Establishment and Serial Quantification of Intrahepatic Xenografts of Human Hepatocellular Carcinoma in Severe Combined Immunodeficiency Mice, and Development of Therapeutic Strategies to Overcome Multidrug Resistance

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ABSTRACT
A murine model in which to study multiple drug resistance in human hepatocellular carcinoma was developed. PRF/PLC/5 hepatoma cells (Alex 0) and an induced multidrug resistant clone (Alex 0.5) were injected intrasplenically into severe combined immunodeficiency mice. In 70% of injected mice, hepatoma cells engrafted in the liver and grew as intrahepatic metastasis. Since Alex cells contain an integrated hepatitis B virus genome and secrete hepatitis B surface antigen (HBsAg), the serum HBsAg concentration in tumor-bearing mice was used to quantitate tumor burden. Tumor wet weight determined at necropsy was directly proportional to the serum HBsAg concentration.

In Alex 0 cells, IC50 for doxorubicin, vinblastine, and cis-platinum were 0.35 μM, 0.029 μM, and 3.70 μM, respectively. Alex 0.5 cells were 25-, 14-, and 1.4-fold more resistant to doxorubicin, vinblastine, and cis-platinum, respectively. Immunoblotting of Alex 0 cell membranes with an anti-P-glycoprotein antibody (C219) revealed small amounts of P-glycoprotein, whereas Alex 0.5 membranes overexpressed the protein. Concurrent exposure to verapamil (10 μM) sensitized both cell lines to the cytotoxic action of vinblastine and doxorubicin but had no effect on the cytotoxicity of cis-platinum. Mice bearing intrahepatic xenografts derived from Alex 0 and 0.5 cells had no response to treatment with i.v. vinblastine or doxorubicin, as was anticipated from in vitro drug testing. Addition of verapamil to vinblastine treatment did not improve the success of in vivo chemotherapy. Immunotherapy with a human anti-P-glycoprotein antibody (MRK16) suppressed the in vivo growth of tumors derived from both cell lines. The effect was most pronounced in mice bearing Alex 0.5 tumors. Immunoblotting of tumors which initially responded to MRK16 therapy, but subsequently relapsed, revealed a marked decrease in P-glycoprotein expression when compared to results in tumors that were untreated or treated with vinblastine or control antibody. In summary, we have developed an intrahepatic tumor xenograft model of human hepatocellular carcinoma in mice that permits noninvasive serial quantification of tumor burden by determination of serum HBsAg levels and demonstrated a positive response to immunotherapy with anti-P-glycoprotein antibodies.

INTRODUCTION
HCC, a prevalent tumor worldwide, is often intrinsically resistant to multiple chemotherapeutic agents (1). When response to chemotherapy does occur, it is often quickly followed by acquired drug resistance and clinical relapse (1).

Little is known concerning factors responsible for multiple chemotherapy drug resistance (MDR) in HCC. One proposed mechanism involves overexpression of P-glycoprotein, a plasma membrane protein, which functions as an ATP-dependent drug efflux pump (for review, see Refs. 2 and 3). In cancer cell lines, P-glycoprotein is responsible for efflux of a variety of structurally and functionally unrelated chemotherapeutic drugs including anthracyclines, Vinca alkaloids, epipodophyllotoxins, and taxanes.

Several lines of evidence suggest a role for P-glycoprotein in drug resistance in HCC. P-glycoprotein is found on the apical biliary canalicular membrane in normal liver (4) where it mediates ATP-dependent chemotherapeutic drug transport (5). Overexpression of MDR1, the human P-glycoprotein gene, has been demonstrated in drug-resistant human hepatoma cell lines and clinical cases of human HCC (6–8). Increased P-glycoprotein expression accompanies transformation of hepatocytes in rodent models of hepatocarcinogenesis (9, 10) and can be induced in rodent cell lines by exposure to cytotoxic agents (12).

The extent to which P-glycoprotein contributes to intrinsic or acquired MDR in HCC has important clinical implications, since several therapeutic strategies to circumvent P-glycoprotein-mediated MDR are under investigation. Many drugs, including verapamil, cyclosporine, quinidine, and progesterone, inhibit P-glycoprotein-mediated drug efflux in cancer cell lines (2, 3, 13). These so-called chemosensitizing agents correct drug accumulation defects and restore drug sensitivity in MDR cell lines. In mice harboring tumor xenografts from MDR cancer cell lines, concurrent administration of chemotherapy agents with chemosensitizing drugs restored drug sensitivity and was associated with tumor regression (13). Clinical trials to evaluate the

Received 8/22/95; revised 12/11/95; accepted 12/21/95.
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3 The abbreviations used are: HCC, hepatocellular carcinoma; MDR, multidrug resistance; SCID, severe combined immunodeficiency; IC50, 50% inhibitory concentration; ADCC, antibody-dependent cell-mediated cytolysis.
efficacy of these chemosensitizers in reversing MDR in human malignancies have shown modest success in hematological malignancies, but results with solid tumors, such as colon and breast cancer, have been disappointing (14). A small clinical trial evaluating the effect of verapamil combined with doxorubicin therapy in unresectable HCC failed to show any beneficial effect (15).

Monoclonal antibodies against external epitopes of P-glycoprotein also modulate the growth of cancer cells which overexpress P-glycoprotein (16–24). When two of these antibodies (MRK16 and HYB-241) were administered in combination with cytotoxic agents, drug sensitivity was restored in mice bearing human tumor xenografts derived from MDR cancer cell lines (18, 21, 24). In addition, MRK16 given alone was capable of modulating the growth of murine tumor xenografts (17, 20).

