Subtractive Immunization Yields Monoclonal Antibodies that Specifically Inhibit Metastasis

Peter C. Brooks, Jian-Min Lin, Deborah L. French, and James P. Quigley
Department of Pathology, State University of New York at Stony Brook, Stony Brook, New York 11794

Abstract. Subtractive immunization allowed the isolation and characterization of monoclonal antibodies that specifically inhibit metastasis but not proliferation of highly metastatic human tumor cells. The tolerizing agent cyclophosphamide was used to suppress the immune system in mice to dominant immunodominants present on a non-metastatic variant (M-) of the human epidermoid carcinoma cell line (HEp3). Mice were then inoculated with a highly metastatic variant (M+) of HEp3 to enhance an immune response to antigenic determinants present on metastatic cells. Hybridomas were generated and screened by ELISA for differential reactivity to M+ HEp3 over M- HEp3 cells. This experimental approach, termed subtractive immunization (S.I.), was compared to a control immunization protocol, which eliminated the cyclophosphamide treatment. The S.I. protocol resulted in an eight-fold increase in the proportion of mAbs that react with molecules enriched on the surface of the M+ HEp3 cells. Two of the mAbs derived from the S.I. protocol, designated DM12-4 and IA5, were purified and examined for their effect in a metastasis model system in which chick embryos are transplanted with primary HEp3 tumors. Purified mAbs DM12-4 and IA5, inoculated i.v. into the embryos, inhibited spontaneous metastasis of HEp3 cells by 86 and 90%, respectively. The mAbs are specifically anti-metastatic in that they have no effect on the growth of HEp3 cells in vitro nor did they inhibit primary tumor growth in vivo. The mAbs recognize M+ HEp3 cell surface molecules of 55 kD and 29 kD, respectively. These data demonstrate that the S.I. protocol can be used for the development of unique mAbs that are reactive with antigenic determinants whose expression is elevated on metastatic human tumor cells and which function mechanistically in the metastatic cascade.

Metastatic dissemination of malignant tumor cells is a complex process which involves a multitude of steps, including migration of primary tumor cells through the local underlying stroma and invasion of the cells into blood vessels and lymphatics to gain access to the host circulation. The tumor cells survive in the vasculature, arrest, and finally invade and grow at distant secondary sites (8, 22, 23, 32). Due to the multiplicity and biochemical complexity of the steps involved in the spread of tumor cells, an understanding of the metastatic process at a molecular level is yet to be fully obtained.

In order for the tumor cells to complete the malignant process successfully they must express a unique set of characteristics. Many of these traits appear to be associated with molecules on the cell surface (16, 29, 33, 39, 42, 47). Changes in the level of expression of these surface molecules could contribute to the distinct phenotypic differences between metastatic and non-metastatic tumor cells (7, 14, 21, 25, 40, 43). In an effort to identify and characterize cell surface molecules that play a functional role in the metastatic cascade, immunological approaches for the generation of specific mAbs have been attempted (2, 11, 15, 17, 45, 51). One approach to develop mAbs to cell surface molecules that play a functional role in metastasis involves direct immunization of mice with intact metastatic tumor cells. However, this technique can be inefficient since the tumor cell surface provides a large array of antigenic determinants that can be immunodominant and may play no functional role in metastasis. Reducing the recognition of common, immunodominant cell surface molecules would increase the chances of producing antibodies to low abundant but functionally relevant cell surface molecules. One way of achieving this goal is through the use of the immunosuppressive agent cyclophosphamide (19, 24, 38, 44, 49) coupled to immunizations with two phenotypically distinct cell variants used as sequential immunogens. Cyclophosphamide selectively kills activated B cells (24, 38) and can be used to suppress the immune system in mice to common cell surface antigens present on a non-metastatic cell variant used as the initial immunogen. After treatment with cyclophosphamide, a phenotypically distinct but related metastatic cell variant is inoculated into mice to generate mAbs that are reactive to unique molecules on the cell surface of the second cell variant. Cyclophosphamide has been used in suppressive immunization procedures...
to produce mAbs to antigens on closely related neuronal cells (26, 27) and to specific antigens on tumor cells (18, 45, 48). In addition, the cyclophosphamide immunosuppression method has been shown to yield significantly more mAbs to specific antigens than a neonatal tolerization method (37).

In the present study a variation of the subtractive immunization (S.I.) approach was used (49). Immunizations were performed with human epidermoid carcinoma (HEp3) cells from which a highly metastatic variant, metastatic HEp3 Cell Variant (M⁺ HEp3), and a non-metastatic variant, Non-Metastatic HEp3 Cell Variant (M⁻ HEp3), can be isolated (12, 31, 34, 35, 46). Cyclophosphamide was employed at various times after M⁺ HEp3 cell inoculations to suppress the immune response in mice to common antigenic determinants present on HEp3 cells. This suppression procedure was followed by multiple inoculations with the M⁺ HEp3 variant. Hybridomas were made from the immunoresponsive mice and screened for mAbs that were differentially reactive to M⁺ HEp3. This S.I. approach resulted in the generation of specific mAbs that react with molecules that are enriched on the surface of the M⁺ HEp3 cells as compared to their M⁻ variants. Some of these mAbs are uniquely anti-functional and inhibit metastasis in an in vivo metastasis model system.

