

TARGETED CHEMOTHERAPY: EVALUATION
OF MONOCLONAL ANTI-CEA ANTIBODY
DRUG CONJUGATE EFFICACY

CENTRE FOR NEWFOUNDLAND STUDIES

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ALAN GRAHAM CASSON



TARGETED CHEMOTHERAPY :
EVALUATION OF MONOCLONAL ANTI-CEA ANTIBODY DRUG CONJUGATE EFFICACY

BY

© Alan Graham Casson, M.B. Ch.B.

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Abstract

Targeted chemotherapy, a potentially clinically relevant method of increasing the selective delivery of cytotoxic agents to tumor cell populations has been evaluated.

The model used in these studies comprised a target, the tumor associated carcinoembryonic antigen, which is expressed by a wide range of human solid tumors, and conjugates of vindesine, a potent vinca alkaloid, covalently linked to a specific monoclonal anti-CEA antibody.

Characterisation of human tumor cell lines by immunocytochemistry and radiobinding assays established a range of CEA expression enabling selected cell lines to be used for the assessment of conjugate efficacy in vitro, and to be grown as xenografts in nude mice enabling a relevant pre-clinical model to be developed. The effect of conjugate treatment upon the growth of xenografts with a range of CEA expression was then assessed.

These studies demonstrated the efficacy and selectivity of immunochemotherapy both in vitro and in vivo using this model, confirming theoretical predictions. In addition, prolonged survival of mice treated with conjugate and reduced toxicity of conjugated compared to free drug was demonstrated in one experimental protocol.

Further investigations and the clinical potential of this approach are discussed.

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Abbreviations

CEA	Carcinoembryonic antigen
Ig	Immunoglobulin
11-285-14	Designation for a monoclonal anti-CEA antibody used extensively in these studies - see text
FCS	Fetal calf serum
PBS	Phosphate buffered saline

Abbreviations used in tables 14 - 48

R	Right flank
L	Left flank
*	Day of injection
M	Missing data
MTV	Mean tumor volume

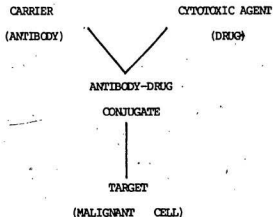
CHAPTER I : INTRODUCTION

I 1.0 INTRODUCTION

I 1.1 Concepts

One of the major limitations of present day cancer chemotherapy is the lack of selectivity of cytotoxic drugs for malignant cells. Consequently, in therapeutic doses, toxic side effects are often seen, especially with rapidly dividing cells of the bone marrow, gonads and gut mucosa.

Targeted chemotherapy offers a method of improving drug delivery to the tumor, while sparing the host toxicity, and has potential application in the clinical setting. The concept of linking cytotoxic agents to antibodies directed against cancer cells as tumor specific delivery agents has appealing theoretical simplicity. The inter-relationship of these factors may be represented in the following scheme, and these will be discussed in Chapter 1.



I 1.2 Paul Ehrlich : Evolution of the Concept

The research of Paul Ehrlich (1854-1915) (plate 1), provided many of the foundations upon which modern immunology, pharmacology and haematology could be based. By extrapolating concepts he proposed for immunology and pharmacology, we may attribute the concept of targeted chemotherapy to this great scientist. Ehrlich is also of interest for his early work in oncology and these aspects of his research will be discussed.

Paul Ehrlich was born in March 1854 at Strehlen, Upper Silesia (Garrison, 1929; Baldry, 1976; Lechevalier & Solotorovsky, 1974), now known as Strzelin, Poland. Although an "indifferent student" (Garrison, 1929) he demonstrated a natural aptitude for certain subjects, while showing little talent for others such as German Literature (Baldry, 1976; Lechevalier et al, 1974). During his medical training at the Universities of Breslau, ~~Strasbourg~~ and Leipzig, he developed a profound interest in histology and indeed his doctoral thesis of 1878 was entitled "Contributions to the Theory and Practice of Histological Staining" (Lechevalier et al, 1974).

Dale has suggested that Ehrlich's research career be divided into three phases (Dale, 1957a). During the first, 1877-1891, Ehrlich's work followed his early histological interest, relating to the staining of cells and micro-organisms with the newly synthesised dyes of the German chemical industry. He made several contributions to this area, including the staining of the tubercle bacillus identified by Koch in 1882.

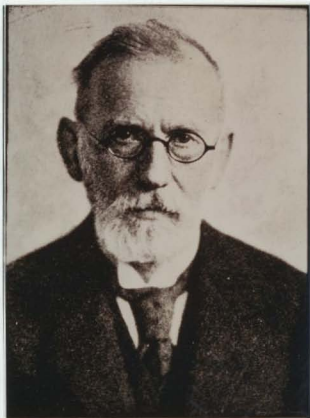


Plate 1. Paul Ehrlich (1854-1915)

Following a two year sojourn in Egypt to recover from tuberculosis, Ehrlich returned to Berlin in 1889. The discovery of the diphtheria toxin by Roux and Yersin in 1886 and the toxin and anti-toxin of tetanus by von Behring and Kitasato in 1890 stimulated the next phase of Ehrlich's research. Thus, between 1891 and 1903 the central theme of his research became the study of immunity, the nature of the toxin-anti-toxin reaction, and the practical aspects of treatment of infectious diseases.

The famous "side-chain" theory, was an attempt to explain the toxin-antitoxin interaction, and although many of his early papers hinted at this concept it was first fully published in 1897 (Ehrlich, 1897) and presented at the opening of the new Institute for the Investigation and Control of Sera, in Frankfurt (Baldry, 1976) and as the Croonian Lecture to the Royal Society of London in 1900 (Dale, 1957b).

Essentially, Ehrlich proposed that each cell of the body exhibited several side chains, with the specific function of "....attaching to themselves certain food-stuffs". During active infection, a toxin would bind to the side chain and render the cell susceptible to damage inflicted by the toxin, either directly, or by the prevention of uptake of nutrients. He stressed the specific nature of the toxin-"side-chain" reaction, and used this to explain the origin and action of the anti-toxin.

"Antitoxins represent nothing more than the side chains, reprod-

used in excess during regeneration, and therefore pushed off from the protoplasm - thus coming to exist in a free state" (Dale, 1957b).

Therefore, binding of the antitoxin, or the secreted "side-chain", to the toxin in the serum would represent a specific reaction and render the toxin harmless to the cells of the host. Ehrlich later referred to these antitoxins as antibodies, or 'magic bullets'. "These antibodies are exclusively 'parasitotrophic', and so it is not surprising that they seek out their targets like magic bullets. In this way I can explain the miraculous cures that are sometimes obtained" (Brock, 1961).

If such substances could be made with the body, reasoned Ehrlich, it should be possible to produce similar specific substances in the laboratory (Baldry, 1976; Lechevalier et al, 1974; Brock, 1961).

"Such substances would then be able to exert their full action exclusively on the parasite harboured with the organism, and would represent, so to speak, magic bullets, which seek their targets of their own accord" (Lechevalier et al, 1974).

The search for such substances stimulated the final phase of Ehrlich's research between 1903 and 1915. This era of chemotherapy led to the testing of organic arsenicals developed by the German Chemical Industry, in the treatment of syphilis. In 1909, substance 606, later called Salvarsan, was found to be active against trypanosomes and was first given to a patient in 1910, marking the beginning of modern chemotherapy.

It was also during this final phase that Ehrlich and his co-worker Apolant, conducted experiments on transplantable murine tumors (Dale, 1957a). They found that pre-inoculation of mice with certain types of tumor enhanced resistance to subsequent inoculation and in some cases led to tumor regression. These early experiments led to interest in the application of immunology to oncology, which declined in later years when it was found that these results were related to allogenic responses to histocompatibility antigens present in normal and tumor tissues.

Just as Ehrlich extrapolated the concepts of 'side chains' from Kekule's Benzene Ring Hypothesis, it is justified to credit Ehrlich with the first concept of targeted chemotherapy. Considering the antibody-drug conjugates as the magic bullets he often referred to, the 'parasitotrophic' effect would be satisfied if one considers the 'parasite' to be the neoplasm to which the antibody is directed, while sparing the host cells any 'organotrophic' consequences.

I 1.3 Present Scope of Chemotherapy for Solid Tumors

Estimates of the incidence of cancer in 1980 from the National Institutes of Health (USA) suggest that of the 785,000 patients with newly diagnosed malignancies for that year, approximately 60 percent are potential candidates for chemotherapy (DeVita, 1983). This figure excludes the 445,000 localised skin, or "in situ" tumors that are cured by local surgical excision alone. Of the patients receiving chemotherapy, an estimated 46,000 would expect to be cured either by drug treatment alone or as an adjuvant to local control of disease by either surgery or radiotherapy, with a further 20,000 patients expecting significant prolonged disease-free survival, while the remainder would receive chemotherapy for palliation. Cure in this context refers to life expectancy of a treated cancer patient to be similar to that of a 'normal' matched cohort of the population.

Although chemotherapy has had its greatest impact upon the prognosis of disseminated haematological malignancies, such as acute lymphocytic leukaemia, where a 50 percent longterm disease-free survival is expected compared to 30 percent in 1973, other tumors, too, are potentially curable such as Hodgkin's Disease, testicular cancer, Burkitt's lymphoma, choriocarcinoma, and diffuse histiocytic lymphoma (Chabner, Fine, Allegra, Yeh & Curt, 1984). The percentage of patients with ovarian and head and neck cancers showing a response or even cure appears to be increasing also.

However, the use of drugs in the treatment of many solid tumors,

especially in the adjuvant setting, is generally disappointing, although there are a few notable exceptions. Of the pediatric solid tumors, cure rate for Wilms' tumor increased significantly following the addition of actinomycin D to surgery and radiotherapy (D'Angio, Evans, Breslow, Beckwith, Bishop, Feigl, Goodwin, Leape, Sinks, Sutow, Tefft & Wolff, 1976), and similarly adjuvant vincristine, actinomycin D and cyclophosphamide have been shown to improve survival for embryonal rhabdomyosarcoma (Maurer, Donaldson, Gehan, Hammond, Hays, Lawrence, Lindberg, Moon, Newton, Ragab, Raney, Ruymann, Soule, Sutton & Teft, 1978). Adjuvant chemotherapy for Ewing's sarcoma has also been shown to significantly increase length of survival (Rosen, Caparros, Mosende, McCormick, Huvo & Marcove, 1978) although this has not yet clearly been demonstrated for osteosarcoma (Rosen, Huvo, Mosende, Beattie, Exelby, Caparros & Marcove, 1978). The current status of chemotherapy in the treatment of early breast cancer has recently been reviewed following an attempt to assess the results of over 80 prospective randomised studies addressing this question (Editorial, 1984a, 1984b). Although it is suggested that adjuvant chemotherapy prolongs disease-free survival of pre-menopausal patients with stage II disease with one to three positive nodes, inconsistent published results and the need to further optimise drug regimens and duration of treatment currently preclude any definite statement as to its exact role in the treatment of breast cancer (Bona-donna & Valagussa, 1985).

By contrast, chemotherapy has not resulted in significant benefit for patients with gastric, colorectal or non-small cell lung cancers. For small cell lung cancers, ten to fifteen percent of patients are now

expected to achieve longterm survival, despite a reported increase in response rate of patients from 30 percent in 1973 to 90 percent in 1983 (Chabner et al, 1984).

A number of ways of improving chemotherapy have been proposed, including optimisation of dosages and schedules of currently available agents, the development of new analogues with reduced toxicity, and a proper assessment of regional chemotherapy, which although based on sound pharmacological principles has received inadequate evaluation through poorly designed studies lacking adequate controls (Chabner et al, 1984). In addition it is expected that as the mechanisms of action of drugs and of drug resistance are better understood, that this too will influence the administration of chemotherapy. The recent establishment of a panel of murine solid tumors and human tumor xenografts in nude mice at the National Cancer Institute (USA), for screening drugs will allow better evaluation of new drugs which may also indirectly improve results. Screening previously utilised the single P388 murine leukemia model whose relevance to human neoplasia was debatable. In addition, it is likely that biologic compounds in the form of lymphokines, monoclonal antibodies, and others will find an increasing role in cancer treatment over the next ten years and may eventually displace conventional cancer chemotherapy (Oldham, 1983b; Baldwin, 1984; Chabner et al, 1984).

Although it is not expected that targeted chemotherapy will, or should, displace currently used drugs, it may well provide a useful method of increasing selectivity of drug delivery, or reducing toxicity, and therefore requires proper and adequate evaluation.

I 2.0 TARGETS

I 2.1 Introduction

The ability to identify and subsequently exploit differences that exist between tumor cells and normal cells would be of value both for the diagnosis of malignancy and for targeting, where such discrimination is a necessary prerequisite of selectivity. However, many claims for "tumor specific markers" have subsequently remained unsubstantiated in the light of further investigation, although these biological substances may well eventually provide further insight into the nature of malignant change and the control of cellular differentiation.

Such suggested tumor markers, which may be detected in the sera of cancer patients or as a cellular component of the tumor itself, have included tumor antigens; oncodevelopmental products, including alpha-fetoprotein, human chorionic gonadotrophin and carcinoembryonic antigen; immunoglobulins, as markers for multiple myeloma and malignant lymphoma; hormones, including ectopically produced calcitonin, insulin, glucagon and catecholamines; and several non-specific enzymes such as lactate dehydrogenase and acid phosphatase, where circulating levels may be elevated in a variety of malignancies. It is apparent that many of these proposed cancer markers are too ubiquitous and non-specific to act as potential targets, and attention has focused upon the use of immunological and oncodevelopmental markers for this role.

1 2.2 Tumor Antigens

An immunological basis of tumor specificity was proposed by Ehrlich in the 1900's, following the demonstration that serum obtained from mice inoculated with tumor could inhibit the growth of similar tumors in other mice. This was thought to be a result of an immune response to tumor antigens and although similar results encouraged further studies into cancer immunology and immunotherapy, subsequent data was less convincing and it is now apparent that such results were obtained from allogeneic responses to histocompatibility antigens present in the tumor. Interest in this field rapidly declined until the 1940's, when a number of investigators were able to demonstrate the presence of tumor specific antigens in methylchloranthrene induced sarcomas in syngeneic mice and other tumor specific antigens were also subsequently demonstrated in a variety of chemically induced experimental animal tumors (Gross, 1943; Prehn & Main, 1957; Klein, Sjogren, Klein & Hellstrom, 1960).

The distinction between tumor specific and tumor associated antigens is important as no tumor specific antigens have so far been demonstrated for human tumors with the possible exception of the human B-cell idiotypes (Stevenson, Elliot & Stevenson, 1977; Qld, 1981). There are, however, a wide range of tumor associated antigens; this distinction arising from the fact that although elevated levels were initially identified in the circulation of cancer patients, improved assay techniques later demonstrated elevated levels in healthy controls and patients with benign disease, as well as in normal tissues also.

Although the origin of these antigens is of fundamental importance to the cancer or cellular biologist, it has not been settled as to whether they represent, for example, differentiation structures arising as a result of derepression of fetal genes or whether they are new molecules arising as a result of neoplasia itself. No attempt will be made to address these questions, rather, to accept their existence and examine their nature and distribution as potential antigens for targeting.

The application of monoclonal antibodies (see later) to this area of tumor immunology has enabled cell surface antigens to be probed with a high degree of specificity and has almost completely replaced the use of absorbed polyclonal antisera. Their use has allowed various aspects of antigenic structure to be investigated in addition to accurate quantitation of cell surface antigen density in tumor and normal cells. With the narrow range of epitope definition by monoclonals, many normal tissues must be screened not only for the distribution of the target antigen, but also to detect any additional and unexpected cross-reactions.

Although heterogeneity of antigen expression has been described using polyclonal antibodies (Sloane & Omerod, 1981) its occurrence has been confirmed following the use of monoclonal antibodies to define cell surface antigens of breast tumors (Arklie, ~~Papadimitriou~~ Papadimitriou, Bodmer, Egan & Millis, 1981), colonic tumors (Arends, Verstijnen, Bosman, Hilgers & Steplewski, 1983), lung tumors (Wagenaar, Hilgers, Moulin, Schol, Verstijnen, Hilkens, Osanto & Vanderschueren, 1983), melanoma (Carrel, Schreyer, Schmidt-Kessen & Mach, 1982) and between primary and

autologous metastatic gastric tumors (Hockey, Stokes, Thompson, Woodhouse, MacDonald, Fielding & Ford, 1984). Many questions remain as to the cause of heterogeneity, whether it reflects neoplastic cellular differentiation or whether it occurs as a result of variation in antigenic structure alone. The implications of heterogeneity upon the localisation of monoclonal antibodies are similarly debated.

Difficulties with immunohistochemical studies upon tumor sections have already been encountered in the studies referred to, which has led to the use of panels of monoclonal antibodies to more precisely define the range of antigens expressed (Gatter, Abdulaziz, Beverley, Corvalan, Ford, Lane, Mota, Nash, Pulford, Stein, Taylor-Papadimitriou, Woodhouse & Mason, 1982; Hand, Nuti, Colcher & Schlom, 1983). Similarly, if as suggested, antigenic heterogeneity appears to limit the overall efficacy of antibody mediated therapy, the use of "cocktails" comprising several monoclonals directed against the range of expressed antigenic determinants may prove useful. It has also been suggested however that the heterogeneity of antigen expression is not an all or none phenomenon, and in fact a range of antigen expression is present (Burchiel, Martin, Imai, Ferrone & Waarner, 1982). If such a differential expression exists, compared to expression in normal tissues, sufficient selectivity may already exist within such a system.

Monoclonal antibodies recognising cell surface antigens have now been described for a variety of tumors including melanoma (Brown, Woodbury, Hart, Hellstrom & Hellstrom, 1981), lung (Cuttitta, Rosen, Gazdar & Minna, 1981), prostate (Frankel, Rouse & Herzenberg, 1982),

colon (Herlyn & Koprowski, 1981), pancreas (Metzgar, Gaillard, Levine, Tuck, Bossen & Borowitz, 1982), acute lymphoblastic leukemia (Ritz, Pesando, Notis-McConarty, Lazarus & Schlossman, 1980), and kidney (Ueda, Ogata, Morrissey, Finstad, Szkudlarek, Whitmore, Oettgen, Lloyd & Old, 1981). Others may also show reactivity not only with the cells of the immunizing tumor, but also with a variety of other malignant cells (Embleton, Gunn, Byers & Baldwin, 1981), which may limit their usefulness in certain situations. In addition, monoclonal antibodies with different epitope specificities may be used to further define complex macromolecules such as carcinoembryonic antigen (CEA) and its related glycoproteins (Primus, Newell, Blue & Goldenberg, 1983; Wagener, Petzold, Kohler & Totovic, 1984; Koga, Kuroki, Matsunaga, Shinoda, Takayasu & Matsuoka, 1985).

Further consideration will be given to carcinoembryonic antigen as it represents the model target of this thesis, applicable to a variety of solid tumors, making it potentially clinically relevant.

I 2.3 Carcinoembryonic Antigen (CEA)

"Carcinoembryonic" antigens of the human digestive system were described in 1965 as specific cancer antigens of the gastrointestinal tract and in order to explain their presence in fetal tissues and gastrointestinal tumors, it was proposed that they represented cellular components which were repressed during normal cellular differentiation, becoming derepressed during neoplasia (Gold & Freedman, 1965a). Initial

hopes of using CEA as a tumor marker for the diagnosis of gastrointestinal tumors were encouraged by the subsequent development of radioimmunoassays capable of detecting nanogram quantities of CEA in serum (Thomson, Krupey, Freedman & Gold, 1969).

However, using increasingly sophisticated immunological techniques it soon became apparent that CEA exhibited substantial molecular heterogeneity (Coligan, Henkart, Todd & Terry, 1973; Vrba, Alpert & Isselbacher, 1975) and with accumulation of data from clinical studies, it was found that elevated plasma levels of CEA or CEA-like substances were detected in a variety of other malignancies including breast (Laurence, Stevens, Bettelheim, Darcy, Lees, Turberville, Alexander, Johns & Neville, 1972), lung (Laurence et al, 1972), pancreas (Khoo & Mackay, 1973), medullary carcinoma of the thyroid (Ishikawa & Hamada, 1976), genitourinary tract including bladder (Guinan, John, Sadoughi, Ablin & Bush, 1974), uterus and cervix (DiSaia, Morrow, Haverback & Dyce, 1976), as well as for a variety of benign diseases. These included rectal polyps (Doos, Wolff, Shinya, DeChabon, Stenger, Gottlieb & Zamcheck, 1975), inflammatory bowel disease (Moore, Kantrowitz & Zamcheck, 1972), hepatic cirrhosis (Moore, Dhar, Zamcheck, Keeley, Gottlieb & Kupchik, 1972), chronic bronchitis (Laurence et al, 1972), and in the serum of heavy cigarette smokers (Stevens & Mackay, 1973).

Subsequent identification of molecules antigenically cross-reactive with CEA stimulated further research into determining more precisely the nature of the molecule, obtaining a satisfactory CEA

standard, developing a reliable method of assay and re-evaluating its clinical potential, and these questions have been addressed at international meetings (Alpert, 1978; Burtin, Gold, Chu, Hammarstrom, Hansen, Johansson, von Kleist, Mach, Neville, Shiveley, Stroebel, Zamcheck, Goldenberg & Westwood, 1978). The use of highly specific monoclonal antibodies has undoubtedly been of value in helping to understand the origin, structure, distribution and function of this molecule although many questions remain unanswered twenty years following its discovery.

CEA was originally identified by immunoprecipitation techniques using highly absorbed antisera (Gold & Freedman, 1965b), and shown to be a glycoprotein of molecular weight 200,000 (Krupey, Gold & Freeman, 1967), confirmed by SDS-polyacrylamide gel electrophoresis to be a single diffuse band (Banjo, Shuster & Gold, 1974). Its method of extraction from hepatic metastases of colonic tumors using perchloric acid followed by gel filtration provided a CEA standard for many studies (Krupey et al, 1967) and an immunogen for the production of anti-CEA antibodies for use in CEA immunoassays. Physicochemical studies demonstrated the molecule to have a range of parent molecular weights (Slayter & Coligan, 1975) and its carbohydrate content, which approximates to between 50 and 75 percent of the molecule by weight is comprised largely of branched chains of N-acetylglucosamine, mannose, galactose, fucose and sialic acid residues (Terry, Henkart, Coligan & Todd, 1972; Terry, Henkart, Coligan & Todd, 1974; Banjo et al, 1974). The variability of these residues, especially sialic acid, has been proposed to partially account for the heterogeneity displayed by the molecule. By contrast,

each single polypeptide chain, stabilised by six disulphide bridges (Westwood & Thomas, 1975), appears to have a relatively constant amino acid composition, with aspartic acid or asparagine, glutamic acid or glutamine, threonine and serine as predominant residues (Terry et al, 1972).

Sequencing of amino acid residues at the N-terminal end of the peptide backbone has been performed using Edman degradations (Terry et al, 1972; Chu, Bhargava & Harvey, 1974), confirming the terminal amino acid residue to be lysine. The region also contains a sequence of residues comprising leucine-leucine-leucine-valine (residues 18, 19, 20, 21) which is identical to a hydrophobic sequence of an immunoglobulin precursor and is thought to be involved in membrane attachment and secretion of the molecule (Burstein, Zemell, Kantor & Schechter, 1977). Interestingly, other areas of homology in the N-terminal region have also been demonstrated between CEA preparations and other CEA-like molecules.

The need to define the unique antigenic determinants specific for CEA is essential in order to improve the specificity of its assay, although it is not clear whether these determinants reside in the carbohydrate or protein component of the molecule, or possibly both (Kania, Wintzer & Uhlenbruck, 1978). Recent studies employing tunicamycin to inhibit glycosylation of the molecule in vitro by cultured cells, leaving only its protein backbone, followed by immunoprecipitation using monoclonal antibodies, suggest that the main antigenic determinants reside in the protein portion, whose molecular weight was

determined to be $80,000 \pm 2,000$ (Kuroki, Kuroki, Ichiki & Matsuoka, 1984). As conventional antisera probably react with several determinants, it is in this area that highly specific monoclonal antibodies will find increasing application (Bedin, Hammarstrom & Larsson, 1982; Primus, Newell, Blue & Goldenberg, 1983).

Consideration must now be given to the CEA-like glycoproteins immunologically cross reactive with CEA, although it must be stated at the outset that it is still not known with certainty whether they represent precursors, cleavage products or indeed individual molecules (Primus Newell et al, 1983).

The first of these to be described was "normal glycoprotein" (NGP) (Mach & Pusztaszeri, 1972), found in perchloric acid extracts of lung and spleen and was of lower molecular weight at 60,000. A similar "non-specific cross reacting antigen" (NCA-1) was also described with widespread distribution in malignant and normal tissues including myeloid cells and neutrophils, but although its immunological and physicochemical properties were similar to CEA, it could be distinguished by virtue of its lower molecular weight (Von Kleist, Chavanel & Burtin, 1972). Subsequently described antigens, including NGP have now been shown to be identical to NCA-1, and these were originally termed CEX (Darcy, Turberville & James, 1973) or CCEA-2 (Turberville, Darcy, Laurence, Johns & Neville, 1973). Similarly, the cross reacting antigen TEX appears to be similar, if not identical, to NCA-1 (Kessler, Shivaly, Pritchard & Todd, 1978).

A further family of cross reacting antigens extracted from

meconium and feces have been described which appear to be more closely related to CEA, and have been designated NCA-2 (Burtin, Chavanel & Hirsch-Marie, 1973), normal fecal antigens, (NFA-2) (Matsuoka, Kuroki, Koga, Kuriyama, Mori & Kosaki, 1982) and meconium antigen (Primus, Freeman & Goldenberg, 1983).

The CEA-like antigen CELIA identified in the gastric juice of normal individuals appears to resemble CEA closer than NCA, although further studies are required to characterise this molecule (Vuento, Ruoslahti, Pihko, Svenberg, Ihmaki & Siukala, 1976). Biliary glycoprotein-1 (BGP-1), found in hepatic bile of normal individuals appears distinct from both CEA and NCA although it is more closely related to the latter (Svenberg, 1976; Hammarstrom, Svenberg, Hedin & Sundblad, 1978).

Although anti-CEA monoclonal antibodies are discussed in a later section they are of considerable importance in characterisation of this molecule both in immunomolecular terms and in determining its distribution in tissues and tumors, in addition to being used in CEA assay systems. Patterns of binding reactivity of panels of anti-CEA monoclonal antibodies with differing epitope specificities are being used to investigate the immunological heterogeneity of CEA, and determinants common to CEA-like glycoproteins (Primus, Newell et al, 1983; Wagener, Petzold, Kohler & Totovic, 1984). Immunohistochemical studies, i.e. immunoperoxidase, employing specific anti-CEA monoclonal antibodies with minimal or no NCA cross-reactivity, remain essential for the evaluation of the distribution of CEA in tumors and normal tissues. Localisation of the antigen has been assessed in human tumor cell lines

in culture, grown as xenografts in nude mice and in fresh surgically resected specimens (Primus, Kuhns & Goldenberg, 1983; Motoyama & Watanabe, 1983; Wolf, Thompson & Von Kleist, 1984; Wagener et al, 1984). A consistent finding of these studies is the heterogeneity of antigen expression by tumors, which has in itself stimulated further studies into the synthesis, distribution and expression of CEA. Variation in membrane associated CEA content is described for colonic cancer cell lines, depending on the degree of tumor differentiation, and appears to be high for well differentiated lines (LS174T, SKCO1) and low for poorly differentiated cells (SW620, HRT18) (Shi, Tsao & Kim, 1983). While immunocytochemical studies have shown that up to eighty percent of CEA is membrane associated (Shi et al, 1983), further data on its subcellular localisation is scarce and attempts to investigate this using immunoelectron microscopy have met with a number of technical difficulties (Wolf et al, 1984). The rate of synthesis and release of CEA also appears to be dependent on the growth phase of these cells, decreasing with LS174T and SKCO1 as cell density increases. Studies have also been extended to xenograft models in vivo, and it has been shown that while CEA production per gram of T-380 xenograft (human colonic tumor) remained constant, serum CEA levels increased as tumor growth occurred (Martin & Halpern, 1984). Differences were also found for the clearance of circulating CEA obtained from patients sera (half-life, two and a half hours) and that extracted from tumors (rapid), suggesting a difference in the membrane expressed and circulating forms of the molecule. Other studies have also demonstrated a rapid clearance of CEA by the liver, which appears to represent the major clearance pathway, involving receptor mediated endocytosis by hepatic Kupffer cells; following the removal of sialic

acid residues, the molecule is transferred to the hepatocyte for further metabolism (Toth, Thomas, Broitman & Zamcheck, 1985). Such studies attempting to investigate the kinetics of CEA are still somewhat preliminary in nature although they represent the first attempts to answer such fundamental questions. Similarly, studies attempting to map the gene coding for CEA using somatic cell hybridization in order to investigate the genetic control of malignancy are also in their infancy (Sheer, Brown & Bobrow, 1982).

There is little doubt that the measurement of serum CEA levels is of little value in the diagnosis or screening of gastrointestinal malignancy (Summary of NIH consensus statement, 1981) although it has been suggested that preoperative levels correlate well with prognosis after curative surgical resection for gastrointestinal cancers (Wanebo, Rao, Pinsky, Hoffman, Stearns, Schwartz & Oettgen, 1978; Staab, Anderer, Brummendorf, Stumpf & Fischer, 1981), and for patients undergoing radical surgery for lung cancers (Ford, Stokes & Newman, 1981). The role of monitoring CEA levels postoperatively in order to detect pre-clinical recurrence of tumor following curative surgical resection has been shown to be of value in colorectal carcinoma (Boey, Cheung, Lai & Wong, 1984) although the pattern of rise of CEA is of no practical value in distinguishing between local and distant recurrence of these tumors (Hine & Dykes, 1984a). Indeed, postoperative elevated serum CEA levels have been proposed as a guide to further therapeutic intervention, in the selection of patients for second look laparotomy in gastrointestinal malignancies (Attieyeh & Stearns, 1981; Staab, Anderer, Stumpf, Hornung, Fischer & Kieninger, 1985) or for chemotherapy (Hine & Dykes, 1984b). However

this is still subject to criticism and a more rewarding approach would be an attempt to correlate immunohistochemical grading of tumors based on localisation of CEA. This has been performed for lung cancer, where 82 percent of specimens showed localisation of anti-CEA antibody by immunoperoxidase, which although correlating with pre and postoperative serum CEA levels, was of little prognostic value (Ford et al, 1981). Similar correlations between immunohistochemical CEA grading of colorectal tumors with serum CEA levels have been reported, although prognostic significance has not been addressed (Hamada, Yamamura, Hioki, Yamamoto, Nagura & Watanabe, 1985).

I 3.0 CARRIERS

I 3.1 Introduction

Although a wide variety of carrier systems for delivery of therapeutic agents to target cell populations have been proposed, the theoretical prerequisites of each should be similar. That is, the carrier should guide the warhead to its target and allow it to exert its effect at that site with minimal or no interference, while protecting the host from any possible toxic effects of the agent. Additionally, protection of the therapeutic agent from the host's natural defence mechanisms would be a desirable property, thereby preventing premature inactivation of the warhead, and reduction in efficacy.

I 3.2 Carrier Systems

Although not specifically addressing the problems of targeting anti-neoplastic agents to tumors, a recent review listed several carriers of potential use in drug targeting, ranging from naturally occurring cellular components to a variety of synthetic compounds (Gregoriadis, 1981). Present experience relates to a limited number of carrier systems, in particular antibodies and liposomes, and apart from isolated case reports and preliminary in vivo studies, the role of other carriers has not yet been defined fully. It is likely, however, that few will have application in practical drug targeting, and even fewer in the delivery of anti-cancer agents, although they provide models for the evaluation of the concept of drug targeting.

As the use of antibodies will be considered in detail in subsequent sections, these alternative carriers will be reviewed first. Interest in the use of liposomes for selective drug delivery has paralleled that of their antibody counterparts and their potential as targeting agents is based upon a number of their characteristic properties (Gregoriadis, 1976a,b). As small spheres consisting of one or more concentric phospholipid bilayers separated by an aqueous phase, a variety of substances, including drugs, enzymes and hormones, may readily be incorporated within either phase depending upon their relative water or lipid solubility, while the final size of the organelle may also be determined by sonication during the process of their preparation in the laboratory (Gregoriadis, 1980). Manipulation of membrane polarity may result in variable degrees of membrane "porosity" allowing drug leakage to proceed at a set rate which may also be dependent upon the local milieu of a target tissue, organ or tumor (Yatvin, Kreutz, Horowitz & Shinitzky, 1980). However, despite these seemingly advantageous properties, the range of cells that associate and fuse with liposomes is small and appears to be related to the cells' intrinsic endocytic potential. It appears therefore, as if cells of the reticulo-endothelial system are chiefly responsible for liposome clearance in vivo (Gregoriadis, 1976a), although this may be advantageous for the selective delivery of agents to cells of the monocyte-macrophage series involved in disease (Schroit, Hart, Madsen & Fidler, 1983). In order to improve the selective targeting of these structures, incorporation of antibodies into their outer layers has been performed and improvement of uptake of liposomal bleomycin by cultured HeLa cells has been demonstrated when anti-HeLa immunoglobulin G (IgG) is incorporated into the liposome, compared to a

non-specific IgG (Gregoriadis, 1976b). Targeting of liposomes to cells expressing CEA has also been achieved by incorporating an anti-CEA monoclonal antibody into liposomes containing a fluorescent dye, carboxy-fluorescein, which could then be demonstrated to be incorporated within the cell by fluorescence microscopy (Guidoni, O'Hara, Price, Shuster & Fuks, 1984).

Problems with this approach to targeting in vivo arise at the capillary level, where transport of these relatively large structures to target cells is hindered by the capillary membrane, and also by a rapid uptake of the liposomes by the liver and spleen. It has also never been demonstrated convincingly that true receptor-mediated targeting occurs using this model in vivo, and the local release of incorporated drugs in sufficient concentration may be one mechanism of action that would produce a response independent of true targeted delivery, as would their capacity to act as a circulating drug depot providing a slow, even release of the drug over a prolonged period. Encouraging studies using the anti-neoplastic drug cytosine arabinoside incorporated into liposomes demonstrated prolonged survival of mice with the L1210 leukaemia (Kobayashi, Tsukagoshi & Sakurai, 1975). The overall reduction in drug toxicity may be a reflection of its possible mechanism of action, if acting as a circulating drug depot.

In addition to cytosine arabinoside, other anti-neoplastic agents such as actinomycin D, 5-fluorouracil, methotrexate and bleomycin have been incorporated into liposomes (Gregoriadis, 1976a,b), and also a number of immunomodulators or immunostimulants (Schroit et al, 1983).