A major limitation in the design of strategies to circumvent P-glycoprotein-mediated drug resistance in solid tumors, such as HCC, is lack of an appropriate animal model in which to study the phenomenon. Present murine models involve growth of human tumor xenografts as ascitic or subcutaneous tumors which limit extrapolation of results to solid tumors. Intraperitoneal ascitic tumors, which are often treated by i.p. drug injections, fail to consider the efficiency of vascular drug delivery to the tumor site and the need for drug penetration through multiple cell layers, which are critical determinants of successful clinical therapy of solid tumors. In ascitic models, it is impossible to verify whether tumor exists on initiation of treatment or to quantitate tumor burden during therapy. Ascitic tumor models rely on prolongation of survival as the end point with which to judge efficacy. In mice bearing subcutaneous tumor xenografts, tumor size can be directly measured using calipers and response to therapeutic intervention monitored quantitatively. However, when some cell lines are injected s.c., resultant tumors grow as well-encapsulated, poorly vascularized masses so that early tissue necrosis and hemorrhage may complicate evaluation of tumor volume. Both ascitic and subcutaneous models fail to consider the rapidly emerging concept that the microenvironment in which tumor cells grow can profoundly influence their response to cytotoxic therapy (25–27). Such limitations may explain, in part, the discrepancy between successes reported with in vivo chemosensitization protocols in murine models and failure of these protocols in clinical trials with solid tumors.

A recently described murine human myeloma tumor model in SCID mice overcomes one of the limitations of an ascitic tumor model in that it permits serial quantification of tumor engraftment (28). Mice injected i.p. with myeloma cells developed peritoneal tumor implants and metastasis. Because the myeloma cells secrete immunoglobulin light chain, urinary light chain concentrations progressively increased in injected mice, presumably reflecting an increasing tumor burden, although tumor mass was not measured.

We have developed a murine xenograft model of human HCC in which the role of P-glycoprotein in intrinsic and acquired drug resistance can be investigated. We sought a model in which human HCC grows within the hepatic parenchyma and contains a quantifiable marker permitting serial noninvasive monitoring of tumor burden. Several investigators demonstrated the feasibility of establishing intrahepatic metastasis of human carcinoma cell lines after intrasplenic injection in immunodeficient mice (29–31). Therefore, we injected the human HCC cell line PRF/PLC/5 (32) intrasplenically into SCID mice. This cell line contains an integrated hepatitis B viral genome and selectively secretes viral surface antigen (HBsAg), but not infectious virion or other viral protein (33). In tissue culture, secretion of HBsAg into the medium is proportional to cell mass (34). Mice bearing subcutaneous tumors from PRF/PLC/5 cells contain circulating levels of HBsAg which correlated positively with tumor size (34, 35). We exploited the secretion of HBsAg by PRF/PLC/5 cells to monitor the growth of HCC xenografts introduced into SCID mice by intrasplenic injection.

Intrasplenic injection of PRF/PLC/5 cells into SCID mice resulted in reproducible engraftment of tumor cells within the hepatic parenchyma. Secretion of HBsAg by tumor cells enabled noninvasive and serial quantification of tumor burden. We explored several therapeutic strategies to treat tumors derived from the parental HCC cell line and an MDR clone which was induced to overexpress P-glycoprotein.

**MATERIALS AND METHODS**

**Animals.** Four- to 6-week-old male and female C.B-17 SCID mice were obtained from Charles River Laboratories, Inc. (Wilmington, MA). All mice were maintained under sterile conditions. Serum samples (20 μl) were obtained from mice at 4 weeks of age for determination of immunoglobulin levels to detect significant breakthrough of the SCID phenotype (36). Animals with IgG or IgM levels greater than 5 μg/ml or 2 μg/ml, respectively, were excluded from the study.

**Drugs.** Doxorubicin HCl (Adriamycin) was obtained from Adria Laboratories (Columbus, OH). Vinblastine sulfate was obtained from Lyphomed (Deerfield, IL). R-verapamil was a gift from BASF BioResearch Corporation (Worcester, MA). cis-Platinum, racemic verapamil, and the mouse myeloma protein UPC 10 were obtained from Sigma (St. Louis, MO). MRK16, a murine monoclonal antibody to human P-glycoprotein, was generously supplied by Professor Takashi Tsuruo (University of Tokyo, Tokyo, Japan). C219, a polyclonal antibody to hamster P-glycoprotein, was purchased from Centocor (Malvern, PA). [3H]Daunorubicin was obtained from New England Nuclear (Boston, MA).

**Cell Lines.** The human HCC cell line PLC/PRF/5 was obtained from Professor David Shafritz (Albert Einstein College of Medicine, Bronx, NY). The parental cell line was designated Alex 0. A MDR clone was developed by sequential selection in increasing concentrations of doxorubicin. This clone, Alex 0.5, has stable resistance to growth in 0.5 μg/ml doxorubicin, overexpresses P-glycoprotein, and exhibits a MDR phenotype (37, 38). Alex cells were cultured in Eagle’s MEM supplemented with 2 mM l-glutamine, 0.01 mM nonessential amino acids, 10% FCS, penicillin (100 units/ml), and streptomycin (0.1 mg/ml). Alex 0.5 cells were grown in doxorubicin (0.5 μg/ml) until 2 weeks before experiments were conducted.

**CH'C5**, Chinese hamster ovary cells which overexpress P-glycoprotein, were obtained from Professor Victor Ling (Ontario Cancer Institute, Toronto, Ontario, Canada) and maintained in α-MEM supplemented with 10% FCS, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (0.1 mg/ml).

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All cell lines were maintained in a humidified environment at 37°C in an atmosphere of 5% CO₂/95% air.

**In Vitro Cytotoxicity Studies.** Alex 0 and 0.5 cells were plated at 1 and 1.5 × 10⁶ cells/35-mm dish, respectively. Cells were allowed to attach for 24 h, after which fresh media containing cytotoxic drug and/or reversing agent were added. After incubation for 72 h, cells were harvested using a 0.05% trypsin/0.002% EDTA solution prepared in 0.9% NaCl. Viable cell counts were by trypan blue dye exclusion using a hemocytometer. IC₅₀ was calculated.