Materials and Methods

Cell Lines

Human breast adenocarcinoma cells (MDA-MB 231), human cervix epidermoid carcinoma cells (M5751), human cervical carcinoma cells (HT-3) and human foreskin fibroblasts were obtained from American Type Culture Collection (Rockville, MD). Human primary keratinocytes were a gift from Dr. Lorne Taichman (SUNY, Stony Brook, NY). HEp3 cells were maintained as monolayer cultures and were obtained from solid HEp3 tumors that were serially passaged on the chorioallantoic membranes (CAMs) of chick embryos (34-36). The metastatic variant of HEp3 cells, M⁺ HEp3, was cultured for 25 d or less before use. The nonmetastatic variant, M⁻ HEp3, was maintained in culture for at least 60-80 d before use. Cells were maintained in DMEM (GIBCO BRL, Gaithersburg, MD) supplemented with 10% FBS (Hyclone, Logan, UT) and sodium pyruvate, and pen/strep (Sigma Chem. Co., St. Louis, MO). All cells were grown in a humidified 5% CO₂ atmosphere at 37°C in 100-mm tissue culture plates (Falcon, Becton Dickinson Labware, Lincoln Park, NJ).

Subtractive Immunization

C57 BL6 female mice, 6-8 wk old and 16-18 grams, were obtained from Charles River (Montreal, Quebec). The S.I. protocol was performed as follows. On day 1, subconfluent M⁺ HEp3 cell cultures were washed 3× with sterile PBS and harvested by the addition of 3 ml of nonenzymatic cell dissociation solution (Sigma Chem. Co., St. Louis, MO) for 1 min. Single cell suspensions were washed and resuspended in sterile PBS (4 x 10⁶ cells/ml). HEp3 M⁺ cells (2 x 10⁶) were inoculated i.p. into mice on day 1. On days 2 and 3, mice were injected i.p. with 200 mg/kg cyclophosphamide (Sigma Chem. Co., St. Louis, MO) in sterile PBS. On day 15 sera were collected and screened by the whole cell ELISA. Hybridomas were made from the immunoresponsive mice and screened for mAbs that were specific to tumor antigens on closely related neuronal cell lines and various other cell types. S.I. protocols were carried out in which cyclophosphamide was given either at 120 and 96 h before the M⁺ HEp3 cells or 1 and 24 h after the M⁻ HEp3 cells. A control protocol was performed that was exactly the same as the S.I. protocol except that no cyclophosphamide was given.

Production of Hybridomas

Hybridomas were produced by standard methods (6). Briefly, the spleens and accessory lymph nodes were removed, washed, and fused (4:1 spleen to myeloma) with the mouse myeloma cell line NSO (10). The cells were plated (2.5 x 10⁶ cells/0.1 ml HAT medium/well) in 96 well tissue culture plates (Falcon, Becton Dickinson Labware). Culture supernatants were screened by the whole cell ELISA and hybridomas from each of the immunization protocols were cloned by soft agar and limiting dilution. Each positive hybridoma was cloned twice.

Whole Cell ELISA

Subconfluent monolayer cultures of HEp3 cells as well as various other cell types were washed 3× in sterile PBS, removed with nonenzymatic cell dissociation solution, washed in sterile PBS, and resuspended in DMEM containing 10% PBS. Cells were added (2 x 10⁶/ml) to 96 well tissue culture plates and allowed to attach for 2 h at room temperature. The plates were washed and the substrate, phenyl phosphate disodium (2 mg/ml in 25 mM NaHCO₃, and 1 mM MgCl₂ pH 9.8), was added (50 µl/well) and incubated for 30 min at 37°C. ELISA plates were read at 405 nm on a Titer Trek Multiscan plate reader. Non-specific binding was determined using preimmune sera or normal mouse IgG (Cappel) and subtracted from the experimental samples. ELISA ratios were calculated as follows: (OD₄⁰₅ M⁺ HEp3/OD₄⁰₅ M⁻ HEp3).

Monoclonal Antibody Purification

Hybridoma conditioned media was centrifuged at 3,000 g for 30 min, concentrated 10× with an Amicon Stirred Cell Concentrator (Amicon, Beverly, MA) and filtered through a 0.22-µm filter (Acrodisc, Gelman Sciences, Ann Arbor, MI). The concentrated conditioned medium was applied to a protein G Superose column (Pharmacia LKB Biotechnology, Piscataway, NJ) connected to a FPLC (Pharmacia LKB Biotechnology) and mAbs were eluted with 0.1 M glycine pH 3.0. Purified mAbs were immediately neutralized with 1.0 M Tris, pH 9.0, dialyzed against PBS, and stored at -20°C.

