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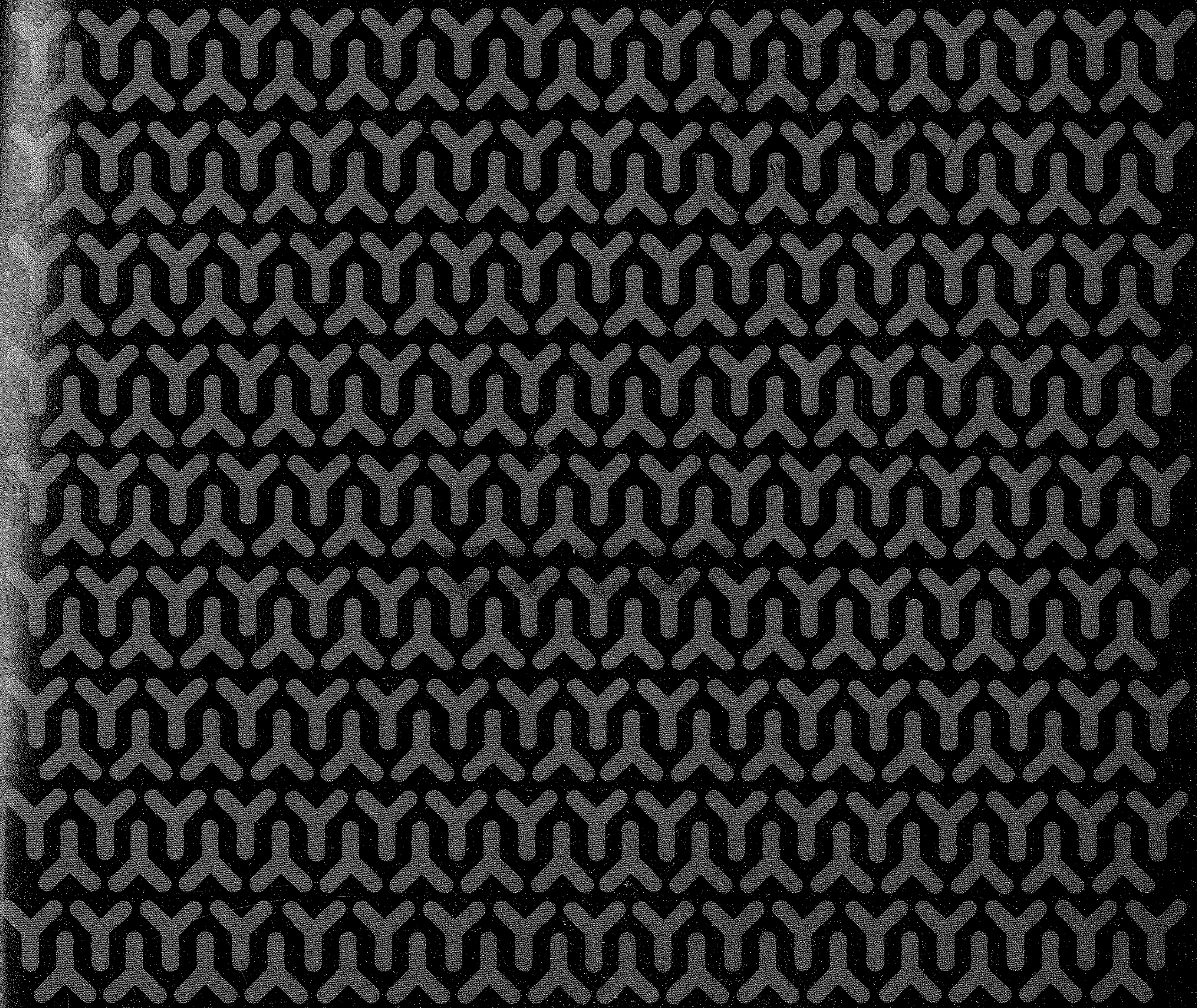

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MOLECULAR BIOTHERAPY

THE INTERNATIONAL JOURNAL FOR THE APPLICATION OF BIOLOGICALS IN CLINICAL OR VETERINARY PRACTICE

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Phase I Trial of Mitomycin C Immunoconjugates Cocktails in Human Malignancies

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Nineteen patients with refractory solid malignancies received individualized combinations of mitomycin C conjugated murine monoclonal antibodies selected by immunohistochemical and flow cytometric screening of tumor specimens. There were no responses in this Phase I study. Thrombocytopenia precluded escalation above a mitomycin C dose of 60 mg per treatment cycle. Preclinical, clinical, and toxicity experiences with this investigational approach to the treatment of cancer are discussed.