However, the efficacy of these liposome-drug complexes in the treatment of cancer still remains to be defined.

A variety of cellular carriers have also been proposed for drug targeting although it is difficult to envisage their use in the specific targeting of anti-cancer drugs to tumors at the present time. These systems include erythrocytes, or their ghosts, which have the advantages of availability and non-immunogenicity although they generally lack true targeting ability, unless specific antibodies can be incorporated into their plasma membranes as has been described with liposomes. It is certainly possible that these drug containing erythrocyte ghosts may act as a circulating store of drug as has also been proposed as one mechanism of liposome action, and it is conceivable that toxicity would also be reduced.

The localisation of neutrophils in inflammatory lesions provided a model for the use of these cells in drug targeting, and it has been demonstrated that cells containing In-111 oxine will selectively image inflammatory lesions in dogs (McAfee, Gagne, Subramanian, Grossman, Thomas, Roskopf, Fernandez & Lyons, 1980) and a similar model has been used to image patients with acute pancreatitis (Anderson, Spence, Laird, Ferguson & Kennedy, 1983). However, it is unlikely that such systems will be of value in tumor targeted chemotherapy or imaging unless a significant inflammatory component is associated with the tumor.

Similarly, a model for the selective delivery of vinblastine to macrophages has been successful in vitro and in the treatment of patients

with idiopathic thrombocytopenic purpura (Ahn, Byrnes, Barrington, Cayer, Smith, Brunskill & Pall, 1978; Agnelli, DeCunto, Gresele, Nenci, Fedeli & Moretti, 1984). It is also unlikely that this model would find any practical application involving the targeting of these cellular components to tumors, although it provides an interesting application of drug targeting in haematology.

The targeting potential of other proteins, enzymes or synthetic compounds such as dextrans has been investigated even less than those systems mentioned. A model illustrating the targeting potential of hormones utilized a conjugate of daunomycin and melanotropin, a tropic hormone binding surface receptors of mouse myeloma cells, to demonstrate selective cell killing (Varga, Asato, Lande & Lemmer, 1977). Fluorescence microscopy demonstrated that the conjugate was internalised preferentially by melanoma cells compared to control fibroblasts lacking melanotropin receptors and could be blocked by free melanotropin. Free daunomycin demonstrated less toxicity to control melanoma cells and murine fibroblasts at similar concentrations to those used in the conjugate, although both cells were equally susceptible to the free drug at much higher concentrations. This study also postulated the importance of receptor mediated internalisation of the conjugate for the specific nature of targeting, as demonstrated indirectly by the absence of effect upon the receptor deficient fibroblasts and the capacity of free hormone to block the response to the conjugate. It is conceivable that advantage could be made of the presence of hormone receptors in certain solid tumors, notably breast, in an effort to target antineoplastic drugs to selected tumors.

I 3.3 Antibodies as Carriers

The use of antibodies directed against tumor associated antigens to target chemotherapeutic agents has great theoretical appeal. Although a number of criticisms of this approach were raised following their early use, these are becoming less valid as work in this field expands. Criticism regarding the nature of the target to which they are directed has been discussed in the section on tumor markers, and criticisms regarding the nature of the conjugation process itself and its sequelae in vivo will be addressed in the later section on conjugates.

Until the mid-1970's, another major criticism related to the techniques used to produce antibodies of required specificity. Conventional methods of antibody production essentially often involved the immunization of unrelated species with a preparation of the antigen in question, followed by collection of the animals' sera after an appropriate period during which immunoglobulin was being produced by the process of active immunization. Refinements of this basic technique include the administration of a suitable adjuvant and determination of the optimum period following antigenic challenge at which to collect the greatest yield of antibody, in accordance with conventional immunologic technique.

The immunoglobulins present within these sera were of a variety of classes, predominantly IgG subclasses with a proportion of IgM, resulting in a heterogeneous antiserum, of variable specificity and affinity. In addition, the proportion of specific immunoglobulin could not be guaranteed when the process was repeated. In order to improve specificity of

these polyclonal antisera, extensive absorption of the sera was required, although it is essential that the absorption process itself is not excessive and is stopped prior to the non-specific loss of the tumor specific antibody (Davies & O'Neill, 1973).

The development of hybridoma technology, with the production of monoclonal antibodies, has largely allowed several of the limitations imposed by polyclonal antibodies to be overcome, in addition to providing tools with which to further probe the structure of complex molecules such as the tumor antigens.

These biotechnology products have also led to further interest in the use of antibodies as therapeutic tools in their own right (immunotherapy), in addition to their use as carriers by linkage to cytotoxic agents such as toxins (immunotoxins), drugs (immunotherapy), radionuclides (immunoradiotherapy and immunoradiodiagnosis) and other potentially biologically useful agents.

I 3.4 Monoclonal Antibodies

In 1975, Kohler and Milstein reported a method of producing monoclonal antibodies in large quantities following the fusion of spleen cells from an immunized donor with cells from a murine myeloma cell line (1975). Their method essentially involved the fusion of immune murine spleen cells with cells of the murine myeloma cell line P3-X63-Ag8, utilising inactivated Sendai virus as a fusing agent, creating hybridomas that contain genetic material from both parent cell

lines. The parent myeloma cell line lacks the enzyme hypoxanthine phosphoribosyl transferase (HPRT) and therefore dies when grown in selective medium containing hypoxanthine, aminopterin and thymidine (HAT) while the unfused spleen cells die spontaneously in culture. This enables the hybridoma cells, which are not HPRT deficient (as these obtain the HPRT from the spleen fusion partner) to grow in culture, in which they may be detected and screened for antibody production. It has been estimated that only one myeloma cell in 1000 fuses with a spleen cell to produce a viable hybrid and that an average fusion will yield up to 500 hybrids if the donor is well immunized (Secher, 1980). It has also been estimated that only half will produce immunoglobulin and of these, one to ten percent will produce specific antibody that reacts with the immunizing antigen. This underlies the rationale for using high ratios (10:1) of spleen to myeloma cells in later fusions, in comparison to the original fusions which used equal cell numbers.

Screening hybrids for antibody activity is usually performed by either radioimmunoassay (RIA) or enzyme linked immunoabsorbant assay (ELISA). Interesting hybrids secreting antibodies of required specificity are cloned and maintained in tissue culture, or injected intraperitoneally into mice to produce an ascites with greater antibody yield. Alternatively, clones may be frozen and stored for subsequent use. The method of fusion has changed little in principle although a number of modifications to the technique have become established, for example, the replacement of the inactivated Sendai virus with polyethylene glycol (PEG) as the fusion promoting agent. Although the basic technique is successful for the majority of applications, additional

problems have arisen.

In general, viral or PEG induced fusions exhibit some degree of randomness and poor yield of specific high affinity antibody is not uncommon. Attempts to improve the yield of high affinity antibody have included the use of electrically induced cell fusion, which promotes cell alignment and increased biomembrane permeability prior to fusion, and promising results have been obtained when this procedure incorporates cell surface receptors to increase fusion selectivity (Lo, Tsong, Conrad, Strittmatter, Hester & Snyder, 1984).

Yield of monoclonal antibodies to weak antigens remains a persistent problem and a promising approach is the transformation of such antigens into highly immunogenic species by coupling them to adjuvant proteins or following chemical modification (Sakato & Eisen, 1975).

More recently a further application of DNA technology has been described in the production of monoclonal antibodies (Roth, Scuderi, Westin & Gallo, 1984). A transforming oncogene, N-ras, from human acute lymphoblastic leukaemia ALL 1-69, was shown to transform NIH 3T3 fibroblasts which then exhibited a tumor-associated antigen, to which a murine monoclonal antibody (IgM) was raised. Studies then related antibody binding specificity and pattern to the distribution of the oncogene-induced cell surface molecule among the parent cell lines, transfected fibroblasts and a range of normal and malignant tissues, using ELISA and immunoperoxidase techniques. It was demonstrated that binding occurred to the parent ALL 1-69 and the secondary transfectant,

but not to the parent fibroblasts, and additionally, the antigen was also demonstrated to be present in a variety of tumors and some normal tissues. The importance of this study relates to the potential use of DNA technology in the generation of selective monoclonal antibodies to oncogene products and the use of these monoclonals as probes for the further elucidation of the nature of oncogenes.

Most hybridomas are the result of murine or rat cell fusions and a number of approaches have been investigated for the production of human monoclonal antibodies. A number of human myeloma cell lines have now been established in culture although rate of immunoglobulin production is poor compared to their murine counterparts and loss of human genetic material is a problem following fusion (Olsson & Kaplan, 1980; Pickering & Gelder, 1982). It is not generally feasible to immunize normal human subjects with the antigens in question. Approaches that have been explored include the use of the Epstein Barr virus to transform the peripheral lymphocytes of patients to produce monoclonal antibodies, although the yield of clones producing sufficient antibody is low (Steinetz, Klein, Koskimies & Makela, 1977), as well as fusion of peripheral blood, intra-tumoral or lymph node lymphocytes from patients with cancer with a human myeloma derived line (Sikora, Alderson, Ellis, Philips & Watson, 1983).

I 3.5 Anti-CEA Monoclonal Antibodies

Using the hybridoma technology described in the preceding section, a number of investigators have attempted to produce monoclonal antibodies

against carcinoembryonic antigen. The reasons for this illustrate clearly the potential application of monoclonal antibodies, and are essentially two-fold. Using these highly specific antibodies, the molecular and immunological complexity of CEA, or indeed other heterogeneous macromolecules may be probed, which in addition to the characterisation of this antigen, offers the potential of developing a standard CEA assay which may eventually prove to be of value in the clinical evaluation of malignancy. Secondly, they offer great potential as specific carriers in a number of targeting systems.

The first successful fusions yielding monoclonal anti-CEA antibodies were reported by Acolla, apparently following a long series of negative results (Acolla, Carrel & Mach, 1980). Using standard fusion technique, spleen cells from mice immunised with 15 ug of purified CEA and Freund's adjuvant were fused with myeloma cells, to obtain some 400 hybrids from 7 different fusions. A radioimmunoassay was used to screen for anti-CEA activity and of 9 positive hybrids, only 2 retained activity after subculture and were subsequently cloned. These IgG1 and IgG2 monoclonals were shown to recognise different antigenic determinants of CEA following a competitive inhibition binding assay. The same group has subsequently utilised the IgG1 monoclonal antibody in an enzyme immunoassay and demonstrated good correlation of CEA quantitation with conventional radioimmunoassay, for 380 serum samples from both patients and normal individuals (Buehgger, Phan, Rivier, Carrel, Acolla & Mach, 1982).

Using modified immunisation schedules, other groups have also

successfully produced monoclonal anti-CEA antibodies, each recognising different epitopes of CEA and exhibiting a range of cross reactivity with NCA or other related antigens (Rogers, Rawlins & Bagshawe, 1981; Hedin et al, 1982; Primus, Newell et al, 1983; Rogers, Rawlins, Kaidana, Gibbons & Bagshawe, 1984).

As a result of a collaborative project between the Surgical Immunology Unit, Birmingham, England and the Lilly Research Centre Windlesham, England, a number of murine monoclonal anti-CEA antibodies were produced by hybridoma technology in the early 1980's (Rowland, Simmonds, Corvalan, Marsden, Johnson, Woodhouse, Ford & Newman, 1982; Corvalan, Axton, Brandon, Smith & Woodhouse, 1984). BALB/c mice were immunised using purified CEA with Freund's adjuvant, or as an alum precipitate with Bordetella pertussis adjuvant or using the CEA producing cell line HT 29. Spleen cells were harvested and fused with the P3-NSI-1-Ag4-1 mouse myeloma cell line in the standard manner described. Hybridomas were screened for anti-CEA activity using an enzyme-linked immunoassay (Woodhouse, Ford & Newman, 1982), and interesting hybrids were cloned and investigated further for CEA or NCA binding, and against a number of tumors and normal tissues using histochemical methods. Of the 14 or so anti-CEA monoclonals obtained, three, designated 11-285-14 (IgG1), 11-35-75 (IgG1), 14-95-55 (IgG2a), demonstrated binding to colorectal carcinoma tissue sections but no reactivity with spleen cells, and competitive inhibition binding studies performed using ELISA have demonstrated that different epitopes of CEA are recognised by each of these antibodies (Corvalan et al, 1984). Further histochemical characterisation of 11-285-14 and the distribution of its target antigen, CEA,

demonstrated positive reaction in normal and malignant gastric and colonic epithelium, and interestingly also in the superficial non--keratinising crypt epithelium of the tonsil (Gatter et al, 1982). No binding was seen for a range of other normal tissues such as brain, skin, spleen, kidney, pancreas, bile duct, liver, lung etc. This paper also suggests the potential use of panels of specific monoclonal antibodies as an adjunct to the diagnosis of neoplasia when histological examination appears unhelpful, in addition to their potential use for the characterisation and distribution of potential target antigens. Subsequent sections will discuss the potential of 11-285-14 for targeting studies.

1'3.6 Monoclonal Antibodies in Cancer Diagnosis and Treatment

It is therefore apparent that these highly specific antibodies have found a wide range of applications, fulfilling Kohler and Milstein's closing statement that "such cultures could be valuable for medical and industrial use" (1975). Several such applications exist within the field of cancer diagnosis and therapy in addition to helping answer fundamental questions regarding cellular differentiation and control, and the structure of a variety of cell surface molecules including oncodevelopmental antigens. These applications will now be discussed with the exception of their application to immunochemotherapy which will be considered in a later section.

I 4.0 IMMUNOTHERAPY

The role of the immune system in the development of cancer and the use of antibodies in its treatment may be traced back to the turn of the century when the first attempts at passive and active immunisation to tumors were made by Hericourt and Coley respectively (Newman, 1978; Mastrangelo, Berd & Maguire, 1984). Since then, interest in this area has been cyclical, although with the advent of monoclonal antibodies, a better appreciation of tumor immunology and the host-tumor relationship, the pessimism that was present in the 1960's and early 1970's has been replaced with a renewed interest in this area.

Until the highly specific monoclonal antibodies became available, only heterogeneous antisera were available to immunotherapists and early results, with often inappropriate animal models, were frequently disappointing. In addition, the use of polyclonal antibodies in the clinical setting led to a variety of toxic reactions along with similarly consistent poor overall results. Other factors that contributed to the negativism that shrouded this approach included the often premature and incorrect extrapolation of early preclinical studies and case reports which often failed to show any significant improvement using antibodies.

Some degree of consensus regarding the present status of immunotherapy is found in a number of recent reviews dealing with this subject (Levy & Miller, 1983; Oldham, 1983a; Mastrangelo et al, 1984; Dillman & Royston, 1984; Baldwin, 1984). The role of antibody in tumor cell destruction was initially focused upon a direct antibody mediated

reaction although it is now being appreciated that antibodies may exert their effects in other ways, in particular in the regulation of the immune response and through their interaction with the various cellular components of the immune system. Indeed there has been a greater recent emphasis on the effect of these cellular components upon tumor growth, through both specific and non-specific mechanisms, although much is still speculative. Biological response modifiers, therapeutic agents or approaches used directly, to augment, or to modify the host's biological response to its tumor, are also being re-evaluated in the light of recent biotechnological advances, and this term has now come to include the use of agents such as Bacillus Calmette-Guerin (BCG), effector cells such as macrophages or natural killer cells, lymphokines and interferons (Oldham, 1983b).

As mentioned, the results of early studies using polyclonal antisera were disappointing, both in the laboratory and clinical settings (Rosenberg & Terry, 1977). With the exception of tumors induced by the Moloney sarcoma virus and the polyoma virus, marginal therapeutic benefits were seen only when laboratory animals were treated prior to tumor inoculation (immunoprophylaxis) or just following tumor challenge, usually within 24 hours. Another factor that appeared to be important in determining response to treatment was the tumor burden, which appeared to exhibit an almost inverse relationship to outcome and the subsequent explanation offered was that immunotherapy could only deal with a critical tumor burden. In the mouse, this was estimated to be 10^6 cells, and therefore mice with tumors of greater cell numbers would not be expected to show a response to immunotherapy. There are also additional

implications arising from this concept regarding the host-tumor relationship and the theory of immune surveillance, which proposes that spontaneously appearing tumor cells are destroyed by the host's immune system, provided tumor burden is below a similar critical value. The role of the immune system in neoplasia in man is at best circumstantial. Several well documented observations are often quoted in support, for example the phenomenon of spontaneous tumor regression, regression of metastases following surgical resection of the primary lesion, the increased incidence of tumors with age, in immune deficiency states, and in immunosuppressed renal transplant patients, although the precise number of cases in which such phenomena have been recorded is actually quite small and inconsistent. The question of whether or not patients with cancer are immunosuppressed is also open to speculation. Several studies have reported this to be the case although others have failed to demonstrate any significant suppression following critical analysis of peripheral lymphocyte levels in cancer patients (Baldwin, 1984).

Antibody destruction of tumor cells may occur in a number of different ways, although the actual mechanism operating in vivo is not known with certainty. Complement mediated cytotoxicity, involving binding of antibody to the cell surface target antigens, followed by fixation of complement through the Fc portion of the antibody with subsequent cell lysis is thought to play a minor role in tumor cell killing and animal studies have failed to demonstrate that complement fixation is essential (Dillman et al, 1984). The role of antibody dependent cell mediated cytotoxicity however has assumed a greater importance and this mechanism has also been supported by early studies

using monoclonal antibodies, which have indicated the importance of antibody isotype. The IgG2a isotype has been found to be most effective *in vivo* (Bernstein, Tam & Nowinski, 1979; Herlyn & Koprowski, 1982) whereas IgM, a strong complement fixer, has been shown to be practically ineffective in tumor cell killing. Other IgG isotypes (G2b, G1, G3) were shown to have a range of efficacy, although all required the binding of mononuclear effector cells to their Fc portions in order to produce cell lysis.

Clinical trials involving treatment with monoclonal antibodies have only become established within recent years and although patient numbers are small, some landmark responses have been reported. Haematological malignancies such as leukaemia and lymphoma are more amenable to this approach although it has also been used to treat a limited number of patients with solid tumors (Sears, Atkinson, Mattis, Ernst, Herlyn, Steplewski, Bayry & Koprowski, 1982; Levy & Miller, 1983; Goodman, Beaumier, Hellstrom, Fernyhough & Hellstrom, 1985). One particularly encouraging report is from Miller who used a murine IgG2b monoclonal anti-idiotypic antibody to treat a patient with advanced B-cell lymphoma (Miller, Maloney, Warnke & Levy, 1982). Complete remission for over four years has been obtained, although there is still speculation regarding the mechanism by which this response was obtained, through either direct cytotoxicity or as a regulatory response. Other investigators have utilised monoclonal antibodies directed against the common acute lymphoblastic leukaemia antigen (Ritz, Pesando, Sallan, Clavell, Notis-McConarty, Rosenthal & Schlossman, 1981). Following administration of such antibodies to patients with leukaemia, a fall in peripheral leukaemic

cell levels was noted, although the result was often of a transient nature and leukaemic cells reappeared without expression of the antigen. This has been attributed to a temporary initial clearance of leukaemic cells from the blood, followed by a number of secondary responses including antigenic modulation (likely), antibody blocking by circulating free antigen or as a result of the patient's immune response to the murine monoclonal antibody.

Similar brief therapeutic responses have also been seen in patients with T-cell leukaemias treated with monoclonal antibodies reacting with malignant T-cell surface antigens (Miller, Maloney, McKillop & Levy, 1981). A response to antibody has been shown in the skin plaques and lymph nodes of a patient with T-cell lymphoma (Miller & Levy, 1981). The response to other solid tumors generally appears to be less encouraging (Sears et al, 1982; Goodman et al 1985) and adequate evaluation is still awaited (Dillman et al, 1984), although the presence of antibody in tumor has been demonstrated after antibody infusion (Oldham, Foon, Morgan, Woodhouse, Schroff, Abrams, Fer, Schoenberger, Farrell, Kimball & Sherwin, 1984).

Early clinical studies such as these have re-emphasized a number of important fundamental questions regarding antibody administration in vivo. It is most likely that antigenic modulation is the reason for the reappearance of leukemic cells not expressing target antigens following serotherapy and it appears as if this may be a more frequently occurring consequence to treatment than was initially anticipated (Mastrangelo et al, 1984). The decreased expression of cell surface

antigen following antibody treatment occurs rapidly but antigen will reappear when antibody is removed from the system with no apparent permanent cellular sequelae. It has been demonstrated that following antigen antibody interaction, the complex localises to one portion of the cell surface ("capping") followed by a process whereby the complex is internalised by the cell (Pesando, Ritz, Lazarus, Tomasell & Schlossman, 1981). This property is also dependent on the nature of the cell surface antigen itself (Levy et al, 1983). This phenomenon also stresses the importance of antibody binding in order to produce an effect upon the tumor cell, and several studies have attempted to relate immunotherapeutic response to antigen saturation.

Obviously many other factors will influence this in addition to antigenic modulation. The tumor cells themselves will generally exhibit a heterogeneous population of target antigens and therefore therapy with one monoclonal antibody recognising one particular determinant may in itself be suboptimal, and this may be overcome by using a combination of monoclonals each identifying separate determinants or different surface antigens. The use of murine monoclonals in this regard may offer therapeutic benefit if the determinants recognised by antibodies of another species are unrecognised by species specific antibodies, or the host's own immune system (Oldham, 1983a).

It is generally agreed that in order to achieve antigen saturation of a critical level, and hence therapeutic effect, large quantities of antibody must be given. This was quantified to be in the order of milligrams for the treatment of murine leukaemias (Bernstein et al, 1979)

and indeed doses used in clinical studies have ranged from microgram quantities to 12 grams (Newman, 1978).

Theoretically many problems arise in the treatment of solid tumors using this approach, where target cell populations are much more inaccessible than for the disseminated haematological malignancies. This may be overcome by using much larger doses of antibody to achieve saturation or by the use of two or more antibodies, as has been suggested by animal studies (Capone, Papsidero & Chu, 1984). Not only would such cells be relatively inaccessible to antibody, the infiltration of tumor bulk by effector cells may also prove to be a problem. However despite these theoretical objections, indirect evidence of antibody localisation is available from studies using this approach in the diagnosis of tumors (immunoscintigraphy), both in animals and in patients. Further optimisation of this approach in the treatment of solid tumors may be achieved if it is used in an adjuvant setting following tumor debulking or in the treatment of subclinical metastatic deposits. Although the use of immunotherapy in these settings is presently not adequately defined, it may well prove to be a most rewarding approach.

The shedding of tumor antigens into the circulation may affect the in vivo response in two ways. Antigen antibody complexes free within the circulation may account for diminished therapeutic response as antibody will effectively be blocked, and in order to overcome this, larger infusions of antibody would be required. The circulating complexes also present a potentially toxic threat as they will be removed from the circulation by reticuloendothelial cells, including the liver and if

renal excretion also occurs, it is possible that a glomerulonephritis may also result, although these side effects have not been seen in the limited numbers of patients studied to date (Dillman et al, 1984).

The potential toxicity of administering monoclonal antibodies received much attention in early studies. True hypersensitivity has been noted in ten to fifteen percent of patients who have received this mode of therapy so far, ranging from fever and chills to urticaria, bronchospasm and anaphylactic shock. Other toxic side effects noted appear to relate to the nature of the treatment itself, and possibly occur as a result of the removal of target cells from the blood. When given as a rapid infusion in the presence of a large circulating tumor load, respiratory distress has been seen in mice (Dillman et al, 1984). This may be explained by the trapping of cell antibody complexes by the lung which would then react characteristically producing a respiratory distress type picture and ultimately respiratory insufficiency. Similar pulmonary symptoms have been reported in patients involved in some clinical trials (Dillman et al, 1984), but not in others (Oldham et al, 1984).

The regulatory effect that antibodies may exert upon the immune response, in particular through idiotypic anti-idiotypic interaction, has been better appreciated since monoclonals became available and these concepts have also been applied to the field of tumor immunology. An ideal target would be the B-cell tumor idiotypes, the only human tumor specific marker presently known, and the use of anti-idiotypic antibodies in the regulation of proliferation of malignant B-cell clones has been proposed (Dillman et al, 1984). Indeed, this mechanism of action has

been suggested as an explanation of the successful treatment of a patient with B-cell lymphoma (Miller et al, 1982). Monoclonals may also act as immune regulators if directed against a number of growth factors or their receptors, such as the transferrin receptor or that of epidermal growth factors, but full evaluation of this mechanism of action is still awaited.

The antibody independent cell mediated response to tumors has also received increased attention recently and this approach broadly encompasses adoptive cellular immunotherapy, utilising specific cytotoxic lymphocytes, or enhancement of the non-specific cytotoxic cells, the macrophage or natural killer cell (Mastrangelo et al, 1984; Rosenberg, 1984).

Early experiments transfusing large numbers of immune lymphocytes into tumor-bearing rats were able to demonstrate tumor regression (Delorme & Alexander, 1964) but the unavailability of suitable cells for human use largely prevented clinical studies. Tumor-immune pig lymphocytes have been used for the adoptive immunotherapy of 24 patients with recurrent bladder cancer, with remission reported for 11 patients (Symes, Mitchell, Eckert, Roberts, Feneley, Tribe & Lai, 1978). Recent work has been encouraged by the demonstration that suppressor T-cells could inhibit a cytotoxic T-cell response in a tumor bearing host and therefore by controlling the suppressor T-cell population, immunotherapeutic response would be facilitated (Mills & North, 1983). In addition, exposure of T-cells to interleukin 2 in vitro could produce large numbers of cytotoxic T-cells (Morgan, Ruscetti & Gallo, 1976) that

would be required for successful adoptive immunotherapy, and using this technique, Rosenberg has demonstrated that lymphocytes obtained from patients with cancer have antitumor activity in vitro following expansion of cytotoxic T-cell clones by exposure to interleukin 2 (1982). Although it is still too early to assess the practical use of such findings, it is quite possible that such techniques would enable the production of large numbers of cytotoxic T-cells to be obtained from individual patients, which could then be used as adoptive immunotherapy to treat the donor patient's tumor.

The role of the non-specific cell mediated immune response is even more speculative and there is no generally accepted view regarding the importance of each cell line proposed in this capacity. The macrophage however appears to have several roles, including an antigen presentation function, as the suggested effector cell mediated specifically through the IgG2a monoclonal antibody (Herlyn et al, 1982) and apparently also a non-specific cytotoxic function (Mastrangelo et al, 1984). Its activation by *Bacillus Calmette-Guerin* (BCG) and *Corynebacterium Parvum* (*C. Parvum*) has led to trials of these agents being used in cancer therapy. In summary it appears as if systemic BCG administration is ineffective in systemic macrophage activation, and this translates into no overall effect upon tumor growth in experimental animal models. However, although its local application has been demonstrated to produce tumor regression in some settings, such as in the treatment of malignant melanoma (Morton, Eilber, Holmes, Hunt, Ketcham, Silverstein & Sparks, 1974) and intra-pleurally for lung cancer (McKneally, Maver & Kausel, 1976), it has never convincingly been demonstrated that this is a result

of specific macrophage activation.

Natural killer cells have been proposed as one of the principle cell types responsible for host resistance to tumors or metastases in a normal individual, although there is still no conclusive supporting evidence of this (Baldwin, 1984). In vitro they appear to exhibit cytotoxicity but to what extent this is important in vivo is not known. Present research is directed at ways of augmenting natural killer cell activity, including the role of interferons, in an attempt to suppress tumor growth.

In summary, the role of immunotherapy in the treatment of cancer remains controversial and is to a great extent unproven. The availability of monoclonal antibodies has not only provided a renewed interest in this approach but has also helped to answer fundamental questions regarding tumor immunology and regulation of the immune system, although this too is far from complete. Additionally, interest is also being focused upon the role of the cellular components of the immune system in tumor cell killing but this area of immunotherapy is even more speculative at present.

I 5.0 IMMUNODIAGNOSIS

Radiolabelled monoclonal antibodies have also been used for imaging which, in addition to immunohistochemical and binding studies, provides indirect supportive evidence of antibody localisation at target tumor sites, and also offers potential as a new diagnostic modality in its own right. As this targeting system has now seen use in patients, it is hoped that experience of administering such antibody-radioisotope conjugates may well define problems that may arise when antibody drug conjugates are subsequently administered for therapy. For example, this approach has already provided interesting information on the percentage of intravenously administered antibody that actually localises in tumors, which may have great relevance to drug targeting systems.

As with other antibody targeting systems, early investigators utilised polyclonal antibodies raised against tumors. In 1953, Pressman reported that radiolabelled rabbit antibodies to the mouse Wagner osteogenic sarcoma localised in mice bearing these implanted tumors, although localisation in several of the normal tissues such as liver and kidney was also noted (Pressman & Korngold, 1953). Such early studies emphasised the requirements of high specificity of the antibody carrier and the presence of tumor associated target antigens. Despite the similar limitations of polyclonal antibodies referred to in preceding sections, they have successfully been used to localise tumors both in laboratory animal models and in patients, with over 80 percent accuracy being reported (Goldenberg, DeLand, Kim, Bennett, Primus, van Nagell, Estes, DeSimone & Rayburn, 1978; Mach, Carrel, Forni, Ritschard, Donath &

Alberto, 1980; Dykes, Hine, Bradwell, Blackburn, Reeder, Droic & Booth, 1980). Although CEA was the target antigen for these studies, other groups have utilised antibodies raised against alpha-fetoprotein (Kim, DeLand, Nelson, Bennett, Simmons, Alpert & Goldenberg, 1980), human chorionic gonadotrophin (Goldenberg, Kim, DeLand, van Nagell & Javadpour, 1980) and for imaging metastases of renal cell cancer (Belitsky, Ghose, Aquino, Tai & MacDonald, 1978).

Estimates of the proportion of injected antibody that actually localises in tumors range from 0.01 to 2.6% (Mach, Carrel, Forni, Ritschard, Donath & Alberto, 1980; Epenetos, Britton, Mather, Shepherd, Granowska, Taylor-Papadimitriou, Nimmon, Durbin, Hawkins, Malpas & Bodmer, 1982). This, in addition to antigen present within the circulation and in normal tissues, accounts for high background levels of radiation detected when conventional scanning procedures are used to image the distribution of labelled antibody. This has largely prevented its widespread clinical evaluation as a diagnostic tool and rapid developments in the other diagnostic modalities of CT scanning and ultrasound at the same time have diminished some investigators' enthusiasm to pursue this diagnostic approach.

In order to overcome these problems, a number of further approaches were explored including the use of monoclonal antibodies, fragments or combinations of antibodies to increase specificity, improved computerised subtraction scanning techniques, the administration of second antibodies to promote clearance of the primary labelled antibodies, and the use of radionuclides with improved imaging characteristics such as Indium-111 or

Iodine-123.

In vivo experiments using radiolabelled monoclonal antibodies have demonstrated localisation of human tumor xenografts in several model systems (Houston, Nowinski & Bernstein, 1980; Moshakis, McIlhinney, Raghavan & Neville, 1981; Stuhlmiller, Sullivan, Verlaert, Coker, Harris & Seigler, 1981; Epenetos et al, 1982; Colcher, Zalutsky, Kaplan, Kufe, Austin & Schlom 1983; Smedley, Finan, Lennox, Ritson, Takei, Wraight & Sikora, 1983) and many of these studies have now seen clinical evaluation. Following the demonstration of monoclonal antibody localisation in HT29 (colorectal) xenografts in mice, 500 micrograms of labelled monoclonal antibody (Iodine-131) was administered to 45 patients with colorectal cancers with good correlation between the extent of clinical disease and scanning after 48 hours (Smedley et al, 1983). Similarly, a monoclonal antibody raised against an osteogenic sarcoma cell line has also been shown to localise in a number of tumors including colorectal primaries, and has been investigated in 60 patients of whom 4 had benign colorectal lesions and 17 non-colonic gastrointestinal cancers (Farrands, Perkins, Pimm, Hardy, Embleton, Baldwin & Hardcastle, 1982; Armitage, Perkins, Pimm, Farrands, Baldwin & Hardcastle, 1984). In this study, 200 micrograms of I-131 labelled antibody was administered and imaging performed between 48 and 72 hours post injection. Correlation was attempted between results obtained from scanning and counts obtained from resected specimens of patients who were treated surgically. Localisation was shown for all 29 patients with primary colorectal cancer in addition to successful imaging of metastatic deposits in 13 of 15 patients with disseminated disease. No positive scans were

obtained for the patients with benign disease or for 10 of the patients with non-colonic gastrointestinal malignancies. Although encouraging results have been obtained from such studies, little is known about the actual mechanism of localisation of such antibodies, and further investigation into the nature, extent, and distribution of the target antigen is warranted.

By contrast, studies targeting tumors expressing relatively well characterised antigens such as CEA may provide more information. Of particular interest are studies utilising the highly specific anti-CEA monoclonal antibody 11-285-14. I-131 labelled 11-285-14 has been shown to localise in nude mice bearing CEA expressing human tumor xenografts (MacDonald, Lazenby, Allum & Fielding, 1985) in addition to current evaluation in patients as a guide to second look laparotomy and in the staging of gastrointestinal and pancreatic tumors (Allum, Anderson, MacDonald & Fielding, 1985a, 1985b).

A number of investigations have utilised radiolabelled $F(ab')_2$ fragments to enhance localisation in tumor tissue, and this has been confirmed in studies with mice xenografted with human tumor cells (Herlyn, Powe, Alavi, Mattis, Herlyn, Ernst, Vaum & Koprowski, 1983). Although it has been suggested that such fragments may show greater intrinsic selective and specific binding compared to the intact antibody, other factors such as increased clearance from the blood may also account for the better imaging as there appears to be less complexing of these $F(ab')_2$ conjugates with circulating antigen, with subsequently less uptake by reticuloendothelial cells in liver, lung, and spleen.

Localisation of a ^{131}I labelled monoclonal antibody, which was raised against a colonic tumor antigen, in a study of 52 patients with colorectal adenocarcinoma, yielded positive scans in 51 percent of cases, with localisation confirmed from assessment of radioactivity in resected specimens where the average antibody concentration varied from 3.6 to 6.3 times higher than in normal tissue (Mach, Chatal, Lumbroso, Buchegger, Forni, Ritschard, Berche, Douillard, Carrel, Herlyn, Stepelwski & Koprowski, 1983). Although only one third of patients received labelled F(ab')_2 fragments, the yield of positive scans obtained was only marginally increased to 61 percent, although it was not stated whether this was a statistically significant difference.