Chick Egg Embryos Metastasis Assay

Fertilized COFAL—negative eggs were obtained from SPAFAS (Norwich, CT) and incubated in 37°C with 60% humidity for 10 d. The chick embryo metastasis assay is a modification of procedures previously described (12, 34). Briefly, a small hole is made through the shell at the end of the egg directly over the air sac with the use of a small crafts drill (Dremel, Division of Emerson Electric Co., Racine, WI). A second hole is drilled on the broad side of the egg directly above embryonic blood vessels, which are located previously by candling the egg. Negative pressure is applied to the original hole, resulting in the choriovallantoic membrane (CAM) pulling away from the shell membrane and creating a false air sac over the CAM. A 0.5 cm x 0.5 cm square window is cut through the shell with the use of a small model grinding wheel (Dremel, Division of Emerson Electric Co.) which allows direct access to the underlying CAM. M⁺ HEp3 cells (2 x 10⁶/ml serum-free DMEM) are placed on top of the CAM through the small window. The window is sealed with sterile tape and the embryos are placed back in the incubator. 24 h later a second small window is carefully cut in the side of the egg shell directly over prominent blood vessels and the outer egg

The Journal of Cell Biology, Volume 122, 1993
shell is carefully removed leaving the embryonic membranes intact. A small drop of paraffin oil is added to the exposed membrane to allow the membrane to become transparent and blood vessels to be easily visualized. Purified mAbs are inoculated (200 µg/0.1 ml PBS) directly into the blood vessels with a 30 gauge needle. The windows are sealed with sterile tape and the embryos are allowed to incubate until day 17. On day 17 the embryos are sacrificed and the primary HEp3 CAM tumors are removed, trimmed, and weighed. The embryonic lungs are removed, finely minced with a pair of small sterile dissection scissors, transferred to the CAMs of a second set of 10-d old embryos prepared as described above, and incubated until day 17. The embryonic lung transfer allows for the expansion of micrometastasis that might be present in the original chick embryo lungs. The second set of embryos are sacrificed at day 17 and the resulting lung tumors are removed from the CAM. The lung tumors are finely minced and morphologically assayed for the presence of HEp3 cells. The HEp3 cells, easily identified due to their large size and morphology compared to the chick lung cells, are counted using a phase contrast microscope. Five randomly selected fields are counted and the number of HEp3 cells per field is calculated. The lung metastases are also quantitated by a human plasminogen activator assay (uPA) (see below).

**Plasminogen Activator Assay**

Lung tumor minces were placed on ice, homogenized with a tissue grinder (Biospec Products, Bontlesville, OK) in 0.9 ml of extraction buffer (25 mM Tris, 0.5% TX-100, pH 8.1) and insoluble material was removed by centrifugation at 10,000 g for 10 min. The concentrations of protein in the lung tumor extracts were determined by the BCA method (Pierce). Lung tumor extracts at a protein concentration of 0.1 mg/ml were assayed for human uPA activity. The uPA assays (9) were performed in 96 well flat bottom ELISA plates (NUNC, Roskilde, Denmark). Lung tumor extracts (10 µl of the 0.1 mg/ml) were incubated with purified human plasminogen (3 µg/well) for 2 h at 37°C. The S2251 chromogenic substrate (Kabi Vitrum, Stockholm, Sweden) was added (15 µg/3-µl extraction buffer) to each well and the ELISA plates were incubated at 37°C for 0, 15, 30, 60, and 90 min. The absorbance at 405 nm was read at each time point with a multiscan plate reader. The human uPA activity was calculated by comparison of the sample values with a standard curve derived from human urokinase standards (Leo Pharmaceutical Products, Denmark). uPA activity was calculated as milli units of human uPA activity per mg of lung protein.

**Immunofluorescence**

HEp3 M+ cells (1.5 × 10⁶) were grown for 24 h on sterile glass coverslips (Rochester Scientific, Rochester, NY) in an atmosphere of 5% CO₂ at 37°C. The media was removed and the cells were washed 2× with PBS. The cells were fixed with 3% paraformaldehyde for 5 min, washed 3× with serum free DMEM and incubated with 1% gelatin for 10 min, and washed 2× with serum free DMEM. Primary mAbs were added at a concentration of 20 µg/ml and incubated for 30-45 min at room temperature. Cells were washed 5× with serum free DMEM for 5 min each. FITC-conjugated goat anti-mouse IgG (TAGO, Burlingame, CA) was added at a 1:20 dilution in DMEM for 30-45 min at room temperature. Cells were washed 5× with DMEM and 3× with PBS for 5 min each. One drop of mounting medium containing 50% glycerol, 50% PBS was added to the coverslip and the coverslip was inverted on a glass slide. The slides were analyzed for FITC immunofluorescence by confocal microscopy.

**Western Blot Analysis**

HEp3 cells were grown to confluence in 100-mm tissue culture plates and washed 3× with PBS. Whole cell lysates were prepared by the addition of 1 ml of lysis buffer (0.1 M Tris pH 8.0, and 1% TX-100 supplemented with a protease inhibitor mixture including 5 mM EDTA, 20 µg/ml Leupeptin, 30 µg/ml TPCK, 20 µg/ml SBTI and 20 U/ml Aprotinin). The lysates were incubated with agitation for 10 min at 4°C and centrifuged at 3,000 g for 10 min to remove detergent insoluble materials. M⁺ HEp3 lysates (20 µg protein) were electrophoresed through either a 10 or 7.5% SDS-PAGE gel under nonreducing conditions (20). The proteins were transferred by electroblotting onto nitrocellulose membranes. The blots were blocked with 5% milk, incubated with either mAbs DM12-4, IA5, or normal mouse IgG at 4°C overnight, and washed 4× with 0.1% Tween-20 in PBS for 15 min. Peroxidase-labeled goat anti-mouse IgG was added as secondary antibody. The immunoblots were washed as before and developed with the chemiluminescence ECL system (Amersham International, UK) as per manufacturer's protocol.

**Statistical Analysis**

Statistical analysis was carried out by applying ANOVA and Newman-Keuls multiple range tests (50).