**Drug Accumulation and Retention.** Alex 0 and 0.5 cells were plated in 35-mm dishes at 4 × 10⁵ cells/dish and incubated overnight under standard tissue culture conditions. Accumulation and efflux of [³H]daunorubicin (1 μM) were determined (39). In brief, 1 μM daunorubicin containing a tracer amount of [³H]daunorubicin was added to fresh media with or without addition of 10 μM verapamil. Dishes were incubated under tissue culture conditions and at 0, 15, 30, and 60 min after drug addition, were quickly washed in ice-cold PBS, and cells were removed by treatment with trypsin. The cell suspension was diluted with 1 ml cold PBS, and cells were counted in a hemocytometer. The tubes were spun at 10,000 rpm at 4°C in a microfuge, and the pellet was lysed in PBS containing 0.05% SDS. Radioactivity in the lysate was determined by liquid scintillation counting. For efflux studies, cells were plated as described above and then loaded with 1 μM daunorubicin in glucose-free medium containing 10 mM sodium azide and 10% dialyzed FCS. After loading for 1 h under tissue culture conditions, the cells were washed with PBS, and regular culture media were added with or without 10 μM verapamil. The amount of daunorubicin remaining in the cells was determined at 0, 5, 15, 30, and 60 min as described above.

**Tumor Induction.** Subconfluent cultures of Alex 0 or 0.5 cells were harvested from monolayer culture after trypsinization. Cells were collected by centrifugation, washed twice with PBS, and resuspended to a final concentration of 12 × 10⁶ cells/ml in PBS.

Six- to 8-week-old male and female SCID mice were anesthetized with Avertin (0.024 mg/kg of a 1.2% tribromoethanol solution i.p.), placed in left lateral recumbancy, and the right paracostal area aseptically prepared for surgery. The spleen was exteriorized through a right paracostal incision and injected subcapsularly into the spleen. After allowing 2 mm for movement of cells out of the spleen, splenectomy was performed. The splenic pedicle was ligated with 6-0 polyglactin. The splenic pedicle was ligated with 6-0 polyglactin (1007D; ALZET, Palo Alto, CA) were implanted i.p. under Avertin anesthesia as previously described (41). Pumps were preloaded with 100 μl of a 125 mg/ml solution of R-verapamil dissolved in 0.9% saline and operated at 0.1 μl/h to deliver a continuous i.p. infusion. Mice with i.p. pumps received a single i.v. injection of vinblastine (5 mg/kg) 4–6 h after pump placement. Immunotherapy with MBK16 or UPC 10 was administered at 500 μg i.v. weekly for 3 weeks.

During therapy, serum HBsAg titers were monitored weekly. Partial response was defined as stabilization of or decrease in HBsAg titer. Complete response to therapy was defined as disappearance of HBsAg titer at two or more assay points. Treatment cures were documented by gross and histological absence of tumor at necropsy. All mice that underwent treatment were necropsied at the time of relapse, the presence of gross hepatic tumors was recorded, and tumor wet weight was obtained. Representative samples of Alex 0 and 0.5 tumors from animals in each treatment group were frozen at −80°C for subsequent analysis of P-glycoprotein expression.

**Immunoblotting.** Plasma membrane preparations were made from subconfluent cultures of Alex 0, Alex 0.5, and CH'CS cells and from mouse tumor xenograft tissue obtained at necropsy. Tumor samples were thawed and minced, whereas tissue culture media were collected from hypotonic lysis buffer (10 mM Tris-HCl, 10 mM MgCl₂, 1.5 mM NaCl, 1 mM dithiothreitol, pH 7.4) containing 2 μM pepstatin, 2 μg/ml leupeptin, and 1 μM phenylmethylsulfonyl fluoride. The cells remained on ice for 10 min and then were homogenized with 15 strokes of a Wheaton A dounce homogenizer. Cell homogenates were spun at 400 × g for 10 min at 4°C to remove nuclei. The resultant supernatant was further spun at 22,000 × g for 30 min at 4°C. The resultant membrane-rich pellet was resuspended in hypotonic lysis buffer in 50% glycerol and stored at −70°C. Protein concentrations were determined according to the method of Lowry et al. (42) using BSA as a standard.

Representative mice with positive HBsAg titers varying from 1 to 2500 ng/ml were euthanized and necropsied. Grossly visible liver tumors were dissected free from normal liver tissue, and tumor wet weight was obtained. Neoplastic tissue and remaining normal liver were fixed in formalin, processed for histopathology, stained with H&E, and evaluated using light microscopy by a veterinary pathologist. Histological evaluation verified tumor in the excised tissue and failed to detect additional evidence of tumor in the remaining liver.

**Drug Treatment Protocols.** Mice with positive HBsAg titers for 2 or more consecutive weeks were entered into chemotherapy or immunotherapy treatment protocols. For chemotherapy trials, mice were treated with single i.v injections of doxorubicin (6–8 mg/kg) or 3 weekly i.v. injections of vinblastine (5 mg/kg) with or without concurrent i.p. administration of racemic verapamil (25 mg/kg) or R-verapamil (100 mg/kg). The vinblastine dose was selected on the basis of pharmacokinetic data in mice, which showed that this dose results in serum vinblastine concentrations (40) within the range associated with *in vitro* cytotoxicity in Alex 0 cells. In addition, this schedule of *Vinca* alkaloid administration has been successfully employed in published studies on MDR reversal in solid tumor models (18, 21, 24). The verapamil doses utilized were the maximum tolerated doses in our mice. In several mice, osmotic pumps (Model 1007D; ALZET, Palo Alto, CA) were implanted i.p. under Avertin anesthesia as previously described (41). Pumps were preloaded with 100 μl of a 125 mg/ml solution of R-verapamil dissolved in 0.9% saline and operated at 0.1 μl/h to deliver a continuous i.p. infusion. Mice with i.p. pumps received a single i.v. injection of vinblastine (5 mg/kg) 4–6 h after pump placement. Immunotherapy with MBK16 or UPC 10 was administered at 500 μg i.v. weekly for 3 weeks.