In order to overcome the problem of nonspecific uptake of isotopes, which creates the high background radioactivity in the circulation and reticuloendothelial tissues, Goldenberg utilised a second labelled antibody to image the blood pool and using a computerised subtraction technique, improved the ratio of tumor detection (Goldenberg, Kim, DeLand, Bennett & Primus, 1980). However, further evaluation of this technique has suggested that precise background correlation is required to prevent false negative or false positive results and that, with a low tumor/background count ratio, the technique is quite unreliable (Ott, Grey, Zivanovic, Flower, Trott, Moshakis, Coombes, Neville, Ormerod, Westwood & McCready, 1983). Despite these limitations, some variation of this computerised subtraction technique has been adopted by most investigators in this field.

Other methods aimed at reducing background radioactivity have

included the use of second antibodies directed against the primary radiolabelled antibody in order to accelerate its clearance from the circulation. These may be administered in the form of liposomally entrapped second antibodies (Begent, Keep, Green, Searle, Bagshawe, Jenkes, Jones, Barratt & Ryman, 1982) or directly as free antibodies into the circulation (Sharkey, Primus & Goldenberg, 1984). The latter approach was investigated in hamsters bearing human colonic xenografts and was found to enhance the clearance of the primary antibody without subsequent concentration of radioactivity in the liver and spleen, as was noted using liposomally entrapped second antibodies. The clearance of the labelled antibody from the tumor was not seen until 24 hours after injection of the second antibody, although maximum association of the primary antibody with the tumor may not have occurred as a result of its rapid clearance from the circulation.

The studies outlined in this section have attempted to demonstrate antibody localisation at target tumor sites, which as indicated earlier, provides only indirect evidence that other antibody conjugates will similarly localise. However, studies have also directly addressed the problem of antibody drug conjugate localisation by labelling either the antibody or drug directly with radioisotope.

Using a I-131 labelled polyclonal anti-CEA antibody-vindesine conjugate, 8 patients with advanced metastatic ovarian and colorectal cancers were investigated for conjugate localisation (Ford, Newman, Johnson, Woodhouse, Reeder, Rowland & Simmonds, 1983). Positive scans, using subtraction technique, were obtained for 5 patients up to 48

hours following the administration of the labelled conjugates suggesting that drug conjugation does not impair the ability of antibodies to localise in vivo, and complementary in vitro studies using ELISA confirmed the anti-CEA binding activity of the antibody following conjugation.

Although immunoradiodiagnosis represents an area of research in its own right, it is hoped that information obtained from studies similar to those described in this section may have relevance to the targeting of antibody drug conjugates when used for therapy.

I 6.0 WARHEADS

I 6.1 Introduction

From the previous section, it appears as if antibodies, especially monoclonal antibodies, offer the greatest potential as target specific delivery agents in order to increase the selectivity of the number of cytotoxic agents, including toxins; radionuclides, which may be used for immunoradiodiagnosis in addition to immunoradiotherapy; anti-cancer drugs, and a variety of other cytotoxic agents such as venom and phospholipases. Such warheads will be discussed in the following section, with the exception of anti-cancer drugs which will be considered under immunochemotherapy, section I 7.0.

I 6.2 Immunotoxins

The advantages conferred upon a targeting system by the use of toxins as warheads result from their extreme potency, which theoretically offers the greatest chance of a total cell kill of malignant tumor cell populations that are either inaccessible to conventional drugs or lack sufficient cell surface antigen density for immunotherapy or immunochemotherapy to be expected to be beneficial (Thorpe, Edwards, Davies & Ross, 1982; Raso, 1982; Sikora, Snedley & Thorpe, 1984). However, the potential for non-specific toxicity also exists using such molecules, emphasizing the need for highly specific delivery agents. Although such immunotoxins have demonstrated selective cell killing in in vitro models, there have been relatively few in vivo studies supporting this to date, and fears of non-specific toxicity have also precluded their use in the

clinical setting to date. ———

A relatively limited number of toxins have been investigated for potential conjugation to antibodies in targeting systems, and these include diphtheria toxin, and the plant toxins abrin and ricin, in addition to other plant-derived polypeptides which although lacking in potency appear to exhibit some degree of "toxin-like" effect in vitro (Thorpe et al, 1982; Sikora et al, 1984).

The structure of the toxins of diphtheria, abrin and ricin is essentially similar, consisting of two polypeptide chains, A and B, each having specific functions, and linked through a disulphide bond. Toxin interaction with cells is mediated through the B chain, which binds to ubiquitous cellular receptors, which for abrin and ricin have been identified to be any surface carbohydrate chain terminating in a galactose residue (Baenziger & Fiete, 1979). This receptor mediated interaction is responsible for entry of the A chain into the cell, although the precise mechanism is still unclear. It is thought that cytotoxicity arising from the A chain entry occurs from its interaction with ribosome components, thereby inhibiting protein synthesis and it has been estimated that the entry of one molecule into a cell is sufficient to kill it (Yamaizumi, Mekada, Uchida & Okada, 1978). A similar A chain effect has been seen with the polypeptide gelonin isolated from the seeds of *Gelonium multiflorum*, which inhibits isolated erythrocyte ribosomes with similar potency to other plant toxins, although its low overall in vivo potency arises from the absence of a B chain (Stirpe, Olanes & Pihl, 1980).

The properties of antibody toxin conjugates are essentially similar to those required of any conjugates used for targeting purposes, namely retention of antibody target antigen binding ability, stability of the linkage in vivo, maintenance of warhead potency and the ability of the conjugate to allow the warhead to exert its action at the target site without interference. Early conjugates using coupling agents such as glutaraldehyde resulted in polymerisation of the conjugate with consequent loss of toxin potency, thought to be due to the formation of irreducible bonds between the toxin A and B chain. Subsequent improved methods of conjugation using coupling agents such as the anhydride or ester derivatives of chlorambucil, "SPDP" reagent (N-succinimidyl 3-(2-pyridyldithio) propionate), or following oxidation of B chain residues into aldehydes capable of reacting with amino groups on the antibody molecule, have proved to be successful for the production of active immunotoxin conjugates (Carlsson, Drevin & Axen, 1978; Thorpe, Ross, Cumber, Hinson, Edwards & Davies, 1978; Thorpe et al, 1982). These methods have now allowed a number of available toxins to be linked to polyclonal and monoclonal antibodies and even to hormones (Chang, Dazord & Neville, 1977).

Initial studies performed in the early 1970's using immunotoxins demonstrated selective cytotoxicity of cells bearing viral or chemically-induced antigens in vitro, when treated with a conjugate of intact diphtheria toxin and antibody raised against these induced antigens (Moolten & Cooperband, 1970; Philpott, Bower & Parker, 1973a). Encouraging results from early animal studies were also obtained as it was demonstrated that survival of hamsters bearing subcutaneous sarcoma tumors expressing chemically-induced antigens, could be prolonged by

treatment with similar conjugates comprising intact diphtheria toxin and antibody directed to tumor antigens (Moolten, Capparell & Cooperband, 1972). However there is presently little evidence that such conjugates can act upon human tumors (Moolten, Schreiber & Zajdel, 1982).

Immunotoxin conjugates utilising intact toxins inevitably show considerable non-specific toxicity in vivo as a result of binding to normal cells through intact B chains, in addition to exhibiting a high degree of potency. Attempts to reduce these undesirable toxic properties of abrin and ricin conjugates in vitro, have utilised free galactose or lactose in culture to block non-specific binding, by competition with cellular receptors for the available B chains (Thorpe, Umber, Williams, Edwards, Ross & Davies, 1981; Sikora et al, 1984). Alternatively, the use of monoclonal antibodies directed at the binding site on the B chain would also prevent non-specific toxicity without affecting efficacy (Sikora et al, 1984).

Direct conjugation of the effector toxin A chain to antibodies or their fragments, has also been performed in order to increase selectivity, although in general potency of such conjugates is much less than that seen using the intact toxin. Immunotoxins formed from coupling the A chains of diphtheria or ricin toxin to rabbit Fab fragments directed against the Ll210 murine leukemia have been demonstrated to show selective cytotoxicity of target Ll210 cells in vitro although potency was diminished (Masuho & Hara, 1980). Selective cytotoxicity of immunotoxins comprising ricin A chain and a polyclonal goat antibody raised against the human tumor associated antigen, CEA, has also been demonstrated for

colonic adenocarcinoma cell lines expressing this antigen (Griffin, Haynes & DeMartino, 1982). In vivo studies using ricin A chain and intact anti-tumor antibodies have also convincingly been shown to reduce tumor growth in mice (Jansen, Blythman, Carriere, Casellas, Gros, Gros, Laurent, Paolucci, Pau, Poncelet, Richer, Vidal & Voisin, 1982).

The use of conjugates of gelonin have been investigated in attempts to reduce toxicity while maintaining selective action, and it has been demonstrated, in common with A chain conjugates, that potency of such immunotoxins is also reduced (Thorpe et al, 1982).

Vitetta has demonstrated that separate delivery of ricin A and B chain antibody conjugates confers advantage in cell killing, even when A chain conjugates have been demonstrated to be ineffective alone (Vitetta, Cushley & Uhr, 1983). It remains, however, to be seen whether this synergism seen between conjugates in vitro, can be reproduced in the in vivo animal model. A further approach to improve efficacy of ricin A chain immunotoxins has utilised the addition of standard chemotherapeutic agents (doxorubicin and cyclophosphamide) to the treatment regime of mice with L1210 leukemia. Combined immunotoxin and chemotherapy was shown to be more effective in prolonging survival of mice than with either treatment modality alone (Sironi, Canegrati, Romano, Vecchi & Spreafico, 1984).

In summary, the efficacy of immunotoxins has been well demonstrated in vitro, although problems related to the intrinsic potency of intact toxins have emerged from the relatively limited numbers of animal

studies performed. Methods of increasing selectivity which have been considered include increasing the specificity of the carrier in addition to reducing non-specific binding of toxins to normal cells through their B chains, or direct coupling of the A chain to a carrier. The latter, however, has in general also resulted in reduction of potency and selectivity of these conjugates must further be defined in experimental models prior to clinical application.

I 6.3 Radionuclides

Although radiation therapy is a well established treatment modality for certain cancers, the systemic administration of radioisotopes has found limited application. One particular exception is in the treatment of thyroid cancer where the selective uptake of radioactive iodide by thyroid tissue, both malignant and normal, is exploited. The concept of conjugating radionuclides to antibodies directed against tumor associated antigens has the potential for increasing the range of tumors that may be treated using radioisotopes in addition to the selective targeting of these agents to radiosensitive tumor cell populations. In addition to this therapeutic approach (immunoradiotherapy) radionuclides have also been conjugated to antibodies for the purpose of diagnosis (immunoradiodiagnosis) as has been discussed in other sections.

As with all potential targeting systems, there are a number of apparent advantages and disadvantages in using high energy radioisotopes as the warhead. Isotopes emitting alpha particles such as Bismuth-212 and Lead-212, have been used infrequently owing to their

general instability, although Astatine-211, an almost pure alpha emitter, has successfully been conjugated to a monoclonal antibody (Bloomer, Lipsztein & Dalton, 1985), and therefore attention has been focused upon such beta emitters as Iodine-131 or Phosphorus-32 (Sikora et al, 1984). The ability of beta radiation to penetrate several cell layers is of advantage in treating tumors with poor vascularity and generally inaccessible target cells. The antigenic heterogeneity of a tumor cell population is theoretically less of a problem using this approach as it would require relatively few cells expressing antigen to target the radioisotope conjugate to the tumor where a cytotoxic effect would be exerted upon surrounding cells regardless of antigen expression. The major disadvantage of this approach is that of non-specific irradiation of normal tissues during the distribution of the conjugate throughout the body, an intrinsic property of the warhead independent of the specificity of the carrier.

Preliminary studies investigating the potential of immunoradiotherapy in treating human colonic tumor xenografts in an animal model have been encouraging, the target antigen being CEA (Goldenberg, Gaffar, Bennett & Beach, 1981). Polyclonal goat anti CEA immunoglobulin, conjugated to I-131, still retaining CEA binding activity, was used to treat hamsters bearing CEA expressing tumor xenografts and a single injection of ^{131}I I-131 was shown to inhibit tumor growth in addition to increasing survival of hamsters receiving conjugate.

Such an approach has also been applied to treat a limited number of patients for whom conventional treatment has failed (Order, Klein,

Ettinger, Alderson, Siegelman & Leichner, 1980). Based on the ability of radiolabelled antibodies to localise in antigen expressing tumors both in animals and in patients, conjugates of anti-CEA or anti-ferritin antibodies and I-131 were administered to 9 patients. Significant tumor regression (CT scan analysis) was seen in 3 of 4 patients with hepatic cancers within one month of treatment, whereas no therapeutic effect was seen in 5 patients with non-hepatic tumors (colon, lung, floor of mouth), when up to 100 mCi of radiolabelled antibody was administered in addition to conventional cancer treatment (combined irradiation and chemotherapy). Only one patient treated with radioimmunoglobulin developed fever, suggesting relative lack of toxicity associated with this treatment administered at these doses.

In order to prevent potential systemic toxicity, a I-131 labelled monoclonal antibody directed against a tumor associated antigen has been administered by the intracavity route (intrapleurally, intrapericardially and intraperitoneally) in three patients (Hammersmith Oncology Group, 1984). Selective delivery of radiation to malignant tissues was demonstrated with evidence of pericardial tumor regression on CT scan seen in one patient, and absence of tumor cells from pleural or peritoneal fluid being found following treatment in the remaining patients. Absence of toxicity was also noted following this treatment.

Such studies suggest the potential of targeting radionuclides to tumor cells for therapy, and encouraging results are apparent from the limited numbers of patients treated using this approach. A further approach to immunoradiotherapy has been investigated by Goldenberg who

used an anti-CEA antibody to target Boron-10 to CEA expressing human colonic xenografts in hamsters where selective localisation was demonstrated in these tumors. The rationale for using Boron-10 lies in its ability to absorb thermal neutrons with the subsequent release of alpha particles, providing a practical application of neutron capture therapy suggested by Bale over 30 years ago (Goldenberg, Sharkey, Primus, Mizusawa & Hawthorne, 1984). Further reports of the efficacy of such approach in the animal models are to be expected as this technique is investigated further.

I 6.4 Drugs

In comparison to the range of alternative warheads described, a relatively large number of anti-cancer drugs have been utilised in a variety of targeting systems, including adriamycin, methotrexate, chlorambucil, vindesine, daunomycin, bleomycin, cisplatin, neocarzinostatin, phenylene diamine-mustard, and the range is expected to increase as new analogues are developed. This approach may well reflect the opinion of several investigators that drugs currently represent the most clinically relevant warheads as they have generally already seen widespread clinical use and much information on the kinetics, distribution and toxicity of the free drugs is available providing a useful baseline for the assessment of conjugate efficacy. By contrast, the limited information available on the in vivo effect of toxins has delayed the administration of immunotoxin conjugates to patients. However, whereas the potency of toxins and radionuclides make these agents appealing for targeting, it has been suggested that a relatively larger number of drug

molecules would require delivery to target cells in order to produce a comparable effect. Although the requirements of antibody drug conjugates will be discussed in following sections it is apparent that the use of the most highly potent of these agents would be advantageous. This underlies the rationale for the use of the vinca alkaloids in the studies in this thesis, which on a molar basis are the most potent anticancer drugs in clinical use. Aspects of the vinca alkaloids will now be discussed.

1 6.5 Vinca Alkaloids

Vinca rosea or common periwinkle, has seen widespread use by herbalists for several years, especially for the healing of chronic wounds, in scurvy, and for the relief of toothache, although its anti-neoplastic potential was not discovered until the 1960's, when investigations into its alleged hypoglycemic properties were undertaken by Lilly (Bender, 1981). Of 70 alkaloids subsequently extracted from this plant, nine are cytotoxic and of these, vinblastine and vincristine have been used widely in the drug treatment of cancer. They have been found to be particularly useful in the treatment of haematological malignancies, although activity against many solid tumors has also been reported, including Breast cancer, testicular cancer, renal cancer, malignant melanoma, and sarcomas.

The molecular structure of these alkaloids is essentially similar, consisting of two linked multi-ringed compounds, vindoline and catharanthine, which in isolated preparations appears to have no anti-

tumor activity (Bender, 1981). Vindesine (desacetyl vinblastine amide), one of two semi-synthetic derivatives of vinblastine, appears to have a different spectrum of anti-tumor activity and on a milligram per kilogram basis, exhibits less toxicity than the other alkaloids, making it an attractive agent for preliminary clinical trials (Creasey, 1981). It has been shown to be active in patients previously unresponsive to vincristine, suggesting lack of cross resistance between these agents (Valdivieso, Richman, Burgess, Bodey & Freireich, 1981).

At the molecular level, it has been demonstrated that these agents require an energy-dependent active transport system in order to cross the cell membrane to enter the cell (Bleyer, Frisby & Oliverio, 1975), and it has been suggested that receptors for the vinca alkaloids, in particular vindesine, may be present on certain human leukaemia cell lines (Totsuka, Oshimi & Mizoguchi, 1982). Once free within the cytoplasm, these agents are thought to exert their effect upon tubulin, inhibiting its polymerisation, and therefore preventing the formation of microtubules, essential for several cellular functions including mitosis, structural integrity and transport of solutes (Bender & Hamel, 1984). A notable feature is that very low concentration of drugs are required to inhibit microtubule formation and hence produce cytotoxicity (Jackson & Bender, 1979) and it is now apparent that cells may be killed at any phase of the cell cycle, although they appear to be most susceptible during the late S phase (Stohr & Fischinger, 1978). In addition to this direct cytotoxic action the alkaloids also cause delay of proliferating cell populations at G₂ and it has been suggested that they may be incorporated into chemotherapy regimens for synchronising tumor cell

populations, with additional agents being used to achieve maximum cell kill at the phase preceeding the block (Hill & Whelan, 1980). Within the kinetic classification of anti-tumor drugs, the vincas are regarded as class II (cell cycle phase-specific) agents.

The toxicity of the vincas appears to vary between individual agents, with neurotoxicity occurring in greater than 30 percent of patients receiving up to 2 milligrams of vincristine as a single dose, but being rarely seen with vinblastine or vindesine (Creasey, 1981). Although dose dependent, these neuropathies (motor, sensory, autonomic) are often irreversible. By contrast, it is myelosuppression that is the limiting toxicity of vinblastine, with a reversible leukopenia and thrombocytopenia seen in greater than 30 percent of patients within 10 days of treatment.

The small doses in which these drugs are used, largely precluded pharmacokinetic studies of these agents until a sensitive radio-immunoassay was developed (Owellin, Root & Hairs, 1977; Sethi, Burton & Jackson, 1980). The pharmacokinetic patterns for the alkaloids appears essentially similar, with rapid drug distribution into extracellular compartments from plasma and biliary excretion following hepatic metabolism of these drugs. Extensive reversible binding to blood elements has also been noted, in the order of greatest binding to plasma proteins, platelets, red and white blood cells (Bender et al, 1984). The relatively short plasma half-life of these agents has stimulated several studies to investigate the value of long term infusion of these drugs, especially for vinblastine and vindesine, which as mentioned, are less limited by neurotoxicity (Bender et al, 1984), and encouraging responses have been reported in patients with advanced breast cancer (Yap, Blumenschein,

Mertobagyi, Tashima & Loo, 1979). Studies by Mathe similarly suggested the value of infusion of vindesine over bolus injection for haematological malignancies (Mathe, Misset, DeVassal, Gouveia, Hayat, Machover, Belpomme, Pico, Schwarzenberg, Ribovo, Musset, Jasmin & DeLuca, 1978), and similar although preliminary studies have also confirmed the value and relative safety of this approach (Bodey, Yap, Yap & Valdivieso, 1980; Gilby, 1980).

The use of vindesine in the treatment of solid tumors is being evaluated further by many phase II studies, and encouraging results of its efficacy in the treatment of malignant melanoma (Carmichael, Atkinson, Calman, Mackie, Naysmith & Smyth, 1982), advanced breast cancer (Walker, Raich, Fontana, Subramanian, Rogers, Knost & Denning, 1982) and non-small cell lung cancer (Furnas, Williams, Einhorn & Cobleigh, 1982) have been reported, although its use in the treatment of renal cancer has not been recommended (Fossa, Denis, van Oosterom, DePauw & Stoter, 1983). Although these studies have utilised the drug as a single agent given weekly by intravenous bolus injection, it has also been successfully used in several multi-drug regimens, and even pre-operatively, with cisplatin and bleomycin, for the treatment of carcinoma of the oesophagus (Bains, Kelsen, Beattie & Martini, 1982).

I 6.6 Miscellaneous Warheads

Although the major classes of warheads investigated in targeting systems have included toxins, drugs and radionuclides, a limited number of other cytotoxic agents have also been reported for use in this setting.

A covalent complex comprising a murine monoclonal antibody, raised against a human melanoma associated antigen, and the C3b-like glycoprotein of cobra venom, was shown by Vogel to exhibit selective cytotoxicity against melanoma cells when mixed with other cell lines, in the presence of serum, determined by a ^{51}Cr -release cytotoxicity assay (Vogel & Eberhard, 1981). The active venom factor, itself non-toxic, is thought to be targeted to cells expressing appropriate antigens, which in the presence of complement factors of the alternative pathway within the serum, cause cell lysis. An advantage of this approach is that the conjugate is active at the surface of target cells and does not require internalisation. Reports regarding the efficacy of this approach in the animal model are awaited however.

The targeting of the enzyme glucose oxidase to tumor cell populations has also been performed, the rationale of this approach being that free radicals formed from reaction products between peroxidases and compounds such as iodide, arspenamine or luminol will cause selective cell killing (Philpott, Bower & Parker, 1973b; Philpott, Bower, Parker, Shearer & Parker, 1974; Parker, Aach & Philpott, 1975). Similarly the targeting of the enzyme phospholipase C in vitro has been shown to lyse target leukaemic cells without killing normal spleen cells (Flickinger & Trost, 1976).

I 7.0 IMMUNOCHEMOTHERAPY

The criteria alluded to throughout this chapter regarding targeting systems and their components are equally applicable to the targeting of drugs to tumors: immunochemotherapy.

These requirements may be summarised as follows: one, that stable conjugates can be prepared, using covalent linkage between the cytotoxic drug and its antibody carrier. Two, that the properties of each component are retained following conjugation. Three, that specificity of the conjugate relates to the specificity of the antibody carrier. Four, that the cytotoxic drug must be sufficiently potent, and must retain activity at the target site.

Similarly, suggested limitations of other targeting systems are also relevant to immunochemotherapy and many questions remain unanswered. These include the ability of such conjugates to reach target cell populations by virtue of vascularity of tumors or their ability to escape from the blood vessels; the mechanism of action of these conjugates, whether they dissociate in the region of the tumor or require internalisation of the conjugate in order to exert their action; the effect of circulating antigens in reducing conjugate efficacy, the role of antigenic heterogeneity and the possibility of the host developing an immune response to such conjugates.

With the widespread use of monoclonal antibodies, targeting with drugs may well prove to be a practical additional mode of cancer chemo-

therapy and the present status of immunochemotherapy will now be discussed.

The modern era of targeted chemotherapy began with a report by Mathe in 1958 (Plate 2), of the effect of an antibody drug conjugate of a murine leukemia (Mathe, Loc & Bernard, 1958). Antibodies specific for the L1210 leukemia were coupled by diazotisation to methotrexate and the resulting conjugate used to treat mice inoculated with the leukaemia. A significant lengthening of survival was found in the group of mice treated with a single bolus injection of conjugate compared to those treated with drug or antibody alone. These encouraging results were followed by other reports of drug targeting in the 1970's.

Ghose utilised a conjugate of chlorambucil and antibody against Ehrlich Ascites Tumor to demonstrate suppression of tumor growth and survival of mice treated with the conjugate, compared to mice treated with free antibody or with chlorambucil bound to a non-specific protein (Ghose & Nigam, 1972). The method of conjugation involved adsorbing the drug physically to the antibody in the cold, resulting in the formation of a non-covalent complex. Using this method, the alkylating properties of chlorambucil were retained and antibody activity demonstrated by immunofluorescence on smears of Ehrlich Ascites Carcinoma Cells. However, despite the convincing anti-tumor effect seen in these experiments, the use of non-covalent bound antibody drug conjugates as a drug targeting system has been criticised following the demonstration of complex dissociation in vivo (Davies & O'Neill, 1973; Rubens & Dulbecco, 1974). Therefore, despite lack of true "homing" or targeting of such

ÉDECINE EXPÉRIMENTALE. — *Effet sur la leucémie 1210 de la Souris d'une combinaison par diazotation d'A-méthoptérine et de γ -globulines de hamster porteurs de cette leucémie par hétérogreffe.* Note de MM. **GEORGES MATHÉ**, **TRAN BA LOC** et **JEAN BERNARD**, présentée par M. Léon Binet.

Il est montré que la combinaison par diazoreaction d'un antimétabolite tel que l'A-méthoptérine avec les γ -globulines de hamsters porteurs de leucémie 1210 par hétérogreffe a une activité sur la leucémie 1210 nettement supérieure à l'A-méthoptérine ou aux γ -globulines seules ou à l'addition des deux (non combinés).

Plate 2. Photograph of Title and Abstract of Mathe's
1958 paper.

conjugates in these studies, it was suggested that the additive or synergistic effects of drug and antibody demonstrated, likely resulted from antibody destroying drug affected cells (Davies et al, 1973).

Using a similar non-covalent chlorambucil anti-tumor antibody conjugate, Ghose also demonstrated suppression of growth of EL4 lymphoma in mice compared to groups treated with the antibody alone (immune rabbit globulin), chlorambucil alone, or chlorambucil bound to non-immune rabbit globulin (Ghose, Norvell, Guclu, Cameron, Bodurtha & MacDonald, 1972). However, these experiments did not include a group of mice treated with a mixture of unbound drug or antibody, an important control if the dissociation of non-covalent complexes does indeed occur in vivo.

Despite these criticisms, the paper does also report the use of a conjugate in the treatment of a patient with metastatic malignant melanoma, who, despite surgery, chemotherapy and direct BCG injection of metastatic cutaneous nodules, showed no evidence of tumor regression until treated with the intravenous chlorambucil-goat antimelanoma antibody conjugate.

The method of coupling drugs to antibodies to form covalent complexes is therefore of importance, and although Mathe's original paper (1958) referred only fleetingly to the process of diazotisation, reports as early as 1964 attempted to define the chemical problems of coupling such agents as methotrexate to immunoglobulin molecules (DeCarvalho, Rand & Lewis, 1964). The range of chemical reactions has been widened to include, in addition to diazotisation, carbodiimide condensat-

ion between carboxyl and amino groups, glutaraldehyde coupling between amino groups, oxidation of sugar residues and ester formation of carboxyl groups (Rowland, In Press). It was thought that direct conjugation of drugs to immunoglobulin molecules would limit the efficacy of such conjugates as a result of physicochemical changes induced in the antibody and the inability to link sufficient drug molecules (Rowland, O'Neill & Davies, 1975). Therefore in order to minimise interference with the antibody, and increase the number of associated drug molecules (i.e. conjugation ratio), inert intermediate carriers were employed, such as polyglutamic acid. p-Phenylenediamine mustard complexed via polyglutamic acid to rabbit immunoglobulin against murine EL4 lymphoma cells was shown to prolong the median survival time of mice inoculated with the EL4 lymphoma (Rowland et al, 1975).

Conjugates obtained from the direct coupling of desacetyl vinblastine hydrazide, a precursor of vindesine, to polyclonal anti-CEA antibodies have been evaluated for targeting efficacy *in vitro*, in a 72 hour exposure microcytostasis assay, using Calu-6, a human tumor lung cell line as the target (Johnson, Ford, Newman, Woodhouse, Rowland and Simmonds, 1981). The results clearly demonstrated the efficacy of such conjugates over free antibody, drug, or a mixture of both in this system. This radiolabelled conjugate has also been administered to patients with advanced cancers and localisation has been confirmed with 5 of the 8 patients studied (Ford, Newman, Johnson, Woodhouse, Reeder, Rowland & Simmonds, 1983). In addition, no obvious toxicity was seen even with the administration of up to 42 mg of a single dose of conjugated antibody, suggesting the feasibility of this approach.

Such studies stimulated further work into the coupling of the vinca alkaloids to monoclonal antibodies, and covalent complexes were prepared between the drug and the anti-CEA monoclonal, 11-285-14; anti-melanoma antibody, 96.5 (Brown, Woodbury, Hart, Hellstrom & Hellstrom, 1981); anti-neuroblastoma antibody, U13A (Kemshead, Fritschy, Asser, Sutherland & Greaves, 1982); and anti-osteogenic sarcoma monoclonal, 791T/36 (Embleton, Gunn, Byers & Baldwin, 1981). In general two methods of conjugation have been employed; azide conjugation and active ester conjugation, the latter being more efficient in addition to achieving higher conjugation ratios (Rowland, In Press).

The efficacy of these conjugates in suppressing tumor growth has been investigated in in vivo systems utilising the antibodies mentioned above. Of particular interest are targeting studies using the CEA expressing colorectal tumor MAWI, maintained by serial passage as xenografts in nude mice (Rowland, Corvalan, Axton, Gore, Marsden, Smith & Simmonds, 1984). Mice bearing these xenografts were treated, on the day of tumor inoculation, with free antibody (175 mg/kg-1 twice weekly for five weeks) and conjugates of 11-285-14 and vindesine (6 mg/kg-1 with respect to vindesine concentration using a similar schedule), the latter resulting in suppression of tumor growth for the 90 days of the experiment, whereas free antibody had only slight effect. Parallel experiments using the anti-CEA monoclonal 14-95-55 (an IgG2a) and its vindesine conjugate, also confirmed conjugate efficacy, although suppression of tumor growth was also obtained with free antibody.

Drug targeting studies have also been pursued by other investigators along similar lines, and a recent comprehensive review summarises experience in vitro and in vivo with conjugates of polyclonal and monoclonal antibodies directed against rat tumor antigens, and alpha-fetoprotein, using daunomycin and its hydroxy-derivative adriamycin (Arnon & Sela, 1982). Targeting of daunomycin with anti-CEA polyclonal antibody conjugates has also been demonstrated in vitro using human tumor cell lines, where conjugate efficacy was also found to be greater than that of free drug, antibody, or a mixture of both (Belles-Iles & Page, 1981).

Adriamycin conjugated to anti-Sp 4 monoclonal antibodies, shown to localise in vivo in tumor bearing rats, has also been shown to retard tumor growth compared to free drug, a conjugate of adriamycin and a non-specific immunoglobulin, or a mixture of free drug and anti-Sp 4 monoclonal (Pimm, Jones, Price, Middle, Embleton & Baldwin, 1982).

Clinical studies utilising antibody-drug conjugates are few and have generally involved limited number of patients, often with advanced disease. In addition to demonstrating clinically the synergistic effect between drug and antibody in five patients with metastatic malignant melanoma, prolonged median survival time of a patient treated intermittently over a year with a covalent melphalan-immunoglobulin conjugate has also been reported (Everall, Dowd, Davies, O'Neill & Rowland, 1977). Using adsorbed, non-covalent, complexes of chlorambucil and anti-melanoma antibodies, Ghose was also able to obtain prolonged survival times in patients with melanoma, compared to those receiving

conventional chemotherapy (Ghose, Norvell, Guclu, Bodurtha, Tai & MacDonald, 1977).

However, further clinical studies using covalent complexes of drug and antibody are scarce, and none have been published to date where monoclonal antibodies have been used as carriers.

I 8.0 AIMS OF THESE STUDIES

From the foregoing sections, it appears as if targeted chemotherapy offers potential as a method of improving the selective delivery of drugs to tumors, which may be clinically relevant.

As part of the evaluation of this approach, the aims of these studies were as follows:

1. Using CEA as a model, to characterise a range of human tumor cell lines for the expression of this target antigen with an anti-CEA monoclonal antibody, using immunocytochemical and radiolabelling studies.
2. To evaluate targeting efficacy and selectivity of anti-CEA monoclonal antibody vindesine conjugates in vitro using selected cell lines (from 1) in a microcytostasis assay.
3. To establish a xenograft pre-clinical model for the in vivo assessment of targeting using the lines selected from 1 above.
4. To assess the efficacy and selectivity of anti-CEA monoclonal antibody vindesine conjugates on the growth of human tumor xenografts with a range of target/antigen (CEA) expression, in the xenograft model (3 above).
5. In addition to these principle aims, the generation of additional anti-CEA monoclonal antibodies, using hybridoma technology, was attempted.

CHAPTER II : MATERIALS & METHODS

II 1.0 HUMAN TUMOR CELL CULTURE

II 1.1 Introduction

Just as a progress in microbiology was slow until 1881, when Koch proposed a method of selective bacterial culture, suitable methods of culture of human tumors have allowed rapid progress to be made in the field of oncology (Sykes, 1975).

Since the first permanent cell culture was prepared from a cervical neoplasm in 1952 (Gey, Coffman & Kubieck, 1952), other human solid tumors have been grown in vitro as monolayers on glass and plastic surfaces.

This has not been without problem however, and attempts are still being made to overcome such fundamental difficulties as initiating the culture, selective growth of tumor cells free of fibroblasts and other 'contaminant cells', suitable nutritional conditions for optimal cell growth and characterization of the cells. Against these difficulties, the threat of infection remains a potential hazard and techniques of handling these cultures under sterile conditions, with judicious use of antibiotics, have also required development.

II 1.2 Cell Lines

Human tumor cell lines utilised in this study are listed in Table 1 along with the type of tumor, and were already established in culture in the Oncology Research Laboratory, where they were undergoing characterisation. They were obtained initially from the American Type Culture Collection, Maryland, U.S.A., with the exception of BENN which was obtained from Dr. M. Ellison at the Ludwig Cancer Institute, London; U.K.

TABLE 1. Human Tumor Cell Lines used at Oncology Research, with source

CELL LINE	SOURCE
LS174T	Adenocarcinoma, colon
SKCO1	Adenocarcinoma, colon
HT29	Adenocarcinoma, colon
COLO320DM	Adenocarcinoma, colon
LoVo	Adenocarcinoma, colon
SW1116	Adenocarcinoma, colon
SW837	Adenocarcinoma, rectum
A549	Lung carcinoma
Calu 6	Lung carcinoma
BENN	Lung carcinoma

The cell lines were grown as monolayers in 75 cm² perspex tissue culture flasks (Falcon, Becton-Dickinson), and maintained at 37 degrees Celsius. Selected cell lines i.e. BENN required incubation in a 5% carbon dioxide atmosphere and were kept in a separate gassed humidified incubator.

Cell growth was checked daily by viewing the monolayer under an inverted viewing phase microscope (Diavert, Leitz). Characteristic patterns of cellular morphology were seen, i.e. epitheloid, pleomorphic, fibroblastic, and the proportion of the monolayer covering the surface of the flask was determined, in addition to other characteristics such as clumping or piling-up of cells. Plate 3 illustrates growth of a tumor cell line in culture.