**Results**

**Immune Suppression of M⁻ HEp3 Cells by Cyclophosphamide Treatment**

A number of cyclophosphamide-treatment protocols were employed to suppress the immune system of mice to highly reactive immunodeterminants present on M⁻ HEp3 cells, inoculated as the initial immunogen. In two protocols, mice were inoculated with M⁻ HEp3 cells followed a short time later with two injections of cyclophosphamide. In another immunization protocol the mice were pretreated with cyclophosphamide 120 and 96 h before the inoculation with M⁻ HEp3 cells. The control protocol employed the same sequence of inoculations except that no cyclophosphamide was used. In all of the protocols, sera were collected 15 d after inoculation.

**Table I. Suppression of Immune Response to M⁻ HEp3 Cells**

| Mouse number | Cyclophosphamide treatment | Dilution of day 15 Sera | ELISA OD U (A₉₀) |
|--------------|----------------------------|------------------------|------------------|
| D-1          | None                       | 1:50                   | 0.276 (+ 0.086)  |
|              |                            | 1:100                  | 0.161 (+ 0.033)  |
|              |                            | 1:500                  | 0.064 (+ 0.011)  |
| D-2          | None                       | 1:50                   | 0.211 (+ 0.001)  |
|              |                            | 1:100                  | 0.140 (+ 0.007)  |
|              |                            | 1:500                  | 0.070 (+ 0.003)  |
| A-1          | -120 and -96 h             | 1:50                   | 0.114 (+ 0.001)  |
|              |                            | 1:100                  | 0.063 (+ 0.006)  |
|              |                            | 1:500                  | 0.033 (+ 0.003)  |
| A-2          | -120 and -96 h             | 1:50                   | 0.083 (+ 0.004)  |
|              |                            | 1:100                  | 0.051 (+ 0.007)  |
|              |                            | 1:500                  | 0.033 (+ 0.004)  |
| B-1          | 1 and 24 h                 | 1:50                   | 0.094 (+ 0.023)  |
|              |                            | 1:100                  | 0.059 (+ 0.013)  |
|              |                            | 1:500                  | 0.021 (+ 0.005)  |
| B-2          | 1 and 24 h                 | 1:50                   | 0.096 (+ 0.020)  |
|              |                            | 1:100                  | 0.038 (+ 0.017)  |
|              |                            | 1:500                  | 0.015 (+ 0.015)  |
| C-1          | 24 and 48 h                | 1:50                   | 0.000            |
|              |                            | 1:100                  | 0.000            |
|              |                            | 1:500                  | 0.000            |
| C-2          | 24 and 48 h                | 1:50                   | 0.010 (+ 0.004)  |
|              |                            | 1:100                  | 0.000            |
|              |                            | 1:500                  | 0.002 (+ 0.001)  |

Mice were inoculated i.p. with 2 × 10⁶ M⁻ HEp3 cells (prepared as described in Materials and Methods) and treated with cyclophosphamide (200 mg/kg) as indicated. The mice from group A received cyclophosphamide 120 and 96 h before M⁻ HEp3 inoculation. Sera from each mouse was collected on day 15 and diluted 1:50, 1:100, and 1:500 with PBS. The sera dilutions were added (100 µl) to duplicate wells of ELISA plates containing 2 × 10⁶ M⁻ HEp3 cells/well. Whole cell ELISAs were carried out as described in Materials and Methods. ELISA values (OD at 405 nm) were determined from duplicate experiments and corrected by subtracting OD U resulting from binding of preimmune sera. The SD is given in (+).
after the initial inoculation with M\(^{-}\) HEp3 cells and screened in whole cell ELISAs for reactivity to M\(^{-}\) HEp3 cells.

As indicated in Table I, control sera from mouse group D (no cyclophosphamide treatment) had a positive reactivity toward the M\(^{+}\) HEp3 cells at all dilutions tested. Sera collected from mice that were pretreated with cyclophosphamide (group A) still showed reactivity toward the M\(^{+}\) HEp3 cells but their titer was reduced 2–3-fold as compared to the control mice. Sera collected from mice treated with cyclophosphamide 1 and 24 h after the initial inoculation (group B) exhibited low reactivity towards M\(^{+}\) HEp3 cells while the sera from mice treated 24 and 48 h with cyclophosphamide (group C) showed negligible or no detectable reactivity towards the M\(^{-}\) HEp3 cells. The 24 and 48 h cyclophosphamide treatment protocol thus results in a pronounced reduction of the immune response to the M\(^{-}\) HEp3 cells indicating a suppression to the antigenic determinants on the M\(^{-}\) HEp3 cells. A series of similar experiments using differently timed injections (data not shown) confirmed that the most efficient immunosuppression resulted from cyclophosphamide treatment that was performed 24 h and 48 h after the initial inoculation of antigen.