For all cell lines, media were added with or without 10 μM verapamil. Dishes were incubated for 1 h under tissue culture conditions and at 0, 15, 30, and 60 min as described above.

**Tumor Detection.** After tumor cell injection, mice were bled (100–200 μl) from the tail vein. Serum was analyzed for HBsAg by RIA (Austria II; Abbott Laboratories). A HBsAg standard curve from 2.5 to 20 ng/ml was constructed using the positive serum control included in the kit. When necessary, mouse serum samples were diluted to fall within this standard curve. HBsAg titers were expressed in ng/ml.

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Membrane protein preparations were subjected to electrophoresis on 8% SDS-PAGE gels. Gels were electrophoretically transferred to nitrocellulose overnight at 30 V at 4°C. Blots were
Table 1. *In vitro* chemosensitivity patterns in Alex cells

<table>
<thead>
<tr>
<th>Chemotherapy agent</th>
<th>Alex 0 -vrp</th>
<th>Alex 0 +vrp</th>
<th>Alex 0.5 -vrp</th>
<th>Alex 0.5 +vrp</th>
<th>P388*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>0.35*d,e</td>
<td>0.077</td>
<td>8.61*d</td>
<td>0.98</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>(0.079)</td>
<td>(0.006)</td>
<td>(2.49)</td>
<td>(0.69)</td>
<td></td>
</tr>
<tr>
<td>Vinblastine</td>
<td>0.029*d,e</td>
<td>0.0015</td>
<td>0.41*d</td>
<td>0.038</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>(0.006)</td>
<td>(0.0002)</td>
<td>(0.010)</td>
<td>(0.006)</td>
<td></td>
</tr>
<tr>
<td>cis-Platinum</td>
<td>3.70</td>
<td>3.23</td>
<td>5.22</td>
<td>5.52</td>
<td>0.075</td>
</tr>
<tr>
<td></td>
<td>(0.329)</td>
<td>(0.421)</td>
<td>(0.925)</td>
<td>(0.876)</td>
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*IC_{50} represents the concentration of drug necessary to cause 50% inhibition of cell growth. Values are means +/−SE from three independent experiments.

*From Refs. 40 and 41.
*d Significantly different (p < 0.01) than IC_{50} in the presence of verapamil.
*e Significantly different (p < 0.05) than IC_{50} in Alex 0.5 cells.

RESULTS

**In Vitro Testing.** *In vitro*, the parental cell line Alex 0 exhibited low levels of resistance to doxorubicin and vinblastine (Table 1) when compared to the well-characterized chemosensitive murine leukemia cell line P388 (43, 44). Alex 0.5 cells were 25 times more resistant to doxorubicin and 14 times more resistant to vinblastine than were Alex 0 cells. This level of drug resistance was maintained even when Alex 0.5 cells were grown in the absence of doxorubicin for up to 45 days (data not shown). *In vitro* resistance to doxorubicin and vinblastine in Alex 0 and 0.5 cells was decreased by exposure to 10 μM verapamil (Table 1). In both cell lines, verapamil reversed vinblastine resistance better than it reversed doxorubicin resistance. Verapamil concentrations as low as 2.5 μM showed similar chemosensitizing ability in both cell lines. An equipotent chemosensitizing effect was seen with the racemic mixture of verapamil or the R-isomer (data not shown). When compared to P388 cells, both hepatoma cell lines showed *in vitro* resistance to cis-platinum, which was not altered by the addition of verapamil (Table 1).

Fig. 1A shows the effect of verapamil on the accumulation of daunorubicin in Alex 0 and 0.5 cells. Alex 0 cells had a time dependent increase in daunorubicin accumulation, and the addition of verapamil increased accumulation at all time points (P < 0.01 at t = 60 and 90 min). Alex 0.5 cells accumulated appreciable amounts of daunorubicin only in the presence of verapamil (P < 0.05 at all times points). The inability of Alex 0.5 cells to accumulate daunorubicin in the absence of verapamil was associated with enhanced drug efflux as reflected in the rapid loss of preloaded daunorubicin (Fig. 1B). Addition of verapamil significantly increased the retention of daunorubicin in Alex 0.5 cells at all time points (P < 0.01). Alex 0 cells retained greater amounts of daunorubicin than did Alex 0.5 cells (P < 0.001 at all time points) and manifested improved retention upon addition of verapamil. These results are consistent with the expression of P-glycoprotein-mediated drug resistance in both cell lines, albeit at different levels. Since verapamil was not capable of completely reversing either increased daunorubicin efflux or *in vitro* doxorubicin cytotoxicity, it is likely that alternative methods of anthracycline drug resistance exist in Alex 0.5 cells. This would not be an unexpected finding in a cell line that was selected for drug resistance by growth in doxorubicin.

**Expression of P-Glycoprotein in Alex Cells.** Immunoblotting of crude membrane fractions prepared from Alex 0, Alex 0.5, and CHF(C5 was performed with C2l9, an anti-P-glycoprotein antibody (Fig. 2). Low-level P-glycoprotein expression was detected in Alex 0 cells consistent with previous studies (6); Alex 0.5 cells had substantially greater amounts of P-glycoprotein, but less than that seen in the P-glycoprotein overexpressing cell line CHF(C5. Expression of P-glycoprotein was maintained in Alex 0.5 cells even when the cells were grown in doxorubicin-free media for 45 days (data not shown).

**Secretion of HBsAg.** Both Alex cell lines secreted HBsAg into the culture medium in direct proportion to cell mass (data not shown; Ref. 34).

**Induction of Tumor Xenografts.** More than 180 mice were entered into the study over a 3-year period. Mice received 1.5–20 × 10⁶ cells by intrasplenic injection. When larger cell inocula were attempted, death from portal thromboembolism occurred. Even at these cell doses, occasional thrombotic complications developed, especially with the Alex 0.5 cells. These episodes were minimized by passing cells through a 30-gauge needle to break up cell aggregates just before injection into the spleen. This treatment had no effect on cell viability as assessed by trypan blue exclusion.

