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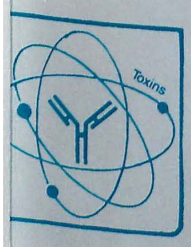

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Pre-Clinical and Clinical Studies with N-Acetyl Melphalan Immunoconjugates and Tumor Necrosis Factor α

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ABSTRACT

Melphalan (MEL), a potent alkylating agent has been modified to a non-toxic derivative, N-Acetyl Melphalan (N-AcMEL) which can be conjugated to monoclonal antibodies (MoAbs) and used for immunotherapy. On average, 20 molecules of N-AcMEL can be attached to each antibody molecule and this conjugate has greater activity than free N-AcMEL. The N-AcMEL conjugates satisfy the basic requirements of specificity and potency for immunoconjugate use *in vitro*. *In vivo* in mice, tumors growing in ascites fluid, and 20% of small subcutaneous tumors can be totally eradicated by N-AcMEL conjugates. For larger tumors, a major problem exists in achieving access to the tumor by the immunoconjugate. To increase immunoconjugate localization we have used recombinant human tumor necrosis factor-α (r-TNF-α) to incite an inflammatory response in the tumor. Under these circumstances where N-AcMEL conjugates or r-TNF-α each give a proportion (20%) of cures, the combination of N-AcMEL-antibody and r-TNF-α leads to partial or complete regression of the majority of tumors. Further improvement was found when recombinant mouse interferon-γ (IFN-γ) was added to the regimen. On the basis of our preliminary preclinical data on N-AcMEL-MoAb conjugates, a Phase I clinical trial was done in patients with advanced colorectal cancer using MoAbs to colon carcinoma conjugated with N-AcMEL. Thus far eight patients have been examined and in excess of 1gm MoAb used conjugated with 35mg of N-AcMEL. No patient showed any side effects of importance as 20mg/m^2 is the maximum tolerated dose (MTD) for MEL in its free form. Only 6 of the 8 patients had evaluable disease (total of 9 courses of treatment as one patient had two courses). Subjective improvement was noted in almost all of the patients examined but only 29% (2/7) of the treatments showed response by strict objective criteria.

INTRODUCTION

Interest in the use of MoAbs to target cytotoxic drugs has grown enormously in recent years from the original concept of "magic bullets" of Paul Ehrlich earlier this century (1,2). Since the early work of Mathè (3) and Ghose (4) with methotrexate and chlorambucil, a number of other drugs (5), toxins (6) and radioisotopes (7) have been used as warheads for the "magic bullet". These

Ehrlich earlier this century (1,2). Since the early work of Mathè(3) and Ghose (4) with methotrexate and chlorambucil, a number of other drugs (5), toxins (6) and radioisotopes (7) have been used as warheads for the "magic bullet". These conjugates are specifically toxic to antigen positive cell lines *in vitro*. *In vivo* efficacy of the immunoconjugates in animal models is varied, but the general pattern emerging is that ascites tumors growing in the peritoneum can be cured, while relatively poor responses are observed with solid subcutaneous tumors (8,9).

Studies using radiolabeled MoAbs show that only a small fraction of the injected dose (<20% in animal models and 0.01% in humans) localized to the tumor, although a higher dose of radiolabeled MoAb fragments localized (10,11, 12). Therefore, the lack of efficient permeation of immunoconjugate into the tumor tissue could be the cause of the less satisfactory response found with solid tumors. To increase blood flow through the tumor, and therefore antigen binding by immunoconjugate, we have used vasoactive agents with the immunoconjugate and an increase in efficacy was noted (13). However, immunoconjugates made using $F(ab')_2$ fragments were only marginally more effective than the corresponding conjugates made using intact antibody (14). It is known that the permeability of the endothelium to immunoglobulin is enhanced during bacterial infection, produced by factors released in the inflammatory process (15); would such an event (increase in capillary permeability) at the tumor site increase the amount of immunoconjugate that enters the tumor? TNF, a monokine with a myriad of biological activities has the capability of causing an inflammatory response in tumors (16) and this paper reports the results of preclinical studies on the combination therapy of N-AcMEL-MoAb immunoconjugate with r-TNF-α and clinical studies in colon cancer using the immunoconjugate.

MATERIALS AND METHODS

Mice

(C57BL/6xBALB/c)F_1 ($B6CF_1$) mice were produced in the Department of Pathology at The University of Melbourne.