**Enhancement of Immune Response to M\(^{+}\) HEp3 Cells**

Immunization with M\(^{+}\) HEp3 cells was performed on all mice on day 18, 15 d after the last cyclophosphamide treatment, and again on day 39. On day 42, sera was collected and screened for reactivity to both M\(^{+}\) and M\(^{-}\) HEp3 cells in duplicate matched whole cell ELISAs. The M\(^{+}\)/M\(^{-}\) ELISA ratios were calculated for all of the mice (Table II). The results indicated that mouse C-1, which had been treated with cyclophosphamide 24 and 48 h after M\(^{+}\) HEp3 inoculation, had the highest M\(^{+}\)/M\(^{-}\) ELISA ratio of 1.9 while the sera from the control mice (no cyclophosphamide treatment) had a M\(^{+}\)/M\(^{-}\) ELISA ratio of 1.2–1.3. The mice pretreated with cyclophosphamide yielded M\(^{+}\)/M\(^{-}\) ratios indistinguishable from that of the control, while the mice treated with cyclophosphamide at 1 and 24 h after M\(^{-}\) HEp3 inoculation were intermediate between the control and the 24 and 48 h cyclophosphamide-treated animals. The sera data indicates that cyclophosphamide suppressed the immune response to M\(^{-}\) HEp3 cells (Table I) and permitted an enhanced differential response to M\(^{+}\) HEp3 cells (Table II).

**Table II. Differential Immune Reactivity of M\(^{-}\) HEp3- Suppressed Mice after Inoculation of M\(^{+}\) HEp3 Cells**

| Mouse number | Cyclophosphamide treatment | Serum ELISA ratio (M\(^{+}\) HEp3/M\(^{-}\) HEp3) |
|--------------|----------------------------|----------------------------------------------|
| D-1          | None                       | 1.22 (± 0.22)                                |
| D-2          | None                       | 1.27 (± 0.03)                                |
| A-1          | -120 and -96 h             | 1.26 (± 0.10)                                |
| A-2          | -120 and -96 h             | 1.35 (± 0.14)                                |
| B-1          | 1 and 24 h                 | 1.65 (± 0.06)                                |
| B-2          | 1 and 24 h                 | 1.47 (± 0.07)                                |
| C-1          | 24 and 48 h                | 1.88 (± 0.07)                                |
| C-2          | 24 and 48 h                | 1.86 (± 0.09)                                |

The groups of M\(^{-}\) HEp3-inoculated mice that had been treated or pretreated with cyclophosphamide were inoculated i.p. with 2 × 10\(^{6}\) M\(^{+}\) HEp3 cells on day 18 and again with 3 × 10\(^{6}\) M\(^{+}\) HEp3 cells on day 39. On day 42, serum from each mouse was collected, diluted 1:100 with PBS, and tested in duplicate matched whole cell ELISA plates coated with either 2 × 10\(^{6}\) M\(^{+}\) HEp3 cells or 2 × 10\(^{4}\) M\(^{-}\) HEp3 cells. The ratio of the OD\(_{450}\) values for M\(^{+}\) HEp3/M\(^{-}\) HEp3 was calculated. The SD values are given in parentheses.

**Table III. Summary of Total HEp3- Reactive Hybridomas**

| Mouse number | Cyclophosphamide treatment | Whole cell ELISA ratio | M\(^{+}\) HEp3/M\(^{-}\) HEp3 (OD\(_{450}\)/OD\(_{450}\)) | % of Total hybridomas* |
|--------------|----------------------------|------------------------|-----------------------------------------------|------------------------|
| D-1          | None                       | 2.0 or >2.0            | 0.5–1.9                                       | 94%                    |
|              |                            |                        | <0.5                                          | 3%                     |
| B-1          | 1 and 24 h                 | 2.0 or >2.0            | 0.5–1.9                                       | 82%                    |
|              |                            |                        | <0.5                                          | 0%                     |
| C-1          | 24 and 48 h                | 2.0 or >2.0            | 0.5–1.9                                       | 74%                    |
|              |                            |                        | <0.5                                          | 0%                     |

Conditioned media (100 µl) from hybridoma clones prepared from the three indicated mice were screened in the whole cell ELISA for reactivity with both M\(^{+}\) HEp3 and M\(^{-}\) HEp3 cells. Only hybridoma clones having ELISA OD\(_{450}\) or OD\(_{450}\) U of greater than 0.050 and at least 3 × the background (100 µl of medium alone) were included in the calculation of percentages. ELISA ratios were calculated as follows: OD\(_{450}\) U for binding of conditioned media to M\(^{+}\) HEp3 cells were divided by the OD\(_{450}\) U for binding of conditioned media to M\(^{-}\) HEp3 cells. Data were derived from triplicate experiments.

* n value for mouse D-1 = 350; B-1 = 211; and C-1 = 50.

**Generation of mAbs from Control and Experimental Mice**

Hybridomas were generated from mice C-1, B-1, and control D-1. Conditioned media from all of the hybridoma clones (611 total clones) were screened in comparative whole cell ELISAs for binding to both the M\(^{+}\) and M\(^{-}\) HEp3 cells. A summary of these results are shown in Table III. The C-1 mouse that was treated with cyclophosphamide at 24 and 48 h after the initial inoculation and had exhibited the greatest immune suppression towards M\(^{-}\) HEp3 cells (Table I), yielded 26% hybridomas with M\(^{+}\)/M\(^{-}\) ELISA ratios of 2.0 or greater. The B-1 mouse, that was treated with cyclophosphamide at 1 and 24 h after the initial inoculation of HEp3 M\(^{-}\) cells, yielded hybridomas of which 18% had M\(^{+}\)/M\(^{-}\) ELISA ratios of 2.0 or greater. The great majority (94%) of the hybridomas produced from the control mouse D-1 had M\(^{+}\)/M\(^{-}\) ELISA ratios between 0.5 and 1.9 with only 3% of the hybridomas showing M\(^{+}\)/M\(^{-}\) ELISA ratios of 2.0 or greater. The S.I. protocol thus resulted in a substantial increase in the proportion of hybridomas that were differentially reactive with molecules on the surface of M\(^{+}\) HEp3 cells.