II 1.3 Media

Table 2 lists the media and supplements required by each cell line. The addition of the supplements to the basic stock solution was performed in the laboratory under sterile conditions.

Media was added to the culture flasks or replaced as necessary, but, on average, 30 - 50 ml of medium was changed bi-weekly, depending on cell growth. This was performed under sterile conditions, using a laminar air flow enclosure (Contamination Control Inc.), sterile equipment and standard micro-biological techniques such as flaming the open mouth of a container prior to pouring.

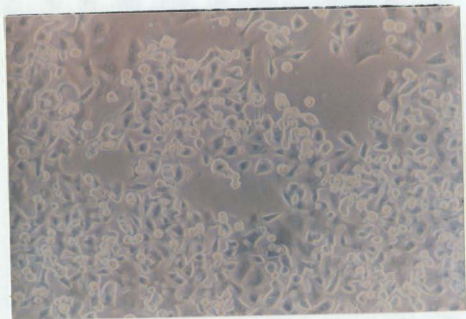


Plate 3. Growth of cell line SKC01 monolayer eight days post-trypsinization, as seen under an inverted viewing phase microscope (Diavert, Leitz)
(Eyepiece, x10 ; Objective, 10/0.25)

TABLE 2. Media required for Cell Culture, including supplements
(All materials supplied by Flow Laboratories)

<u>CELL LINE</u>	<u>MEDIUM</u>	<u>SUPPLEMENTS</u>
HT29	McCoy's (500 ml)	100 ml fetal calf serum (15.6%) 6 ml glutamine 6 ml non-essential amino acids 12 ml penicillin-streptomycin (60000 U penicillin, 60 mg streptomycin) 15 ml IM HEPES Buffer
LoVo	HAM'S F-10 (500 ml)	100 ml fetal calf serum(16.18%) 6 ml glutamine 12 ml penicillin-streptomycin
COL0320DM	RPMI-1640 (500 ml)	50 ml fetal calf serum (8.8%) 6 ml glutamine 12 ml penicillin-streptomycin
SW1116 / SW837	L-15 (500ml)	50 ml fetal calf serum (8.8%) 6 ml glutamine 12 ml penicillin-streptomycin

Table 2 -continued

Calu 6	Dulbecco's (500 ml)	100 ml fetal calf serum (16.02%) 6 ml glutamine 6 ml non-essential amino acids 12 ml penicillin-streptomycin
BENIN	M199. (250 ml) + Dulbecco's (250 ml)	50 ml fetal calf serum(8.8%) 6 ml glutamine 12 ml penicillin-streptomycin
A549	Dulbecco's 500 ml	50 ml fetal calf serum(8.8%) 6 ml glutamine 12 ml penicillin-streptomycin
LS174T / SKCO1	Minimum essential medium (500 ml)	50 ml fetal calf serum(8.8%) 6 ml glutamine 6 ml non-essential amino acids 12 ml penicillin-streptomycin

II 1.4 Trypsinization

When cell growth in the tissue culture flask reached confluence, a cell suspension was prepared by trypsinizing the cell monolayer. This enabled a cell count to be performed and based on the number of viable cells present in suspension, aliquots of known cell number were prepared for use in subsequent experiments.

II 1.4 (a) Materials

Phosphate buffered saline (PBS) pH 7.2, 0.15M prepared from PBS tablets (Oxoid Ltd., England), dissolved in distilled water as per manufacturer's instructions, and sterilized by autoclaving.

Trypsin-EDTA 10x (Gibco) 1/10 dilution in PBS

II 1.4 (b) Method

1. Under sterile conditions, the medium in the culture flasks was poured off and discarded.
2. The cell monolayer was washed twice with 20 ml sterile PBS at room temperature, and the PBS discarded.
3. 5.0 ml trypsin-EDTA solution (1:10 dilution in PBS) was added to the flask, which was then incubated at 37 degrees Celsius (°C) for 5 minutes.
4. The trypsin solution containing the cells was then poured into a sterile centrifuge tube and 2.3 ml PBS added to the flask to wash any remaining cells. This was then poured into the centrifuge tube also.
5. The cells were then centrifuged for 5 minutes at 200g.
6. The supernatant was carefully poured off the cell pellet and the cells re-suspended by tapping the tip of the centrifuge tube.
7. 10.0 ml of medium was then added to the cells and a cell count performed.

II 1.5 Cell Count

The concentration of cells in suspension was calculated using a haemocytometer. Cell viability was assessed by using a solution of acridine orange (which stains viable cells brilliant green) and ethidium bromide (which stains non-viable cells orange or dull green) when viewed with a fluorescence microscope.

II 1.5 (a) Materials

Haemocytometer (Neubauer), with coverslip

Acridine Orange (Sigma chemicals, USA) *

Ethidium Bromide (Gurr, London, U.K.) *

*used as a 0.001% solution AO/EB

Fluorescence Microscope (Ortholux II, Leitz), with a 50 watt mercury vapour lamp.

II 1.5 (b) Method

One drop of the cell suspension, at appropriate dilution, was mixed with one drop AO/EB solution and the mixture placed beneath the coverslip of the haemocytometer, which was then viewed under the fluorescence microscope. Viable and non-viable cells were counted and the percentage of viable cells calculated.

II 1.6 Cell Dispensing

Following the preparation of a cell suspension and determination of a viable cell count, aliquots of cells at known concentrations were prepared.

At every trypsinization, cell smears were routinely prepared for immunocytochemistry. A cell suspension of 10^6 cells in 1 ml 1% bovine serum albumin (Sigma) in PBS was prepared and 50 μ l dispersed onto each of fifteen, alcohol cleaned, microscope slides. These were allowed to air dry for 24 hours prior to fixation in methanol for 20 minutes.

New flasks were established using a minimum of 10^6 viable cells, which were initially placed in 10-20 ml of medium prior to incubation. Subsequent maintenance of these cell cultures has been described.

Extra cells were frozen in order to obtain a stock supply for the laboratory. For this purpose, a 10% solution of Dimethyl Sulphoxide (BDH Chemicals) in fetal calf serum (Flow Laboratories) was sterilized by passage through a 22 μ m micropore filter, and 1.0 ml of this solution added to $4 - 6 \times 10^6$ viable cells, prior to transfer to a Nunc vial (Intermed). These vials were cooled for 24 hours in a -70°C Reyco freezer (Rheem Manufacturing Co., Asheville, N.C.) prior to storage in liquid nitrogen. (Liquid nitrogen cannister: APOLLO SX 35, MVE).

Cells were retrieved by rapidly thawing the vial in a 37°C water-bath until a little ice remained and then adding the cells, under sterile conditions, to 9.0 ml of medium in a sterile centrifuge tube. The suspension was then centrifuged at 200g for 5 minutes, the supernatant discarded, 5.0 ml of fresh medium added, and following a cell count, the suspension was added to a tissue culture flask.

The concentrations of cells used in individual experiments, uridine assay, fusions, xenograft inoculation, etc. will be described in the appropriate section.

II 2.0 GENERATION OF ANTI-CEA MONOCLONAL ANTIBODIES

II 2.1 Introduction

The method of fusion outlined for the generation of anti-CEA monoclonal antibodies followed that of Kohler (1975) and Woodhouse (1982).

II 2.2 Mouse Myeloma Cell Line, NS-1

The NS-1 mouse myeloma cell line was obtained from the 'Human Genetic Mutant Cell Repository' (Camden, N.J.) It was grown in suspension in 75cm² tissue culture flasks (Falcon, Becton-Dickinson) at 37° C in a 5% carbon dioxide humidified atmosphere. Its medium, RPMI-1640 (500 ml) was supplemented with 50 ml fetal calf serum (FCS), 6.0 ml glutamine, 2.0 ml penicillin/streptomycin (10000 U penicillin, 10 mg streptomycin), 10.0 ml glucose (4.5 g/l) and 1.25 ml thioguanine. (All materials supplied by Flow Laboratories). To maintain exponential cell growth, medium was decanted from the flask under sterile conditions and replaced with fresh medium at approximately 2 day intervals.

One day prior to fusion, cells were diluted 1:10 following a cell count and assessment of viability, in order to maintain exponential growth at the time of fusion. A cell count was repeated on the day of fusion and following a sterile PBS wash, a suspension of 10⁷ viable cells ml⁻¹ was prepared.

II 2.3 Spleen Cell Donor

Table 3 indicates the immunisation schedules for spleen cell donors.

To harvest the spleen cells, mice were sacrificed using carbon dioxide and the spleen removed under sterile conditions. A cell suspension was prepared by pushing the spleen through a fine sterile sieve using a rubber tipped syringe plunger. The spleen cells were collected in sterile PBS, and the suspension allowed to stand in order to allow large clumps to settle. A pipette was used to remove the supernatant, consisting of spleen cells in suspension.

Table 3. Immunisation Schedule for Spleen Cell Donors

(performed by members of the Oncology Research Laboratory)

FUSION	DAY	AGENT	QUANTITY (ug, cells)	ADJUVANT	ROUTE
1.	0	CEA	20	ALUM/B.P.	IP
	31	CEA	20	ALUM	IP
	42	CEA	20	ALUM/B.P.	IP
	129	CEA	20	ALUM/B.P.	IP
	346	CEA	26.6	ALUM/B.P.	IP
	355	CEA	20	ALUM	IV
	409	CEA	20	ALUM/B.P.	IP
	454	CEA	20	ALUM/B.P.	IP
	465	CEA	20	ALUM	IP
	469	CEA	25	ALUM	IP
472		FUSION			
2.	0	LoVo	107	B.P.	IP
	52	LoVo	107	PBS	IP
	355	LoVo	4.58x10 ⁶	PBS	IP
	377	LoVo	3.45x10 ⁶	PBS	IP
	386	CEA	30	ALUM	IP
390		FUSION			

II 2.4 Fusion

II 2.4 (a) Materials

SPLEEN CELL SUSPENSION 10^7 cells ml⁻¹

MOUSE MYELOMA CELL SUSPENSION (NS-1) 10^7 cells ml⁻¹

RPMI-GLN: RPMI 1640 100 ml

GLUTAMINE (GLN) 1.0 ml

PENICILLIN/STREPTOMYCIN 1.0 ml

PEG-DMSO-RPMI (44% PEG): POLYETHYLENE GLYCOL 8.0g

DIMETHYLSULPHOXIDE 1.5 ml

RPMI-GLN 8.5 ml

RPMI-GLN-FCS: RPMI-GLN 100 ml

FCS .11 ml

RPMI-GLN-HAT: RPMI-GLN 100 ml

HAT x 50 2.0 ml

RPMI-GLN-HAT-FCS: RPMI-GLN-FCS 100 ml

HAT x 50 2.0 ml

RPMI-GLN-HT-FCS: RPMI-GLN-FCS 100 ml

HT x 50 2.0 ml

TISSUE CULTURE PLATES, 96 and 24 WELL (Linbro, Flow)

All materials from Flow Laboratories, except PEG (BDH, Poole, England) and DMSO (BDH, Toronto).

HAT : Hypoxanthine Aminopterin Thymidine

HT : Hypoxanthine Thymidine .

II 2.4 (b) Method

1. Spleen cells and myeloma cells were mixed in a ratio of 10:1 and centrifuged at 500g for 5 minutes.
2. Following removal of the supernatant, the cell pellet was warmed in a beaker containing water at 37° C which in turn was placed within a larger beaker containing water at similar temperature.
3. 1.0 ml PEG-DMSO-RPMI was added to the cell pellet dropwise over one minute, stirring gently with a pipette tip. This was continued for a further minute and 1.0 ml RPMI-GLN-HAT added over one minute in a similar manner, followed by one further minute of gentle stirring. 8.0 ml of RPMI-GLN-HAT was added over 3 minutes and cells then centrifuged for 5 minutes at 200g.
4. Following removal of supernatant, cells were resuspended in 25.0 ml RPMI-GLN-HAT-FCS and plated out into five, 96 well plates which were incubated at 37° C in a humidified 5% carbon dioxide atmosphere.
5. The plates were undisturbed for 5 days. The medium was then changed by using a 19 gauge sterile hypodermic needle (Yale, Becton-Dickinson) attached to a vacuum line to remove half, which was replaced by fresh RPMI-GLN-HAT-FCS. The medium was changed every 3 days and after two weeks, the hybridoma selective medium (RPMI-GLN-HAT-FCS) was replaced by amino-pterin depleted medium, RPMI-GLN-HT-FCS, for a minimum of

three changes. This was then replaced by RPMI-GLN-FCS.

6. Microscopy using an inverted viewing phase microscope was performed on a daily basis after the first medium change in order to detect growth of colonies. Supernatants from promising wells were removed under sterile conditions and tested for anti-CEA activity by ELISA.
7. Positive hybrids were either expanded by transfer into 24 well culture plates or cloned immediately by limiting dilution (10 and 5 cells ml⁻¹), followed by dispensing 100ul aliquots into the wells of 96 well culture plates.

II 3.0 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

II 3.1 Introduction

The detection and quantitation of small amounts of biologically active substances, even within the nanogram per millilitre range, with a high degree of specificity, sensitivity and reproducibility has been achieved using methods that utilize the unique nature of the antigen-antibody response. Radioimmunoassay has been widely used for such purposes since its introduction over 20 years ago (Yalow & Berson, 1959). The use of radioisotopes however has several disadvantages, related to the handling and safety of these reagents, their cost, availability and limited shelf life (O'Sullivan, Bridges & Marks, 1979; Ekins, 1980).

These disadvantages have been overcome by using enzymes as alternative labels to radioisotopes in an enzyme immunoassay. Horse-radish peroxidase and alkaline phosphatase are widely used enzyme labels, although others such as glucose oxidase, B-D galacto-oxidase and glucosylase have also been used (O'Sullivan et al, 1979).

Enzyme activity may then be assessed in a quantitative or semi-quantitative manner following catalysis of a simple reaction, which may involve a color change or thermal response. Several techniques of enzyme immunoassay have been described with reaction sequences related to labelling of antigen or antibody, phase of reaction and the number of stages involved.

The antibody ELISA used in screening hybridoma supernatants in this study was essentially a modification of the sandwich assay. Excess

antigen was bound to a solid phase and reacted with antibody, which in turn was detected by an anti-mouse immunoglobulin enzyme labelled antibody. The color reaction was then quantitated spectroscopically, and it has been shown that 50 pg per cuvette of anti-CEA monoclonal antibody may be detected using this assay (Woodhouse et al, 1982).

II 3.2 Antibody Assay

II 3.2 (a) Materials

Carbonate Buffer (0.1M, pH 9.8)

Sodium Chloride 8.76 g l⁻¹

Sodium Carbonate 10.6 g

Sodium Bicarbonate 8.4 g

1% BSA Carbonate Buffer

BSA (Sigma, USA)

Carbonate Buffer, 0.1M, pH 9.8

Citrate Phosphate Buffer (0.1M, pH 4)

Citric Acid 0.906g

Disodium Hydrogen Phosphate 1.017g

Dissolve in 100 ml distilled water

BSA-PBS-Tween Diluent

BSA, 1%

PBS pH 7.2

Tween 20 (BDH, Toronto) 0.1%

Hydrogen Peroxide, 0.0024%

Saline Tween

Sodium Chloride 0.15M

Tween 20 0.1%

CEA - Each cuvette requires 250 μ l of a 2.5 μ g ml⁻¹ concentration.

2,2' azino-01 [3ethyl-Benzthiazoline sulphonic acid] (ABTS)

ABTS Stock, 27.8 ng ml⁻¹ (Sigma, St.Louis)

Peroxidase conjugated rabbit anti-mouse immunoglobulins (Dako, Denmark)

Gilford ELA50 processor, with Reader and Thermal Printer

Cuvettes (ELA Cuvettes, Gilford)

II 3.2 (b) Method

1. To coat cuvettes, 250 μ l of a 2.5 μ g ml⁻¹ solution of CEA in carbonate buffer was dispensed using the Gilford ELA 50 at appropriate setting into each well of cuvette strips. These were incubated for 3 hours in a water bath at 37° C, followed by storage at 4° C. The supernatant was decanted prior to use.
2. To block non-specific binding, 300 μ l of 1% BSA-carbonate buffer was dispensed into each cuvette followed by an incubation period of 1 hour at 37° C.
3. Test reagents were prepared in appropriate dilution in BSA-PBS-TWEEN, and following removal of the 1% BSA-carbonate buffer, 200 μ l was pipetted into each labelled cuvette, with appropriate controls. A further incubation period of 2 - 3 hours at 37° C followed.

4. Rabbit anti-mouse immunoglobulin horse-radish peroxidase at 1:1000 dilution was prepared and 250 μ l added to each cuvette after removal of the test reagents and controls. The cuvettes were incubated for 3 hours in a water bath at 37° C. The substrate was prepared to provide a concentration of 0.44 g/l ABTS in citrate-phosphate buffer, with 0.0024% hydrogen peroxide.
5. The Gilford ELA50 was set to produce an absorbance reading of zero and stabilized. Conjugate was removed from the cuvettes, followed by 6 washes with saline-tween and the substrate dispensed into each cuvette (250 μ l). The time of reaction was noted, and the absorbance read spectroscopically using a 405 nm filter and displayed by the printer.
6. For calculation of results, the blank absorbance value obtained when ABTS was initially dispensed was subtracted from the final absorbance reading for each cuvette.

II 3.3 Antibody Assay for Testing Conjugates

The assay outlined above was used to test antibody activity of iodinated and vindesine conjugated 11-285-14, with 11-285-14 as a control. Concentrations tested were 1000, 100, 10, 1 ng ml⁻¹ and tests were performed in triplicate. Standard curves of absorbance (abscissa) with antibody concentration (ordinate) were constructed.

II 3.4 Antigen Assay

For quantitation of CEA, the initial sequence of the assay was modified. Cuvettes were coated using 250 μ l of a 1/1000 dilution of rabbit immunoglobulins to human CEA (Dako, Denmark) in carbonate/bicarbonate buffer, and incubated at 37°C for 3 hours.

Following the incubation with 1% BSA/carbonate, 200 μ l of test sample dilutions were added to the cuvettes in triplicate, with CEA standards as controls, and incubated for 2 hours at 37°C.

11-285-14 at a concentration of 2.5 μ g ml^{-1} was dispensed into each cuvette (250 μ l) as the next stage, and following a 2 hour incubation at 37°C, the assay was as for stage 4 of the antibody assay, outlined above.

Standard curves of absorbance (abscissa) against CEA concentration (ordinate) were constructed to quantitate CEA.

II 4.0 IMMUNOPEROXIDASE

II 4.1 Introduction

The immunoperoxidase technique utilizes a specific immunological reaction to detect a variety of cell products including onco-developmental antigens such as CEA. Although several methods have been described (Heyderman, 1979), the essential feature of the test involves the polymerisation of a substrate e.g. diaminobenzidine at the site of antigen-antibody interaction in the presence of peroxidase and hydrogen peroxide.

The test used in this thesis was an indirect method (Heyderman & Neville, 1977) with modification (Ford, Stokes & Newman, 1981). The first antibody (11-285-14) was a monoclonal anti-CEA antibody while the second antibody was a rabbit anti-mouse immunoglobulins (heavy and light chain) horseradish peroxidase conjugate.

With appropriate controls and highly specific antibodies, the test is extremely reliable, with the additional advantage of its permanence and preservation of cellular and tissue morphology (DeLellis, Sternberger, Mann, Banks & Nakane, 1979).

II 4.2 Materials

SPECIMEN

either methanol fixed smears of human tumor cells prepared following trypsinization, or formalin-fixed, paraffin embedded tissue sections.

XYLENE Stock solution.
 ETHYL ALCOHOL Absolute; 75%, 60%, 30%; stock solution.
 HYDROGEN PEROXIDE 7.5% in distilled water supplied as 30% solution (Anachemia Ltd., Ontario).
 BOVINE SERUM ALBUMIN 1% in PBS (Sigma, USA)
 PERIODIC ACID 2.28% in distilled water supplied as 95% solution (BDH, Toronto).
 POTASSIUM BOROHYDRIDE 0.02% in distilled water (BDH, U.K.)- prepared fresh.
 PHOSPHATE BUFFERED SALINE pH 7.2
 stock reagents
 sodium chloride (NaCl) 36.0g
 di sodium hydrogen phosphate (Na_2HPO_4) 7.4g
 potassium di hydrogen phosphate (KH_2PO_4) 2.15g
 Dissolve in distilled water and make up to 5.0 litres; adjust pH to 7.4 as required.
 BRIJ 35 0.001% solution in PBS pH 7.2 supplied as 30% solution (Technicon, New York).
 NORMAL RABBIT SERUM 1:25 dilution in 1% BSA in PBS
 TEST ANTISERUM 11-285-14, 10, 1 ug ml⁻¹
 RABBIT ANTI-MOUSE IMMUNOGLOBULINS,
 CONJUGATED TO HORSE RADISH PEROXIDASE (Dako, Denmark)
 CONTROL ASCITES (BRL MOLECULAR DIAGNOSTICS) 10, 1 ug ml⁻¹
 3'3' DIAMINOBENZIDINE (Sigma, U.S.A.) prepared fresh
 10 mg Diaminobenzidine (DAB)
 20 ml PBS

20 ul 30% hydrogen peroxide

Mayer's Haemalum (Harleco Diagnostics, BDH)

One litre contains:

- 1.0 haematoxylin
- 20 ml 1% sodium iodate
- 50g ammonium alum

Lithium Carbonate - Saturated Aqueous Solution

Permunt (Fisher, NJ)

II 4.3 Method- Cell Smears

1. Methanol fixed cell smears were bleached by covering the slide with 7.5% hydrogen peroxide for 5 minutes, followed by a tap water wash.
2. Endogenous peroxidase was blocked using 2.28% periodic acid for 5 minutes, followed by a tap water wash.
3. Aldehyde groups were blocked with 0.02% potassium borohydride for 2 minutes, the slides were then washed with tap water followed by PBS pH 7.4.
4. Non-specific conjugate binding was prevented by application of 50 ul of a 1:25 solution of normal rabbit serum, in a moist chamber, for 10 minutes, since the second antibody was a rabbit anti-mouse reagent.
5. The slides were washed in 0.001% BRIJ for 5 minutes.
6. Incubation with 50 ul of the first antibody (anti-CEA) at test concentrations following appropriate dilution in 1% BSA in PBS,

within the moist chamber for 30 minutes. Negative controls included were 1% BSA and, when available, control ascites at the same concentration as the first antibody. Positive controls included a known CEA expressing cell line or liver metastases from a CEA producing colorectal adenocarcinoma.

7. Wash in BRLJ 0.001% for 15 minutes.
8. Incubation with 50 μ l of the second antibody (rabbit anti-mouse immunoglobulins peroxidase conjugate) for 30 minutes. A 1:50 or 1:100 dilution in 1% BSA in PBS was prepared depending on the batch.
9. Wash in BRLJ 0.001% for 15 minutes.
10. DAB solution was prepared and immediately applied to the slides for 5 minutes, then gently washed with PBS to clear the slide.
11. 2 drops of Mayer's Haemalum were added for 5 minutes to counterstain, then rinsed with distilled water.
12. Slides were placed in the saturated aqueous solution of lithium carbonate for 5 minutes.
13. The slides were then dehydrated through alcohols to xylene.
14. A coverslip was mounted in permount prior to assessment of the slide.

II 4.4 Modifications

A number of modifications to the technique were used when tissue or xenograft sections were studied.

1. Any paraffin embedded section was initially dewaxed in xylene and rehydrated by passage through alcohols to water, prior to stage 1

of the method.

2. Xenograft sections were also washed in 0.001% BRIJ solution for 10 minutes at this point to reduce murine serum components.
3. The volume of reagents used at each stage was 100 ul.
4. Handling of sections required extra care during each stage, especially during washing or rinsing to prevent the section lifting off the slide.
5. Appropriate controls for xenograft sections included a section of liver with metastatic colorectal adenocarcinoma expressing CEA as a positive control, and the use of a mouse ascites at corresponding protein concentration to the first antibody as a negative control.

II 4.5 Assessment

Slides were viewed under a microscope (x16 objective) and assessment of staining determined subjectively by two assessors, one who is experienced at the technique. The results were expressed as a percentage of cells stained in comparison to the negative controls. Background staining was also assessed.

II 4.6 Cautionary Note

Many of the reagents used in the test are toxic, or have even been demonstrated to be powerful carcinogens, such as diaminobenzidine. The investigator is at particular risk when the reagent is in powder form and therefore the preparation of its solution and application to slides was performed with mask and gloves at a sink.

II 5.0 IMMUNOFLOURESCENCE

II 5.1 Introduction

The technique of immunofluorescence has been used extensively to detect cell surface antigenic determinants since its introduction over 40 years ago (Coons, Creech, Jones & Berliner, 1942). Its use in the detection of cell surface expressed CEA provides a complementary method of immunocytochemistry to immunoperoxidase staining.

II 5.2 Materials

Test antiserum (11-285-14)

PBS pH 7.2

Goat anti-mouse immunoglobulins fluorescein isothiocyanate (FITC) conjugate (Kirkegaard & Perry Laboratories) 1:30 dilution in PBS

Fluorescence Microscope

Mounting solution - 50% Glycerol in PBS

II 5.3 Method

1. Cell smears were incubated with 50 ul of the first antibody at various concentrations in a moist chamber for 15 minutes. Appropriate controls were included and no blocking stage was required.
2. Wash for 15 minutes in PBS pH 7.2

3. 50 μ l of a 1:50 dilution in PBS of a goat anti-mouse immunoglobulins fluorescein conjugate, in appropriate dilution was applied to the slide for 20 minutes and incubated in the chamber.
4. PBS wash for 15 minutes.
5. Slides were then mounted and assessed immediately by two investigators under the fluorescence microscope (x 25 objective).

II 6.0 ANTIBODY RADIOLABELLING

II 6.1 Introduction

The following method of iodination of the monoclonal antibody 11-285-14 is based upon oxidation of the immunoglobulin with Iodine-125 using the oxidising agent Chloramine T (McConahey & Dixon, 1966). This method minimised protein denaturation, while maintaining maximum efficient iodination of the antibody.

II 6.2 Materials

Phosphate buffered saline (PBS) pH 7.4

1% Bovine serum albumin in PBS

Potassium iodide 1mg ml⁻¹ in PBS

Sodium bisulphite 10 mg ml⁻¹ in PBS - fresh

Sodium phosphate buffer (pH 7.5, 0.5M)

Trichloroacetic acid (TCA) (Baker, N.J.) 20% w/v

Chloramine T (Sigma, St.Louis) 4 mg ml⁻¹ in sodium phosphate buffer, prepared at the time of use.

Sephadex G25-medium (Pharmacia, Uppsala, Sweden)

2g-standing in 20 ml PBS overnight

I-125 (Amersham) 250uCi/2.5ul

11-285-14 (Anti-CEA monoclonal antibody) 100ug (Stock:

1.149mg ml⁻¹)

Gamma counter, Gamma 7000 (Beckman Scientific Instruments, Irvine, Ca)

Eppendorf tubes 1.5ml

Glass test tubes marked at 0.5ml (12 x 75mm)

II 6.3 Method

1. A disposable 10ml glass pipette was plugged loosely with glass wool and sephadex, after standing overnight in PBS, poured into the column ensuring homogeneous distribution. 20 ml of 1% BSA in PBS was used to equilibrate the column. The remaining procedures were performed in a fume cupboard designated for the use of radioisotopes, ensuring appropriate precautions.
2. 100ug of antibody was pipetted into an eppendorf tube and 250uCi of ^{125}I added. Chloramine T solution was prepared fresh by adding 1.0 ml of sodium phosphate buffer to 4.0 ml of the powder, and 50ul of chloramine T solution added to the eppendorf tube which was shaken gently for one minute. 50ul of sodium bisulphite, 100ul of potassium iodide and 200ul of 1% in PBS were then added in sequence, and the resulting solution applied directly to the column.
3. Elution was performed using 1% BSA in PBS, and 0.5 ml fractions were collected to a total of 20 ml. Radioactivity present in each tube was determined using a gamma counter, ensuring that two peaks were present, the first representing iodinated protein and the second, free iodide.
4. Total counts obtained from tubes over the first peak were expressed as a percentage of total overall counts (i.e. from

the start of the first peak to the start of the second) to obtain the percentage of protein iodinated (Amersham).

5. The fractions that comprise the first peak of radioactivity were pooled and 5.0ul added to 1.0 ml of 1% BSA/PBS, mixing well. 250ul of the resulting solution was removed and placed in an eppendorf tube labelled "A".

250ul of TCA was added to the initial solution, and following adequate mixing using a vortex, was allowed to stand for 15 minutes. The tube was centrifuged at 4000g for 5 minutes and 250ul of supernatant removed and placed into an eppendorf labelled "B". Both 'A' and 'B' were placed in the gamma counter for counting.

To calculate the percentage bound cpm (counts per minute), the following formula was employed.

$$\text{Percentage Bound (cpm)} = \frac{100 - 2B \times 100}{A}$$

II 7.0 BINDING ASSAY

II 7.1 Materials

Iodinated anti- CEA monoclonal antibody (11-285-14)

- see previous section
- calculated antibody concentration: 0.041 mg ml⁻¹

TEST CELL LINES

CEA positive LSI74T ; BENN

CEA negative COLO320DM

CELL CONCENTRATION

2.5 x 10⁶ viable cells ml⁻¹ in 1% F.C.S./PBS

1% foetal calf serum (FCS) in PBS pH 7.4 - Fresh

GAMMA 7000 (Beckman Scientific Instruments, Irvine, Ca)

Centrifuge, Beckman TJ6 with refrigeration unit, and TH4 rotor

Eppendorf tubes 1.5 ml

12 x 75 mm glass test tubes

II 7.2 Method

1. The saturation assay employed seven test antibody concentrations, at 200, 1000, 1400, 1600, 1800, 2000 and 2400 ng/tube, in duplicate. 100ul of the cell suspension was dispensed into eppendorf tubes, to which the calculated volume of iodinated antibody, together with 1% FCS/PBS diluent to obtain a total volume of 100ul, was added. This was performed for each cell line except BENN, where antibody concentrations used were 200, 400, 1400, 1800, 2400 ng/tube. Controls comprised blank tubes to which 200ul of 1% FCS/PBS was added.

2. Tubes were then incubated at 40°C for 30 minutes. 1.0 ml of 1% FCS/PBS was then added to each of the tubes, which were then vortexed to ensure adequate mixing, and then centrifuged at 1520g, 40°C for 10 minutes.
3. Supernatants were then removed and 1.0ml 1% FCS/PBS added, tubes vortexed and centrifuged for a further 10 minutes. Following centrifugation, supernatants were removed, 1.0 ml of 1% FCS/PBS added, and after mixing, the contents of the eppendorf tubes were transferred individually to glass test tubes. Further centrifugation was performed for 10 minutes and following removal of the supernatant, 0.5 ml of 1% FCS/PBS added prior to counting with the gamma counter.
4. Mean counts were obtained for each cell line at each antibody concentration, following the subtraction of control counts from test counts. Results were then expressed graphically; antibody concentration (ng)/tube along the abscissa and cpm along the ordinate.

II 8.0 IN VITRO EFFICACY

II 8.1 Introduction

Chemosensitivity of cell lines to vindesine or the monoclonal antibody-vindesine conjugate was assessed using a microcytostasis assay (Smith & Nicklin, 1979) with modification (Johnson, Ford, Newman, Woodhouse, Rowland & Simmonds, 1981).

Essentially the test involves application of the test reagent to cell monolayers growing within wells of microtitre plates and following a period of recovery, radiolabelling surviving cells determining inhibition of cell protein synthesis. It has been shown that uptake of ^3H uridine into the RNA precursor pools of surviving cells correlates well with cell adherence and survival (Smith et al, 1979; Gadiot, Hoedemaeker & The, 1982).

The method also has the additional advantage that the cell monolayer is not destroyed on completion of the assay, permitting additional histological examination.

II 8.2 Materials

Cell Suspension 10^5 cells ml^{-1} in appropriate medium
following trypsinization

Medium - sterile

Tissue culture multiwell plates, 96 wells (Linbro, Flow
Laboratories)

Trichloroacetic Acid (JT Baker Chemicals, New York)

Scintillation Vials, 20 ml (Fisher Scientific)

Aquasol 2 scintillation fluid (New Research Products, Boston, Mass.)

^3H -Uridine (New England Nuclear, Maine) supplied 1 uCi = 1 ul
Vindesine Sulphate (Lilly, Indianapolis, USA) supplied 1 mg ml⁻¹
11-285-14 Vindesine conjugate (Lilly Research Centre, Windlesham, U.K.) Specific Vindesine concentration and immunoglobulin concentration varied in different batches (See Appendix A).

II 8.3 Method

1. Under sterile conditions, 100 ul of the cell suspension was dispensed into wells of the microtitre plate, such that each well contained 10⁴ cells.
2. The plate was incubated for 24 hours at 37°C in a humidified atmosphere of 5% carbon dioxide.
3. Under sterile conditions, a sterile Pasteur pipette was used to remove medium from the wells and doubling dilutions of drug or conjugate were added in a volume of 100 ul to each well. The test was performed in quadruplicate for each dilution, with appropriate control wells that received 100 ul of medium only. The plate was incubated for 24 hours at 37°C as before.
4. The supernatants were discarded in a similar manner to previously and each well washed three times with sterile PBS at 37°C. 100 ul of medium was then added to each well and a 24 hour recovery period followed.

5. Following removal of the medium under sterile conditions after recovery, 50 ul of medium containing 1 uCi of ^3H uridine was added to each well.
6. A 3 hour incubation period in the gassed humidified incubator at 37°C followed.
7. The supernatant was then discarded and the wells washed with PBS. 150 ul of 5% trichloroacetic acid was added to each well and the plate incubated on ice for 20 minutes. This stopped the reaction.
8. 125 ul of the solution within each well was added to 7.0 ml of scintillation fluid, and each vial mixed thoroughly and labelled.
9. The vials were placed in a Beckman LS8100 counter for counting.
10. Results from the counter were expressed as follows:

$$\text{Percentage Cell Survival} = \frac{\text{Mean Count at Each Dilution} \times 100}{\text{Mean Control Count}}$$

11. A dose response curve of percentage cell survival (ordinate) against drug concentration (abscissa) was then constructed.

II 9.0 HUMAN TUMOR XENOGRAFTING

II 9.1 Introduction

The growth of human tumor xenografts in immunodeficient laboratory animals provides a model for assessment of in vivo drug efficacy.

Animals used in such experiments may be rendered immunodeficient by thymectomy and irradiation prior to xenografting or immunodeficiency may be a consequence of an inherited immunological defect. Such animals in their own right have provided the immunologist with information on the role of the thymus and T-lymphocytes in the immune response in addition to their use in grafting experiments (Davies, 1980).

