Guinea Pig Line 10 Hepatocarcinoma Model: Characterization of Monoclonal Antibody and in Vivo Effect of Unconjugated Antibody Conjugated to Diphtheria Toxin A Chain

Michael I. Bernhard, Kenneth A. Foon, Thomas N. Oeltmann, Marc E. Key, Kou M. Hwang, Gregory C. Clarke, Wayne L. Christensen, Lawrence C. Hoyer, Michael G. Hanna, Jr., and Robert K. Oldham

ABSTRACT

Monoclonal antibodies were raised against the guinea pig line 10 (L10) hepatocarcinoma, and an IgG1-producing hybridoma (D3) was selected for further study. D3 is a true monoclonal antibody as demonstrated by two-dimensional gel electrophoresis. Radioimmunoassays on live cells revealed no cross-reactivity with normal tissues or with the line 1 hepatocarcinoma which was used as a control. Membrane immunofluorescence assays demonstrated similar specificity. Immunoperoxidase staining of cryostat sections of tumor and normal tissues of both adult and fetal animals and fetuses showed that the D3 monoclonal antibody reacted primarily with the L10 tumor, but some cross-reactivity with smooth muscle, placenta, fetal skeletal muscle, and fetal liver was also demonstrated. Radioimmunoprecipitation of detergent extracts of iodinated L10 cells showed that the antigen is present on the cell surface as a dimer of 290,000 (unit size, M, 148,000). Therapy studies with unconjugated D3 antibody demonstrated a minor dose-dependent effect on tumor growth. D3 antibody conjugated to the A chain of diphtheria toxin (10\(^{-7}\) M) was cytotoxic to 100% of L10 cells in vitro. Animals treated with a single 1-mg i.v. injection of this immunoconjugate on Day 7 following the intradermal injection of 10\(^5\) tumor cells demonstrated a highly significant inhibition of tumor growth compared to control animals and those treated with unconjugated antibody.

INTRODUCTION

The chemically induced, transplantable L10 strain 2 guinea pigs (34) is a valuable model for tumor immunotherapy. This tumor is weakly immunogenic and after i.d. injection forms a localized tumor mass with subsequent spontaneous metastases to draining lymph nodes and visceral organs. Injections i.t. of viable Bacillus Calmette-Guérin induce regression of established tumors and can eliminate regional lymph node metastases (16, 41). Although this immunotherapeutic approach has been successful only with tumors of limited size in specific locations, systemic tumor immunity was also produced (15, 16). Active specific immunotherapy using Bacillus Calmette-Guérin and irradiated, nontumorigenic yet viable L10 cells resulted in the elimination of established metastatic tumor foci in the lungs (12, 13, 15), curing most treated animals.

We used the L10 model to investigate some of the possible therapeutic applications of monoclonal antibodies. In comparison to murine models, this model provides a solid tumor in a species the immune responsiveness of which more closely approximates that of humans; guinea pigs are exquisitely sensitive to anaphylactic reactions, a consideration in cancer immunotherapy utilizing murine antibodies in humans; and the use of a heterologous monoclonal antibody is more analogous to the treatment of human tumors with murine monoclonal antibodies than the more prevalent allogeneic models.

Monoclonal antibodies were raised against the L10 tumor by immunizing BALB/c mice with viable L10 cells and fusing splenic lymphocytes to the P3X63-NS1-1 mouse myeloma line (17, 18). In this paper, we characterize the monoclonal antibody produced by the D3 clone. We also describe the antigen defined by this antibody and the results of in vivo administration of antibody and antibody:diphtheria toxin A chain conjugates. Subsequent reports will characterize the antigen more fully and will describe the results of various approaches to tumor immunotherapy, including the use of toxins, drugs, and radioisotopes coupled to antibody.

MATERIALS AND METHODS

Immunization and Fusion. BALB/c mice were immunized by i.v. injection with 10\(^6\) viable L10 ascites cells and were boosted after 7 days. Three days later, we removed the spleens, dissociated the tissue, and fused the spleen cells to P3X63-NS1-1 mouse myeloma cells (17, 31). Screening for specific antibody was performed by using a fluorescence assay on the L10 and L1 cells. The L1 hepatocarcinoma, which was induced in guinea pigs in a manner similar to the induction of the L10 tumor and which is antigenically distinct from L10 (4), was used as a control in these studies. Cells producing antibody of appropriate specificity were cloned, restested, and recloned. Early passages of selected clones were frozen in liquid nitrogen. The most promising of these clones, D3, was chosen for use in the studies.