From the C-1 mouse hybridomas a panel of mAbs were purified and evaluated by whole cell ELISA for their reactivity to various human cell types. Table IV illustrates a representative sample of these purified mAbs including a mAb (FM40-4) that exhibited little or no enhanced reactivity to M\(^{+}\) HEp3 cells. The mAbs IA5, DM12-4, and FMZ3-3 exhibited M\(^{+}\)/M\(^{-}\) ELISA ratios of 5.5, 2.4, and 2.5, respectively. All of these mAbs exhibited little or no reactivity to non-malignant human cells including normal fibroblasts and primary keratinocytes. mAb DM12-4 exhibited some reactivity to a series of highly malignant human tumor cells including breast adenocarcinoma, cervical carcinoma, and...
fibrosarcoma cells. The reactivity of this mAb to these malignant cells, however, was 4-10-fold lower than its immunoreactivity to M+ HEp3 cells. mAb IA5 also was highly reactive to M+ HEp3 cells but showed little or no reactivity to the other malignant human tumor cells including the tumorigenic but non-metastatic M- HEp3 cells.

**Anti-Metastatic Effect of mAbs**

To ascertain if the cellular determinants that are recognized by the various mAbs are involved functionally in the metastatic cascade, the mAbs were screened in the chick embryo metastasis assay model system (12, 34). Purified mAbs were inoculated i.v. into 11-d old chick embryos previously implanted on day 10 with M+ HEp3 cells (see Materials and Methods). The mAbs were analyzed for their effect on spontaneous metastasis (HEp3 cells appearing in the lung) as well as tumorigenicity (HEp3 primary tumor growth). The results shown in Table V indicate that inoculation of mAb DM12-4 resulted in a dramatic reduction in HEp3 metastasis, as indicated by an 84% inhibition of the number of HEp3 cells found in the chick lungs and an 86% inhibition of the amount of human uPA activity measured in the lung tumors as compared to controls. A strong linear correlation ($r = 0.870$) exists between the number of HEp3 cells found in the lung and the amount of human uPA activity measured in the lung tumors (12, 31, 34, 35, 46). The mAb DM12-4 exhibited no significant effect on tumorigenicity as compared to the control thus demonstrating its specific effect on tumor metastasis. The mAb IA5 also substantially inhibited HEp3 metastasis to the lung. Inoculation of purified IA5 IgG caused a 93% inhibition of the number of HEp3 cells found in the chick lungs and a 90% inhibition of the appearance of human uPA activity in the lung tumors but had no effect on primary tumor growth. The mAbs FM23-3 and FM40-4 which are isotype matched IgG subtypes that also bind to the surface of M+ HEp3 cells (Table IV), demonstrated no significant effect on lung metastasis and primary tumor growth (Table V). Since both mAbs, FM23-3 and FM40-4, did not inhibit metastasis but bound strongly to M+ HEp3 cells, the pronounced inhibition of metastasis observed with DM12-4 and IA5 mAbs was not due simply to nonspecific or steric interactions caused by mAbs binding to the HEp3 cell surface.

The inhibition of metastasis by mAbs DM12-4 and IA5 also does not appear to be due to a cytotoxic effect of the mAbs on the HEp3 cells because no significant change in primary tumor weights was observed (Table V). Furthermore mAbs DM12-4 and IA5 were examined for any effect on HEp3 cell growth in vitro. Fig. 1, A and B shows that HEp3 cell growth was not affected by incubation with mAb DM12-4 or IA5 in comparison to either HEp3 cell growth in the presence of control normal mouse IgG or no antibody additions.

**Properties of the Antigens Recognized by the Anti-Metastatic Antibodies**

The anti-metastatic mAbs DM12-4 and IA5 were originally detected by whole cell ELISA indicating that they were reactive to M+ HEp3 cell surface immunodeterminants. To confirm the localization of the molecules recognized by mAbs DM12-4 and IA5, M+ HEp3 cells were analyzed by indirect immunofluorescence and confocal microscopy. Fig. 2 a illustrates a transmitted light photo micrograph of M+ HEp3 cells. Fig. 2 b shows a immunofluorescent micrograph illustrating the reactivity of mAb DM12-4 to M+ HEp3 cells in the same field. The staining pattern indicates that mAb DM12-4 recognizes epitopes localized to the lateral and peripheral edges of the surface of M+ HEp3 cells. Fig. 2 c and d is transmitted light and immunofluorescent micrographs of M+ HEp3 cells stained with normal mouse IgG. No distinct staining pattern is observed indicating that the distribution and localization of the molecules recognized by mAb DM12-4 are specific and not due to nonspecific binding of IgG. The mAb IA5 also exhibited distinct localization to the cell surface of M+ HEp3 (data not shown) consistent with its immunoreactivity in whole cell ELISA (Table IV).