Tumor cells

E3, a clonal variant of the thymoma ITT(1)75NS was used (17). The cell line was maintained *in vitro* in Dulbecco's modified Eagles medium (DMEM), supplemented with 10% heat-inactivated newborn calf serum (Flow Laboratories, Sydney, Australia), 2mM glutamine (Commonwealth Serum Laboratories (CSL), Melbourne Australia), 100µg streptomycin/ml (Glaxo Laboratories, Melbourne) and 100IU penicillin/ml (CSL). *In vivo* E3 was maintained by serial passage in ascitic fluid in $B6CF_1$ mice. For *in vivo* experiments the cells from the ascitic fluid were washed and centrifuged (1,500rpm for 5 min.) twice in DMEM and PBS, resuspended in Phosphate buffered saline (PBS), and injected subcutaneously (s.c.) into $B6CF_1$ mice.

Monoclonal Antibodies

Several MoAbs were used in this study (Table 1). A rosetting assay (21) or an immunoperoxidase assay (22) was used to determine antibody activity of MoAbs and immunoconjugates. The choice of MoAb for use in drug conjugation was made by immunoperoxidase staining on frozen (for 250-30.6, I-1 & JGT MoAbs) and/or formalin-fixed (for I-1 and JGT MoAbs) tissue sections of colon cancer tissue of individual patients. The sections were then assessed by light microscopy to estimate the percentage of colon carcinoma cells stained with each of the antibodies and the result expressed on the scale 0 to 4 according to whether nil (0), up to 25% (1), 25-50% (2), 51-75% (3) or >75% (4) of carcinoma cells stained (The intensity and the distribution of stain in the cancer cells and the staining of extracellular material were not taken into account) (23). The MoAbs selected for use in drug conjugation for an individual patient had a staining score of 3 or 4. Therapy given to patient 1, the second time, and patients 3 and 6 had at least two MoAbs in the final drug-MoAb conjugate preparation, given in equivalent proportions.

TABLE 1
Details of MoAbs used in Experimental Studies.[1]

Antibody	Reference	Immunoglobulin Subclass	Specificity
anti-Ly-2.1	18	IgG2a	murine Ly-2.1
250-30.6	19	IgG2b	Colon cancer
I-1	20	IgG1	Carcinoembryonic antigen (CEA)
JGT	20	IgG1	CEA

[1] All antibodies were purified using Protein-A Sepharose.

Preparation of N-AcMEL-MoAb Conjugates

N-AcMEL was coupled to various MoAbs by a previously described procedure (24). For clinical studies, the final preparation after drug conjugation was batch tested for pyrogens and sterility (Pharmacology Department, University of Melbourne; and Sigma Pharmaceuticals, Clayton, Victoria, Australia).

Drug Activity

Cytotoxicity of the free drug and immunoconjugates was determined by measuring the inhibition of DNA synthesis using [^{3}H]-thymidine as previously described (24).

***In Vivo* Experiments**

E3 (Ly-2.1^{+ve}) tumor cells were injected subcutaneously into the abdominal wall of B6CF$_1$ (Ly-2.1^{-ve}, Ly-2.2^{+ve}) mice and allowed to develop into a palpable lump (0.1-0.6cm^2) depending on the experiment before commencing treatment. Conjugates were administered intravenously and the tumor size measured daily with a caliper square, measuring along two perpendicular axes of the tumor, and the data recorded as mean tumor size (product of two diameters ± standard error of the mean). Experimental groups of 10-20 mice, all of the same sex and age, were used in each experiment. The details of the preclinical studies of antibodies used in the clinical studies are provided elsewhere (25,26).

Recombinant Human-TNF-α (r-TNF-α) and Recombinant Mouse-IFN-γ (r-IFN-γ)

Both human r-TNF-α (6x10^7 U/mg, purity >99%, ≤1.0ng endotoxin/mg) and mouse r-IFN-γ (1-2x10^7 U/mg) were generously supplied by Boehringer Ingelheim; these were diluted in PBS and stored in single dose aliquots.