Preparation of Guinea Pig Tissues. Two-month-old strain 2 guinea pigs were sacrificed, and brains, livers, spleens, and kidneys were immediately removed and placed on ice. The tissues were rinsed in PBS,
tissed free of connective tissue and fat, and minced with scissors and scalpels. Minced tissues were forced through a fine stainless steel mesh and washed 3 times in PBS. The viable cells were counted by means of trypan blue exclusion, and cytospin slides were prepared for examination. Cells for radioimmunoassay were used immediately; tissue blocks for immunoperoxidase studies were cut into 1 cu cm pieces and either fixed in formalin or frozen in liquid nitrogen.

**Ascites Production.** BALB/c mice (6-week-old females) were treated with pristane (2,5,10,14-tetramethylpentadecane; Aldrich Chemical Co., Milwaukee, Wis.) 5 to 7 days prior to use. Twenty million tissue-culture-grown hybridoma cells were injected i.p., and ascites fluid was harvested 1 to 2 times beginning 7 to 14 days after inoculation. Lots of ascites fluid consisted of pooled fluids from groups of 20 to 50 mice inoculated simultaneously.

**Concentration of Ascites Fluid.** Fluids were clarified by centrifugation at 12,500 × g for 30 min and precipitated with 20.4% (w/v) sodium sulfate. Precipitated material was pelleted by centrifugation at 12,500 × g for 30 min, resuspended in 0.1 M Tris (pH 7.3), precipitated in 14% (w/v) sodium sulfate, and pelleted as above. The pellet was resuspended in Tris and exhaustively dialyzed against PBS; the concentrated monoclonal antibody was then clarified by centrifugation at 100,000 × g for 1 hr, passed through a 0.2-μm Millipore filter for sterility, divided into aliquots, and stored at -85°C at concentrations >5 mg/ml. The Bio-Rad protein assay was used to determine total protein before and after concentration, and IgG1 levels were quantitated by radial immunodiffusion.

**Live Cell Assay Using a Radioimmunoassay.** This assay is a modification of the procedure of Morgan et al. (28). Live L1 and L10 cells were washed 3 times in PBS, and 2 × 10^6 cells were placed in each well of Dynatech (Alexandria, Va.) polyvinyl 96-well U-bottomed plates that had been blocked by overnight incubation (4°C) in PCS. Ascites fluid or tissue culture supernatants containing monoclonal antibody were added (100 μl/well, diluted in PCS) and placed on a shaking platform at 4°C for 1 hr.

**Immunoglobulin Subclass of Monoclonal D3.** Subclass analysis was performed using subclass-specific antisera (Miles-Yeda, Israel; Litton Bionetics, Kensington, Md.) in a live cell assay on L10 cells, using the anti subclass reagents as the second antibody. In addition, surface immunoglobulin on D3 cells was isotype by the use of subclass-specific antisera followed by 125I-Protein A on the hybridoma cells.

**Preparation of Iodinated Protein A.** Purified Protein A (Pharma Fine Chemicals, Piscataway, N. J.) was iodinated by the lactoperoxidase method using the NEZ 151 system (New England Nuclear, Boston, Mass.). Following iodination, free iodide was removed by passing the protein through a Sephadex G-25 column (Pharmacia). Nonbiologically active 125I-Protein A was removed by passing the material through an affinity column of purified rabbit IgG (DAKO; Accurate Chemical Co., Westbury, N. Y.) coupled to Sepharose 4B (Pharmacia) followed by elution of the biologically active material with phosphate buffer, pH 3.0 (27). Up to 20% of total 125I counts were incorporated into the biologically active Protein A, and 98% of these counts were bound in live cell assays with D3 antibody and rabbit anti-mouse IgG.

**Immunofluorescence Assays.** Indirect immunofluorescence assays for membrane antigens were performed on live L1 and L10 ascites cells and on single-cell suspensions prepared from adult guinea pig livers and spleens. Ascites and tissue cells were washed 3 times in PBS. The erythrocytes were hypotonically lysed by adding 8 ml of distilled water to the cell suspension, agitating for 60 sec, and then adding 2 ml of 3.5% NaCl solution. After 3 additional washes in PBS, cells were adjusted to 2 to 2.5 × 10^6 live cells per 12-× 75-mm polycarbonate tube and centrifuged at 700 × g for 5 min. Cells were resuspended in 0.1 ml of D3 or control P3 tissue culture supernatant at equimolar concentrations of IgG1 equivalent to 1:200 and 1:1000 dilutions of D3 in PBS containing 1% BSA. After 30 min on ice, cells were washed twice in PBS:BSA, and 0.1 ml of fluorescein isothiocyanate-conjugated rabbit anti-mouse serum (1:5; Litton Bionetics, Inc.) was added at 1:20 PBS:BSA. After an additional 30-min incubation on ice, cells were washed twice in PBS:BSA, and fluorescence was assessed as described below.