Keywords: Mitomycin C; immunoconjugates; antibody cocktails

Introduction

The signal achievement of hybridoma technology described by Kohler and Milstein[1] has provided researchers a virtually unlimited repertoire of molecular probes of high purity and unequaled specificity with which to characterize and purify macromolecules and to unscramble heretofore obscure biologic processes (*e.g.*, immunologic networks). Unfortunately, with a few notable exceptions,[2–4] the specificity afforded by murine monoclonal antibodies (MoAb) has not yet been successfully exploited in the treatment of cancer. Responses in hematologic malignancies to unconjugated murine MoAb have generally been transient and nonsustainable.[5,6] Attempts to utilize unmodified antibodies recognizing tumor-associated antigens in the treatment of solid tumors have likewise been discouraging,[7] although antibody delivery to tumor deposits has consistently been demonstrable.[8,9] Antibodies have been utilized to serve as carriers of toxic agents to tumor cells. Examples include radionuclide immunoconjugates,[10–13] immunotoxins,[4,14] and chemotherapy-antibody conjugates.[15,16] These studies have demonstrated that antibodies are, in general, well tolerated, that the risk of major allergic reaction to these foreign proteins on first exposure is small, that myelosuppression may accompany radioimmunoconjugates and chemoimmunoconjugates, that edema and a vascular leak syndrome may follow immunotoxin infusions, and that the emergence of human antimurine antibodies may limit ongoing treatments.

The broad clinical experience with and the well-characterized response and toxicity profiles of chemotherapeutic agents make them attractive as conjugates to antibodies. Drug immunoconjugates have been shown to retain both *in vitro* cytotoxicity and antibody specificity.[17] However, few clinical studies have as yet been reported with chemotherapy immunoconjugates. We have previously studied 23 patients who each received individualized cocktails of component doxorubicin-conjugated murine MoAb; results are reported elsewhere.[15] Five minor responses (healing of skin ulcers, minor shrinkage of tongue nodules, shrinkage of lymphadenopathy in chronic lymphocytic leukemia) were observed, but there were no major responses. This demonstration of biologic activity, although modest, did occur in clinical situations of bulky tumor and previous doxorubicin resistance. Evidence for deconjugation of doxorubicin from antibody prior to tumor cell binding and internalization prompted us to investigate alternative conjugation methodologies and screen other possible chemotherapeutic candidates. Another commonly used cytotoxic agent, mitomycin C, has been reported to demonstrate both *in vivo* and *in vitro* activity following conjugation to an anticolon cancer murine MoAb.[18] Our preclinical studies also suggested potentially improved conjugation characteristics of the mitomycin C–antibody preparation. Like the doxorubicin–antibody linkage, the mitomycin C–antibody conjugate is acid labile and, following endocytosis, subject to cleavage within the acidic environment of the lysosome. In this study, individual tumor specimens were typed against a

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panel of previously generated murine MoAb, which recognize tumor-associated antigens. Two to five antibodies exhibiting *in vitro* binding to tumor were selected for each patient. A fundamental hypothesis examined in this trial proposes that distinct permutations of antibodies individually specified for each patient will be needed to encompass a heterogeneic tumor cell population. In this report, we describe tumor processing and antibody selection, conjugation methodology, and our clinical experience in 19 patients treated with individualized mitomycin C immunoconjugates.

Materials and Methods

Patient population

Ten females and nine males, from 28 to 74 years of age (median age, 59 years) and with a variety of solid tumors, participated in this trial (*Table 1*). All patients were screened and counseled by a board-certified medical oncologist following referral upon failure of standard treatment modalities. Performance status (PS) distribution was as follows: PS 0 (n = 3); PS 1 (n = 8); PS 2 (n = 4); and PS 3 (n = 4). Seventeen patients had received prior chemotherapy (three prior mitomycin C); 13 prior radiotherapy and nine prior biotherapy. One of the patients with breast cancer had earlier received a doxorubicin immunoconjugate. A patient with cancer of the jejunum had 9 months previously been exposed to diagnostic murine antibody when administered an anti-CEA radiolabeled antibody for staging purposes. Duration of illness ranged from 1 to 10 years, with a median of 3.3 years.