Biodistribution

B6CF$_1$ mice bearing subcutaneous E3 tumors (0.2-0.5cm^2) were used to compare the distribution of ^{125}I-anti-Ly-2.1 in the presence or absence of therapeutic levels of r-TNF-α (5μg). Groups of 4 mice were sacrificed 24hrs after the injection of labeled anti-Ly-2.1 and the biodistribution of ^{125}I-anti-Ly-2.1 was determined by counting the radioactivity of blood, heart, spleen, liver, kidneys and tumor. The distribution of isotope is reported as the % injected dose/gm tissue. All mice received 1.5x10^6 cpm of ^{125}I activity (60μg protein) by tail vein injection 24 hours after the intraperitoneal (i.p.) administration of r-TNF-α.

Clinical Studies

Patients Seven patients with disseminated colorectal carcinoma and one patient who previously had a curative colonic resection (Duke C) were entered into the study. All (except patient 6) had previous resection of the primary colorectal carcinoma and all (except patient 8) presented with progressive metastatic disease: Two patients had pulmonary and hepatic metastases and five patients had extensive hepatic metastases. Two patients previously had failed intensive chemotherapy. Table 2 summarises the characteristic and clinical features of the patients. The ages ranged from 38 years to 62 years. All

patients (except patient 8) had measurable disease (hepatic metastases), a performance status (ECOG) of at least 3, and had not received anti-cancer therapy for at least 4 weeks and no such therapy was administered during evaluation (at least 9 weeks after therapy). This Phase I study was approved by the Medical Research Board of the Royal Melbourne Hospital and written informed consent was obtained from every patient.

TABLE 2
Characteristics and Clinical Features of Patients

Patient No.	Age	Disease	Performance Status (ECOG)	Previous Treatment
1	59	Hepatic metastases	2	HAI[a] with Cisplatinum
2	57	Hepatic and Pulmonary metastases	2	-
3	61	Hepatic metastases	2	-
4	58	Hepatic and Pulmonary metastases	2	-
5	62	Hepatic metastases	3	HAI with Cisplatinum
6	46	Hepatic metastases	3	-
7	62	Hepatic metastases and unresected primary colon carcinoma	3	-
8	38		1	-

[a] HAI = Hepatic Artery Infusion

Administration of N-AcMEL-MoAb Conjugates

Using the Seldinger technique, an external arterial catheter was introduced percutaneously into the left axillary or high brachial artery. The catheter was placed into the common hepatic artery under fluoroscopic guidance. The N-AcMEL-MoAb conjugates were administered by oxymetric pump through the hepatic artery cannula in 100ml of normal saline over 2 hours per day for 2 days. All patients had 3 doses of the immunoconjugates (t=0, t=24hrs, t=48hrs). In between infusion of the immunoconjugates, the patency of the hepatic artery catheter was accomplished by heparinised saline (5000 I.U. aqueous heparin in 1 litre normal saline at the rate of 50ml/hour). The arterial catheter was removed at the end of the 2-day infusion. A dose escalation protocol of 5mg/m^2, 10mg/m^2, 15mg/m^2, 20mg/m^2 N-AcMEL conjugated to MoAb was used. One patient received 5mg/m^2, and two months later, 10mg/m^2, two received 10mg/m^2, two received 15mg/m^2 and three received 20mg/m^2 N-AcMEL conjugated to MoAbs. The study was closed at the 20mg/m^2 dose of N-AcMEL conjugated because of the financial cost involved.

All patients had dexamethasone 8mg administered intravenously just before each infusion of immunoconjugates and oral prednisolone 10mg daily for 7 days after completion of therapy to diminish hypersensitivity phenomena.

Evaluation of Responses

Patients were monitored clinically including noting changes in temperature, pulse rate, blood pressure and respiratory function during and after the infusion. Blood studies (full blood count, serum tests of renal and liver function and human anti-mouse antibody response) were performed before, during and weekly for 9 weeks after the therapy to assess potential toxicity. The tumor responses were evaluated by weekly physical examinations, weekly blood tests (liver function test, carcinoembryonic antigen level) and computed tomography (CT) scans of the abdomen, performed before therapy, 1 month and 2 months after therapy. Complete response (CR) is defined as a reduction of at least 50% in the sum of the products of the two greatest diameters of measured lesions; minor response (MR) is a reduction of greater than 25% but less than 50% in the size of measurable lesions in the absence of progression or occurrence of new lesions elsewhere; stable disease (SD) is no objective change of all measurable tumors; and progressive disease (PD) is the appearance of new lesions or increase in size of one or more measurable lesions by at least 25%.

Serological Tests Human IgM and IgG antibodies to the murine MoAbs were measured by an indirect enzyme linked immunosorbent assay (ELISA). Ninety-six

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