**Flow Cytometry.** Fluorescence analysis by flow cytometry was performed on a FACS IV fluorescence-activated cell sorter (Becton-Dickinson Co., Mountain View, Calif.) and held at 500 milliwatts, scatter gain at 1 x 0.5, fluorescence gain at 1 x 0.5, and the photomultiplier tube at 720 V. Fifty thousand viable cells were counted by light scattering and analyzed for fluorescence intensity.

**Immunoperoxidase Staining.** Analysis of formalin-fixed, paraffin-embedded sections was performed as described previously (37) using the D3 murine monoclonal antibody and the Vectastain ABC immunoperoxidase kit (Vector Laboratories, Burlingame, Calif.) for the detection of murine immunoglobulin.

Similar analyses were performed on unfixed cryostat sections by modification of this procedure. Unfixed cryostat sections were maintained at -85°C until used. Slides were incubated for 5 min in PBS and pretreated for 10 min with PBS containing 10% normal horse serum and 0.1% BSA. Sections were reacted with the monoclonal antibody (diluted in PBS containing 0.1% BSA) for 30 min, washed three times in PBS, and then incubated in methanol containing 0.3% H2O2 to block endogenous peroxidase activity. The remainder of the assay was performed according to the procedure outlined in the Vectorstain ABC kit.

Live ascites L1 and L10 cells were treated in a similar fashion. Thoroughly washed cells were treated with the D3 monoclonal antibody and fixed in Saccamono’s solution. Cytospin preparations were made and then processed in the same manner as were the cryostat sections. In all of the peroxidase procedures (fixed, cryostat, and live cell), P3 ascites fluid controls (at the same concentrations as the D3 monoclonal antibody preparations) and PBS controls were included as negative controls. All assays were performed at 1:1,000, 1:5,000 and/or 1:10,000 dilutions.

**Isoelectric Focusing.** D3 hybridoma cells were grown in serum-free medium for 72 hr. After clarification of the supernatant (10,000 × g for 30 min), samples at various dilutions (from undiluted to 1:100) were electrofused on an LKB polyacrylamide gel electrophoresis plate (pH 3.5 to 9.5) on an LKB Multiprobe (LKB Instruments, Inc., Rockville, Md.). Components were stained with Coomassie Blue R250.

**Two-Dimensional Gel Electrophoresis.** Strips containing the stained band from isoelectric focusing (covering pH range from 3.5 to 9.5) were cut out and equilibrated overnight in SDS-polyacrylamide gel electrophoresis buffer containing 10% 2-mercaptoethanol. A single strip was placed in an appropriately cut area of the stacking gel of a 10% SDS: polyacrylamide slab gel and run as described by Laemmli (20).

**Radioimmunoprecipitation.** Fresh L1 and L10 ascites cells were washed 3 times in PBS and iodinated by the lactoperoxidase technique or metabolically labeled with [3H]leucine (1 mCi/2 × 10^7 cells; specific activity, 110 Ci/mmol). Cells were washed 4 times in 50 ml of PBS and pretreated with 100 μl of 1:20 diluted ascites fluid and held at 4°C.

**Supernatants.** Supernatants were clarified by centrifugation at 100,000 × g for 1 hr and held at 4°C.

**PAS (Pharmacia) was coupled to monoclonal antibody for immunoprecipitation.** Four hundred μl of PAS as a 10% suspension in TTW were mixed with 20 μl of D3-concentrated ascites or an equivalent concentration of P3 control ascites fluid (Bethesda Research Laboratories, Rockville, Md.). The volume was brought to 1.0 ml with TTW, and tubes were
rotated at 4°C for 2 hr. After washing 3 times in TTB, D3 and P3 pellets were kept dry at 4°C.