Patients participating in this trial were required to have a confirmed diagnosis of metastatic cancer not or no longer amenable to standard therapy. Patients exhibited normal renal (creatinine <2 mg/dl) and hepatic (bilirubin <2 mg/dl) functions, full ambulatory capability, a life expectancy of greater than 3 months, adequate hematologic parameters, and evaluable or measurable disease. A history of irreversible mitomycin C-related toxicity (hemolytic/uremic syndrome, pulmonary toxicity, unacceptable myelosuppression) precluded participation.

Table 1. Patient characteristics

Patient no.	Age (yr)	Sex	Tumor type
1	68	M	Prostate
2	74	F	Hepatoma
3[a]	37	F	Breast
4	53	F	Ovary
5	61	F	Breast
6	48	F	Ovary
7	59	F	Uterus (leiomyosarcoma)
8	34	M	Lung
9	64	M	Kidney
10	42	M	Schwannoma
11[b]	63	M	Jejunum
12	65	M	Prostate
13	57	F	Uterus (carcinosarcoma)
14	57	M	Head and neck
15	36	F	Breast
16	64	M	Colon
17	27	F	Breast
18	64	F	Colon
19	59	M	Penis

[a] Had previously received doxorubicin immunoconjugate (murine).
[b] Had previously received diagnostic murine radioimmunoconjugate.

Antibody generation, characterization, and purification

The broad representation of tumor-associated antigens among common solid tumors permits the systematic screening of tumor specimens from a panel of selected murine MoAb.[19] These antibodies have been generated against adenocarcinomas (breast and colon) and melanomas using standard hybridoma methodology (*Table 2*). The designation BT indicates an antibody developed within the laboratories of Biotherapeutics, Inc.; the designation BA indicates antibodies acquired from outside sources. The hybridomas secreting MoAb BA-Br-1 (B38.1) and BA-Br-5 (B72.3), originally developed by Horan Hand *et al.* at the National Cancer Institute,[20] were provided by American Type Culture Collection, Rockville, MD. Characteristics of MoAb BA-Br-3 (BTMA8), BA-Me-4 (140.72), and BA-Me-5 (140.240) have previously been published.[21–23] A description of antibody-binding patterns to selected tumors *in vitro* has been reported elsewhere.[19] Liao *et al.* observed that such binding crosses organ-specific boundaries, *e.g.*, BA-Br-1 bound not only to all 35 breast cancers examined *in vitro* but also to all 15 colon, 11 lung, 4 prostatic and 2 pancreatic cancers tested. Each antibody in the panel used in this study has been extensively tested against normal adult tissues, including blood components (polymorphonuclear neutrophils, monocytes, lymphocytes, erythrocytes, platelets), kidney, lung, heart, breast, and skin.[19] Some binding within liver cells, to renal tubules, to lung tissue, and to sweat glands was observed, but to date no hepatotoxicity, nephrotoxicity, pulmonary toxicity, nor cutaneous injury has been attributed to any of this panel of antibodies. Murine MoAb were expanded in ascites of BALB/c mice or by means of a bioreactor and then submitted for purification using one or more of several methodologies [caprylic acid, ammonium sulfate, protein-A sepharose CL-4b, or high performance liquid chromatography (HPLC)].

Selection of individualized antibody cocktails

Frozen tissue specimens (from fresh or cryopreserved primary or metastatic tumors) were provided the Monoclonal Antibody Division for assessment of binding by a panel of murine monoclonal antibodies. Five-micron-thick sections of frozen tissue embedded in OCT compound (polyvinyl alcohol, benzalkonium chloride, polyethylene glycol, d.H_2O; Miles Labs, Elkhart, IN), then cut and placed on gelatin-coated slides, were evaluated by means of the avidin-biotin-

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Table 2. Murine MoAb used in this study