The nude mouse was first mentioned in 1962 and it was subsequently shown that the normally recessive "Nu" gene was expressed by the homozygote (Helson, 1980). These mice were also shown to be athymic (Pantelouris, 1968), to account for their congenital immunodeficiency.

The first report of xenografting appeared in 1969 when a colonic adenocarcinoma was maintained in the nude mouse model by serial passage for four years (Rygaard & Povlsen, 1969).

II 9.2 The Nude Mouse Colony

The nude mouse colony at Memorial University was established in May 1984 by Dr. C.H.J. Ford, operating under the regulations of the Memorial University Biosafety Committee.

Mice were supplied by Charles River (U.S.A.) and were obtained via the University of Toronto Hospital for Sick Children. All were homozygous nu/nu upon a BALB/c background.

The mice were kept under sterile conditions in an animal enclosure (Canadian Cabinets Co) fitted with high efficiency particulate air (HEPA) filters. All cages and bedding was autoclaved, and changed on a weekly basis. Food and water was also autoclaved and supplied ad libitum. Multivitamins (MVI 1000) were added to water bottles under sterile conditions following autoclaving in a dosage of 1.0 ml/litre water. Antibiotics were not added.

Breeding cages consisting of two females and one male were encouraged to maintain stocks of mice. Offspring were weaned at 28 days following sexing.

Gowns, masks and footwear were worn upon entering the room, and sterile surgical gloves worn for any procedure requiring handling of the mice. Work surfaces were sterilized using a solution of 8% formaldehyde, and 70% alcohol, in PBS.

II 9.3 Inoculation

Preliminary experiments demonstrated that mice inoculated with 10^6 viable tumor cells failed to exhibit tumor growth (Ford, 1984, personal communication). Subsequently inoculation of 10^7 viable, washed tumor cells were used in all experiments in order to establish tumor growth.

Following trypsinization of a cell line and cell count, the volume of cell suspension containing 10^7 viable cells, or multiples thereof, was centrifuged at 200g and the cell pellet washed in sterile PBS. This was re-centrifuged and following re-suspension of the cell pellet in sterile PBS ($200\mu\text{l}$ - 10^7 viable cells), the cell inoculum was

transferred to a sterile 1.0 ml syringe with a 26 gauge 3/8" needle (Becton Dickinson, Ontario).

Mice were to be inoculated following anaesthesia with ether (BDH Chemicals), at a subcutaneous site on either flank or bilaterally.

II 9.4 Mensuration

Calipers were used to measure the tumor dimensions of maximum length and maximum width in order to quantitate tumor growth. In order to determine tumor volume, the formula for an oblate spheroid was used in view of the predicted irregularity of tumor growth.

$$V = \frac{\pi L (D)^2}{6}$$

(Evans, Smith & Millar, 1983)

where V represents tumor volume (mm)³

L represents maximal tumor length (mm)

D represents maximal tumor width (mm)

II 9.5 Characterisation

The following human tumor cell lines were inoculated for characterisation as xenografts in the nude mouse model: CLO320DM, LoVo, BENN, 8837, HT29, LS174T, CALU 6, SW1116, A549, SKCO1.

Mice bearing these tumors were sacrificed according to the following criteria.

1. Number of days post inoculation. Following tumor growth,

sacrifices were performed at various time intervals.

2. Tumor size. When tumor growth reached the point where it was felt that the animal would be disabled, or the tumor would ulcerate, the mice were sacrificed.
3. Overall host status. If a tumor bearing animal appeared debilitated as a consequence of the tumor, or exhibited pathogenic infection, the mouse would be sacrificed.

Following the sacrifice of any animal, the dimensions of the tumor were taken and it was then dissected noting any obvious morphological features such as necrosis or invasion of deep structures. The tumor was then weighed (g) and preserved in 10% formalin in PBS. At a later stage, tumors were washed in PBS prior to formalin preservation, to remove murine serum components.

Characterisation of selected xenograft tumors was performed by:

1. Volume weight correlation,
2. Histological examination by haematoxylin and eosin staining,
3. CEA expression by immunoperoxidase,
4. CEA extraction and quantitation (selected cell lines).

II 9.6 CEA Extraction (Xenografts)

II 9.6 (a) Materials

Xenograft specimen

Phosphate buffer (0.05M phosphate in 0.15M sodium chloride
pH 5.5)

Polytron (Brinkmann Instruments, N.Y.)

Centrifuge, Sorval RC2B with SS34 rotor (Sorval, Newtown, Conn.)

Centrifuge tubes (50 ml), 'Oak Ridge' (Nalgene Labware, Rochester, N.Y.)

GF/A glass fibre Whatman filter, 25 mm (Balston, England)

Filter holder, 25 mm (Millex, Bedford, Ma.)

Ultrafiltration Cell (model 12), with PM10 membrane (Amicon, Lexington, Mass.).

II 9.6 (b) Method

1. A known weight of xenograft tumor was minced with phosphate buffer (10 ml buffer to 1.0g tumor) using the polytron (setting 11) for 2 minutes.
2. The homogenate was stirred on ice for 30 minutes and then centrifuged at 20000g for 30 minutes at 40C, using the Sorval centrifuge.
3. The supernatant was then filtered through the glass fibre sheet and its CEA quantitated by ELISA.
4. The solution may be concentrated using the ultrafiltration cell prior to ELISA. Both the pellet and supernatant were stored at -200C.

II 9.7 Haematoxilin and Eosin Staining

II 9.7 (a) Materials (All Stock Reagents)

Hematoxilin:	Haematoxilin	24.0g
	95% alcohol	150 ml

Ammonium Alum Sulphate 360g

Dissolve in 2400 ml distilled water and expose to air and light for one month. Then add,

Glycerin	600 ml
95% alcohol	600 ml

Allow to stand until the color is sufficiently dark

Eosin:

Eosin (Water soluble)	9.0g
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Distilled water	450 ml
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Saturation Solution Picric Acid	45 ml
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95% alcohol	60 ml
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Scotts Tap Water Substitute:

Sodium Bicarbonate	14.0g
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Magnesium Sulphate	80g.
--------------------	------

Tap Water	4000 ml
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Acid Alcohol:	70% alcohol	1000 ml
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Hydrochloric acid (M)	10.0 ml
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Graded Alcohols:	Absolute, 80%, 70%, 50%
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Xylene

Acetone

II 9.7 (b) Method

The standard method used for haematoxinilin and eosin staining included the following stages.

1. Xylene (5 minutes)
2. Absolute alcohol (5 minutes)
80% alcohol (2 minutes)
70% alcohol (2 minutes)
50% alcohol (2 minutes)
3. Tap water rinse
4. Haematoxinilin (5 minutes)
5. Tap water rinse
6. Acid alcohol (4 dips)
7. Tap water rinse
8. Scotts Tap Water Substitute (1 minute)
9. Tap water rinse
10. Eosin (3 minutes)
11. 50% alcohol (3 dips)
70% alcohol (3 dips)
80% alcohol (3 dips)
Absolute alcohol (1 minute)
12. Acetone (2 minutes)
13. Xylene (1 minute)
14. Slides were then mounted with a coverslip and read.

II-10.0 PRELIMINARY EXPERIMENTS

II 10.1 Introduction

Initial experiments using the model of the nude mouse bearing human tumor xenografts were performed with limited numbers of animals in order to:

1. Establish the system for later use in assessment of efficacy of antibody-drug conjugates and,
2. To assess the effect of free vindesine upon xenograft growth.

II 10.2 Experiment A

INOCULATION Four nude mice (4-8 weeks) received an inoculation of 10^7 viable HT 29 cells in each flank, bilaterally.

RADIOMISATION Eleven days post inoculation, the mice were randomly allocated into two groups, weighed and individual tumors measured. One group was to receive treatment with free vindesine (Lilly, Indianapolis) while the remaining group acted as control.

TREATMENT The two mice randomised to receive free drug were injected with 2 mg kg^{-1} of vindesine, given as a 1.0 ml intraperitoneal injection after dilution in sterile PBS. The injections were initiated on day eleven post inoculation, and continued on a twice weekly basis, for 5 weeks. The vindesine dosage was adjusted on the basis of weekly weights obtained for each mouse. Mice randomised as the control group were injected with 1.0

ml sterile PBS intraperitoneally on a similar schedule.

MENSURATION

Tumor dimensions were obtained twice weekly by using calipers to measure the maximum tumor length and maximum width. The tumor volume was calculated from these measurements as described, and was used to assess tumor growth. Upon termination of the experiment, final tumor measurements were obtained and the tumor was dissected, weighed and characterised as previously described.

II 10.3 Experiment B

In this second preliminary experiment, the above protocol was followed, using a different cell line and numbers of mice.

Eight nude mice (4 - 8 weeks) received bilateral flank inoculations of 10^7 viable cells/flank of the cell line LS174T.

Five days post inoculation, mice were randomised into two groups, consisting of four mice/group, and weighed. The mice were maintained as two animals/cage in order to facilitate individual identification.

One group received free vindesine, 2 mg kg^{-1} on a twice weekly basis while the control group received 1.0 ml sterile PBS on a similar schedule.

Mensuration of tumors was performed twice a week as described.

II 10.4 Toxicity Study

This preliminary study was performed to assess the toxicity of free vindesine at higher doses to those used in experiments A and B,

using a limited number of mice.

Five nude mice (6-12 weeks) were included in this experiment, weighed and randomized into two groups. The group of three mice received individual 1.0 ml intraperitoneal injection of vindesine at a dose of 10 mg kg⁻¹, diluted in sterile PBS. Y

The group of two mice received vindesine at a dose of 6 mg kg⁻¹. Each group was inspected daily, noting any morbidity or mortality.

II 11.0 TARGETING STUDIES

II 11.1 Introduction

These experiments were designed to assess the effect of the monoclonal anti-CEA vindesine conjugate (11-285-14-VDS) on growth of human tumor xenografts in nude mice. Cell lines studied demonstrated a range of CEA expression as determined by immunoperoxidase and immunofluorescent techniques, and in vitro efficacy for each cell line had previously been assessed (See results).

II 11.2 Experiment 1

INOCULATION: Fourteen nude mice (4-8 weeks) were inoculated with 107 viable cells from the cell line COLO320DM into right and left flanks. One mouse received a unilateral inoculation (right flank), making a total of fifteen mice used in the experiment.

RANDOMISATION: Three days post inoculation, the mice were randomly allocated into one of three groups, consisting of five mice each. The mice were weighed and individual tumors measured, using the dimensions of maximum length and maximum width of the tumor.

One group was to receive treatment with the free drug, vindesine, one to receive the antibody drug conjugate (code #8 - see appendix A) while the third group acted as a control.

TREATMENT: The regimen for treatment with vindesine was a

2 mg kg⁻¹ intraperitoneal injection of free drug in PBS, given at two-weekly intervals for three doses.

The regimen for treatment with conjugate was an intraperitoneal injection of 10 mg kg⁻¹, with respect to the vindesine concentration, of the conjugate, given every two weeks for three doses. Mice in the control group received a 1.0 ml intraperitoneal injection of sterile PBS at similar intervals. Treatment was initiated at the time of randomisation and the mice weighed at appropriate intervals in order to calculate the correct drug dosage.

MENSURATION: Twice weekly, tumor dimensions of maximum length and maximum width were obtained using calipers, and the tumor volumes calculated. Upon termination of the experiment, the tumors were characterised as described previously. Serum from selected mice was also obtained at sacrifice.

II 11.3 Experiments 2,3,4 and 5

The protocol for the following experiments differed in the following ways.

- a) Mice were inoculated unilaterally (right flank) in all cases.
- b) Cell lines used were: LS174T, experiment 2; SW1116; experiment 3; BENN, experiment 4; SKCO1, experiment 5.
- c) Conjugates used were: #8, experiment 2; # 10, experiment 3; # 8, experiment 4; # 8, experiment 5.

CHAPTER III : RESULTS

III 1.0 IMMUNOCYTOCHEMISTRY

III 1.1 Immunoperoxidase

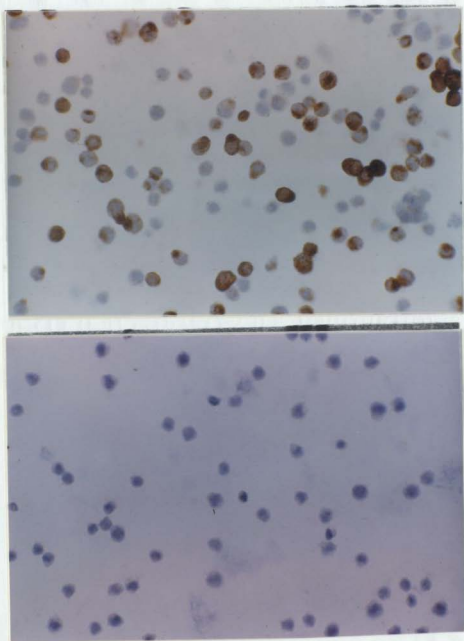
Immunoperoxidase assays were performed routinely on human tumor cell lines, using cell smears prepared at the time of trypsinizing the cell cultures.

Plates 4 to 9 illustrate results obtained with these assays.

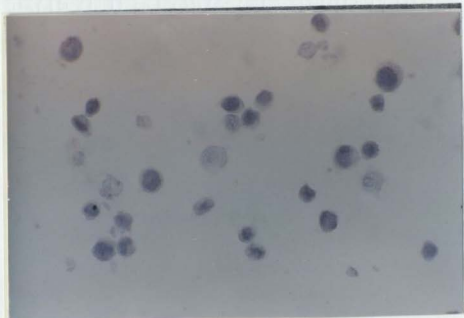
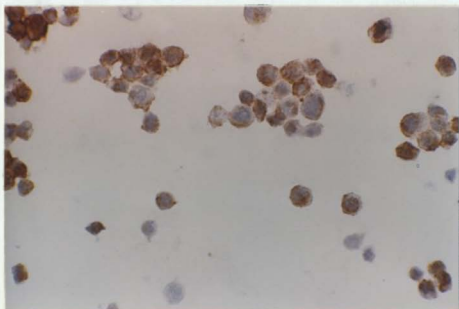
For the cell lines LS174T, plate 4 and BENN, plate 6, the intense brown staining, which is predominantly membrane associated with only minimal cytoplasmic staining, represents expression of CEA by these tumor cells, as detected using the monoclonal anti-CEA antibody - 11-285-14 at a concentration of 10 ug ml^{-1} . Control slides, using an equivalent concentration of control ascitic fluid are shown for both cell lines (LS174T, plate 5; BENN, plate 7).

At this antibody concentration, no staining is seen for COLO320DM plates 8 and 9, indicating that this cell line is a non-CEA expressor, within the sensitivity of the immunoperoxidase assay.

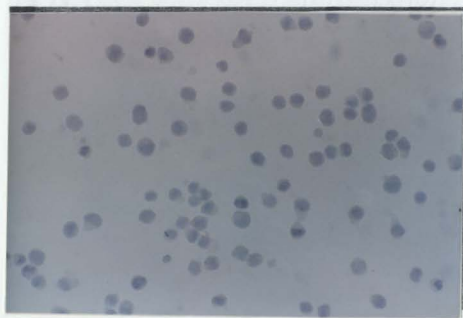
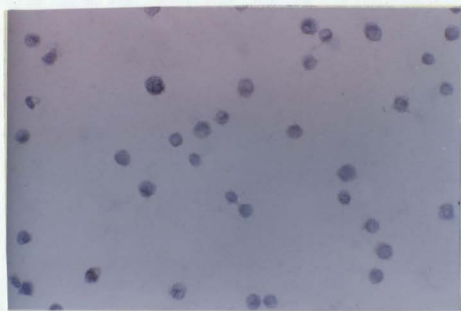
The range of CEA expression of the cell lines used in these studies is shown by the histogram in figure 1, where the mean percentage of cells expressing CEA is represented along the ordinate. Two antibody concentrations were used to test each cell line, 10 ug ml^{-1} and 1.0 ug ml^{-1} , and these are represented along the abscissa by the left and right bars for each cell line respectively. Also indicated are the number of serial passages, or trypsinizations each cell line had undergone to calculate their average CEA expression.



Plates 4 (above) & 5 (below). Immunoperoxidase of LS174T cell smears using 11-285-14 at $10 \mu\text{g ml}^{-1}$ (above), and control ascites at corresponding concentration (below).



Plates 6 (above) & 7 (below). Immunoperoxidase of BENN cell smears using 11-285-14 at 10 ug ml^{-1} (above), and control ascites at corresponding concentration (below).



Plates 8 (above) & 9 (below). Immunoperoxidase of COL0320DM cell smears using 11-285-14 at 10 ug ml⁻¹ (above), and control ascites at corresponding concentration (below).

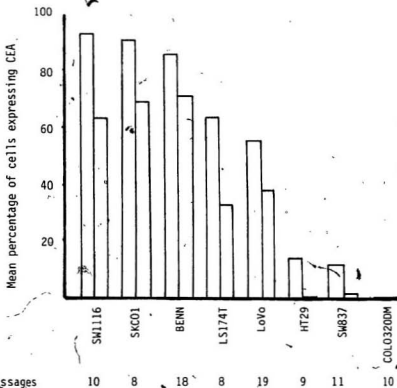


Figure 1. Histogram showing range of CEA expression by cell lines tested by immunoperoxidase. Antibody concentration $10 \mu\text{g ml}^{-1}$, (right bar, $1.0 \mu\text{g ml}^{-1}$).

Consistently high CEA expressors were SW1116, SKCO1, BENN and LS174T, whereas COLO320DM was found to be a non-CEA expressing cell line. Although not included on this chart, the lung tumor cell line A549 was also found to be a consistent non-CEA expressor.

III 1.2 Immunofluorescence

Immunofluorescence assays were also routinely performed following trypsinization of cell lines using the test antibody (11-285-14) at the same concentrations (10,1 ug ml⁻¹).

Figure 2 is a histogram with similar format to that described in the preceeding section, demonstrating CEA expression of cell lines assessed using this technique.

A similar range of antigen expression to that described for the immunoperoxidase assay is seen, with BENN, SKCO1, SW1116 and LS174T as consistently high CEA expressing cell lines, whereas HT29, COLO320DM and A549 (not shown) were consistent non-expressors using this assay.

Immunocytochemical determination of CEA expression of cell lines utilized for the individual experiments referred to in subsequent sections is summarized in appropriate sections of this chapter.

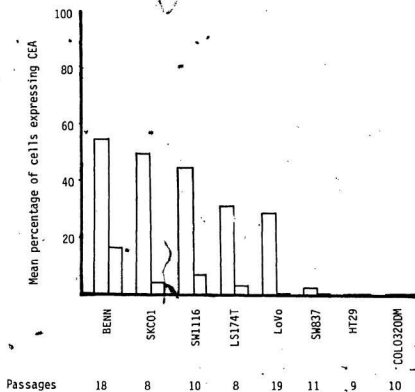


Figure 2. Histogram showing range of CEA expression by cell lines tested by immunofluorescence. Antibody concentration 10 $\mu\text{g ml}^{-1}$, (right bar, 1.0 $\mu\text{g ml}^{-1}$).

III 2.0 ANTIBODY RADIOLABELLING

III 2.1 Calculation of Percentage Protein Iodinated

III 2.1 (a) Eluted Protein

Total overall counts of the protein peak comprising the sixteen tubes collected up to the beginning of the second peak - see II 6.3 - were 364587215 cpm.

Four of these tubes comprised the major peak of radioactivity eluted from the column and the total counts of these pooled tubes, volume 2080 ul, was 324736115 cpm.

Therefore, the percentage of total protein recovered from the column and present in the four pooled tubes was calculated as:

$$\frac{\text{Counts in 4 pooled tubes}}{\text{Counts in protein peak}} = \frac{324736115}{364587215} \times 100 = 89.1\%$$

From the 100ug protein (antibody) used at the start of the experiment, a yield of 89.1 ug was obtained.

III 2.1 (b) Percentage Bound Radioisotope

The percentage bound isotope (cpm) was determined following T.C.A. precipitation (see II 6.3, stage 5)

Counts in tube 'A' : 194183 cpm

Counts in tube 'B' : 1370 cpm

$$\% \text{ bound cpm} = 100 - \frac{(2 \times 1370 \times 100)}{194183} = 98.5\%$$

Therefore, 98.5% of the 89.1 ug antibody eluted from the column following iodination was iodide bound.

III 2.2 Confirmation of Anti-CEA Binding

To confirm that radiolabelled 11-285-14 retained anti-CEA binding activity, an antibody ELISA was performed as outlined in Section II 3.3

Figure 3 compares standard curves obtained for the iodinated antibody and 11-285-14, confirming anti-CEA binding had been retained.

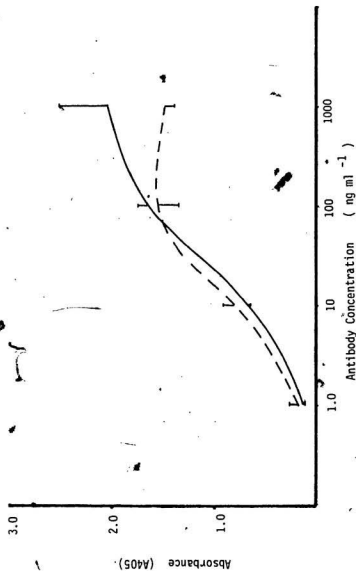


Figure 3. Standard curves comparing anti-CEA binding of iodinated 11-285-14 — with present 11-285-14 —, by ELISA.

III 3.0 QUANTITATIVE RADIOLABELLED ANTIBODY BINDING ASSAY

III 3.1 Binding to Cell Lines

The range of antibody binding to cell lines of varying CEA expression is illustrated in figure 4 , for two CEA expressors LS174T and BENN, and the non-expressor COLO320DM.

III 3.2 Estimate of Number of Binding Antibodies

Of the 100 ug of antibody used for the iodination, 89.1% or 89.1 ug was recovered from the column, and of this, 98.5% was iodide associated or 87.8 ug.

To calculate the number of antibody molecules this represents, Avogadro's number (6.023×10^{23}), the amount of antibody in grams ($87.8 \times 10^{-6}g$) and the molecular weight of mouse immunoglobulin G1 (1.5×10^5) was used.

$$\text{Number of iodinated antibody molecules} = \frac{(87.8 \times 10^6) \times (6.023 \times 10^{23})}{1.5 \times 10^5}$$

$$3.53 \times 10^{14}$$

This equated to a count of 324736115 cpm, section III 2.1(a), or 3.25×10^8 cpm corrected to two decimal places.

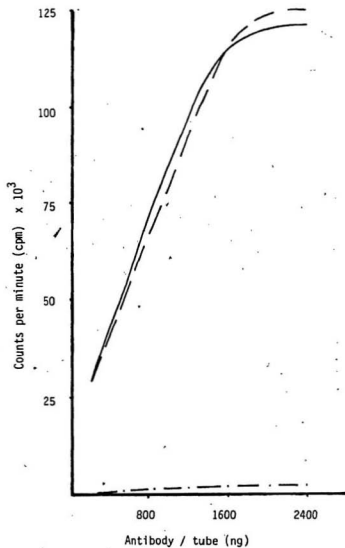


Figure 4. Antibody binding curves for LS174T

BENN and COL0320DM

Therefore, the number of antibody molecules associated with 1 cpm

$$= \frac{\text{number of antibody molecules}}{\text{total cpm}}$$

$$= \frac{3.53 \times 10^{14}}{3.25 \times 10^8}$$

$$= 1.09 \times 10^6 \text{ antibody molecules}$$

As the binding assay was performed 4 days following iodination, the decay of .0955 was accounted for, so that on the day of assay

$$1 \text{ cpm} = 1.14 \times 10^6$$

The number of antibody molecules is then calculated by multiplying the cpm obtained at saturation (start of the plateau portion of the binding curve) by this figure (antibody molecules associated with 1 cpm)

$$\begin{aligned} \text{For example, for the cell line BENN, this was } 125,628 \times 1.14 \times 10^6 \\ = 1.432 \times 10^{11} \text{ antibody molecules} \end{aligned}$$

The number of antibody molecules binding to each cell (assuming equal antigen density)

$$= \frac{\text{number of antibody molecules}}{\text{number of cells}} = \frac{1.432 \times 10^{11}}{2.5 \times 10^5}$$

$$= 5.73 \times 10^5$$

Using similar calculations the number of antibody molecules binding to the two other cell lines were:

LS174T: 5.55×10^5

COLO320DM, 1.31×10^4

III 4.0 IN VITRO EFFICACY

III 4.1 Cell Lines

Of the ten cell lines available, eight were tested in vitro (CALU 6 and LoVo were excluded), and efficacy of monoclonal anti-CEA vindesine conjugates is illustrated in figures 5 to 10, where dose-response curves are presented for six target cell lines, four colorectal and two lung. Percentage survival of cells is shown along the ordinate and drug and antibody concentration of conjugates, using a logarithmic scale, along the abscissa.

The assays presented in this section are shown to illustrate results using selected cell lines, and represent approximately one fifth of the total performed. The mean counts for each assay with one standard deviation are also included, tables 4 to 9.

An effect of conjugate was seen for the high CEA expressing lines LS174T, figure 5; SKCO1, figure 6; BENN, figure 7; and the intermediate expressor, HT29, figure 8, whereas no effect was seen upon the non-expressors COLO320DM, figure 9, or A549, figure 10.

An effect of free drug, vindesine, upon these cell lines is also seen, and is represented by the broken line.

Immunocytochemical evaluation of individual cell lines used in these assays is shown in table 10. Cell smears were prepared at passage of cell lines used in microcytostasis assays, but those from A549 were not available.

In addition, SW1116 and SW837 were also assayed three times each, noting no effect of free drug or conjugate (data not shown).

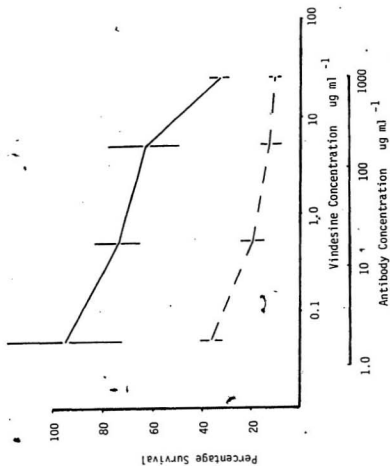


Figure 5. Dose-Response curves for Vindesine — and 11-285-14 Vindesine Conjugate — on cell line LS174T, with standard deviation.

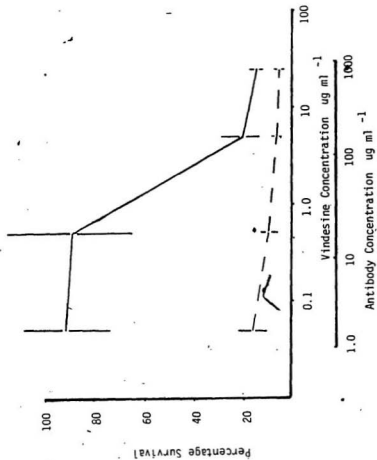


Figure 6. Dose-Response curves for Vindesine — and 11-285-14 Vindesine Conjugate — on cell line SKCO1, with standard deviation.

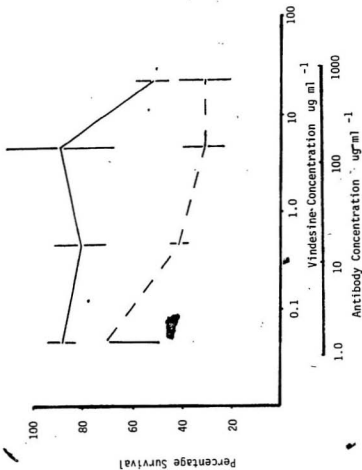


Figure 7. Dose-Response curves for Vindesine — — — and 11-285-14 Vindesine Conjugate — — — on cell line BENN, with standard deviation.

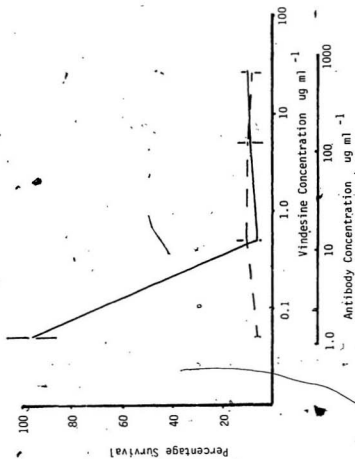


Figure 8. Dose-Response curves for Vindesine — and 11-285-14 Vindesine Conjugate — on cell line HT29, with standard deviation.

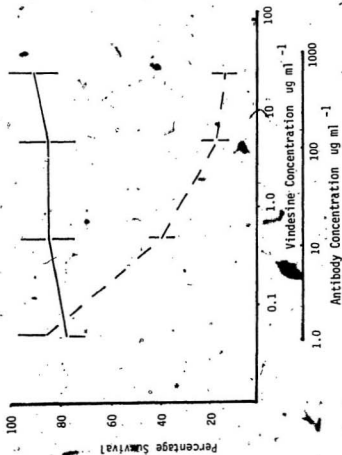


Figure 9. Dose-Response curves for Vindesine — and 11-285-14 Vindesine conjugate — on cell line COLO320DM, with standard deviation.

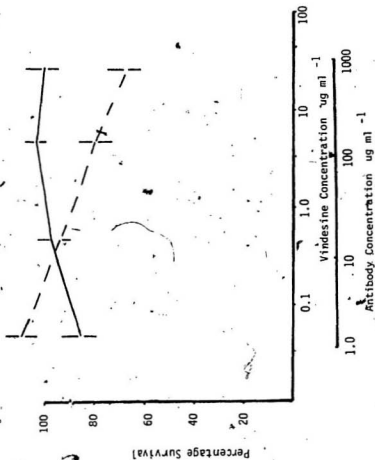


Figure 10. Dose-Response curves for Vindesine — and 11-285-14 Vindesine Conjugate — on cell line A549, with standard deviation.

Table 4. COUNTS OBTAINED FROM MICROCYTOSATIS ASSAY FOLLOWING 24 HOUR EXPOSURE OF LS174T CELLS TO VINDESINE AND 11-285-14-VINDSINE CONUGATE (STANDARD DEVIATION, S.D.)

VINDESINE CONCENTRATION ng ml ⁻¹	VINDESINE		CONUGATE	
	MEAN COUNTS (S.D.)	PERCENTAGE OF CONTROL (S.D.)	MEAN COUNTS (S.D.)	PERCENTAGE OF CONTROL (S.D.)
24,000	9,234 (1,150)	11.1 (1.4)	27,810 (3,423)	33.4 (4.1)
4,800	11,232 (3,498)	13.5 (4.2)	52,042 (12,231)	62.5 (14.7)
480	17,100 (4,259)	20.5 (5.1)	61,394 (7,405)	73.8 (8.9)
48	30,762 (3,950)	37.0 (4.7)	78,249 (18,930)	94.0 (22.7)

CONTROL COUNTS: 83,236 ± 23587

Table 5. COUNTS OBTAINED FROM MICROCYTOSTASIS ASSAY FOLLOWING 24 HOUR EXPOSURE OF SKOOL CELLS TO VINDESINE AND 11-285-14 - VINDESINE CONUGATE (STANDARD DEVIATION, S.D.)

VINDESINE CONCENTRATION	VINDESINE		CONUGATE	
	MEAN COUNTS (S.D.)	PERCENTAGE OF CONTROL (S.D.)	MEAN COUNTS (S.D.)	PERCENTAGE OF CONTROL (S.D.)
ng ml ⁻¹				
24,000	9,796 (2,334)	7.1 (1.7)	20,514 (4,006)	14.8 (2.9)
4,800	9,103 (1,967)	6.6 (1.4)	29,352 (11,938)	21.2 (8.6)
480	13,631 (3,992)	9.9 (2.9)	123,966 (35,313)	89.6 (25.5)
48	22,222 (8,104)	16.1 (5.9)	125,024 (23,963)	91.1 (17.3)

CONTROL COUNTS: 138,374 \pm 37,135

Table 6 COUNTS OBTAINED FROM MICROCYTOSTASIS ASSAY FOLLOWING 24 HOUR EXPOSURE OF BENN CELLS TO VINDESINE
 5, 11-285-14 - VINDESINE CONTIGATE (STANDARD DEVIATION, S.D.)

VINDESINE CONCENTRATION ng ml ⁻¹	VINDESINE		CONTIGATE	
	MEAN COUNTS (S.D.)	PERCENTAGE OF CONTROL (S.D.)	MEAN COUNTS (S.D.)	PERCENTAGE OF CONTROL (S.D.)
24,000	1,506 (507)	31.7 (10.7)	2,476 (353)	52.2 (7.4)
4,800	1,484 (399)	31.3 (8.4)	4,238 (1,040)	89.3 (21.9)
480	1,983 (190)	41.8 (4.0)	3,861 (503)	81.3 (10.6)
48	3,341 (1,165)	70.4 (24.5)	4,201 (303)	88.5 (6.4)

CONTROL COUNTS: 4,747 ± 878

Table 7. COUNTS OBTAINED FROM MICROCYTOSTASIS ASSAY FOLLOWING 24 HOUR EXPOSURE OF HT29 CELLS TO VINDESINE & 11-285-14- VINDESINE CONJUGATE (STANDARD DEVIATION, S.D)

VINDESINE CONCENTRATION ng ml ⁻¹	VINDESINE		CONJUGATE	
	MEAN COUNTS (S.D.)	PERCENTAGE OF CONTROL (S.D.)	MEAN COUNTS (S.D.)	PERCENTAGE OF CONTROL (S.D.)
24,000	11,125 (5,300)	9.1 (4.3)	12,259 (3,124)	10.0 (2.5)
4,800	12,373 (6,052)	10.1 (4.9)	11,755 (6,886)	9.6 (5.6)
480	13,450 (5,160)	10.9 (4.2)	8,088 (3,024)	6.6 (2.5)
48	7,320 (872)	6.0 (0.7)	119,467 (12,304)	97.2 (10.0)

CONTROL COUNTS: 122,896 \pm 11,640

Table 8. COUNTS OBTAINED FROM MICROCYTOSTASIS ASSAY FOLLOWING 24 HOUR EXPOSURE OF COL-320M CELLS TO VINDESINE & 11-285-14- VINDESINE CONJUGATE (STANDARD DEVIATION, S.D.)