**Pass: monoclonal antibody and control ascites (PASS:D3 and PASS:P3)** were then incubated overnight at 4°C with L10- and L1-labeled detergent extracts. The pellets were washed 3 times with TTB and 3 times with 0.1 M Tris (pH 8.5):0.5% Tween 20, boiled in SDS-polyacrylamide gel buffer with and without 2-mercaptoethanol, and run on 5 or 7.5% SDS-polyacrylamide slab gels (20).

**Serotherapy Trials with Unconjugated D3 Antibody.** Two experiments were designed to study the effect of unconjugated D3 antibody on the growth of L10.i.d. tumors in vivo. In the first experiment, 8 groups of animals (5 guinea pigs per group) were studied. All of the animals were given injections of 10^6 L10 cells i.d. on Day 0. On Day 2, one group of animals was untreated, and the other groups were treated with 0.1, 0.5, 1, 5, or mg of i.v. D3 antibody. One group of animals was also treated with 1 mg of D3 on Day 7, and another group, on Day 7 and Day 11.

In the second experiment, 5 groups of animals (5 guinea pigs per group) were given injections of 10^6 L10 cells i.d. on Day 0. One group of animals served as the control and was given no D3 antibody. The second group was treated i.v. with 10 mg of D3 on Day 2, the third group with 10 mg on Days 2 and 7, and the fourth group with 10 mg on Days 2, 7, and 11. A fifth group of animals was treated with 30 mg of D3 on Day 2 only. Tumor growth was measured every 3 to 4 days.

**Preparation of Diphtheria Toxin A Chain Immunocoujgate.** Crude diphtheria toxin was obtained from Connaught Laboratories, Toronto, Ontario, Canada, and A chain was prepared as described by Chung and Collier (6). D3 antibody was coupled to the purified A chain by the method of Carlsson et al. (5) as described previously by Oettmann and Forbes (30). Briefly, the antibody (5 mg/ml) was diazoylated against 0.1 M sodium phosphate, pH 7.4, which contained 0.1 M NaCl for 16 hr. A 10-fold molar excess of N-succinimidyl-3-(2-pyridyldithio)propionate was added, and the solution was allowed to stand at room temperature for 30 min followed by dialysis against 4 liters of 10 mM sodium phosphate, pH 7.4. Diphtheria toxin A chain was reduced with 100 mM dithiothreitol overnight, passed over a Sephadex G-25 column in the same buffer, and immediately mixed with the N-succinimidyl-3-(2-pyridyldithio)propionate:antibody. This mixture was incubated at room temperature for 30 min. The reaction mixture was directly applied to a NAD:Sepharose column equilibrated with 20 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 6.5. Uncoupled IgG was not bound, while the hybrid was retained by virtue of the interaction of the NAD* binding site of the A chain moiety (Kb 8 M) with the bound NAD*. The hybrid was subsequently eluted with buffer containing 10 mM adenine and 0.5 M NaCl, dialyzed against 10 mM phosphate buffer, and concentrated by pressure dialysis. The mixture was then applied to a Sephadex G-200 column (2.6 x 60 cm) equilibrated with 10 mM phosphate buffer, pH 7.4. The D3:diphtheria toxin A chain-containing fractions were clearly separated from unincorporated A chain. The yield, based on D3 input, was 30%. Analysis of the purified hybrid on 8% polyacrylamide:SDS slab gels in the presence of mercaptoethanol showed the presence of 3 protein bands: IgG heavy chain, M, 50,000; IgG light chain, M, 25,000, and A chain, M, 21,000. There were no bands with molecular weight less than 150,000 in the absence of mercaptoethanol.

**In Vitro Studies with Immunocoujgate.** Line 10 tissue culture cells from several log-phase T-250 tissue culture flasks were combined, and viability was determined by trypsin blue exclusion and flow cytometry analysis. In each experiment, starting cells were > 90% viable. T-30 tissue culture flasks were seeded with 5 ml of RPMI Tissue Culture Medium 1640 containing 15% fetal calf serum and 1% L-glutamine (complete RPMI). 3.5 x 10^4 viable cells/ml. Replicate flasks were set up, and cells were grown for the first 24 hr in complete RPMI as above or complete RPMI containing D3:diphtheria toxin A chain conjugate at 10^-8 to 10^-11 M antibody in log steps (a single flask for each concentration was replicated by using 2 separate lots of D3:diphtheria toxin A chain conjugate) or unconjugated D3 antibody at similar concentrations. Conjugates and antibody were sterile filtered after dilution in complete RPMI (0.22-μm Millex-GV filters; Millipore Corp., Bedford, Mass.). After 24 hr, cells were washed twice in complete RPMI and resedeted in fresh flasks. Flasks were sampled daily (0.5 ml) for 8 days, determining viability (trypsin blue dye exclusion) and total cell number. On Day 4, total volume of each flask was adjusted to 2.5 ml, and all flasks were fed with 2.5 ml of fresh complete RPMI, with all subsequent total cell counts suitably adjusted. In subsequent experiments, the 24-hr pulse with antibody or antibody conjugate was performed in 15-ml sterile plastic centrifuge tubes (Falcon Plastics, Oxnard, Calif.). After washing, cells were transferred to T-30 tissue culture flasks.