Serum samples from mice given injections were analyzed...
A

Fig. 1  Time-dependent accumulation (A) or retention (B) of daunorubicin in Alex 0 or Alex 0.5 cells in the presence and absence of 10 μM verapamil. Daunorubicin accumulation was measured as described in ‘Materials and Methods’ at various time points following the addition of 1 μM daunorubicin. Daunorubicin retention was measured at the times indicated after loading Alex cells in the presence of 1 μM daunorubicin for 60 min and then transferring the cells to drug-free media. Values are the means of three independent experiments; bars, SE. For some time points, the SE bars are not visible due to the small variability.

B

weekly for HBsAg. In initial experiments to determine the association between serum HBsAg and tumor weight, mice with positive titers had grossly visible liver tumors at necropsy. Single or multiple hepatic tumors grew as distinct white to tan nodules within the hepatic parenchyma. Extrahepatic tumor sites were rare and included the root of the mesentery (n = 10), the incision site (n = 2), and the lung (n = 1). Microscopic examination of tumor sections prepared from Alex 0 or 0.5 tumors revealed features of a moderately well-differentiated HCC identical to those previously reported in subcutaneous xenografts derived from the parental cell line (29, 30). Mice that remained HBsAg negative never showed gross or histological evidence of tumor formation.

Tumors consistently developed after intrasplenic injection of Alex 0 or 0.5 cells. Successful engraftment occurred in 73% (89/112) and 71% (50/70) of mice given injections of Alex 0 and 0.5 cells, respectively. The latent period from injection to appearance of HBsAg titers was longer in mice receiving injections of Alex 0.5 cells (39.9 days; range, 28–63 days) than in mice that received Alex 0 cells (30.1 days; range, 14–56 days). The differences in latency may reflect both a lower inoculating dose of Alex 0.5 due to these cells enhanced tendency to clump at the high concentrations and the slightly slower in vitro growth rate noted in this drug resistance cell line (data not shown).

Serial determination of serum HBsAg titers in mice receiving injections revealed progressive increases over a 4–8-week interval (Fig. 3). Fig. 4 shows the statistically significant positive correlation between the serum HBsAg titer and tumor wet weight when wet weight was less than 200 mg. Correlation of the HBsAg titer with tumor weight decreased as tumor mass increased above 200 mg. This disparity was reflected in finding necrosis and hemorrhage in larger tumors, which had been included in the measurement of total tumor weight. Mice appeared to be clinically normal when HBsAg titers were less than 200 ng/ml. Once titers increased over 200 ng/ml, mice started showing mild weight loss. As titers continued to rise, mice exhibited more profound weight loss, lethargy, and an unthrifty coat and were humanely sacrificed in compliance with the UKCCCR guidelines.
Multidrug Resistance in a Murine Hepatoma Model

As monitored by HBsAg titer, tumors derived from Alex 0 or 0.5 cells grew at comparable rates once engrafted, although some variation among mice within experimental groups receiving injections on the same day was noted (Fig. 3).

**Immunotherapy of HCC Xenografts.** Table 2 summarizes the results of immunotherapy with MRK16. Fourteen mice bearing Alex 0-derived tumors (HBsAg titers, 1.5–128 ng/ml; median, 23 ng/ml) and seven mice bearing Alex 0.5 tumors (HBsAg titers, 12–144 ng/ml; median, 14 ng/ml) were treated with MRK16. All mice bearing tumors derived from Alex 0.5 cells responded to therapy. Two mice were cured (HBsAg titers, 1.5 and 15.8 ng/ml) and three had complete responses (HBsAg titers, 4.4, 14, and 12.3 ng/ml), but relapsed at 57, 79, and 119 days after treatment (Fig. 5A). Two mice with the highest HBsAg titers on initiation of therapy had sustained partial responses (38 and 42 days), but relapsed when therapy ended (Fig. 5B). Response to MRK16 in mice bearing tumors from Alex 0 cells was less consistent. Six mice (HBsAg titers, 5.8–128 ng/ml; median, 19 ng/ml) had no response (Fig. 5C). Six mice (HBsAg titers, 9.8–104 ng/ml; median, 25 ng/ml) had partial responses, lasting up to 68 days (Fig. 5D). Two mice with the lowest titers (HBsAg titers, 3.2 and 1.5 ng/ml) had a complete response and were cured.

Ten mice (HBsAg titers, 4.1–118 ng/ml; median, 23 ng/ml) were treated with an isotype-matched control antibody, UPC 10 (Table 3). Two mice with Alex 0-derived tumors had stabilization of titers for the first week, but titers rose sharply thereafter. In the remaining eight mice, there was no response to treatment with UPC 10.

Statistical analysis comparing animals treated with UPC 10 or MRK16 that had no response to treatment with the number of animals that responded (partial responders plus complete responders) revealed a significant increase in the number of responders in MRK16-treated animals (P < 0.05 for Alex 0 tumors and P < 0.001 for Alex 0.5 tumors).

**Chemotherapy of HCC Xenografts.** Initial chemotherapy protocols used doxorubicin at a previously published tolerable dose in mice (8 mg/kg i.v.). Consistent with the finding that SCID mice have a decreased ability to repair DNA breaks (28, 36), doxorubicin, at this dose, caused significant morbidity and mortality. When the dose was reduced to 6 mg/kg i.v., mice tolerated a single dose of doxorubicin. Nine mice bearing tumors (HBsAg titers, 1–209 ng/ml; median, 22 ng/ml) from either cell line were treated with this dose of doxorubicin. Nine mice bearing tumors (HBsAg titers, 12–144 ng/ml; median, 14 ng/ml) were treated with an isotype-matched control antibody, UPC 10.