VINDESINE CONCENTRATION ng ml ⁻¹	VINDESINE		CONJUGATE	
	MEAN COUNTS (S.D.)	PERCENTAGE OF CONTROL (S.D.)	MEAN COUNTS (S.D.)	PERCENTAGE OF CONTROL (S.D.)
24,000	21,033 (8,695)	13.8 (5.7)	137,930 (15,701)	90.8 (10.3)
4,800	26,413 (9,088)	17.4 (6.0)	129,053 (18,499)	85.0 (12.2)
480	60,068 (7,795)	39.5 (5.1)	128,744 (17,199)	84.8 (11.3)
48	130,385 (17,424)	85.8 (11.5)	117,821 (12,921)	77.6 (8.5)

CONTROL COUNTS: 151,900 ± 31,655

Table 9. COUNTS OBTAINED FROM MICROCYTOSTASTIS ASSAY FOLLOWING 24 HOUR EXPOSURE OF A649 CELLS TO VINDESINE & 11-285-14- VINDESINE CONJUGATE (STANDARD DEVIATION, S.D.)

VINDESINE CONCENTRATION ng ml ⁻¹	VINDESINE		CONJUGATE	
	MEAN COUNTS (S.D.)	PERCENTAGE OF CONTROL (S.D.)	MEAN COUNTS (S.D.)	PERCENTAGE OF CONTROL (S.D.)
24,000	179,640 (19,010)	67.7 (7.2)	266,739 (19,021)	100.5 (7.2)
4,800	211,808 (18,875)	79.8 (7.1)	273,806 (11,484)	103.2 (4.3)
480	250,213 (11,488)	94.3 (4.3)	256,421 (15,418)	90.7 (5.8)
48	289,249 (16,809)	109.0 (6.3)	227,084 (17,651)	85.6 (6.7)

CONTROL COUNTS: 266,280 ± 25,396

Table 10. IMMUNOCYTOCHEMICAL EVALUATION OF INDIVIDUAL CELL LINES USED
IN IN VITRO ASSAYS, USING 11-285-14 AT 10ug ml⁻¹

CELL LINE	PASSAGE NUMBER	% POSITIVITY	
		IMMUNOPEROXIDASE	IMMUNOFLUORESCENCE
SKCO1	50	80	80
LS174T	108	35	25
HT29	22	0	0
COLO320DM	90	0	0
BENN	63	> 70	20
A549	80	M	M

'M' denotes missing cell smears for this assay, although this cell line had consistently been a non-CEA expressor.

III 4.2 Assay Reproducibility

Figure 11 illustrates the reproducibility of this assay. Dose response curves for conjugate # 7 upon two different target cell lines SKCO1 and COLO320DM were obtained by two different investigators using the same assay technique, at different times. The interval between the assays using COLO320DM (passages 86 & 90) was 81 days and for SKCO1, (passages 44 & 50), 42 days.

III 4.3 Efficacy of Conjugates

The dose response curves illustrated in figure 12 were obtained from assays in which each conjugate, # 7, # 8, # 9 and # 10 was tested individually against a standard CEA expressing cell line, LS174T, in order to contrast efficacy.

III 4.4 Conjugate Anti-CEA Binding Activity (ELISA)

Retention of anti-CEA binding activity is illustrated for conjugates # 8, # 9, # 10 in figure 13, as determined by ELISA.

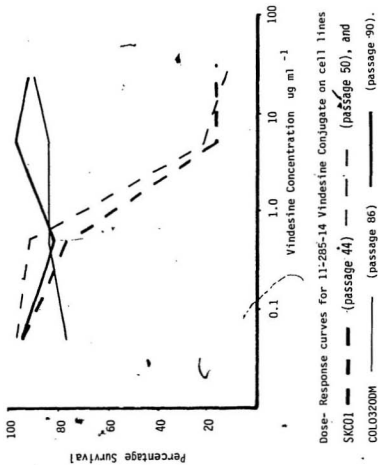


Figure 11. Dose-Response curves for 11-285-14 Vindesine Conjugate on cell lines SKCO1 (passage 44) (---) and COL03200M (passage 86) (—) (passage 50), and (passage 90).

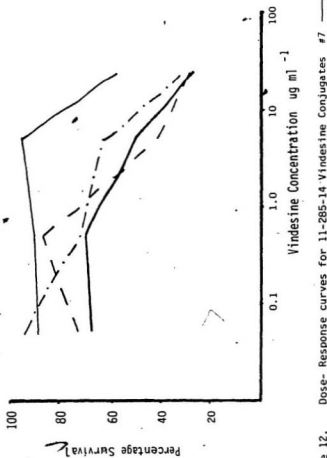


Figure 12. Dose-Response curves for 11-285-14 Vindesine Conjugates #7 ———— #8 - - - - - #9 - . - . - and #10 - - - - - against cell line LS1747.

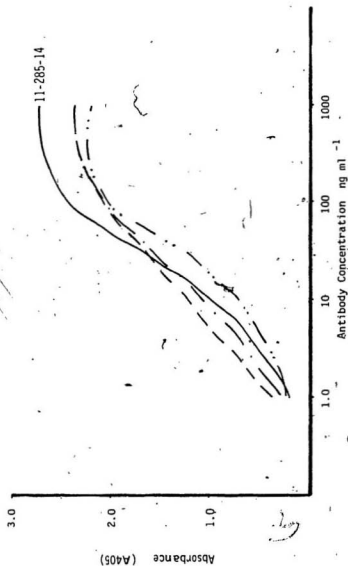


Figure 13. Standard Curves comparing anti-CEA binding of 11-285-14 Vindesine Conjugates #8 — — — — #9 — — — — and #10 — — — — by ELISA, to 11-285-14.

III 5.0 XENOGRAPHING

III 5.1 Tumor Growth

Using an inoculum of 10^7 viable cells, it was possible to grow human tumor cell lines as xenografts in nude mice, either bilaterally or unilaterally.

Cell lines grown as xenografts for the purpose of characterisation prior to performing any in vivo experiments, are listed in table 11. Mice bearing these initial tumors were sacrificed between 18 and 73 days following inoculation of the various cell lines, and tumor dimensions were available for 41 of a possible 44 tumors.

Figure 14 is a scatter graph relating tumor volume to weight for these initial tumors, with linear regression analysis. The correlation coefficient of weight to volume for all tumors was 0.85, with coefficients for individual tumor lines as follows: HT29, 0.85 (fourteen tumors); LoVo, 0.65 (six tumors); LSL74T, 0.98 (six tumors) and BENN, 0.95 (five tumors).

The weight-volume correlation coefficient for all tumors in these studies, including those for characterisation purposes only and tumors obtained from targeting experiments was 0.96 (154 tumors).

Three tumors did not exhibit growth at sixty days post inoculation during the characterisation of these tumors, two from the cell line SW837 and one from the cell line OLC320DM, and therefore the overall percentage of tumor "take" was 93.6 percent, using this tumor cell inoculum.

Table 11. HUMAN TUMOR CELL LINES GROWN AS XENOGRAFTS IN NUDE MICE
FOR CHARACTERIZATION ONLY

<u>CELL LINE</u>	<u>NUMBER OF TUMORS OBTAINED</u>
HT29	14
LoVo	8
LS174T	6
BEN	5
COLCB20DM	4
SW637	4
SW6116	1
CAU6	1
AS49	1
TOTAL	44

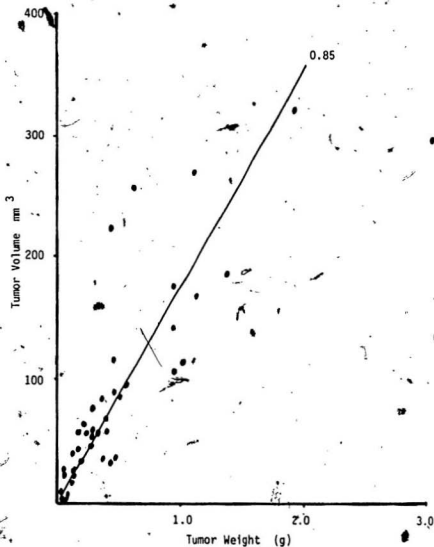


Figure 14. Scatter Graph showing Weight - Volume Correlation for Xenografts, with linear regression.
(Correlation Coefficient, 0.85)

III 5.2 Morphology

The tumors grew as discrete subcutaneous nodules that could easily be palpated and measured when the inoculum became established. Until this point, which was of a variable interval (2-10 days), the inoculum appeared diffuse and plaque-like with ill-defined borders. Growth progressed irregularly in three planes, often giving the impression of individual lobes within the tumor bulk. Satellite nodules were not seen for any xenograft tumor, although it was not uncommon to see tumor extending partially along the needle track used for the inoculation. When this was present, the tumor was noted to be in continuity with the main tumor mass, and was not existing as a discrete nodule.

Upon dissection, the tumors appeared well defined although there was no evidence of a discrete tumor capsule. Tumor surface appeared shiny and smooth, although the 'lobed' appearance gave rise to overall irregularity of shape.

The degree of vascularity of the tumor was extremely variable and inconsistent. Overall, the tumors appeared grossly avascular, with occasional visible blood vessels noted over the tumor surface, but rarely invading the bulk of the tumor itself. One tumor however, COLO 320DM excised 42 days post inoculation, appeared haemorrhagic and gelatinous throughout, with no evidence of necrosis or cavitation, making dissection difficult.

Tethering to skin was a finding that was also variable and appeared to be unrelated to tumor type. This uncommon finding occurred

in two or three early tumors (LoVo) and was probably related to the method of inoculation.

Frank ulceration of the overlying skin occurred with one tumor, LoVo, after 49 days inoculation. Mice exhibiting any suggestion of impending skin breakdown, would generally be sacrificed prior to its occurrence however. This particular tumor also appeared quite necrotic within the centre of the tumor mass, and cavitation had also been established in continuity with the skin breakdown. Varying degrees of central tumor necrosis were noted with all LoVo tumors, although no others had cavitated at the time of dissection.

Spread to deeper layers, in particular of the abdominal wall musculature and the proximal muscles of the hind-limbs was noted on two occasions only, with the tumors LoVo, after 28 days inoculation and HT29, after 30 days inoculation. In the latter case, spread had occurred through the proximal muscle bulk of the right hind limb down to the right femur, resulting in an incomplete dissection, as the lower limit of the tumor could not be adequately defined.

Generally, all tumor bearing mice appeared to retain good general health, with no deaths prior to termination of the experiment. One notable exception however, related to mice inoculated with the cell line BENN. Of the three mice inoculated, two bilaterally and one unilaterally, two mice appeared extremely cachectic at the time of sacrifice 53 days post inoculation. Both mice carried bilateral tumors and total tumor burden for each mouse was 1.359g and 1.140g, compared to the unilaterally inoculated mouse, with a tumor mass of 1.138 grams.

Limited autopsy performed on selected mice bearing representative tumors from each cell line consistently failed to demonstrate

metastatic tumor deposits, with particular reference to the peritoneum and lung.

III 5.3 Histology

Haematoxylin and eosin staining of xenograft sections demonstrated that tumor architecture had been maintained, and this was confirmed by two independent staff pathologists. Histological interpretation is summarised in table 12, and illustrated in plate 10 by LSI74T, a moderately well differentiated adenocarcinoma.

The histology of xenografts from mice in the targeting experiments i.e. those treated with vindesine or antibody-drug conjugate was contrasted to their controls and this is referred to in subsequent sections.

III 5.4 Immunoperoxidase

Immunoperoxidase staining of a section of SW1116 xenograft, with its control slide, is illustrated by plates 11 and 12.

The distribution of CEA, similarly represented by the brown staining was found to be predominantly peri-luminal, although in other sections studied stromal staining was also prominent.

Table 13 summarises immunoperoxidase results obtained with xenografts, with correlation to the CEA expression of the inoculating cell line.

Immunocytochemistry of xenografts from targeting studies is considered in subsequent sections.

Table 12.

SUMMARY OF XENOGRRAFT HISTOLOGY

XENOGRRAFT	HISTOLOGY
COL0320DM	Carcinoma, undifferentiated, widespread necrosis.
LoVo	Adenocarcinoma, poorly-differentiated; non-infiltrating; patchy necrosis.
BENN	Adenocarcinoma; poorly differentiated; non-infiltrating; moderate necrosis.
SW837	Adenocarcinoma, moderate differentiation; non-infiltrating; little necrosis; cribriform pattern from large amounts of fibrosis.
HT29	Adenocarcinoma, moderate differentiation; moderate necrosis.
LS174T	Adenocarcinoma, moderately well differentiated; widespread necrosis; large amount of luminal mucin and debris.
Calu 6	Adenocarcinoma, poorly differentiated; little fibrosis.
A549	Adenocarcinoma, moderate differentiation; moderate fibrosis.
SW1116	Adenocarcinoma, moderate differentiation.

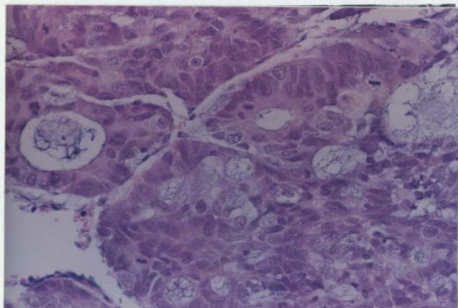
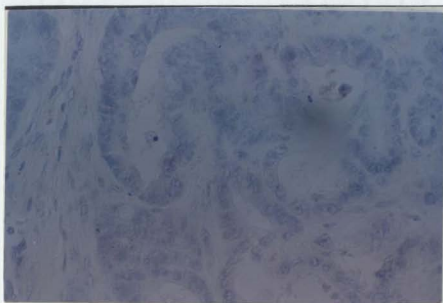
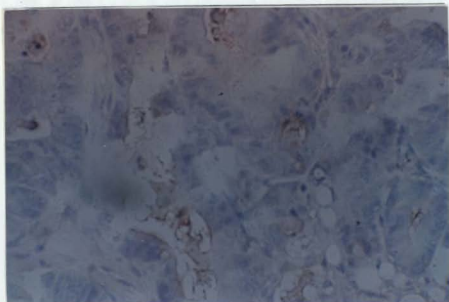


Plate 10. Haematoxylin and Eosin stain of LS1747
Xenograft section (x25 objective ; x10 eyepiece)



Plates 11 (above) & 12 (below). Immunoperoxidase of SW1116
Xenograft sections, using 11-285-14 at 10 ugml^{-1} (above) and
Control Ascites at corresponding concentration (below).

Table 13. CORRELATION OF CELL LINE AND XENOGRFT IMMUNOCYTOCHEMISTRY-
IMMUNOPEROXIDASE ASSAY USING 11-285-14 at 10ug ml-1

<u>CELL LINE/XENOGRFT</u>	<u>% POSITIVITY</u>	
	<u>CELL SMEAR</u>	<u>XENOGRFT</u>
HT29	5	<10
LoVo	30	<10
LS174T	70	30
BEN	90	30
COLC320DM	0	0
SW837	20	20
SW1116	>95	55
Calu 6	0	<5
A549	0	10

III 5.5 CEA Quantitation

To establish the range of CEA content of xenografts, five tumors underwent CEA extraction: LSI174T (CEA positive), three tumors; BENN (CEA positive), one tumor; A549 (CEA negative), one tumor. Xenografts of COL0320DM (CEA negative) were not available for processing.

Tumors were selected from control groups of targeting experiments or from initial characterisation studies, with the exception of one. LSI174T Xenograft (indicated by * below) which was obtained from a nude mouse treated with 11-285-14 vindesine conjugate in the second targeting experiment.

CEA content, quantified by ELISA, is summarised below. A 1:50 dilution of extract was used and results corrected for this dilution.

<u>XENOGRRAFT</u>	<u>CEA CONTENT (ng g⁻¹)</u>
LSI174T	15834.5
LSI174T*	14107.1
LSI174T*	11427.6
BENN	9574.5
A549	4083.3

* Extracted by S. Butt, summer student, Faculty of Medicine

III 6.0. PRELIMINARY EXPERIMENTS

Figures 15 and 16 illustrate the growth of individual tumors and mean tumor volumes of each treatment group for the preliminary experiments A and B respectively. Tumor volumes and their means are presented in tables 14 to 17 for both experiments.

III 6.1 Experiment A

From figure 15 it is seen that, graphically, there is a difference in growth between control group tumors and those treated with free vindesine. In view of the limited numbers of tumors used in this experiment, no attempt was made to interpret this data further, although in terms of the time required to reach a fixed tumor volume (500 mm³), the group receiving vindesine had a growth delay of 8 days compared to the control group.

One vindesine treated mouse, bearing bilateral tumors, died between 35 and 38 days post inoculation from unknown reasons, and the experiment was terminated at this point. No toxicity was seen in mice treated with vindesine during the experiment.

Loss of this mouse made it difficult to meaningfully contrast tumor weights of dissected xenografts at the termination of the experiment, and individual xenograft weights are listed in table 18.

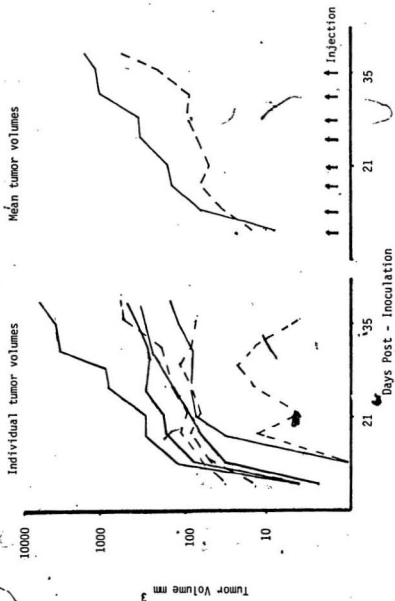


Figure 15. Individual and Mean Tumor Volumes of HT29 Xenografts, Experiment A
 — — — Group receiving Vindesine ——— Control.

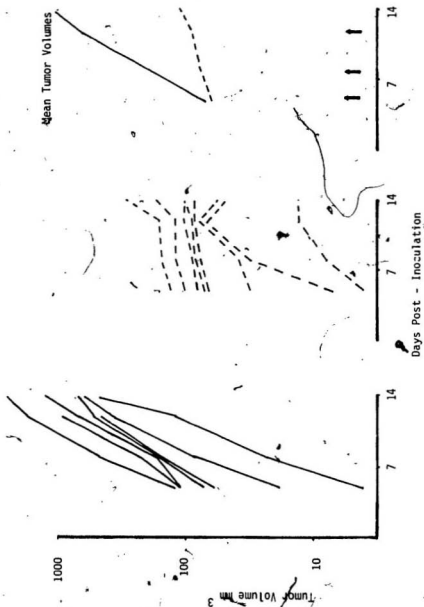


Figure 16. Individual and Mean Tumor Volumes of LS174T Xenografts, Experiment B
 --- Group receiving Vindesine — Control.

Abbreviations used in tables 14-48

R	Right flank
L	Left flank
*	Day of injection
M	Missing data
MTV	Mean tumor volume

Table 14. INDIVIDUAL TUMOR VOLUMES, EXPERIMENT A, (H29 XENOGRAFTS)

TREATMENT GROUP	VINDesine				CONTROL			
	1		2		1		2	
	R	L	R	L	R	L	R	L
MOUSE								
FLANK								
Days Post Inoculation								
11*	0	14.1	33.5	0	4.2	0	4.2	23.6
14*	0	47.7	65.4	0	37.7	0	87.1	144.0
18*	14.1	85.1	113.1	0	68.9	33.5	179.6	333.5
21*	4.2	65.4	95.0	0	91.6	78.5	205.2	333.5
25*	14.1	85.1	143.8	0	150.8	91.6	335.0	932.9
28*	22.5	113.1	176.9	0	216.7	91.6	309.2	1017.7
31*	14.1	87.1	197.9	0	282.2	91.6	318.3	393.6
35*	4.2	78.5	575.9	0	335.0	131.9	416.1	4410.0
38	M	M	628.2	0	378.2	160.2	567.0	6074.6

Table 15. INDIVIDUAL TUMOR VOLUMES, EXPERIMENT B, (LS174T XENOGRAFTS)

MOUSE	TUMOR VOLUMES VINDESINE GROUP (mm ³)							
	1		2		3		4	
FLANK	R	L	R	L	R	L	R	L
Days Post								
Inoculation								
5*	110.9	33.5	87.1	142.5	4.2	7.3	78.5	72.0
8*	131.9	41.9	95.0	169.6	8.2	33.5	91.6	85.1
12*	131.9	87.1	113.1	179.6	14.1	78.5	98.2	95.0
14	188.5	63.3	111.2	318.3	14.1	53.0	110.9	91.0
TUMOR VOLUMES CONTROL GROUP (mm ³)								
5*	126.7	110.9	4.2	74.2	113.1	63.6	18.8	0
8*	502.6	170.1	23.6	169.6	230.9	150.8	85.1	0
12*	1881.3	720.9	117.8	530.0	969.6	472.8	381.6	0
14	2641.6	1330.2	485.2	755.9	H	H	654.5	0

Table 16. MEAN TUMOR VOLUMES, EXPERIMENT A (HT29 XENOGRAFTS)

DAYS POST INOCULATION	TREATMENT GROUP	
	MEAN TUMOR VOLUMES (mm ³)	
	VINDESINE	CONTROL
11*	15.8	8.0
14*	37.7	67.2
18*	70.8	153.9
21*	54.9	177.2
25*	81.0	377.6
28*	104.2	408.8
31*	99.7	1156.4
35*	219.5	1323.3
38	628.0	1795.0

Table 17. MEAN TUMOR VOLUMES, EXPERIMENT B (LS174T XENOGRAPHS)

DAYS POST INOCULATION	TREATMENT GROUP MEAN TUMOR VOLUMES (mm ³)	
	VINDESINE	CONTROL
5*	67.0	73.1
8*	82.1	190.4
12*	99.7	724.9
14	118.9	1173.5

Table 18.

TUMOR WEIGHTS (g) AT TERMINATION OF EXPERIMENT A
(HT29 XENOGRAPHS)

VINDESINE GROUP					
MOUSE	1		2		
	R	L	R	L	
FLANK					
WEIGHT	M	M	0.59	0	

CONTROL GROUP					
MOUSE	1		2		
	R	L	R	L	
FLANK					
WEIGHT	0.12	0.084	0.24	1.34	

III 6.2 Experiment B

The duration of the second preliminary experiment was much shorter, lasting only 14 days, reflecting the rapid growth of LS174T xenografts. Superficial tumor ulceration was noted at this time, and therefore the experiment was terminated. Although mice received only three injections of free drug, a difference between the two groups is seen in figure 16, both for the rate of growth of individual tumors and for mean tumor volumes of each group.

No vindesine toxicity was seen in this experiment, and the death of one mouse in the control group at 14 days was anaesthetically related. Individual and mean tumor volumes are recorded in tables 15 and 17, and tumor weights at the completion of the experiment, in table 19. Mean tumor weight for the control group was 0.533g (5 tumors) and for the vindesine treated group 0.055g (8 tumors).

III 6.3 Toxicity Study

Following the intraperitoneal bolus injection of free vindesine at 6 mg kg⁻¹ (2 mice) and 10 mg kg⁻¹ (3 mice), all mice appeared subjectively cachectic by day 2 post-injection. At day 3, one death had occurred in each group and the remaining mice appeared more cachectic than on the previous day, especially those receiving the higher concentration of free drug. This preliminary experiment was terminated at this point. Mice were not weighed during this experiment.

Table 19. TUMOR WEIGHTS (g) AT TERMINATION OF EXPERIMENT B (LS174T XENOGRAPHS)

MOUSE	VINCESINE GROUP			
	1	2	3	4
FLANK	R	L	R	L
WEIGHT	0.084	0.025	0.052	0.170
			0.009	0.018
			0.051	0.027
MOUSE	CONTROL GROUP			
	1	2	3	4
FLANK	R	L	R	L
WEIGHT	1.258	0.772	0.069	0.28
			M	M
			0.284	0

III 7.0 TARGETING EXPERIMENTS IN VIVO

III 7.1 Experiment 1 - COLO320DM

Individual tumor volumes of COLO320DM xenografts are listed in tables 20 , 21 and 22 and figure 17 contrasts growth of these xenografts in control, conjugate and vindesine treated groups. It was found that one tumor failed to grow at all throughout the experiment - mouse number 3, left flank, vindesine treated group. Although four tumors were not present in the control group at the start of treatment (right and left flanks of mice 5 and 6 of control group), all tumors were subsequently present 10 days post inoculation. One tumor (mouse 1, right flank, control group) became undetectable by day 21 post inoculation.

When mean tumor volume, table 23 , expressed on a logarithmic scale along the ordinate, of xenograft tumors in each group is plotted against time post-inoculation, along the abscissa, it can be seen that little difference exists graphically between the control and conjugate treated group, figure 18 . Despite no conjugate effect upon these non CEA expressing xenografts, a marginal effect of free drug is represented on this graph by the broken line.

The similarity of these human tumor xenografts at the termination of the experiment is illustrated by plate 13 , which shows representative nude mice from each of these treatment groups bearing tumors, and plate 14 showing dissected tumors from each group. The weights of individual tumors are recorded in table 24 , and mean tumor weights for each group are summarised in table 25 . Histological examination of xenograft sections from each group revealed little

Table 20. INDIVIDUAL TUMOR VOLUMES (mm³) EXPERIMENT 1 (CULO): CONTROL GROUP

MOUSE	1		2		3		4		5		6	
	R	L	R	L	R	L	R	L	R	L	R	L
Days Post Inoculation												
3*	63.6	37.7	56.4	23.6	32.1	53.0	72.0	50.3	0	0	0	0
7	58.6	50.3	85.1	44.9	50.3	23.6	91.6	41.9	91.6	9.8	0	10.5
10	41.9	41.9	95.0	46.1	37.7	85.1	179.6	95.0	235.6	243.7	74.2	104.7
14	47.7	72.0	78.5	50.3	33.5	235.6	321.5	150.8	466.4	510.6	104.7	335.0
17*	41.9	58.3	150.8	104.7	87.1	378.2	496.1	368.5	1130.8	1013.5	282.2	614.2
21	0	78.5	218.0	230.9	179.6	551.2	904.9	904.6	2389.6	1846.9	593.6	1539.1
24	0	78.5	472.5	265.5	424.0	572.4	1206.1	1761.1	3562.0	3477.1	950.2	2002.4
28	0	58.3	385.4	397.1	448.8	785.3	1431.1	2002.4	4157.6	4180.3	1415.5	2479.3
31*	0	102.9	628.2	728.4	698.8	1206.1	1761.1	1949.5	6646.4	5202.7	1693.0	3222.7
35	0	126.7	823.5	1055.4	760.1	1592.5	2002.4	3561.9	9161.3	8179.7	1356.9	3799.7
38	0	160.2	808.0	1282.8	1022.5	2641.2	2572.0	5235.0	9488.4	8179.7	2515.4	3404.2

Table-21. INDIVIDUAL TUMOR VOLUMES (mm³) EXPERIMENT 1 (COLO320DM): CONJUGATE TREATED GROUP

MOUSE	1		2		3		4		5	
FLANK	R	L	R	L	R	L	R	L	R	L
Days Post										
Inoculation										
3*	131.9	58.3	78.5	74.2	23.6	18.8	131.9	41.9	37.7	84.8
7	33.5	50.3	78.5	32.1	25.7	54.4	110.9	78.5	62.8	78.5
10	110.9	74.2	104.7	32.1	95.0	141.3	230.9	91.6	58.6	62.8
14	340.4	160.2	269.3	65.4	131.9	351.8	654.4	111.2	150.8	91.6
17*	448.8	301.6	385.3	47.7	160.2	680.6	950.2	294.5	230.9	104.7
21	508.8	575.6	779.2	78.5	309.2	759.1	1846.9	692.6	466.4	335.0
24	654.4	402.8	1055.4	53.0	466.4	1206.1	2636.7	934.6	823.5	385.3
28	680.6	628.2	1130.8	28.9	654.4	1871.1	3762.5	1766.8	823.5	551.2
31*	785.3	855.1	2389.6	41.9	1055.4	2680.3	4606.8	1846.9	1766.8	1145.2
35	1107.7	1017.7	4260.0	65.4	1526.5	3477.1	4535.6	2345.3	2422.9	1526.5
38	1356.9	1431.1	3607.2	53.0	1898.2	4157.6	5830.0	2296.9	2805.6	1816.1

Table 22. INDIVIDUAL TUMOR VOLUMES (mm³) EXPERIMENT 1 (COLO320DM): VINDESINE TREATED GROUP

MOUSE	1		2		3		4		5
FLANK	R	L	R	L	R	L	R	L	R
Days Post									
Inoculation									
3*	63.6	37.7	18.8	85.1	72.0	0	65.4	8.4	72.0
7	91.6	78.5	14.1	33.5	18.8	0	6.3	50.3	25.7
10	122.5	58.6	104.7	78.5	18.8	0	72.0	150.8	50.3
14	98.2	41.9	188.5	179.6	53.0	0	98.2	424.0	33.5
17*	118.8	72.0	265.4	131.9	65.4	0	188.0	779.2	41.9
21	192.4	53.0	732.9	368.5	122.5	0	732.9	1459.8	85.1
24	58.6	33.5	1013.5	628.2	113.1	0	837.6	2002.4	143.8
28	37.7	14.1	1431.4	602.0	131.9	0	1246.2	2680.3	179.6
31*	8.4	0	1769.4	680.6	122.5	0	1545.4	3404.1	154.8
35	0	0	2355.8	667.0	179.6	0	1453.9	3916.3	179.6
38	0	0	5574.2	732.9	279.7	0	2591.3	6049.7	268.0

Tumor Volume mm^3 (logarithmic scale)

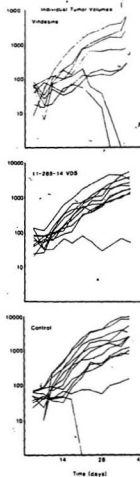


Figure 17. Graphs of individual COL0320DM xenograft tumor volumes, contrasting treatment groups in Experiment 1.

Table 23. MEAN TUMOR VOLUMES (MTV) EXPERIMENT 1 (COLO320DM)

DAYS POST INOCULATION	TREATMENT GROUP MTV (mm ³)		
	VINDESINE	CONJUGATE	CONTROL
3*	52.9	68.2	33.1
7	39.9	60.5	46.5
10	82.0	100.2	106.7
14	139.6	232.7	200.6
17*	207.8	360.5	393.9
21	468.4	635.1	786.4
24	603.8	861.8	1231.0
28	790.4	1189.9	1478.4
31*	960.7	1717.3	1986.5
35	1081.5	2264.5	2701.7
38	1937.0	2525.3	3109.1

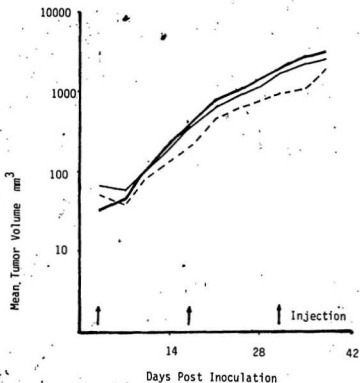
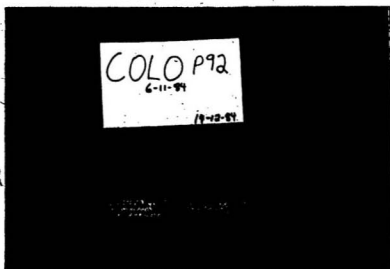


Figure 18. Mean Tumor Volumes of COLO320DM Xenografts, Experiment 1, treated with Vindesine — — —, 11-285-14 Vindesine Conjugate — — —, and Controls — — —



Plates 13 (above) & 14 (below). Nude mice bearing COLO320DM Xenografts, Experiment 1, from .Control (left), Conjugate (middle), and Vinorelbine treated groups (right) : plate 13. Dissected tumors, in corresponding order, plate 14.

Table 24.

TUMOR WEIGHTS (g) AT TERMINATION OF EXPERIMENT 1 (COLO320DM XENOGRAPHS)

VINDESINE GROUP									
MOUSE	1		2		3		4		5
FLANK	R	L	R	L	R	L	R	L	R
WEIGHT	0	0	1.655	0.305	0.156	0	1.478	2.44	0.164

CONUGATE GROUP									
MOUSE	1		2		3		4		5
FLANK	R	L	R	L	R	L	R	L	R L
WEIGHT	0.74	0.566	2.175	0.005	3.07	1.38	0.83	1.58	1.38 0.86

CONTROL GROUP											
MOUSE	1		2		3		4		5		6
FLANK	R	L	R	L	R	L	R	L	R	L	L
WEIGHT	0	0.09	0.51	0.63	0.884	1.244	0.75	2.42	5.03	4.68	1.167 2.264

Table 25. MEAN TUMOR WEIGHTS AT TERMINATION OF EXPERIMENT 1 (COLO320DM)

TREATMENT GROUP	MEAN TUMOR WEIGHT	NUMBER OF TUMORS	PERCENTAGE OF CONTROL WEIGHT
Vindesine	0.689	9	42
Conjugate	1.260	10	77
Control	1.639	11	100

difference between groups, all were undifferentiated carcinomas, although generally greater necrosis was seen in sections of conjugate treated tumors.

No toxicity was exhibited by mice in these groups throughout the course of the experiment.

Table 26 , summarises the immunocytochemical characteristics of xenografts used in each targeting experiment, and contrasts these to the CEA expression of individual inoculating cell lines used in each of these experiments.

Table 26. CORRELATION OF XENOGRAPH SECTIONS WITH INOCULATING CELL
LINE BY IMMUNOPEROXIDASE ASSAY (11-285-14 at 10ug ml⁻¹)

TARGETING EXPERIMENT/ TREATMENT GROUP	% POSITIVITY	
	XENOGRAPH	CELL SMEARS
# 1 - CLO320DM		0
Control	0	
Conjugate	0	
Vindesine	<5	
# 2 - LS174T		70
Control	30	
Conjugate	40	
Vindesine	45	
# 3 - BENN		90
Control	30	
Conjugate	65	
Vindesine	20	
# 4 - SKCO1		90
Control	0	
Conjugate	<2	
Vindesine	<2	
# 5 - SW1116		45
Control)		
Conjugate)	ONGOING	
Vindesine)		

III 7.2 Experiment 2 - LSL74T

Individual tumor volumes of LSL74T xenografts are listed in tables 27 to 29 and contrasted graphically in figure 19. Mean tumor volumes of xenografts in each treatment group are listed in table 30 and represented graphically in figure 20. Two mice in the group receiving vindesine died early in the experiment as a consequence of anaesthesia, and no drug or conjugate toxicity was seen during the experiment.

It can be seen from figure 20 that a difference exists between the vindesine and the conjugate treated groups compared to controls, signifying an effect of both conjugate and vindesine upon xenograft growth. The effect of vindesine appears to begin to diminish towards the end of the experiment however.

Plates 15 and 16 illustrate the difference in tumor growth between groups at the termination of the experiment, for mice bearing tumors and dissected tumors.

Weights of individual tumors are recorded in table 31, and mean tumor weights are contrasted in table 32.