To determine the nature and specificity of the antigens recognized by the anti-metastatic mAbs DM12-4 and IA5, M+ HEp3 whole cell lysates were electrophoresed in nonreducing SDS-PAGE gels and probed by Western blotting (Fig. 3). In Fig. 3 A, M+ HEp3 whole cell lysates were probed with either mAb DM12-4 (lane l) or normal mouse

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**Table IV. Reactivity of Isolated mAbs to M+ HEp3, M- HEp3, and a Number of Malignant and Normal Human Cell Types**

| mAb tested | M+ HEp3 | M- HEp3 | MDA-MB231 | MS751 | HT-3 | HT1080 | HFF | KER |
|------------|---------|---------|------------|-------|------|--------|-----|-----|
| FM40-4     | 0.288   | 0.233   | 0.037      | 0.000 | 0.001| ND     | 0.000| 0.007|
|            | ±0.049  | ±0.113  | ±0.038     |       |      |        |     |     |
| FM23-3     | 0.340   | 0.135   | ND         | 0.030 | 0.031| 0.126  | ND  | ND  |
|            | ±0.048  | ±0.028  | ND         | ±0.007| ±0.003| ±0.007 |     |     |
| DM12-4     | 0.424   | 0.178   | 0.105      | 0.071 | 0.084| 0.041  | 0.012| 0.003|
|            | ±0.008  | ±0.001  | ±0.007     | ±0.013| ±0.004| ±0.025 | ±0.016| ±0.004|
| IA5        | 0.312   | 0.057   | 0.002      | 0.000 | 0.014| ND     | 0.000| ND  |
|            | ±0.003  | ±0.003  | ±0.002     |      |      |        |     |     |

**Legend:**

M+ HEp3, Human Cervix Epidermoid Carcinoma; M- HEp3, nonmetastatic Human Epidermoid Carcinoma; M+ HEp3, metastatic Human Epidermoid Carcinoma; MDA-MB 231, Human Adenocarcinoma of the breast; MS751, Human Cervix Epidermoid Carcinoma; HT-3, Human Cervical Carcinoma; HT1080, Human Fibrosarcroma; HFF, Human Foreskin Fibroblasts; KER, Human Primary Keratinocytes; ND indicates not determined.
The identiﬁcation of speciﬁc molecules involved functionally in the multistep process of tumor metastasis is made difficult due to the complexity of the various in vivo steps in the metastatic cascade. Metastatic processes such as tumor cell growth, migration, invasion, and adhesion have been studied individually in various cell culture model systems (1, 3, 4, 28). A signiﬁcant number of molecules including growth factors, adhesion glycoproteins and proteolytic enzymes have been correllatively, and in some cases functionally linked to phenotypic behavior of the cells in the culture model systems (5, 13, 30, 41). Very few of these molecules, however, have been tested rigorously in the overall process of metastasis and shown to be functionally involved in vivo. In the present study a subtractive immunization approach has been used to immunologically identify speciﬁc molecules, present on the surface of highly metastatic human tumor cells, that function mechanistically in the overall process of metastasis. This subtractive approach, in conjunction with sequential screening of antibody binding to metastatic cells and antibody-mediated inhibition of metastasis, allows the immunological approach itself to select out relevant antigen molecules. The selection is carried out without any experimental bias towards a favored class of molecules such as enzymes, growth factors, or adhesion molecules and instead the relevancy of the antigen is dictated by the results of the screening process. The selective pressure of screening for a monoclonal antibody that speciﬁcally inhibits the final outcome of metastasis, i.e., appearance of tumor cells in a secondary organ, provides strong evidence that the antigen recognized by the antibody is involved in the mechanism of metastasis.

The subtractive approach taken in the present study was used in conjunction with a human tumor cell line Hep3, that had been shown previously to be highly tumorigenic and metastatic in both the chick embryo and nude mouse (12, 31, 34, 35, 36, 46). Specific molecules on the surface of Hep3 cells might indeed contribute to Hep3's metastatic phenotype, and antibodies to such molecules would be extremely useful. Immunizing mice directly with Hep3 cells, however, would likely yield an immune response mainly to immunodominant antigens present on the surface of many human cells as suggested by studies employing direct immunization of target cells (19, 27, 49). It is probable that many of the immunodominant molecules are not directly involved in the metastatic process. Their immunodominance, however, make it difﬁcult to isolate antibodies to rare and possibly low immunogenic molecules that might have a signiﬁcant role in metastasis. The subtractive immunization protocol whereby the non-metastatic variant of Hep3 cells, M- Hep3, is inoculated into mice and the resulting immunoreaction is suppressed by cyclophosphamide, allows for a subsequent shift in the immunological response upon challenging the mice with the highly metastatic variant, M+ Hep3.

A clear suppression of immune response to intact M- Hep3 cells occurred in mice inoculated with M- Hep3 cells when cyclophosphamide was injected 24 and 48 h after the initial inoculation of M- Hep3 (Table I). Upon challenge of the suppressed mice with M+ Hep3, a shift in the immune response was apparent as a greater immune reactivity to M+ Hep3 over M- Hep3 became apparent in the antibody titers of the serum from the cyclophosphamide treated animals (Table II). Although only a modest change in the M+/M- immunoreactivity ratio over the control animals.