Because of the SCID mouse’s unique susceptibility to doxorubicin and in vitro studies with vinblastine, which suggested that the Alex cell lines were more sensitive to this drug than to doxorubicin, vinblastine was used in subsequent drug treatment protocols. Therapy with vinblastine at 5 mg/kg i.v. weekly for 3 weeks was ineffective in treating mice bearing Alex 0 (HBsAg titers, 5.5–64 ng/ml; median, 55 ng/ml) or Alex 0.5 tumors (HBsAg titers, 4.1–68 ng/ml; median, 14.1 ng/ml). Short-term partial responses represented by stabilization of HBsAg titers were seen in both groups (Table 3). Mice tolerated vinblastine chemotherapy with only weight loss noted.

**Chemosensitization with Verapamil.** Reversal of P-glycoprotein-mediated drug resistance was attempted by combining vinblastine chemotherapy with verapamil (Table 4). In the first protocol, mice were given three weekly injections of either racemic verapamil (25 mg/kg) or R-verapamil (100 mg/kg) as a single i.p. bolus at the time of vinblastine administration. These doses of verapamil represented the maximum toler-

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*Fig. 3* Progressive increase in serum HBsAg titer as monitored by RIA in SCID mice bearing tumors derived from Alex 0 (A) or Alex 0.5 tumors (B). The four lines represent the results of serial determination of HBsAg titers in four animals given injections on the same day of either Alex 0 or 0.5 cells.

*Fig. 4* Correlation between mouse serum HBsAg titer and the wet weight of intrahepatic tumor xenografts derived from Alex 0 or 0.5 cells determined at the time of necropsy. The correlation coefficient was 0.976, and the linear association was significant at P < 0.001.
Table 2  Results of immunotherapy with MRK16 in SCID mice bearing human HCC xenografts

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Tumor origin</th>
<th>Therapeutic response</th>
<th>Therapeutic response</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRK16</td>
<td>Alex 0</td>
<td>6/14</td>
<td>2/14</td>
</tr>
<tr>
<td></td>
<td>Alex 0.5</td>
<td>6/14 (14-68)*</td>
<td>2/7 (38-42)</td>
</tr>
<tr>
<td>UPC 10</td>
<td>Alex 0</td>
<td>3/5</td>
<td>3/7 (57-119)</td>
</tr>
<tr>
<td></td>
<td>Alex 0.5</td>
<td>2/5</td>
<td>2/7</td>
</tr>
</tbody>
</table>

* Antibodies were given at 500 μg i.v. weekly for 3 weeks.
* Duration of response in days.

Fig. 5  Response in SCID mice bearing intrahepatic xenografts derived from Alex cells to immunotherapy with anti-P-glycoprotein antibody MRK16. Sequential serum HBsAg titers in mice bearing tumors derived from Alex 0.5 (A and B) or 0 (C and D) cells treated with 500 μg MRK16 i.v. weekly for 3 weeks. The first injection of MRK16 in each mouse was administered at time 0, and the subsequent two doses were administered during the succeeding 2 weeks. A, five mice with Alex 0.5 tumors that had complete responses. B, two mice with Alex 0.5 tumors that had partial responses. C, five mice with Alex 0 tumors that had no response. D, seven mice with Alex 0 tumors that had partial responses. Note the variation in the X- and Y-axes in the four graphs.

ated doses in our mice. In these studies, increased efficacy of vinblastine chemotherapy was not apparent (HBsAg titers, 9.1–68 ng/ml; median, 17 ng/ml). Previous studies suggested that to maximize chemosensitization, verapamil should be administered before and continuously throughout chemotherapy (41, 45) to ensure serum and tissue verapamil concentrations in the range capable of in vitro chemosensitization. Since previous studies demonstrated that i.p. administration of 75 mg/kg racemic verapamil resulted in transient appearance of drug levels which produce in vitro chemosensitization, it was possible that our single i.p. bolus injections of verapamil were adequate. We, therefore, used a previously published protocol (41) to infuse R-verapamil continuously using an i.p. osmotic pump at 150 mg/kg/day, which maintains verapamil concentrations of 10 μM in plasma and 100 μM in liver (41). One course of therapy consisting of verapamil infusion with vinblastine chemotherapy did not significantly improve response to therapy (Table 4). One mouse with an Alex 0 tumor (HBsAg titer, 52 ng/ml) had a complete response for 2 weeks, but the other six mice (HBsAg titers, 8.4–100 ng/ml; median, 24 ng/ml) with Alex 0 or 0.5 tumors had responses no better than those seen when vinblastine was administered alone. Since this chemosensitization protocol was associated with increased morbidity in the mice as noted by increased weight loss, enhanced immobilization, and the occasional development of diarrhea, weekly treatments were not possible in all animals. In two mice, an additional cycle of treatment was administered, but did not improve response.

P-Glycoprotein Expression in Hepatic Xenografts. Immunoblotting for P-glycoprotein expression in tumor samples from untreated mice and mice subjected to various therapeutic protocols was performed (Fig. 6). When gels were overloaded with 300 μg membrane protein derived from untreated Alex 0
tumors (n = 4), low levels of P-glycoprotein were detected. Untreated Alex 0.5 tumors (n = 4) expressed large amounts of P-glycoprotein easily visualized with only 50 μg crude membrane protein. Representative immunoblots of untreated Alex 0 and Alex 0.5 tumors are shown in Fig. 6, Lane 6 and Lane 5, respectively. Tumors from Alex 0 or 0.5 cells treated with vinblastine or UPC 10 control antibody, in which no response to therapy was noted, had P-glycoprotein levels similar to those seen in untreated tumors. However, in mice that had a complete or partial response to MRK16 immunotherapy followed by relapse, relapsed tumor tissue contained considerably less P-glycoprotein than was observed in untreated tumors. In an Alex 0.5 tumor-bearing mouse that had a complete response to MRK16 immunotherapy followed by relapse, relapsed tumor tissue contained considerably less P-glycoprotein than was observed in untreated tumors. In an Alex 0.5 tumor-bearing mouse that had a complete response to MRK16, P-glycoprotein was detected only after six times as much protein was loaded onto the gels. Nonspecific staining in the gel.