Histologically, increased necrosis was seen in sections of xenografts from the conjugate treated group, with otherwise little difference between xenograft sections from control and vindesine treated groups, representing moderately well differentiated adenocarcinomas.

Table 27. INDIVIDUAL TUMOR VOLUMES (mm³) EXPERIMENT 2 (LS174T): CONTROL GROUP

MOUSE	1	2	3	4	5	6	7	8	9
Days Post Inoculation									
4*	169.6	113.1	210.1	63.6	141.3	85.8	131.9	111.2	91.6
7	169.6	144.0	279.7	448.8	210.1	466.4	279.7	210.1	368.5
11	811.4	263.8	602.0	602.0	368.5	1641.7	980.0	469.1	1206.1
14	2814.3	331.8	1022.5	1194.4	823.5	3366.8	2002.4	890.0	2747.3
18*	7236.9	699.7	1846.9	3177.1	2814.3	6233.3	4606.8	2146.7	5967.3
21	9426.9	1384.7	3631.0	5720.0	4120.9	9112.7	8850.6	3563.1	8962.2
25	13439.5	2257.3	6291.7	9498.4	4976.5	12032.1	11324.4	6233.3	11573.8
28	19479.1	3782.3	7615.6	13547.9	7892.5	15596.1	13602.2	8744.5	14969.0
32*	18813.0	4724.6	6841.1	15440.0	9112.7	17610.5	12499.4	8584.9	14528.7
35	19906.1	6002.5	6579.6	17610.5	11396.5	16417.0	15596.1	9950.7	15849.5
38	28216.7	6160.0	7950.7	25719.6	11396.5	23318.8	19788.3	12867.0	17903.7

Table 28.

INDIVIDUAL TUMOR VOLUMES (mm³) EXPERIMENT 2 (LS174T): CONJUGATE TREATED GROUP

MOUSE	1	2	3	4	5	6	7	8
Days Post Inoculation								
4*	160.2	126.7	165.9	230.9	179.6	192.4	141.3	141.3
7	179.6	117.8	265.0	192.4	243.7	230.9	340.4	165.9
11	131.9	508.8	750.3	424.0	230.9	141.3	445.2	207.3
14	131.9	732.9	1168.5	523.5	320.6	199.1	628.2	245.0
18*	179.6	1908.2	2546.3	696.8	1013.5	243.7	1621.9	624.1
21	235.6	2709.1	5540.7	785.3	1725.2	294.5	2238.0	826.8
25	179.6	3933.6	8443.0	1459.8	2881.3	466.4	4479.2	1125.5
28	235.6	8584.9	11324.4	1981.2	4120.4	654.4	8798.5	1635.9
32*	268.0	14252.3	17903.7	2680.3	4920.9	1235.2	10262.7	2473.5
35	268.0	17903.7	23373.8	3561.9	6334.4	1168.5	10142.8	2079.1
38	301.5	20123.3	22076.3	2993.0	7839.9	1431.4	14088.4	3121.4

Table 29. INDIVIDUAL TUMOR VOLUMES (mm³) EXPERIMENT 2 (LS174T) VINDESINE TREATED GROUP

MOUSE	1	2	3	4	5	6	7	8
Days Post Inoculation								
4*	179.0	335.0	402.8	179.0	158.4	150.8	131.9	188.5
7	207.3	169.6	230.9	M	207.3	150.8	98.2	142.5
11	427.0	268.0	M	M	282.2	230.9	169.6	256.5
14	811.4	335.0	M	M	593.6	368.5	282.2	466.4
18*	1013.5	791.8	M	M	2355.8	614.2	785.3	980.0
21	890.0	1055.4	M	M	2355.8	969.3	1076.8	1130.8
25	1432.3	1884.6	M	M	3328.4	1327.1	1681.0	1356.9
28	1769.4	2345.3	M	M	4777.5	1590.4	2355.8	1096.6
32*	2257.3	3025.8	M	M	7805.8	2278.3	3816.3	1813.7
35	2709.1	3177.1	M	M	8433.0	2211.3	4816.2	2003.6
38	2650.2	3206.4	M	M	8744.5	2002.4	5025.6	2308.6

Tumor Volume mm^3 (logarithmic scale)

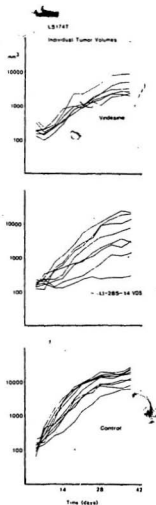


Figure 19. Graphs of individual LS174T xenograft tumor volumes, contrasting treatment groups in Experiment 2.

Table 30. MEAN TUMOR VOLUMES (MTV) EXPERIMENT 2 (LS174T)

DAYS POST INOCULATION	TREATMENT GROUP MTV (mm ³)		
	VINDESINE	CONJUGATE	CONTROL
4*	215.7	167.3	124.1
7	172.4	217.0	286.3
11	272.4	355.0	771.6
14	476.2	493.7	1608.1
18*	1090.1	1104.3	3858.8
21	1246.4	1794.4	6085.8
25	1835.1	2871.1	8625.2
28	2322.5	4667.0	11692.1
32*	3499.5	6749.6	12017.2
35	3891.7	8104.0	13256.5
38	3989.6	8996.9	17035.7

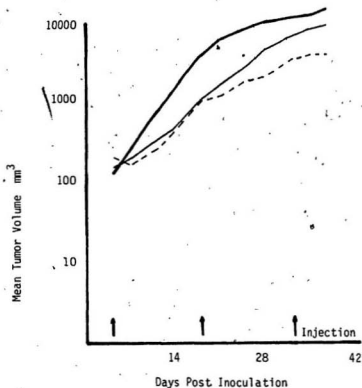
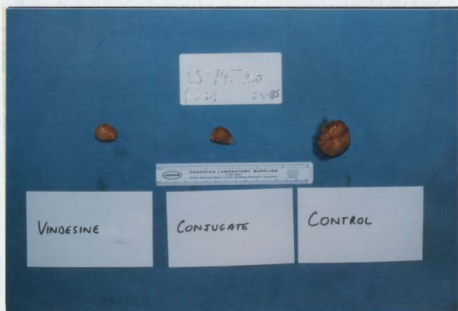


Figure 20. Mean Tumor Volumes of LS174T Xenografts, Experiment 2, treated with Vindesine — — —, 11-285-14 Vindesine Conjugate —————, and Controls —————



Plates 15 (above) & 16 (below). Nude mice bearing LS174T Xenografts, Experiment 2, from Vindesine, Conjugate and Control groups (left - right) : plate 15. Dissected tumors shown in plate 16, below.

Table 31 TUMOR WEIGHTS (g) AT TERMINATION OF EXPERIMENT 2 (LS174T XENOGRAFTS)

		VINESINE GROUP							
MOUSE	1	2	3	4	5	6	7	8	
WEIGHT	2.14	1.46	M	M	5.74	1.30	3.17	1.24	
		CONJUGATE GROUP							
MOUSE	1	2	3	4	5	6	7	8	
WEIGHT	0.07	11.53	17.88	1.80	3.59	0.63	7.57	2.21	
		CONTROL GROUP							
MOUSE	1	2	3	4	5	6	7	8	9
WEIGHT	14.56	12.09	4.90	17.49	8.07	17.12	16.37	8.66	10.46

Table 32. MEAN TUMOR WEIGHTS AT TERMINATION OF EXPERIMENT 2 (LS174T)

TREATMENT GROUP	MEAN TUMOR WEIGHT (g)	NUMBER OF TUMORS	PERCENTAGE OF CONTROL WEIGHT
Vindesine	2.50	6	21
Conjugate	5.66	8	46
Control	12.19	9	100

III 7.3 Experiment 3 - BENN

Individual tumor volumes of BENN xenografts are recorded in tables 33 , 34 and 35 , and contrasted graphically in figure 21 . Mean tumor volumes of xenografts from each treatment group are recorded in table 36 and presented graphically in figure 22 .

An effect of conjugate is seen upon xenograft growth throughout the experiment, apparently greatest within the initial 25 days but persisting until the termination of the experiment. The free drug also had an effect upon tumor growth and the growth curve of these xenografts is seen to lie between that of the control and conjugate treated groups.

At 20 days post-inoculation, one mouse in the control group (number 1) appeared extremely cachectic and was therefore sacrificed. At 30 days post-inoculation, a further mouse (number 4) also appeared cachectic and by 44 days, all mice in the control group exhibited this state, and a further death had occurred (mouse number 7).

Cachexia and mortality was also seen in the group treated with vindesine, one mouse appearing ill at 6 days post inoculation (number 4, vindesine treated group) and by the termination of the experiment at 61 days, only two mice remained alive. One death, however, was a consequence of anaesthesia, and this was accounted for in the survival chart of mice in these groups, figure 23 . All mice receiving conjugate appeared well until 44 days, at which time, mouse number 3 appeared to become cachectic, although even at 58 days, the degree of cachexia exhibited by three mice in this groups (numbers 3, 4, and 5) was subjectively much less than that seen in the other two groups. An

Table 33. INDIVIDUAL TUMOR VOLUMES (mm³) EXPERIMENT 3 (BENN): CONTROL GROUP

MOUSE	1	2	3	4	5	6	7
Days Post Inoculation							
2*	28.3	150.8	91.6	126.7	44.9	110.9	104.7
6	13.6	78.5	95.0	63.6	44.9	85.1	91.6
9	137.4	41.9	91.6	72.0	98.2	78.5	72.0
13	551.2	33.5	46.1	65.4	35.3	65.4	72.0
16*	696.8	41.9	41.9	87.1	38.5	41.9	65.4
20	796.2	58.6	50.3	235.6	28.3	78.5	91.6
23	M	72.0	110.9	321.5	58.6	131.9	113.1
26	M	205.2	110.9	424.0	74.2	131.9	268.0
30*	M	378.2	256.5	549.7	112.2	230.9	381.6
34	M	680.6	487.6	855.1	188.5	530.0	575.9
37	M	823.5	614.2	823.5	256.5	680.6	575.9
41	M	1243.8	886.8	1327.1	333.5	1055.4	823.5
44	M	1281.5	1130.8	1539.1	359.1	1055.4	M

Table 33 - continued

48	M	1846.9	1327.1	2144.3	502.6	1744.3	M
51	M	2326.8	1327.1	1884.6	469.1	1744.3	M
55	M	2238.0	1526.5	2002.4	502.6	2326.8	M
58	M	2921.7	1693.0	1795.6	678.5	2479.3	M
61	M	3561.9	1744.3	2345.3	811.4	2345.3	M

Table 34. INDIVIDUAL TUMOR VOLUMES (mm³) EXPERIMENT 3 (BENN): CONJUGATE TREATED GROUP

MOUSE	1	2	3	4	5	6	7
Days Post Inoculation							
2*	104.7	158.4	126.7	205.2	110.9	141.3	131.9
6	51.3	130.9	74.2	91.6	78.5	74.2	32.1
9	28.3	38.5	50.3	41.9	25.9	41.9	16.5
13	4.2	63.6	68.9	53.0	23.6	18.8	25.7
16*	0	53.0	41.9	41.9	46.1	9.8	14.1
20	4.2	13.1	65.4	53.0	78.5	4.2	78.5
23	33.5	4.2	98.2	72.0	113.1	0	58.3
26	18.8	0	230.9	78.5	131.9	9.8	50.3
30*	46.1	0	496.1	165.9	205.2	6.3	63.6
34	104.7	0	575.9	218.0	416.1	33.5	50.3
37	176.9	0	900.0	282.2	378.2	65.4	91.6
41	199.1	0	1194.4	368.5	628.2	113.1	104.7

Table 34 - continued

44	265.0	0	1130.8	529.5	791.8	78.5	230.9
48	335.0	0	1431.1	508.8	886.8	176.9	169.6
51	294.5	0	1766.8	680.6	969.3	230.9	340.4
55	256.5	0	1415.5	637.8	1282.8	199.1	416.4
58	368.5	0	1693.0	732.9	1349.6	256.5	508.8
61	256.5	0	1884.6	950.2	1548.3	294.5	572.4

Table 35.

INDIVIDUAL TUMOR VOLUMES (mm³) EXPERIMENT 3 (BENN): VINDESINE TREATED GROUP

MOUSE	1	2	3	4	5	6	7
Days Post Inoculation							
2*	131.9	110.9	57.7	179.6	154.8	91.6	41.9
6	98.2	67.0	48.1	41.9	91.6	23.6	67.0
9	28.3	25.9	58.6	33.5	65.4	13.1	28.3
13	46.1	41.9	104.7	33.5	46.1	0	91.6
16*	41.9	46.1	104.7	8.2	38.5	14.1	95.0
20	23.6	18.8	98.2	16.5	18.8	18.8	131.9
23	65.4	91.6	221.2	21.2	104.7	41.9	179.6
26	113.1	98.2	323.9	8.2	117.8	65.4	359.3
30*	M	174.2	418.8	33.5	169.6	104.7	606.0
34	M	282.2	466.4	14.1	150.8	221.2	523.5
37	M	593.6	785.3	33.5	294.5	207.3	1055.4
41	M	1076.8	918.5	33.5	416.1	323.9	934.6
44	M	755.9	M	37.7	466.4	307.8	1327.1

Table 35 - continued

48	M	1266.9	M	M	723.9	402.0	1415.5
51	M	1902.1	M	M	636.1	435.6	1504.0
55	M	2767.0	M	M	708.7	338.6	1504.0
58	M	2701.1	M	M	865.7	256.5	M
61	M	4070.7	M	M	M	279.7	M

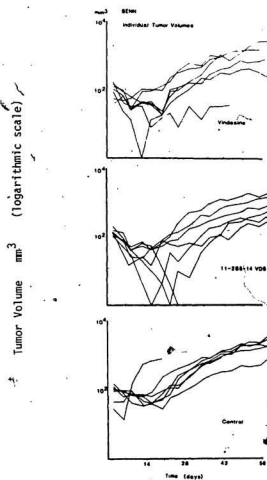


Figure 21. Graphs of individual BENN xenograft tumor volumes, contrasting treatment groups in Experiment 3.

Table 36 MEAN TUMOR VOLUMES (MIV) EXPERIMENT 3 (BENN XENOGRAPHS)

DAYS POST INOCULATION	TREATMENT GROUP MIV (mm ³)		
	VINDesine	CONJUGATE	CONTROL
2*	109.8	139.9	94.0
6	62.5	76.1	67.5
9	36.2	34.8	84.5
13	52.0	36.8	124.1
16*	49.8	29.5	144.8
20	46.7	42.4	191.3
23	103.7	54.2	134.7
26	155.1	74.3	202.4
30*	251.1	140.5	318.0
34	276.4	199.8	553.0
37	494.9	270.6	629.0
41	617.2	372.6	945.0
44	579.0	432.4	1073.2
48	952.1	501.2	1513.0
51	1119.5	611.8	1550.4
55	1329.6	601.1	1719.3
58	1277.1	701.3	1913.6
61	2175.2	786.6	2161.6

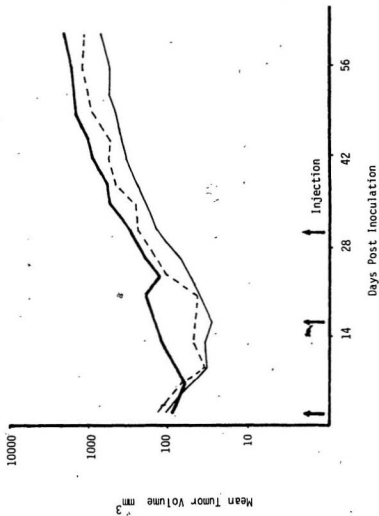


Figure 22. Mean Tumor Volumes of BENN Xenografts, Experiment 3, treated with Vindesine — 11-285-14 Vindesine Conjugate — and Controls - - -

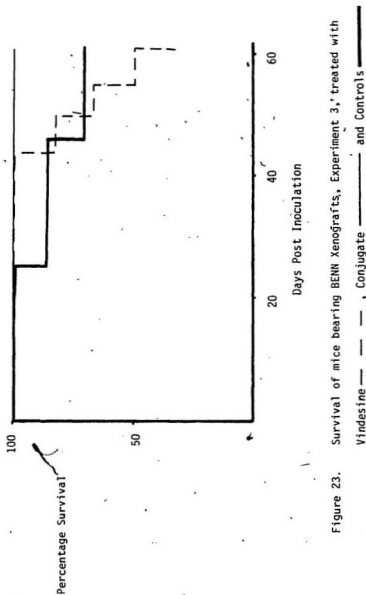


Figure 23. Survival of mice bearing BENN Xenografts, Experiment 3, treated with

Vindesine — — — — — , Conjugate — — — — — and Controls — — — — —

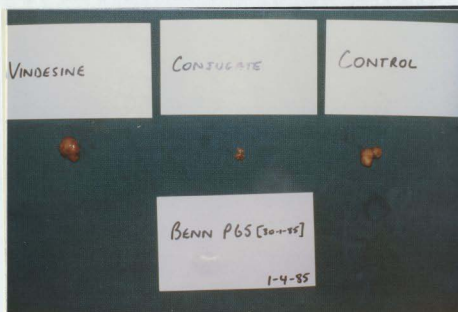
attempt to quantitate cachexia was made, using body weight as an index, and this is summarised below, contrasting mean body weights of mice in each treatment group at the start and termination of the experiment.

Treatment Group	<u>Day 2 Post-inoculation</u>		<u>Day 61 Post-inoculation</u>	
	Mean Body	Number	Mean Body	Number
	<u>Weight (g)</u>	<u>of mice</u>	<u>Weight (g)</u>	<u>of mice</u>
Control	25	7	18	5
Conjugate	25	7	23	7
Vindesine	25	7	21	2

Plate 17 illustrates representative mice from each group bearing tumors and plate 18 illustrates the dissected tumors.

Individual tumor weights are recorded in table 37, and mean tumor weights contrasted between groups in table 38.

Histological examination of xenograft sections, from each group revealed greater tumor necrosis in sections from the conjugated treated group, compared to control or vindesine treated groups. Plates 19, 20 and 21 illustrate histological sections from these three groups, the tumor being a poorly differentiated adenocarcinoma.



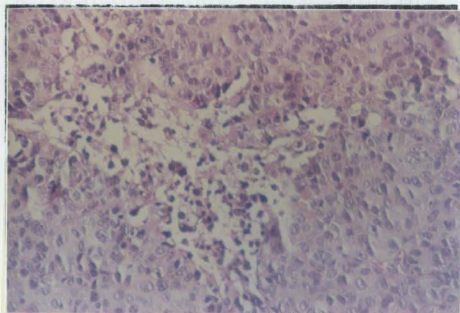
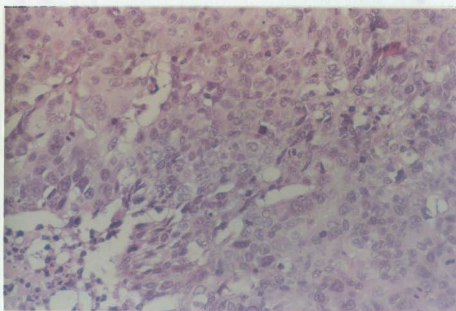
Plates 17 (above) & 18 (below). Nude mice bearing BENN Xenografts, Experiment 3, from Vindesine, Conjugate and Control groups (left - right) : plate 17. Dissected tumors shown in plate 18, below.

Table 37. TUMOR WEIGHTS (g) AT TERMINATION OF EXPERIMENT 3 (BENN XENOGRAFTS)

		VINDESINE GROUP						
MOUSE	1	2	3	4	5	6	7	
WEIGHT	M	1.89	M	M	M	0.12	M	
		CONUGATE GROUP						
MOUSE	1	2	3	4	5	6	7	
WEIGHT	0.15	0	0.93	0.69	0.74	0.12	0.16	
		CONTROL GROUP						
MOUSE	1	2	3	4	5	6	7	
WEIGHT	M	1.21	0.98	1.28	0.31	1.62	M	

Table 38. MEAN TUMOR WEIGHTS AT TERMINATION OF EXPERIMENT 3 (BENN)

TREATMENT GROUP	MEAN TUMOR WEIGHT (g)	NUMBER OF TUMORS	PERCENTAGE OF CONTROL WEIGHT
Vindesine	1.01	2	94
Conjugate	0.4	7	37
Control	1.08	5	100



Plates 19 (above) & 20 (below). Haematoxylin and Eosin stain of BENN Xenograft sections from Control (above) and Conjugate (below) treated mice in Experiment 3. (x16 objective; x10 eyepiece).

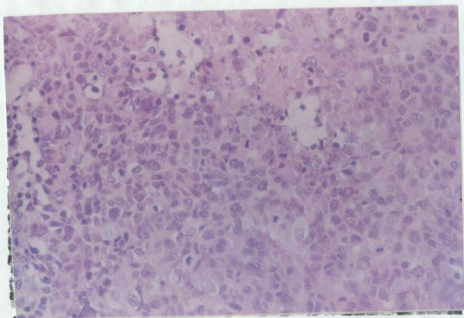


Plate 21. Haematoxylin and Eosin stain of BENN Xenograft
section from Vindesine treated mouse in Experiment 3.
(x16 objective; x10 eyepiece)

III 7.4 Experiment 4 - SKOOL

Tables 39 ,40 and 41 list tumor volumes from the three treatment groups in this experiment, and these are contrasted graphically in figure 24 . Mean tumor volumes are listed in table 42 , and represented graphically in figure 25 .

It can be seen that although a steady no-growth state was reached by all tumors in each of the treatment groups, a conjugate effect was also seen throughout the course of the experiment.

Only one mouse died during this experiment, number 6 of the conjugate group, between 20 and 24 days, with no apparent explanation.

Plates 22 and 23 illustrate the size of the xenografts at the termination of the experiment, and the tumor weights are recorded in table 43 . Mean tumor weights at the termination of the experiment may be contrasted between groups, table 44 .

Histology failed¹ to demonstrate any tumor cells in any slide studied, the only tissue present being of a calcified, necrotic, amorphous nature. As this was the only cell line previously not grown as a xenograft in the preliminary characterization experiments, no further histological details were available.

In a subsequent experiment aimed at establishing growth of this cell line as xenografts, nude mice were inoculated in the usual manner with 2.5×10^7 , 5×10^7 and 7.4×10^7 viable SKOOL tumor cells. No xenograft growth was seen at 21 days post inoculation and indeed there was no detectable nodule at all on the flank of the mouse that had received the largest inoculation. These results were also confirmed at 31 days post inoculation. These mice had also previously been

Table 39. INDIVIDUAL TUMOR VOLUMES (mm³) EXPERIMENT 4 (SKOOL): CONTROL GROUP

MOUSE	1	2	3	4	5	6	7
Days Post Inoculation							
2*	78.5	150.8	104.7	221.2	104.7	58.6	104.7
6	122.5	122.5	104.7	150.8	98.2	65.4	78.5
10	65.4	41.9	78.5	79.5	63.6	54.4	53.0
13	72.0	37.7	53.0	91.6	23.6	33.5	33.5
17*	46.1	9.8	32.1	58.6	13.1	13.5	28.9
20	37.7	8.2	32.1	58.6	13.1	13.1	28.9
24*	32.1	4.2	50.3	58.6	18.8	21.2	21.2
27	33.5	4.2	41.9	74.2	21.2	13.1	23.6
31*	41.9	4.2	41.9	104.7	18.8	23.6	28.9
34	18.8	4.2	32.1	58.6	11.5	18.8	25.7
38	14.1	4.2	16.4	58.6	8.4	13.1	18.8
41	41.9	4.2	23.6	58.6	14.7	18.8	32.1

Table 39 - continued

45	23.6	4.2	23.6	62.8	11.5	16.4	18.8
48	18.8	4.2	23.6	58.6	6.3	13.1	18.8
52	21.2	4.2	32.1	54.4	8.4	11.5	13.1
55	41.9	4.2	28.3	30.6	6.3	11.5	13.1
59	18.8	4.2	32.1	58.6	18.8	13.1	41.9
62	18.8	4.2	25.9	41.7	6.3	18.8	18.8
66	23.6	4.2	37.9	58.6	8.2	18.8	21.2
69	18.8	4.2	28.9	50.3	4.2	16.5	25.7

Table 40. INDIVIDUAL TUMOR VOLUMES (mm³) EXPERIMENT 4 (SKOL): CONJUGATE TREATED GROUP

MOUSE	1	2	3	4	5	6	7
Days Post Inoculation							
2*	65.4	95.0	18.8	41.9	91.6	137.4	118.8
6	33.5	32.1	65.4	53.0	63.6	54.5	28.3
10	14.1	16.5	18.8	25.7	46.1	44.9	32.1
13	6.3	9.6	14.1	16.5	50.3	54.4	23.6
17*	4.2	4.2	18.8	6.3	50.3	50.3	32.1
20	0	0	4.2	14.1	18.8	23.6	11.5
24	0	0	6.3	4.2	50.3	M	10.5
27	0	0	2.4	33.5	23.6	M	8.4
31*	0	0	4.2	7.3	23.6	M	21.2
34	0	0	4.2	4.2	23.6	M	7.3
38	0	0	4.2	4.2	23.6	M	9.8
41	0	0	4.2	1.8	41.9	M	9.8

Table 40 - continued

45	0	0	4.2	4.2	35.3	M	18.8
48	0	0	4.2	4.2	28.3	M	18.8
52	9.8	0	4.2	6.3	23.6	M	18.8
55	4.2	0	13.1	5.2	38.5	M	13.1
59	0	0	8.2	4.2	38.5	M	7.3
62	0	0	5.2	18.8	28.3	M	8.4
66	0	0	6.3	18.8	23.6	M	18.8
69	0	0	9.8	8.4	38.5	M	18.8

Table 41.

INDIVIDUAL TUMOR VOLUMES (mm³) EXPERIMENT 4 (SKOOL): VINDESINE TREATED GROUP

MOUSE	1	2	3	4	5	6	7
Days Post Inoculation							
2*	83.8	78.5	83.8	132.0	58.6	58.6	78.5
6	67.0	25.7	83.8	50.3	84.8	65.4	72.0
10	57.7	16.5	54.4	41.9	74.2	41.9	25.7
13	84.8	14.1	62.8	63.6	58.6	74.2	21.2
17*	41.7	4.2	23.6	28.9	41.9	41.9	4.2
20	62.8	1.8	67.0	41.9	54.4	50.3	9.8
24	58.6	1.8	38.5	38.5	32.1	35.3	14.1
27	67.0	0	58.6	50.3	58.6	58.6	23.6
31*	46.1	0	23.6	37.7	50.3	41.9	4.2
34	58.6	0	44.9	23.6	41.7	18.8	2.4
38	38.5	0	35.3	33.5	35.3	23.6	7.3
41	58.6	0	54.4	41.9	50.3	28.3	6.3

Table 41 - continued

45	58.6	0	35.3	41.9	50.3	28.3	6.3
48	51.3	0	28.3	25.9	50.3	25.9	6.3
52	67.0	0	33.0	41.9	63.6	30.6	6.3
55	44.9	0	58.6	23.6	38.5	25.9	5.2
59	58.6	0	46.1	38.5	46.1	25.9	7.3
62	58.6	0	28.9	47.7	41.9	23.6	5.2
66	50.3	0	38.5	41.9	23.6	32.1	5.2
69	54.4	-0	50.3	32.1	32.1	18.8	6.3

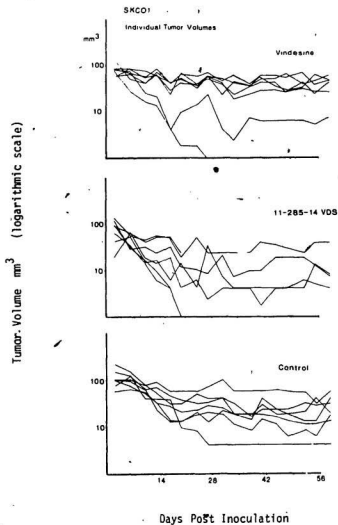


Figure 24. Graphs of individual SKCO1 xenograft tumor volumes, contrasting treatment groups in Experiment 4.

Table 42. MEAN TUMOR VOLUMES (MTV) EXPERIMENT 4 (SKCO1)

DAYS POST INOCULATION	TUMOR GROUP MTV (mm ³)		
	VINDESINE	CONJUGATE	CONTROL
2*	82.0	81.3	117.6
6	64.1	47.2	106.1
10	44.6	28.3	62.3
13	54.2	25.0	49.3
17*	26.6	23.7	28.9
20	41.1	10.3	27.4
24	31.3	11.9	29.5
27	45.2	11.3	30.2
31*	29.1	9.4	37.7
34	27.1	6.6	24.2
38	24.8	7.0	19.1
41	34.3	9.6	27.7
45	31.5	10.4	23.0
48	26.9	9.3	20.5
52	34.6	10.5	20.7
55	28.1	12.4	19.4
59	31.8	9.7	26.8
62	29.4	10.1	19.2
66	27.4	11.3	24.6
69	27.7	12.6	21.2

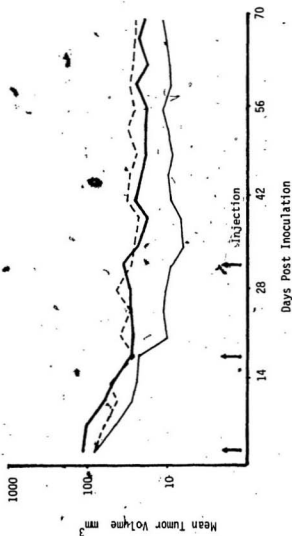
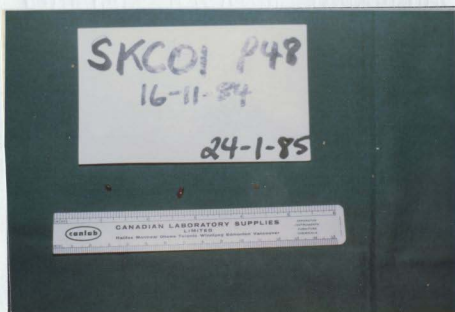


Figure 25. Mean Tumor Volumes of SKC01 Xenografts, Experiment 4, treated with

Vindesine — — —, 11-285-14 Vindesine Conjugate — — —, and Controls — — —



Plates 22 (above) & 23 (below). Nude mice bearing SKCO1 Xenografts, Experiment 4, from Vindesine, Conjugate and Control groups (left - right) : plate 22. Dissected tumors shown in plate 23, below.

Table 43. TUMOR WEIGHTS (g) AT TERMINATION OF EXPERIMENT 4 (SKOL XENOGRAFTS)

		VINDESINE GROUP					
MOUSE	1	2	3	4	5	6	7
WEIGHT	0.018	0	0.015	0.018	0.02	0.016	0.014
		CONJUGATE GROUP					
MOUSE	1	2	3	4	5	6	7
WEIGHT	0	0	0.01	0.01	0.026	M	M
		CONTROL GROUP					
MOUSE	1	2	3	4	5	6	7
WEIGHT	0.026	M	0.017	0.035	0.006	0.014	0.021

Table 44. MEAN TUMOR WEIGHT AT TERMINATION OF EXPERIMENT 4 (SKC01)

TREATMENT GROUP	MEAN TUMOR WEIGHT (g)	NUMBER OF TUMORS	PERCENTAGE OF CONTROL WEIGHT
Vindesine	0.014	7	70
Conjugate	0.009	5	45
Control	0.020	6	100

inoculated with 10^7 viable SKCO1 tumor cells (opposite flank) 100 days previously, which had failed to grow.

III 7.5 Experiment 5 - SW1116

This final targeting experiment is ongoing, and therefore data has been assembled up to 71 days post inoculation of SW1116 tumor cells. Individual tumor volumes are recorded in tables 45 , 46 and 47 , and mean tumor volumes of each treatment group in table 48 . This is represented graphically in figure 26 , where it is seen that there is little tumor growth for control and conjugate treated groups, whereas growth of xenografts in the vindesine treated group appears to be proceeding at a greater rate, shown by the broken line.

One mouse died as a consequence of anaesthesia (mouse number 5, control group, 50 days post inoculation), whereas the two additional deaths, one in the control group and one in the conjugate treated group, occurred without explanation.

No toxicity has been seen in any treatment group to date.