Although unconjugated diphtheria toxin A chain was not available as a control, blocking experiments were performed to determine whether the toxic effects observed were due to specific binding of the D3:diphtheria toxin A chain conjugate. L10 cells were preincubated with unconjugated D3 antibody at 10^-6 M for 2 hr followed by addition of D3:A chain conjugate at 10^-6 M for 24 hr. Cells were then washed, resedeted, and sampled as above.

**In Vivo Studies with Immunocoujgate.** Three groups of 10 animals per group were given injections of 10^6 L10 cells i.d. on Day 0. The first group of animals served as a control and was given no treatment. The second group of animals was given injections of 1 mg of unconjugated D3 antibody i.v. on Day 2. The third group of animals was given injections i.v. with 1 mg of D3 conjugated to the diphtheria toxin A chain on Day 7 when tumors were palpable in all recipients.

**RESULTS**

**Characterization of the D3 Monoclonal Antibody.** Isotyping performed directly on D3 hybridoma cells and on L10 cells showed that the monoclonal antibody is an IgG1. Unfractionated ascites fluids generally titered to a dilution of 1.25 x 10^-7, with saturation binding occurring at 1.6 x 10^-8 M (0.68 μg/2 x 10^8 cells). The titer of sodium sulfate-concentrated ascites was generally several doubling dilutions higher than that of unfractionated ascites fluids tested on either live ascites tumor cells or tissue culture cells. Binding to L10 tissue culture cells and live ascites cells is shown in Chart 1. There was no binding to L1 with D3 (above that of D3 control) at any dilution studied. Radial immunodiffusion analysis indicated that unfractionated ascites fluids contained 3 to 20 mg of IgG1 per ml (concentrated ascites fluids ranged from 37 to 110 mg of IgG1 per ml with IgG1 present as ≥90% of total protein).

Isoelectric focusing of tissue culture supernatants from D3 hybridoma cells grown in serum-free medium for 72 hr revealed only a single band, pl 5.4. Following reduction, second-dimension analysis on 10% SDS-polyacrylamide gels showed only a single approximate M, 51,000 component corresponding to the heavy chain of mouse IgG (Fig. 1). The small amounts of sample run precluded detection of the light chain. However, the light chain was clearly visible on SDS-polyacrylamide gels of more concentrated samples run without prior isoelectric focusing.

Membrane immunofluorescence assays on L1, L10, and fresh guinea pig liver and spleen cells, performed on a FACS IV fluorescence-activated cell sorter, showed intense fluorescence on L10 cells (>80%) and no fluorescence (<5%) on L1, liver and spleen cells, L2C guinea pig leukemia cells, or cells from a spontaneous mammary tumor in guinea pigs (data not shown).

Immunoperoxidase staining of cytopsin preparations of ascites L10 cells showed strong membrane staining with D3 antibody on >90% of L10 cells and no staining of L10 cells in the PBS.
Guinea Pig Model for Monoclonal Antibody Therapy

Chart 1. Titration (radioimmunoassay) of D3 monoclonal antibody on L10 ascites cells (○), L10 tissue culture cells (●), and L1 ascites cells (□). P3 IgG1 ascites backgrounds have been subtracted.

Control (Fig. 2, A and B). Cryostat sections of a dermal L10 tumor treated with D3 antibody demonstrated strong reactivity, while there was no reactivity with the P3 control ascites (Fig. 2, C and D). Cryostat sections of normal guinea pig lung, liver, spleen, and kidney were also treated with D3 monoclonal antibody and were negative, except for some staining of smooth muscle surrounding blood vessels and small bronchioli (Fig. 2, E to H).