**DISCUSSION**

Intrasplenic injection of Alex cells into SCID mice reproducibly produced intrahepatic metastasis. This tumor xenograft model offers several advantages over other murine tumor models. First, the malignant hepatoma cells grow as solid tumors within a microenvironment similar to that seen clinically. Several studies have suggested that neighboring cells and extracellular matrix components profoundly effect tumor cell phenotype (25-27). Second, tumor engraftment can be reliably monitored. Alex cells, which contain an integrated hepatitis B virus genome, secrete large amounts of HBsAg when growing as hepatic xenografts. HBsAg accumulates in the mouse’s circulation and progressively increases over time. Tumor burden, as determined by weight, was proportional to serum HBsAg titer. Regression of tumors was accompanied by a decrease in HBsAg titer. HBsAg titers provide a noninvasive method for serially quantitating tumor burden at initiation of therapy and during chemotherapy. HBsAg is an ideal circulating marker since it is rapidly secreted by tumor cells, is nonpathogenic, has a short half-life, and can be quantified by a highly sensitive reproducible RIA (46). Finally, the fact that the parental cell line Alex 0 is also intrinsically MDR represents an important component of this physiologically relevant HCC tumor model. Since the cellular basis for acquired and intrinsic drug resistance may differ, development of a HCC model in which modulation of both forms of resistance can be evaluated is of considerable importance.

We exploited this murine model to examine MDR in HCC. Clinically, HCC is often resistant to chemotherapy, but identification of important mediators of drug resistance has received little attention. In vitro both Alex cell lines express P-glycoprotein-mediated MDR, as demonstrated by the presence of P-glycoprotein on immunoblots and the finding of in vitro drug resistance to P-glycoprotein substrates that is associated with verapamil-reversible defects in drug accumulation and efflux. The intrinsically drug-resistant Alex 0 cells have a lower level of P-glycoprotein-mediated MDR than did Alex 0.5 cells, which were selected for acquired MDR. Chemoresistance was also seen in vivo because hepatic tumors derived from Alex 0 or 0.5 cells were resistant to treatment with doxorubicin and vinblastine.

**Table 3** chemotherapy in SCID mice bearing human HCC xenografts

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor origin</th>
<th>None</th>
<th>Partial</th>
<th>Complete</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin*</td>
<td>Alex 0</td>
<td>5/6</td>
<td>1/6</td>
<td>(10)*</td>
</tr>
<tr>
<td></td>
<td>Alex 0.5</td>
<td>3/4</td>
<td>1/4</td>
<td>(7)</td>
</tr>
<tr>
<td>Vinblastine*</td>
<td>Alex 0</td>
<td>3/9</td>
<td>6/9</td>
<td>(7-21)</td>
</tr>
<tr>
<td></td>
<td>Alex 0.5</td>
<td>1/5</td>
<td>4/5</td>
<td>(7-23)</td>
</tr>
</tbody>
</table>

* Dxorubicin was given at 6–8 mg/kg i.v. once.
* Vinblastine was given at 5 mg/kg i.v. weekly for 3 weeks.

**Table 4** use of verapamil to chemosensitize human HCC xenografts

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor origin</th>
<th>None</th>
<th>Partial</th>
<th>Complete with relapse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vinblastine/verapamil bolus*</td>
<td>Alex 0</td>
<td>4/6</td>
<td>2/6</td>
<td>(7-26)*</td>
</tr>
<tr>
<td></td>
<td>Alex 0.5</td>
<td>2/4</td>
<td>2/4</td>
<td>(7-23)</td>
</tr>
<tr>
<td>Vinblastine/R-verapamil bolus*</td>
<td>Alex 0</td>
<td>4/6</td>
<td>2/6</td>
<td>(7-28)</td>
</tr>
<tr>
<td></td>
<td>Alex 0.5</td>
<td>4/6</td>
<td>1/6</td>
<td>(7)</td>
</tr>
<tr>
<td>Vinblastine/R-verapamil infusion*</td>
<td>Alex 0</td>
<td>4/6</td>
<td>2/6</td>
<td>(7-26)</td>
</tr>
<tr>
<td></td>
<td>Alex 0.5</td>
<td>4/6</td>
<td>2/6</td>
<td>(7-26)</td>
</tr>
</tbody>
</table>

* Vinblastine was given at 5 mg/kg i.v. and verapamil at 25 mg/kg i.p.
* Duration of response in days.
* Vinblastine was given at 5 mg/kg i.v. and R-verapamil at 100 mg/kg i.p.
* Vinblastine was infused via an i.p. pump at 150 mg/kg/day.
changes in intracellular pH, which promote sequestration of chemotherapeutic agents in cytoplasmic compartments or interfere with their binding to cellular targets, may also contribute to drug resistance (47). Preliminary results in our laboratory show that the Alex cells have increased glutathione S-transferase and glutathione levels.4

Immunotherapy with MRK16 modulated growth of HCC xenografts. MRK16 is a monoclonal antibody against a drug-resistant human myelogenous leukemia cell line (15). Since MRK16 modulates the uptake of anthracyclines and Vinca alkaloids in MDR cell lines (16, 18), it has been used as a chemosensitizing agent in mice bearing MDR human xenografts. In mice with intraperitoneal tumors from human colonic cancer cells, i.p. administration of MRK16 with doxorubicin or vinblastine enhanced survival (18). Administration of MRK16 alone had no effect. In mice bearing subcutaneous xenografts from a MDR human ovarian cancer cell line or a human colorectal carcinoma cell line, i.v. administration of MDR 16 alone prevented tumor appearance when given at the same time as cell injection and inhibited growth of tumors when treatment was started after tumors were palpable (17, 20). No effects of MRK16 immunotherapy were seen when tumors from parental cell lines, which did not express P-glycoprotein, were treated. In our studies, i.v. administration of MRK16 decreased tumor burden and, in some cases, was curative.