### Table V. Effect of Purified mAbs on Hep3 Spontaneous Metastasis

| mAb inoculation | Primary tumor (mg) | % of control | Hep3 cells in lung tumors (Cells/Field) | % of control | Human uPA in lung tumors (mU/mg) | % of control |
|-----------------|-------------------|-------------|----------------------------------------|-------------|-----------------------------------|-------------|
| None            | 322.6 ± 32.9      | 100         | 13.5 ± 1.1                             | 100         | 4,344 ± 816                       | 100         |
| FM40-4          | 323.5 ± 62.9      | 100         | 12.6 ± 3.1                             | 93          | 3,931 ± 683                       | 91          |
| FM23-3          | 347.1 ± 32.1      | 108         | 11.9 ± 1.7                             | 88          | 3,941 ± 921                       | 91          |
| DM12-4          | 307.8 ± 22.8      | 95          | 2.2 ± 0.4                              | 16          | 599 ± 144                         | 14          |
| IA5             | 309.1 ± 10.4      | 96          | 1.0 ± 0.3                              | 7           | 440 ± 86                          | 10          |
Figure 1. Effect of mAbs DMI2-4 and IA5 on M+ HEp3 growth in vitro. M+ HEp3 cells (2 × 10⁵) were added to 60-mm tissue culture plates in 2 ml of DMEM containing 10% FBS and incubated at 37°C for 1 h. The incubation allowed 80-90% of the cells to become adherent. The plates were washed 3x with PBS and 2 ml of DMEM containing 10% FBS supplemented with either 50 µg/ml normal mouse IgG, mAb DMI2-4 (A), or mAb IA5 (B) was added to the plates. Cultures were incubated at 37°C in an atmosphere of 5% CO₂ for 24, 48, and 72 h. At each time point the cells were removed from the plates with nonenzymatic cell dissociation solution, counted in a hemocytometer and calculated for the number of cells per plate. The data in each panel were derived from two separate experiments.

(1.9 vs 1.3) was apparent, it is important to note that serum immunoreactivity represents the total antibody population of the immunized animal. Antibodies reacting uniquely or at enhanced levels to M+ HEp3 would be masked in the overall population of circulating antibodies since even in the S.I. protocol most antibodies would still be reactive to antigens common to both M+ HEp3 and M- HEp3 cells. In the immunosuppressed mice the enrichment of antibodies that recognize antigens expressed specifically, or at elevated levels on M+ HEp3 cells became more apparent when spleen cell hybridomas are analyzed individually (Table III). The results indicate that the cyclophosphamide-treated animals yield a greater proportion of B cells producing antibodies that react differentially with M+ HEp3 over M- HEp3 cells. The subtractive approach was not absolute or complete since antibodies reactive solely to M+ HEp3 cells were never observed. Nevertheless a clear enhancement of the immune response to antigens on the M+ HEp3 cells was obtained (Tables III and IV). The tolerizing effects of cyclophosphamide are not permanent and mice begin to recover from the induced tolerance with time if the initial antigen and cyclophosphamide inoculation schedule is not cyclically repeated (24, 27). It may be possible to complete or prolong the tolerance and enhance the possibility of obtaining antibodies uniquely reactive to the challenged immunogen (M+ HEp3) by repeated inoculation of M-HEP3 followed by 24 and 48 h cyclophosphamide treatments before the M+HEP3 challenge. However, multiple cyclophosphamide inoculations were often lethal to the mice and complete tolerization to M+HEP3 was never achieved.

The distinct success of the subtractive protocol used in the present study was most apparent in the procurement of specific antibodies that are anti-functional in their effect on M+ HEp3's phenotype (Table V). Both DMI2-4 and IA5 mAbs had a pronounced inhibitory effect on the spread of HEp3 cells to the lungs. The inhibition was not due to a cytotoxic effect since the antibodies did not significantly reduce the size of the expanding primary tumors (Table V) nor did...
The subtractive immunization approach has yielded additional monoclonal antibodies that in preliminary experiments also appear to be anti-metastatic but studies on the nature of the antigens recognized by these antibodies are incomplete. Thus far none of the tested monoclonal antibodies that were derived from control animals (no cyclophosphamide treatment) are anti-metastatic. This is consistent with the observation that mAb FM40-4 which recognizes equally M⁺ HEp3 and M⁻ HEp3 (Table IV) is not anti-metastatic (Table V). It would appear that the subtractive approach enriches for antibodies that react with relevant functional antigens. It may be that functional antigens are rare or in low abundance on the cell surface relative to highly immunodominant antigens that only a tolerization or suppression event such as cyclophosphamide treatment allows for their immune recognition upon subsequent challenge with the relevant target cell type. Cyclophosphamide-mediated immune suppression has been used previously to obtain antibodies to rare or low abundance antigens on both normal and malignant cell types (18, 19, 26, 27, 48). The present study using both a low and highly metastatic human tumor cell along with an empirically determined immunization protocol has allowed for the first anti-metastatic antibodies to be procured by this subtractive approach. The protocol could engender a variety of experimental approaches designed to generate unique anti-functional antibodies in various biological systems where two closely related but phenotypically distinct cell or tissue types are available.

The authors gratefully acknowledge Drs. Nancy Reich and Sidney Strickland for their critical reading of the manuscript, Ms. Betty Draskin for her help in preparing the manuscript, and Dr. Paul Patterson for his original discussions.

This research was supported in part by American Cancer Society grant IM565 and a National Institutes of Health grant CA 16740. Received for publication 5 January and in revised form 11 June 1993.

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