Reactivity of D3 antibody with fetal guinea pig tissues was also examined. We prepared cryostat sections of guinea pig fetuses (midterm) by sectioning entire fetuses longitudinally at several different levels. Of all fetal tissues examined, strong reactivity was observed only with placental tissue. This reactivity was equal in strength to the intense reactivity observed with L10 tumors. Slight reactivity was also observed in skeletal muscle and occasionally in liver cells, whereas all other tissues examined were negative. P3 ascites and PBS controls were negative in all tissues showing reactivity with D3 monoclonal antibody.

Molecular Characterization of the D3 Antigen. Radioimmunoprecipitation analyses were performed with detergent extracts of iodinated and metabolically labeled live L10 and L1 ascites cells. Analysis of the immune precipitates on 5 and 7.5% SDS:polyacrylamide gels under reducing conditions revealed a single major band (of M, 148,000). When analyzed by SDS:polyacrylamide gel electrophoresis without reduction, the predominant band was seen at approximate M, 290,000 with small amounts of the M, 148,000 protein present (Fig. 3). In precipitates of control preparations of L1 cells treated with D3 or P3, and L10 cells treated with P3 ascites fluid, no radioactivity was detected. Identical results were obtained when iodinated or metabolically labeled L10 cells were used as the antigen source.

Serotherapy with Unconjugated D3 Antibody. In the first experiment, animals were given injections of 10^5 L10 cells i.d. on Day 0 and i.v. on Day 2 of 0.1, 0.5, 1, 5, or 10 mg of D3 antibody. One group of animals received 2 doses of 1 mg of D3 (Days 2 and 7), and a third group was given injections of 1 mg of D3 on Days 2, 7, and 11. There was significant inhibition of tumor growth (p < 0.05 by Student’s t test) on Day 28 in the groups treated with 5 mg, 10 mg, and 3 doses of 1 mg of D3 compared to the untreated controls (data not shown). In a second experiment, animals were given injections of 10^6 L10 cells i.d. on Day 0 and i.v. on Day 2 of 10 or 30 mg of D3 antibody (Chart 2). One group received an additional dose of 10 mg of D3 on Day 7, and another group received 2 additional doses on Days 7 and 11. There was significant inhibition of tumor growth (p < 0.05 by Student’s t test) on Days 34 and 38 for those animals treated with 3 doses of 10 mg of D3 or with one dose of 30 mg of D3. While an in vivo biological effect of D3 antibody was demonstrated in these experiments, there was clearly not a major impact on tumor growth.

In Vitro and in Vivo Studies with D3:Diphtheria Toxin A Chain Conjugate. The D3:diphtheria toxin A chain conjugate was first demonstrated to specifically bind to L10 cells and not L1 cells in vitro, demonstrating that the covalent conjugate had no demonstrable effect on the antibody-binding site of D3. The D3:diphtheria toxin A chain conjugate or D3 alone was then incubated with L10 tissue culture cells at varying concentrations for 24 hr, after which the cells were washed free of excess immunon conjugate. At 10^{-6} and 10^{-7} m, there was 100% cytotoxicity of L10 cells at Day 7 after the 24-hr pulse, as demonstrated

Chart 2. Serotherapy with unconjugated D3 antibody. Five groups of animals (5 animals per group) were given injections of 10^6 L10 cells i.d. on Day 0. The control group received no D3 antibody (A). The other groups of animals received 10 mg of D3 on Day 2 (●), 10 mg on Days 2 and 7 (○), 10 mg on Days 2, 7, and 11 (□), 30 mg on Day 2 (△). There was significant inhibition of tumor growth (p < 0.05) on Days 34 and 38 for animals treated with 3 doses of 10 mg of D3 or one dose of 30 mg of D3.
by trypan blue exclusion (Chart 3A). There were 80% cell death at $10^{-8}$ M concentration and only a minimal effect at $10^{-11}$ M. D3 alone had no significant effect. Although the experiment was ended at this time, it appeared that cytotoxicity with the conjugate at $10^{-8}$ M would have reached 100% in a few more days, because of both the constantly increasing percentage of dead cells (Chart 3A) and the total lack of cell replication (Chart 3B). Unconjugated D3 antibody at $10^{-6}$ and $10^{-10}$ M did not inhibit cell replication, nor was it cytotoxic in vitro (data not shown).

The specific nature of L10 killing by the immunoconjugate was demonstrated by blocking the effects of the immunoconjugate with a 2-hr preincubation with unconjugated D3 antibody, after which $10^{-6}$ M D3:diphtheria toxin A chain conjugate was added (Chart 4). Cells were washed after an additional 24 hr, and cell growth and cytotoxicity were monitored for 6 days. D3 at $10^{-6}$ M almost completely blocked inhibition of replication and cytotoxicity by the diphtheria toxin A chain conjugate, thus demonstrating the antibody-specific nature of the toxic activity of the conjugate.