Several tumor-related factors could have influenced monoclonal antibody accumulation in tumor xenografts and, thereby, affected efficacy of immunotherapy (48). Our results are consistent with previous observations that the amount of antigen expressed on the surface of the tumor and the size of the tumor are important determinants of the success of monoclonal anti-
body immunotherapy (48, 49). The best response to MRK16 therapy occurred in Alex 0.5 tumors which overexpressed P-glycoprotein. Presumably, the greater the amount of monoclonal antibody bound, the greater the tumor killing activity. We also observed better responses to MRK16 when tumor burdens were small (<20 mg). This finding may be associated with poor penetration of MRK16 into cells deep within large tumors due to decreased vascular supply or permeability in deep tissue. Interaction of MRK16 with P-glycoprotein on surface tumor cells may also retard antibody entrance into the tumor. Large tumors may also have a heterogeneous distribution of P-glycoprotein such that some tumor cells lose expression of the protein and no longer bind MRK16. Our results support suggestions that immunotherapy may be most effective in solid tumors for controlling residual primary or metastatic tumor following surgery, radiation, or chemotherapy (48, 49).

It should be noted that MRK16 does not recognize native mouse P-glycoprotein. If used to treat human tumors, the antibody would recognize and bind to naturally occurring P-glycoprotein present on several normal tissues. This binding would undoubtedly have implications in the design of dosage schedules as well as how an impact on the development of potential side effects.

Several mice initially responded to MRK16 immunotherapy, but relapsed. By immunoblotting, these relapsed tumors had markedly decreased P-glycoprotein expression when compared to untreated tumors or tumors treated with vinblastine or control antibody. If pretreatment tumor cell populations are heterogeneous in P-glycoprotein expression, MRK16 may bind to and mediate cell death only in P-glycoprotein-positive cells, leaving behind a population of P-glycoprotein-negative tumor cells which subsequently proliferate. Hence, tumor cells remaining after MRK16 therapy may be more sensitive to the cytotoxic actions of chemotherapy agents. In two Phase I clinical chemosensitization trials, patients with P-glycoprotein-positive hematological malignancies treated with chemotherapy in combination with the MDR-reversing agent cyclosporine had decreased expression of P-glycoprotein in biopsy samples posttreatment (50, 51). It would be of interest to determine in vivo chemosensitivity of Alex tumor cells which remain after immunotherapy to determine whether they respond to chemotherapy immediately following a course of MRK16 or at the time of clinical relapse.

The mechanism whereby MRK16 modulates growth of murine MDR xenografts is unknown. MRK16 alone has no effect on the growth of MDR tumor cells in culture (16–18). Complement-dependent cytotoxicity has been demonstrated in a MRK16-treated MDR human ovarian carcinoma cell line in culture (17), although we were unable to document this in vitro with the Alex cell lines. Alternatively, ADCC may be responsible for antitumor activity. ADCC effector cells may be lymphocytes, monocyte/macrophages, natural killer cells, or neutrophils. MRK16 induced ADCC in a human ovarian cell line when mouse spleen cells were used as effector cells (17). MRK16 also promoted human monocyte and lymphocyte-mediated tumor killing in ovarian carcinoma and myelogenous leukemia cell lines (52). Since SCID mice lack functional T and B lymphocytes, these cell populations are unlikely effector cells in our study. Natural killer cells may be the effector cells in hepatic immunotherapy (31, 53, 54). These cells are important in the response to immunotherapy in colonic and gastric carcinomas growing as intrahepatic metastasis in immunodeficient mice (31, 53). When murine lymphocyte populations were depleted of natural killer cells, the cytotoxic action of human mouse chimeric MRK16 antibody was significantly diminished (54).

Preliminary studies suggest that magnetic resonance imaging of tumor xenografts with a new asialoglycoprotein receptor-targeted magnetic resonance imaging contrast agent, AG-USPIO (Advanced Magnetics, Inc.; Ref. 55), may provide an additional mechanism for monitoring tumor burden. Alexander cells lack surface expression of the hepatic asialoglycoprotein receptor (56), which is responsible for the removal of desialylated glycoproteins and is abundant on normal hepatocytes (57). AG-USPIO is an ultrasmall superparamagnetic iron oxide particle coated with arabinogalactan, a ligand for the asialoglycoprotein receptor (55). During magnetic resonance imaging of mice bearing tumor xenografts, normal mouse hepatocytes internalized the agent with a resultant decrease in signal intensity while tumor xenografts failed to take up the contrast agent and maintain their signal intensity (58). This imaging sharply demarcated normal and neoplastic hepatic tissue, permitting visualization of very small tumors (1 mm) and accurate computer-generated reconstructions to determine tumor volume. Tumor volume determined by this method appears to be proportional to the serum HBsAg titer.

We developed a physiologically relevant intrahepatic xenograft model of human HCC in SCID mice in which tumor burden can be noninvasively and serially quantified by determination of a soluble, secreted tumor marker. The relative contribution of drug resistance mechanisms, as defined by investigations in cell culture, to clinical drug resistance in vivo can be evaluated in this murine model. Since HCC xenografts grow within the hepatic parenchyma, mechanisms of in vivo drug resistance which may require tumor cell interactions with the extracellular matrix can also be evaluated. Our initial investigations of P-glycoprotein-mediated drug resistance using this model suggest that treatment modalities which target drug delivery to cells expressing P-glycoprotein, such as immunotherapy with anti-P-glycoprotein antibodies, may be a more effective means of overcoming P-glycoprotein-mediated MDR in solid tumors than are protocols employing the combined use of chemosensitizing agents along with chemotherapy drugs.

REFERENCES


Multidrug Resistance in a Murine Hepatoma Model

Establishment and serial quantification of intrahepatic xenografts of human hepatocellular carcinoma in severe combined immunodeficiency mice, and development of therapeutic strategies to overcome multidrug resistance.

C R Leveille-Webster and I A Arias