MoAb	Isotype	Immunogen	Antigen structure
BA-Br-1	IgG1	Membrane extract of breast carcinoma tissue	ND[a]
BA-Br-3	IgG1	Membrane extract of breast carcinoma cell line CAMA-1	>300 kD glycoprotein
BA-Br-5	IgG1	Membrane extract of breast carcinoma tissue	220–400 kD glycoprotein
BT-Br-6	IgG1	Dispersed cells from breast carcinoma tissue	ND
BT-Co-1[b]	IgG3	Dispersed cells from colon carcinoma grown as xenografts in nude mice	29 kD + 31 kD protein
BT-Co-2	IgG3	Dispersed cells from colon carcinoma grown as xenografts in nude mice	ND
BT-Co-3	IgG3	Dispersed cells from colon carcinoma grown as xenografts in nude mice	ND
BT-Co-4	IgG3	Dispersed cells from colon carcinoma grown as xenografts in nude mice	ND
BT-Co-5[b]	IgG3	Dispersed cells from colon carcinoma grown as xenografts in nude mice	29 kD + 31 kD + protein
BT-Co-6	IgG3	Dispersed cells from colon carcinoma grown as xenografts in nude mice	ND
BA-Me-4	IgG1	Melanoma cell line CaCL 78-1	95–150 kD glycoprotein(s)
BA-Me-5	IgG2a	Melanoma cell line CaCL 78-1	p97-like (97 kD) glycoprotein
BT-Me-7	IgG1	Melanoma cell line BUR	110 kD protein
BT-Me-8	IgG1	Melanoma cell line BUR	110 kD + 40 kD protein
BA-Me-11	IgG1	Melanoma cell line	280 kD + >400 kD proteoglycan

[a] ND = Not yet defined, although attempts to determine the molecular mass of antigen involved were made.
[b] Based on epitope blocking and indirect immunoprecipitation experiments. BT-Co-1 and BT-Co-5 recognized different epitopes present on the same or similar molecules.

peroxidase complex technique (Vectastain ABC kit, Vector Laboratories, Burlingame, CA). Following fixation with cold acetone, tissue sections were incubated with normal horse serum for 15 minutes to reduce nonspecific binding of horse anti-mouse IgG antiserum. Murine MoAb selected from the panel were then incubated at a concentration of 5 μg/ml with tissue sections for 1 hour, washed with phosphate-buffered saline (PBS) at *p*H 7.4, and incubated with biotinylated horse anti-mouse IgG antiserum for 30 minutes. Reaction with methanol and hydrogen peroxide for 30 minutes blocked endogenous peroxidase in tissue. Sections were then incubated with a preformed complex of avidin-biotinylated-horseradish peroxidase for 1 hour, oxidized by 3-amino-9-ethylcarbazol (AEC) and 0.002% hydrogen peroxide for 15 minutes, and then counterstained in Gill's hematoxylin (Fisher Scientific, Norcross, GA) for 20 minutes. Positive (anti-HLA-A,B,C monomorphic determinant MoAb W6/32 and anti-epithelial membrane antigen MoAb) and negative (nonspecific murine IgG and PBS) antibody controls were also used. One or two sections from the frozen tissue block were routinely stained with hematoxylin and eosin to permit histopathologic confirmation of adequately preserved tumor cells. The immunohistochemical preparations were scored independently by two observers, using a grading system based on intensity [from absent (0) to very strong (4+)] and the distribution and pattern of the reddish-brown stain. A continuum from homogenous membrane and/or cytosolic to patchwork staining was observed. A designation "±" was given when there were foci of positive antibody binding to tumor within a predominantly negative stain. By this means, antibodies were chosen as potential constituents of an immunoconjugate cocktail.

Enzymatically or mechanically disaggregated tumor cells (when available) were subjected to flow cytometric analysis to determine the percentage of tumor cell coverage and binding intensity by immunohistochemically selected antibodies, either singly or in combination (Cytofluorograph IIS, Ortho Diagnostic System, Inc., Westwood, MA). Tumor cells were prepared enzymatically by incubating finely minced tumor tissue in dispersion solution containing RPMI 1640 medium, 5% fetal bovine serum (Hyclone), and Worthington collagenase II, 31–25 units/10 ml/g tumor tissue. The dispersion was carried out at 37°C for 70 minutes with continuous stirring. DNase (1,000 units, Sigma type I) was added for the final 10 minutes to increase filterability. Single cells were separated from undigested tumor mince using a 60-μm mesh nylon filter. Flow cytometry makes use of single cell suspensions of tumor and fluoresceinated antimurine antibody tracers to quantitate the proportion of tumor cells bound by each selected murine MoAb and the relative intensity of that binding (*i.e.*, reflecting the number of antibodies bound to positive cells), as indicated by the mean peak channel (MPC).[24] The HLE-1 antibody, which binds to leukocytes, corrected for any white blood cell contamination and propidium iodide exclusion staining for nonviable cells. Simultaneous antibody exposures were examined for possible additivity or interference among antibody combinations because one antibody might sterically hinder binding by another antibody recognizing a different epitope on the same or different antigen. Such antibodies would be better used separately.

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