on cyanogen bromide activated paper and the second antibody was conjugated to an enzyme for detection. This assay was sensitive to 0.5 ug of CEA/liter within a range of 0.5-250 ug/liter. Kennel and Lankford (1983b) demonstrated both increased specificity and sensitivity in a two-site assay used to detect fragment D of human fibrinogen. This assay discriminates between human fibrinogen and fragment D by at least 100 fold. It is 10-fold more sensitive than a liquid-phase competition RIA. Binding of antigen to the solid-phase antibody can be driven to near completion by employing a large amount of the solid-phase antibody (Kennel and Lankford, 1983b). Unlike competition RIA, this assay is not as limited by the antibody's Ka. Low affinity antibodies can be used as the solid-phase antigen concentrating antibody and high affinity antibodies for detection.

346-11 MoAb was developed to a second epitope on TSP-180 for future use in a two-site immunoassay with 135-13C MoAb. 346-11 MoAb could also be used with 135-13C MoAb to enhance imaging in the BALB/c mouse. This would elucidate if localization to lung, kidney and spleen is due to a specific distribution of TSP-180 in these organs. It might also enhance the detection of specific binding to the target tumor that is masked by the nonspecific accumulation of control immunoglobulin in the serum pool of the tumor.

The immunogen used to produce 346-11, TSP-180-AC, is not directly identifiable as TSP-180. 346-11 MoAb does, however,

precipitate a protein that comigrates with the protein precipitated by 135-13C MoAb and it was assumed that these proteins are identical. Competition analysis and additive binding on viable line 1 cells demonstrated that 346-11 MoAb binds to a different site on line 1 cells than 135-13C MoAb. 346-11 is a suitable candidate for use in a two-site assay for the detection and quantitation of TSP-180 in detergent lysates of normal tissues.

Conclusions

Monoclonal antibodies have been used to image tumors with some success in human systems (Mach et al., 1981; Larson et al., 1983; van Nagell et al., 1980) Radioimaging of tumors could be performed in other systems, such as in lung cancer, with the appropriate imaging antibodies. Monoclonal antibodies specific for human lung cancers have been developed (Mulshine et al., 1983 and Sikora and Wright, 1981). The lung is a good candidate for successful imaging since it is a highly vascularized organ and delivery of the appropriate imaging antibody through the blood to the target tumor site should be successful. The line 1 alveolar carcinoma of the BALB/c mouse could be used as a model system for lung cancer imaging if an appropriate imaging antibody can be developed. The utility of this system could be enhanced by imaging smaller tumors, prior to the development of serum pools which nonspecifically accumulate serum proteins. It is possible that 135-13C MoAb and 346-11 MoAb could be used together in such a system to demonstrate tumor imaging with specificity for the line 1 tumor.

Tumor imaging can be used to determine the extent and location of metastases for staging of cancer, prognosis, selection of the appropriate therapy and surgical resection of the tumor where appropriate. Imaging antibodies are not as limited as antibodies intended for therapeutic use by strict requirements for specificity to the tumor cells. An antibody directed to an antigen that is expressed predominately by tumor cells could be sufficiently specific for imaging use. Many monoclonal antibodies specific for tumor-associated antigens have been produced. These antibodies could be tested for their imaging potential by *in vivo* clearance kinetics. The appropriate antibodies could then be used to image tumors and metastases.

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