Three groups of 10 animals per group were given injections...
Guinea Pig Model for Monoclonal Antibody Therapy

Monoclonal antibodies may present new opportunities for the treatment of cancer. Recent studies in humans have demonstrated that serotherapy with monoclonal antibodies can reduce the number of circulating tumor cells (24, 25, 29, 32, 33, 36) and the size of skin lesions and lymph nodes (24) in leukemia patients. However, these effects have been transient. Monoclonal antibody serotherapy of solid tumors in humans has yet to be critically evaluated.

Our aim in these studies was to develop a serotherapy model for treating solid tumors with monoclonal antibody which would have possible applicability to humans. The model is being used to study the effects of monoclonal antibody serotherapy alone or monoclonal antibody conjugated (by several procedures) to various toxins, drugs, and radionuclides on primary and metastatic tumors (3, 8–10, 19, 26, 35, 38). The L10 guinea pig tumor was chosen for several compelling reasons: (a) this chemically induced, transplantable metastasizing tumor is sensitive to certain forms of immunotherapy and chemotherapy (1, 12–16, 39, 41); (b) this tumor grows locally, metastasizes to regional nodes and lung, and kills the animal; (c) a similarly derived syngeneic, spontaneously mammary carcinoma in guinea pigs, the L2Cguinea pig leukemia, or normal guinea pig lung, spleen, kidney, and liver.

Furthermore, there was no reactivity with fetal or adult kidney by direct immunoperoxidase staining or radioimmunoassay. In contrast, we have seen localization of D3 in kidney tubules following i.v. infusion of D3 antibody (18). This appeared to be nonspecific as this staining pattern was also seen following the i.v. infusion of an IgG1 control antibody (P3), and no staining in vitro with D3 was noted. While this may be nonspecific reactivity, it nevertheless could cause problems when immunon conjugates with highly toxic drugs and toxins are used for therapy.

Strong reactivity was demonstrated with placental tissue, and much weaker reactivity was seen with fetal muscle and liver cells. The significance of these reactions is unknown. However, the positive reaction observed with some fetal liver cells (but not adult liver cells) is interesting in view of the origin of the L10 tumor from the liver. Thus, the antigen defined by D3 may be an oncofetal and oncoplacement antigen. Heteroantiserum to L10 and L1 described previously have demonstrated that guinea pig embryonic tissues share antigens with both L10 and L1 (4), but D3 appears to recognize a different specificity, since it can discriminate L10 from L1.

Numerous investigators have studied the cell-mediated immunity to tumor-associated antigens in the L10 and L1 systems (4, 5, 7, 11, 21–23, 34, 40). These studies have demonstrated that active specific immunotherapy using whole cells or cell extracts leads to individual tumor-specific immunity. Although the D3 antigen also appears to be restricted to L10, its relationship to the antigens previously described remains to be determined. We are currently attempting to purify the D3 antigen in large enough quantities for active specific immunotherapy experiments to determine if it is immunogenic in syngeneic guinea pigs.

Immunoperoxidase studies of L10 tumor-bearing animals treated with D3 antibody showed that the antibody localized specifically in L10 tumor cells (18), a finding also supported by radioimaging studies using 111In- or 125I-labeled D3 monoclonal antibody (2). D3 antibody, an IgG1, has shown only minimal complement-dependent cytotoxicity or antibody-dependent cellular cytotoxicity in vitro. This may in part explain the inability of unconjugated D3, even at very high dosages (10 and 30 mg), to have a major impact on the growth of L10 in vivo. We have demonstrated specific localization of the D3 antibody in L10 tumors in vivo (18), and our data suggest that there may be some dose-dependent inhibition of growth (p < 0.05 with one dose of 30 mg and with 3 treatments of 10 mg). In contrast, when the same type of experiment was performed using one dose of 1 mg of D3 conjugated to the A chain of diphtheria toxin, there was a highly significant inhibition of tumor growth. Even more impressive was the observation that the tumors palpable on Day 7 actually regressed and were not palpable on Days 10 and 14. The specific cytotoxic nature of this conjugate was also demonstrated in vitro. This work is preliminary to studies using multiple treatments with this and other immunon conjugates at varying dosages and schedules in animals with varying tumor burdens. The effects of immunon conjugates composed of D3 covalently linked to ricin A chain, abrin A chain, e-aminitin, daunorubicin, and various isotopes are also being studied.

These model studies indicate clearly that mouse monoclonal antibody and conjugates can be given i.v. and will specifically traffic to s.c. tumors with therapeutic effects. Information obtained from further studies with this model should prove useful for designing analogous clinical trials with monoclonal antibodies.

I.d. of 10^6 L10 cells on Day 0 and were then given injections i.v. of 1 mg of unconjugated D3 on Day 2, when the tumor was not palpable, or 1 mg of immunon conjugate on Day 7, when tumors were just palpable. One group of animals served as a control. A significant inhibition of tumor growth (Chart 5) could be demonstrated at each examination through Day 43 for animals treated with the immunon conjugate (Day 35, 1.1 ± 0.2 cm; Day 43, 1.4 ± 0.07 cm) compared to the untreated (Day 35, 1.9 ± 0.1 cm; Day 43, 2.1 ± 0.2 cm) control animals (p < 0.001 on Day 35; p < 0.0005 on Day 43 by Student’s t test) and those animals treated with unconjugated (Day 35, 1.8 ± 0.1 cm; Day 43, 2.2 ± 0.1 cm) D3 antibody (p < 0.004 on Day 35; p < 0.0002 on Day 43). Interestingly, tumors that were palpable on Day 7 actually regressed and were no longer palpable on Days 10 and 14 in the immunon conjugate-treated group.

DISCUSSION

Two-dimensional gel analysis showed that the D3 antibody is truly monoclonal. SDS-polyacrylamide gel electrophoresis of radiolabeled immunoprecipitates indicated that the reactive antigen is present on the cell surface as a dimer with a molecular weight of 290,000 and with a unit size of approximate molecular weight of 148,000. Radioimmunoassays and immunofluorescence analysis showed that D3 antibody reacts specifically with L10 tumor cells. In addition, in vitro immunoperoxidase staining demonstrated that the reactivity is specific to an antigen expressed on the surface of only L10 tumor cells, with some cross-reactivity with a cytoplasmic antigen found in guinea pig smooth muscle. No reactivity was demonstrated to cells of the L1 tumor, a spontaneous mammary carcinoma in guinea pigs, the L2C guinea pig leukemia, or normal guinea pig lung, spleen, kidney, and liver.

SEPTEMBER 1983 4425

Downloaded from cancerres.aacrjournals.org on May 31, 2017. © 1983 American Association for Cancer Research.
REFERENCES


Fig. 1. Two-dimensional gel electrophoresis of D3 monoclonal antibody. Column A, molecular weight standards on SDS-polyacrylamide gel. From the top: BSA (M, 66,000); ovalbumin (M, 45,000); carbonic anhydrase (M, 31,000); soybean trypsin inhibitor (M, 21,500). Column B, single component (arrow) resolved in both first and second dimensions.

Fig. 3. Radioimmunoprecipitation analysis of L10 antigen. Iodinated L10 and L1 cells were precipitated with D3 monoclonal antibody or P3 control ascites fluid immobilized on PAS. Left (A to D), unreduced; right (E to I), reduced. A, P3 + L10; B, P3 + L1; C, D3 + L1; D, D3 + L10. E, 14C-labeled molecular weight markers: 1, myosin (M, 200,000); 2, β-galactosidase (M, 116,250); 3, phosphorylase B (M, 92,500); 4, BSA (M, 66,200). F, D3 + L10; G, D3 + L1; H, P3 + L10; I, P3 + L1.
Fig. 2. Immunoperoxidase staining on cytospin preparation of L10 cells reacted with D3 monoclonal antibody showing membrane reaction (A) and the PBS negative control (B). C to H are cryostat sections of L10 tumor with the D3 monoclonal antibody (positive) (C), L10 tumor with P3 control (negative) (D), lung with D3 antibody (negative except for smooth muscle) (E), liver with D3 monoclonal antibody (negative) (F), spleen with D3 antibody (negative except for smooth muscle) (G), and kidney with D3 antibody (negative) (H).
Guinea Pig Line 10 Hepatocarcinoma Model: Characterization of Monoclonal Antibody and in Vivo Effect of Unconjugated Antibody and Antibody Conjugated to Diphtheria Toxin A Chain


Updated version  Access the most recent version of this article at:  http://cancerres.aacrjournals.org/content/43/9/4420

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.