Table 45. INDIVIDUAL TUMOR VOLUMES (mm³) EXPERIMENT 5 (S11116): CONTROL GROUP

MOUSE	1	2	3	4	5	6	7
Days Post Inoculation							
5*	122.5	209.4	192.4	150.8	117.8	144.0	141.3
8	84.8	183.2	188.0	150.8	169.6	216.7	205.2
12	10.5	263.8	74.2	54.8	98.2	111.2	72.0
15	50.3	190.0	131.9	78.5	53.0	M	78.5
19*	63.6	163.6	130.9	98.2	78.5	M	50.3
22	78.5	100.5	169.6	102.9	113.1	M	78.5
26	134.6	176.7	158.4	142.5	150.8	M	78.5
29	62.8	132.5	243.7	98.2	122.5	M	78.5
33*	91.6	226.2	256.5	117.8	205.2	M	91.6
36	63.6	254.4	256.5	111.2	188.0	M	131.9
40	104.7	170.1	307.8	158.4	176.9	M	134.6
43	104.7	117.3	368.5	160.2	301.5	M	113.1

Table 45 - continued

47	67.0	89.8	402.0	142.5	282.2	M	91.6
50	104.7	176.7	351.8	142.5	256.5	M	150.8
54	58.6	61.2	453.9	142.5	M	M	150.8
57	67.0	117.3	567.0	124.3	M	M	205.2
61	117.8	155.2	572.4	307.8	M	M	179.6
64	98.2	113.1	551.2	207.3	M	M	179.6
68	84.8	66.0	785.3	256.5	M	M	230.9
71	130.9	66.0	680.6	188.5	M	M	150.8

Table 46. INDIVIDUAL TUMOR VOLUMES (mm³) EXPERIMENT 5 (SM1116): CONJUGATE TREATED GROUP

MOUSE	1	2	3	4	5	6	7
Days Post Inoculation							
5*	74.2	85.1	62.8	110.9	141.3	131.9	141.3
8	130.9	63.6	141.3	176.9	160.2	131.9	100.5
12	160.2	188.5	150.8	78.5	58.6	118.8	60.9
15	104.7	84.8	46.1	78.5	91.6	72.0	130.9
19*	122.5	91.6	78.5	78.5	67.0	53.0	116.6
22	85.1	54.4	50.3	113.1	50.3	65.4	226.2
26	160.2	91.6	91.6	110.9	104.7	110.5	104.7
*29	117.8	58.6	141.3	126.7	111.2	85.1	92.1
33*	188.5	188.5	122.5	150.8	98.2	102.9	127.2
36	188.5	84.8	58.6	110.9	126.7	102.9	127.2
40	254.4	169.6	91.6	176.9	169.6	142.5	235.6
43	307.8	75.4	424.0	126.7	160.2	110.9	190.0

Table 46 -- continued

47	235.6	282.2	150.8	230.9	179.0	150.8	170.1
50	333.5	188.5	91.6	282.2	104.7	179.0	132.5
54	335.3	254.4	M	323.9	104.7	160.2	100.5
57	320.6	226.2	M	282.2	91.6	160.2	104.7
61	427.0	307.8	M	416.1	117.8	230.9	205.9
64	385.3	256.5	M	637.8	126.7	169.6	235.6
68	333.5	368.5	M	680.6	160.2	179.0	205.9
71	359.1	307.8	M	732.9	169.6	169.6	309.7

Table 47. INDIVIDUAL TUMOR VOLUMES (mm³) EXPERIMENT 5 (S41116): VINOSINE TREATED GROUP

MOUSE	1	2	3	4	5	6
Days Post Inoculation						
5*	117.8	74.2	50.3	150.8	131.9	284.8
8	141.3	169.6	41.9	199.1	254.4	256.5
12	91.6	91.6	54.4	104.7	130.9	205.2
15	46.1	50.3	38.5	111.2	75.4	104.7
19*	65.4	37.7	91.6	110.9	117.9	134.6
22	58.3	98.2	98.2	104.7	130.9	188.5
26	63.6	104.7	104.7	169.6	221.2	169.6
29	91.6	54.4	142.5	188.5	282.2	309.2
33*	165.9	104.7	124.3	323.9	368.5	368.5
36	192.4	84.8	130.9	282.2	323.9	453.9
40	205.2	126.7	188.5	282.2	487.6	628.2
43	256.5	126.7	254.4	418.8	614.2	904.6

Table 47 - continued

47	256.5	104.7	307.8	508.8	680.6	1282.8
50	424.0	104.7	243.3	980.0	732.9	1766.8
54	466.4	169.6	243.3	1194.4	950.2	1949.4
57	523.5	142.5	309.7	1436.5	1013.5	2613.3
61	549.7	207.3	307.8	1943.5	1206.1	2850.5
64	628.2	179.0	333.5	2278.3	1592.5	3607.2
68	466.4	179.0	382.8	2779.2	1621.9	3479.7
71	823.5	221.2	435.6	3392.3	2120.2	4535.6

Table 48. MEAN TUMOR VOLUMES (MTV) EXPERIMENT 5 (SW1116)

DAYS POST INOCULATION	TREATMENT GROUP MTV (mm ³)		
	VINDESINE	CONJUGATE	CONTROL
5*	135.0	106.8	154.0
8	177.1	129.3	171.2
12	113.1	116.6	97.8
15	71.0	86.9	97.0
19*	93.0	86.8	97.5
22	113.1	92.1	107.2
26	138.9	110.7	140.3
29	178.1	104.7	123.0
33*	242.6	139.8	164.8
36	244.7	114.2	187.6
40	319.7	177.2	175.4
43	429.2	199.3	194.2
47	523.5	199.9	179.2
50	708.6	187.4	197.2
54	828.9	213.2	173.4
57	1006.5	197.6	216.2
61	1177.5	284.3	266.6
64	1436.5	301.9	229.9
68	1484.8	321.3	284.7
71	1921.4	341.5	243.4

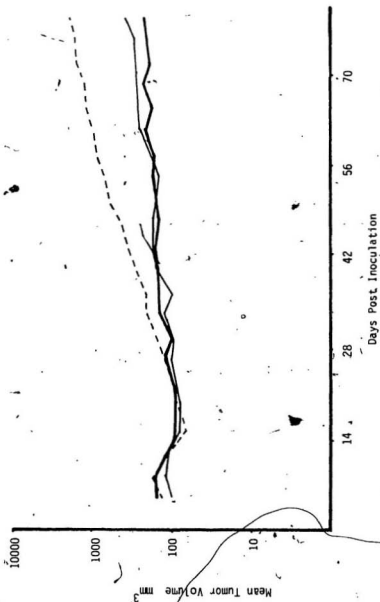


Figure 26. Mean Tumor Volumes of SW116 Xenografts, Experiment 5, treated with

Vindesine — — —, 11-285-14 Vindesine Conjugate — — — and Controls — — —

III 8.0 GENERATION OF MONOCLONAL ANTI-CEA ANTIBODIES

Growth of colonies was noted in several wells following both fusions, and when supernatants from these wells were tested by ELISA, anti-CEA activity was demonstrated. This is illustrated in figure 27, a histogram showing results obtained in a screening ELISA of selected wells from both fusions.

In this example, screening of fusion 1 supernatants was performed at 27 days post fusion, and was the last screening of wells from the original plates. No anti-CEA activity was detected in these remaining wells, as illustrated by the histogram, whereas selected wells from fusion 2, where supernatants were tested for the first time at 10 days post fusion, were generally positive. In particular, strongly positive results were obtained from wells designated A10, G11, A10, B6, D12, H6, H11 and A3. Controls included the monoclonal anti-CEA antibody 11-285-14 (positive); PBS-BSA (negative) and RPMI-HT medium (negative).

When the supernatants were re-tested in ELISA after subsequent cloning of these positive wells by limiting dilution, all anti-CEA activity had disappeared, despite growth of several colonies.

In summary, both fusions did not yield monoclonal anti-CEA antibodies.

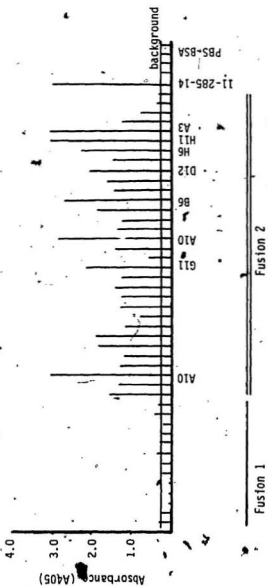


Figure 27. Histogram of screening ELISA of selected wells from Fusion 1 and 2.

III 9.0 STATISTICAL HANDLING OF RESULTS

III 9.1 Introduction

Statistical advice regarding the presentation of data obtained from the in vivo targeting experiments was obtained from a number of sources (see acknowledgements), and the widely differing opinions offered reflected the complexity of the problem, which also was apparent in the literature.

An attempt was therefore made to interpret the data using statistical methods of increasing complexity, and such results will be considered in this section. A full discussion of the problems of statistical interpretation will be considered in Chapter IV.

III 9.2 Growth Delay

For a group of mice bearing xenograft tumors and receiving a particular treatment, growth delay corresponds to the time taken for that particular groups mean tumor volume to reach a pre-determined fixed tumor volume in comparison to controls.

This growth delay may be represented as follows for cell lines COLO320DM, LSI74T and BENN, where the fixed tumor volume was taken as 500 mm³.

XENOGRAFTS	DAY 500 mm ³ REACHED		GROWTH DELAY (DAYS)
	CONTROL	CONJUGATE	
COLO320DM	18	19	1
LSI74T	9	14	5
BENN	33	48	15

It can be seen from figure 25 that in experiment 4 (SRCQ1), the no-growth state reached did not allow meaningful comparison to be made between groups.

III 9.3 Comparison of Curves

Direct visual assessment of the form of the growth curves for each treatment group of mice bearing xenografts, compared to controls, obtained in each experiment was a reliable initial method of assessing the efficacy of treatment.

Growth curves are illustrated in section III 7.0 by figures 18, 20, 24 and 26, where mean tumor volume is expressed in mm^3 on a logarithmic scale along the ordinate, with days post tumor inoculation along the abscissa.

An alternative method of presenting these results would have been to plot the mean tumor volume directly along the abscissa without logarithmic transformation. The form of the curves is visually quite different, although the efficacy of each treatment modality, too, is strikingly apparent. Growth curves plotted in this manner are contrasted in figures 28, 29 and 30 for COLC320DM, LS174T and BENN, respectively. Standard error has been included in these curves, although it would not be meaningful to include such values on the logarithmic plots.

III 9.4 Mean Tumor Volume as a Percentage of Control

The mean tumor volumes of mice treated with the antibody drug conjugate (conjugate MIV) was expressed as a percentage of their corre-

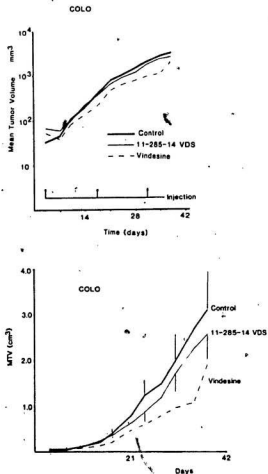


Figure 28. Comparison of format of growth curves for COLO320DM. Mean tumor volume expressed along ordinate in cm³ below; on a logarithmic scale in mm³ above. Groups receiving Vindesine — — — Conjugate ——— Control ———

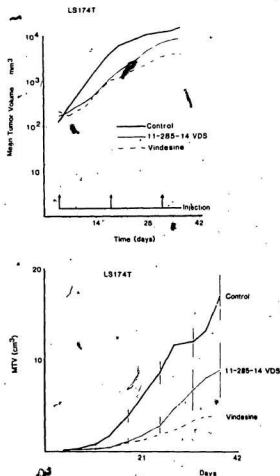


Figure 29. Comparison of format of growth curves for LS174T.

Mean tumor volume expressed along ordinate in cm^3 below; on a

logarithmic scale in mm^3 above. Groups receiving Vindesine — — —

Conjugate ——— Control —————

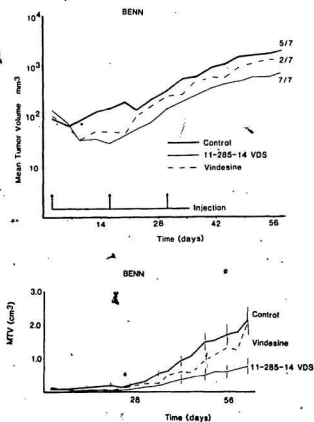


Figure 30. Comparison of format of growth curves for BENN.

Mean tumor volume expressed along ordinate in cm^3 below; on a

logarithmic scale in mm^3 above. Groups receiving Vindesine — — —

Conjugate ——— Control —————

sponding control mean tumor volumes (control MTV), at various times throughout each experiment, viz. at the start of treatment, weekly for four weeks and at the termination of the experiment; experiments 1 and 2 terminated at 5 weeks, experiment 3 at 9 weeks, experiment 4 at 10 weeks, experiment 5 is ongoing.

This is shown as follows for each cell line.

WEEKS POST TREATMENT	COLC320DM	LS174T	BENN	SKCO1	SW1116
0	203	135	149	69	69
1	116	29	30	51	90
2	81	30	22	38	86
3	81	40	37	37	85
4	84	61	36	27	68
TERMINATION (see text)			36	59	140

III 9.5 Student's t test

The student's t test was used to compare the tumor volume means of control and conjugate treated groups of mice in each targeting experiment, as these were normal populations. Calculations were performed using a TI programmable 59 calculator (Texas Instruments).

The following lists the days post inoculation of each cell line used in experiments 1-5 and the significance level as determined from tables of significance levels of the Student Distribution (2 tailed test) (Documenta Geigy, Scientific Tables).

COLO320DM	DAYS	3	14	21	28	35	
	P<	0.01	0.7	0.7	0.7	0.7	
LS174T	DAYS	4	14	21	28	35	
	P<	0.8	0.1	0.005	0.01	0.2	
BENN	DAYS	2	13	20	26	34	61
	P<	0.05	0.3	0.2	0.1	0.02	0.02
SKCOL	DAYS	2	13	20	27	34	69
	P<	0.3	0.1	0.1	0.2	0.1	0.8
SW1116	DAYS	5	15	22	29	36	71
	P<	0.05	0.8	0.7	0.6	0.2	0.5

III 9.6 Analysis of Variance

In order to assess the contribution of a variety of different factors upon the variation of data ie. inter and intra subject variation, time etc. an attempt was made to apply the statistical technique of analysis of variance, using the MANOVA command (repeated measures

multivariate analysis of variance), as outlined in Chapter 28 of the SPSSX (Statistical Package for the Social Sciences - extended) data analysis system manual (SPSS Inc, Chicago).

Accordingly, data files were constructed for each experiment listing individual tumor volumes, in the format of tables 20-22 (COLO320-DM; experiment 1); tables 27-29 (LS174T, experiment 2); tables 33-35 (BENN, experiment 3) and tables 39-41 (SKCO1, experiment 4), using a VT100 terminal (Digital Equipment Corp., Mass), connected to the central University Computer, VAX-11/780 VMS V3.7.

An example of the format of the computer programme designed for the data of experiment (COLO61184.DAT), as designed by R. Cornish (see acknowledgements) is listed in appendix B, and was referred to as PROG.DAT.

This programme was modified accordingly when other data files were used.

To run the SPSSX MANOVA programme, the following command was typed into the VT100 terminal when the dollar sign was showing:

```
$          SPSSX/OUT = PRINT  PROG.DAT.
```

A printed copy of the results could be obtained with the command:

```
$          TY  PRINT.DAT
```

Interpretation of these results (referred to as PRINT.DAT) was performed at the Statistical Consulting Laboratory (see acknowledgements), and following a further Manova 3 way analysis, the following

results were obtained for experiment 1.

1. There was no difference among the means of the three groups (Treatment groups: Vinchesine, Conjugate, Control).
2. There was a significant difference among the means of the time periods ie. this was a time-dependent process.

Attempts were made to apply this programme to other data files of the remaining experiments, but the programme was unable to take account of missing data in the analysis, following the death of a mouse.

The Statisticians proposed that in order to overcome this, mice dying during the course of an experiment should either be totally excluded (as the programme did) or that an extrapolated mean tumor volume, based on the last value obtained, should be substituted.

As neither option was biologically valid, and in the absence of alternative programmes, this approach was aborted.

A full discussion of the problems encountered with the statistical interpretation of results is found in Chapter IV.

CHAPTER IV : DISCUSSION

IV 1.0 INTRODUCTION

Prior to attempting to assess the efficacy of targeted chemotherapy, it was necessary to evaluate certain aspects of the various components of the targeting system employed in these studies. This underlies the rationale for the inclusion of the initial experiments aimed at characterisation of the target, human tumor cell lines expressing the antigen CEA, and the carrier, the monoclonal anti-CEA antibody 11-285-14. Although not a principle aim of these studies, an attempt was also made to generate further anti-CEA monoclonal antibodies for additional characterisation.

Evaluation of the warhead alone, it must be stated at the outset, was not attempted by these studies for several reasons. The vinca alkaloids, as discussed in section I 6.5, have already received extensive characterisation and clinical trial and in addition, there is no exact analogue of the vinca alkaloid (a precursor of vindesine) conjugated to this antibody that exists in free state, although vindesine represents the nearest chemical relative of any practical value. Therefore vindesine has been included in many of these studies as an interesting and relevant parallel rather than as a strict control in view of the difficulty correlating efficacy of free drug to that of conjugated drug.

The studies outlined above enabled logical progression to evaluation of conjugate efficacy proper, which was performed initially in vitro using selected cell lines in an established microcytostasis assay.

However, prior to assessment of in vivo efficacy it was necessary to establish a suitable and relevant pre-clinical model. Accordingly,

cell lines selected on the basis of CEA expression from initial studies were grown as xenografts in nude mice, and characterisation of these xenograft tumors performed confirming the suitability of the model for later studies, ie. that tumor histology and immunocytochemical characteristics had been maintained. Preliminary experiments using this model were designed to ensure that the system would work for later targeting experiments.

Although it is somewhat artificial to divide the preceding studies into stages as above, this has been done for convenience and clarity of discussion. In fact, these studies formed a cohesive whole which eventually led to an evaluation of conjugate efficacy in vivo, where the growth of human tumor xenografts with a range of CEA expression was assessed following treatment of the host with monoclonal anti-CEA antibody vindesine conjugates.

The results obtained at each of these stages will be the subject of discussion in this chapter.

There is little doubt that this approach to cancer chemotherapy is appealing in view of the limitations imposed by many present day cytotoxic agents, and that it offers potential improvement over traditional immunotherapy. The current status of immunochemotherapy was outlined in section I 7.0, where encouraging results were presented from a number of studies employing a range of targeting systems, although it is still somewhat surprising that so little information exists on this approach, twenty-seven years following Mathe's original publication (1958).

These studies are encouraging in their support of this therapeutic approach, where efficacy and selectivity of monoclonal anti-CEA antibody drug conjugates has been demonstrated in vitro and in a relevant pre-clinical in vivo model. However, several questions have also been raised from these studies and these will be addressed in this chapter.

IV 2.0 CHARACTERISATION OF THE TARGETING COMPONENTS

The availability of ten human tumor cell lines provided sufficient material upon which to base these studies, and to select key cell lines for further evaluation. By maintaining these cell lines in culture as described in Section II 1.0, sufficient cells were generally available for experiments to be performed, provided that care was taken in their handling, especially in the prevention of infection. Only two flasks of cultured cells (COLO320DM) were discarded during these studies following infection with *Staphylococcus aureus*, which most likely occurred following a breakdown in sterile technique.

Immunocytochemical studies of the available cell lines using 11-285-14 enabled simultaneous evaluation of both target and antibody, as both components were essentially inter-dependent. The specificity of the monoclonal antibody (11-285-14) for CEA epitope(s), with little NCA or 'normal' cross-reactivity was qualified in Section I 3.5, making it potentially a suitable carrier for such an approach. Its use as a tool to characterise the distribution of the target antigen, CEA, is therefore justified.

The immunoperoxidase technique was preferred, because more information regarding the cellular distribution of CEA (cytoplasmic or surface) could be gained over the immunofluorescence technique, which only exhibited surface reaction. The latter consistently gave a lower mean percentage of cells expressing the antigen for each corresponding cell line tested, as is apparent from figures 1 and 2. In addition, subjective assessment of immunofluorescence slides was more difficult, and the permanent nature of the immunoperoxidase reaction, enabling slides to be stored for later review, was a distinct advantage.

Figures 1 and 2 illustrate the range of CEA expression by cells of each cell line, and consistent high expressors (by either technique) therefore include SW1116, SKCO1, BENN and LSI74T; intermediate to low expressors, LoVo, HT29, SW837; and non expressors, COLO320DM and A549.

In the evaluation of the selectivity of targeting¹⁸, it would be important to contrast results obtained using cell lines of quite different CEA expressivity, such as any of the high expressors (SW1116, SKCO1, BENN, LSI74T), with non-expressors (COLO320DM, A549). A further refinement would be to later evaluate the response using a cell line of intermediate antigen expression.

Binding studies, using radiolabelled 11-285-14, were also performed to confirm the binding of antibody to a range of cell lines expressing CEA, as selected from immunocytochemical assessment, and also to attempt to quantitate the number of antibody molecules binding.

Several attempts were made at radiolabelling, and following the successful iodination of one batch of 11-285-14, with confirmation of its

antibody binding activity by ELISA (section III 2.2), this was employed in a binding study using selected cell lines to illustrate the principle. Figure 4 clearly illustrates the difference in binding of the antibody to two CEA expressing cell lines, LS174T and BENN, compared to the non-expressor, COLO320DM. These results were consistent with those obtained previously by the Oncology Research Laboratory.

Quantitation of the number of antibody binding molecules reveals a similar number binding to the CEA expressing cell lines LS174T and BENN, and a forty fold reduction of binding to COLO320DM. This result may appear surprising at first sight, as it would be expected that antibody binding would not be detected with the non-expressor. This may be explained, however, by the relative insensitivity of the immunocytochemical assay, which may not detect binding at this level of antibody saturation. The essential feature illustrated here is that a range of binding exists over this concentration range used. Also, evidence from other work in Oncology Research indicates the 'background' level of binding of a non-specific IgG (from the Ag8 mouse myeloma line) is at the level of 3×10^4 molecules per cell, which is consistent with the COLO320DM results.

It should also be noted at this point that such studies assume that antigen density is equal for each cell. It may be demonstrated that this is not the case however, and antigenic heterogeneity may be demonstrated by immunocytochemical studies, see plates 4 and 6, where it is seen that not every cell on the slide has been stained, and that this is even heterogeneous within the cell itself.

IV 2.1 Attempt to Generate Further anti-CEA Monoclonal Antibodies

Two fusions were performed in an attempt to generate additional anti-CEA monoclonal antibodies for characterisation as potential carriers.

It can be seen from the histogram of a representative screening ELISA, figure 27, that several wells tested exhibited anti-CEA activity, although this disappeared on subsequent cloning and re-testing. The non-viability of these clones was also apparent on microscopy.

No firm conclusions can be drawn from these attempts at monoclonal antibody production in view of the limited numbers of fusions performed. The demonstration of anti-CEA activity at an early stage suggests that at least initially the fusions had been successful, and it appears as if the problem arose at a later stage, in maintaining viable hybrid clones. Early cloning was performed to prevent overcrowding of cells, but appeared to have little influence on outcome.

These two fusions illustrate the difficulties that arise in the generation of monoclonals using hybridoma technology, and indeed several investigators reported many initial attempts prior to obtaining a successful fusion. The reasons for this, too, are unclear as many factors may influence outcome. No further fusions were attempted as this was not a central theme of this thesis.

IV 3.0 TARGETING IN VITRO

In keeping with the theoretical predictions of targeting, conjugate efficacy in vitro is clearly demonstrated in figures 5, 6, 7 and 8,

where dose-response curves show a progressive reduction in percentage of surviving cells to increasing antibody-drug concentration, when each of the high CEA expressing cell lines LS174T, SKCO1, BENY and the intermediate expressor HT29 are evaluated.

Selectivity is confirmed by the absence of conjugate response upon the non-expressing cell lines COLCO320DM and A549, over a similar concentration range, figures 9 and 10.

Table 10 shows the CEA expression of the individual passages of cell lines used in each of these experiments, although cell smears of A549 were not available.

Interestingly HT29, an intermediate / low CEA expressor (.17%, figure 1) (passage number 22) used in this particular assay illustrated in figure 8, was not demonstrated to express CEA, within the sensitivity of either immunocytochemical assay, (table 10), although a convincing response to conjugate was still obtained. The reason for this is unclear as previous experience showed that on average up to 20% of HT29 cells on cell smears were antigen expressors when tested using 11-285-14 at 10 ug ml⁻¹ by immunoperoxidase. It is possible that for this particular cell passage cells were 'stripped' of antigen by excessive trypsinisation during preparation of cells for assay and that after 24 hours recovery in culture medium, sufficient target sites had been regenerated for a conjugate effect.

Another feature of interest indicated by these results is that regarding the effect of free drug. Although the limitations of its use

as a control or for a direct comparison to conjugate were addressed in the introduction to this chapter, section IV 1.0, it can be seen from each of the dose-response curves obtained that vindesine used alone caused cytostasis with six cell lines showing that the vinca alkaloid was effective upon a range of human tumor cell lines despite their source (colorectal or lung) and that effects were seen in general at lower concentrations than for conjugated drug. Therefore, when a cell line was sensitive to free drug, a conjugate effect was only seen with cell lines expressing the target antigen. This is in contrast to results obtained with SW116 (high expressor) and SW837 (low expressor) which were both insensitive to free drug and consequently also to conjugated drug. The explanation of the observation that vindesine is effective at a lower concentration range is speculative although it may be that the smaller vindesine molecule is internalised faster by the cells in this assay system, compared to its conjugated counterpart. This hypothesis assumes that internalisation is required for the drug to exert its effect, which is likely but not proven.

The value of a microcytostasis assay (similar to the one used in these experiments) as a predictive test of human tumor chemosensitivity, in comparison to other available assays such as the clonogenic assay (Salmon, 1984), has been assessed (Wilson, Ford, Newman & Howell, 1984). Both the clonogenic and monolayer assays were found to be comparable, suggesting that non-clonogenic assays have value in in vitro predictive testing. In order to improve the consistency of results, tests included in this thesis were performed throughout in quadruplicate rather than triplicate.

Other groups, too, have reported similar results of conjugate

efficacy in vitro, and the efficacy of polyclonal anti-CEA antibody vindesine conjugates has been well documented using a similar microcytostasis assay to that used in these studies (Johnson et al, 1981; Rowland et al, 1982). With monoclonal antibody carriers, in vitro efficacy has been reported for conjugates comprising adriamycin and an anti-Sp4 (rat mammary carcinoma) monoclonal antibody (Pimm et al, 1982) and using colony inhibition assays and ^{75}Se -selenomethionine incorporation assays, Rowland has recently reported the efficacy of vindesine conjugated to monoclonal anti-melanoma, anti-osteogenic sarcoma and anti-CEA antibodies (Rowland, Axton, Baldwin, Brown, Corvalan, Embleton, Gore, Hellstrom, Hellstrom, Jacobs, Marsden, Pimm, Simmonds & Smith, 1985). By employing cell lines of both positive and negative target antigen expression, the in vitro studies outlined in this thesis have also confirmed selectivity of the immunoconjugates in addition to efficacy.

Reproducibility of this assay was reported in section III 4.2, and figure 11 clearly shows that the assay was reproducible not only when performed at different times, but also when performed by two different investigators. Results have been included for two different CEA expressing cell lines; SKCO1, a high expressor, where conjugate efficacy is again demonstrated, and COLO320DM a non expressor, demonstrating no conjugate effect and hence the selective nature of targeting.

As all in vitro assays were performed at different times throughout the year, different batches of 11-285-14 vindesine conjugate were employed. Figure 12 illustrates the comparative efficacy of four different conjugates, tested against LS174T, a CEA expressor. The

variation in the dose-response curves likely represents the individual variation of CEA expression by the cell line, as these assays were not performed simultaneously. Ideally, this experiment would be repeated using a cell line of the same passage number, performed on the same date, and testing each of the conjugates together. However, the similarity of each curve demonstrates the efficacy of each of these conjugates, as does the demonstration of their anti-CEA binding activity compared to the parent antibody 11-285-14, when tested by ELISA, figure 13.

These studies have also demonstrated that conjugate efficacy is maintained for at least several months following their preparation, when stored as outlined in appendix A. Periodic evaluation of the conjugates in vitro has confirmed the efficacy of these monoclonal anti-CEA antibody vindesine conjugates for up to seven months post-conjugation, and this had never previously been addressed.

IV.4.0 THE IN VIVO MODEL

As alluded to in section I 1.3, the testing of potentially new anti-cancer drugs has been subject to criticism in view of the established practice of screening these agents against rodent tumors of debatable relevance to the human. Although an acceptable tumor model may never be completely agreed upon, the use of xenografted human tumors in immunosuppressed mice or rats is becoming increasingly used for such purposes (Lancet Editorial, 1978) and has also received further recent support (Atassi, 1984).

Immunosuppression may be achieved as a consequence of congenital

thymic aplasia, as in the nude mouse (Pantelouris, 1968), or following immunosuppressive measures such as thymectomy, the administration of anti-lymphocyte serum, cytotoxic drug treatment and whole body irradiation (Castro, 1972; Steel, Courtenay & Poston, 1978). The increasing use of the nude mouse in such a role is evident by the establishment of International Workshops on Nude Mice, although the immunosuppressed mouse is favoured by many in view of its ease of handling and cost (Steel, Courtenay & Peckham, 1983).

The method of xenografting, too, may vary from the subcutaneous inoculation of tumor cell suspensions as performed in these studies and of others (Fogh, Fogh & Orfeo, 1977), to the direct subcutaneous implantation of fresh surgically resected specimens, which may then be maintained by transplantation between mice when the tumor reaches suitable proportions, a process of serial passage (Houghton & Taylor, 1978a).

Using these methods, a wide variety of tumors have been xenografted into immunodeficient hosts including colorectal tumors (Pickard, Cobb & Steel, 1975; Houghton et al, 1978a), bladder tumors (Kyriazis, Kyriazis, McCombs & Peterson, 1984), lung tumors (Mattern, Wayss, Haag, Toomes & Volm, 1980), breast tumors (Bailey, Gazet, Smith & Steel, 1980), rhabdomyosarcomas (Houghton, Williams, Torrance & Houghton, 1984) and melanomas (Fodstad, Aass & Phil, 1980) in addition to a variety of cell lines including LoVo (Stragand, Bergerat, White, Hokanson & Drewinko, 1980), SW1116 (Klog, Salzman, Quinn, Melincoff, Sedmak, Tubbs & Zurawski, 1984), HT29, LS174T and A549 (Fogh et al, 1977).

Much attention has been given to the characterisation of these transplanted tumors, especially with regard to the maintenance of

original tumor histology (Houghton & Taylor, 1978b; Kyriazis et al, 1984), tumor markers (Thomson, Neville, Phelan, Scanzano & Vandévoorde, 1981) including CEA (Lewis, Smith, Keep & Boxer, 1983) and tumor kinetics (Sprang-Thomsen, Nielsen & Visfeldt, 1980; Sprang-Thomsen & Vindelov, 1984). The extent to which these characteristics are maintained reflects the usefulness of the tumor model, and this has been addressed in most of the studies referred to in addition to reviews (Steel & Peckham, 1980).

Consensus is that such xenografts do maintain the biological tumor characteristics, for at least up to 10 serial passages if transplantation is employed (Houghton et al, 1978a; Steel et al, 1983) and this includes the maintenance of histology, tumor markers, isoenzyme and chromosomal patterns. Host response to the xenograft is essentially minimal and infrequent, with tumor infiltration by host tissue and metastases being uncommon findings. Steel comments on reports of host response to tumor occurring in immunosuppressed mice, possibly following incomplete immunosuppression, and the uncommon occurrence of this phenomenon in the nude mouse (1983).

IV 4.1 Xenograft Characterisation

Despite the initial problems of establishing a nude mouse colony and the precautions necessary for the handling of these mice, it was felt that it was more convenient to work with the nude mouse model rather than attempt immunosuppression at this institution.

Preliminary experiments (Ford, personal communication) had established that an inoculum of 10^6 viable tumor cells did not result in established tumor growth and therefore 10^7 cells were used as the

inoculum size in future experiments. The overall "take" of 93.6% for the initial 44 tumors grown for characterisation was comparable to that of 90% achieved by Fogh establishing xenografts from 127 tumor cell lines (1977). The range of tumor "take" of serially transplanted tumors is reported quite variable but averages at less than 50% (Steel et al, 1983) - a further reason for direct inoculation of tumor cell lines in this model,

Of the ten cell lines available for characterisation, nine were grown as xenografts for characterisation prior to experimentation, with the exception of SKCO1. The cell line SKCO1 was inoculated initially at the outset of experiment 4, and as the results from this experiment indicate, these tumors failed to grow. The growth curves of individual SKCO1 tumors are shown in figure 24. Following this experiment an attempt was made to grow these tumors by using inocula of increasing cell number, up to 7.5×10^7 viable cells, and this too, failed to achieve tumor "take". A criticism of this latter experiment relates to the prior inoculation of mice with 10^7 SKCO1 tumor cells, and ideally inocula of increasing cell number should be injected into 'fresh' nude mice. It is, however, unlikely that prior inoculation would be responsible for any tumor suppression following a later challenge, and it is possible that greater than 10^8 viable cells will eventually be required to establish growth of this cell line as xenografts in the future. Alternatively it may be a cell line that is 'resistant' to growth in the nude mouse.

Serial measurement of tumor volume was not documented during the course of these characterisation studies and the only measurements

recorded were that of tumor volume and weight at the time of sacrifice.

The necessity of following the growth of tumors in preliminary and targeting experiments enabled growth curves to be constructed for the cell lines used in these experiments. Therefore for HT29, LS174T, COLO320DM, B22N and SKCO1, the growth curves of control tumors from experiments utilising these cell lines are represented in figures 15, 20, 18, 22 and 25 respectively.

Particularly striking was the similarity between growth curves obtained for the HT29 xenografts in experiment A (figure 15) and those reported for the cell line HT29R (Warenius, Freedman & Bleehen, 1980). Tumor growth approximated well with the Gompertzian function, and in addition, the spontaneous regression of occasional tumors corresponded with published findings (Warenius et al, 1980; Strang-Thomsen et al, 1980). The explanation of the latter phenomenon is not known, although vascularity at the site of inoculation and the role of local trauma have been proposed as contributing factors.

Figure 14 correlated tumor volume to weight at the termination of the characterisation studies, where a correlation coefficient of 0.85 was obtained for 41 of a possible 44 tumors grown. The poor correlation of weight to volume for the six LoVo xenografts studied (correlation coefficient 0.65) was most likely a reflection of the high proportion of tumor necrosis exhibited by these tumors, as has been reported by others (Stragand et al, 1980). The overall correlation of these two tumor parameters obtained for a total of 154 tumors, including those used in targeting experiments, was good, with a correlation coefficient of 0.96. These results support the view that tumor volume is a suitable guide to

tumor growth in such a model.

The measurement of tumor volume used in these studies followed that of Evans (1983), who used the formula for an oblate spheroid to compensate for the fact that tumors did not grow as true spheres. Measuring the maximum tumor length (L) and maximum tumor width (W) at right angles, it was possible to calculate tumor volume (V) using the formula:

$$V = \frac{\pi^2 \times L \times (W)^2}{6}$$

Several other formulae have been reported in the literature, and although the final volumes derived by each are all of similar magnitude, they assume that growth is equal in all dimensions. Such examples are listed below, where D represents tumor diameter, and H, tumor height.

$$V = (D)^3 \times \pi / 6 \quad (\text{Warenius et al, 1980})$$

$$V = L \times (W)^2 \times 0.4 \quad (\text{Kyriazis et al, 1984})$$

$$V = L \times W \times H / 2 \quad (\text{Rowland et al, 1983})$$

$$V = L \times (W)^2 \times 1/2 \quad (\text{Podstadt et al, 1980})$$

The irregularity of gross tumor morphology has been mentioned, and it was felt that the site and depth of tumor cell inoculation was important in determining final tumor morphology. Intradermal inoculation, either accidental or from the needle track, appeared to account for the skin tethering occasionally seen, and possibly for skin ulceration, although this may also have resulted, in the case of large tumors, from a pressure necrosis effect upon an area of already diminished vascularity.

In only one case was there apparent involvement of the abdominal wall musculature, most likely related to depth of tumor cell inoculation, and in no cases were metastases demonstrated.

Haematoxylin and eosin staining of xenograft tumor sections documented that the histological architecture had been maintained, and this is summarised in table 12. Plate 10 clearly illustrates the preservation of glandular architecture seen with LS174T, a moderately well differentiated adenocarcinoma of colorectal origin.

Expression of CEA, illustrated by plate 11, was evaluated for each cell line, and table 13 contrasts the results of immunoperoxidase assays performed on representative xenograft sections with their inoculating cell lines. This indicates that expression of the antigen by the xenograft is generally reduced, in keeping with published results (Lewis et al, 1983), with the exception of A549 where 10% of cells appeared to express CEA on tumor sections, compared to no expression by the inoculating cell line. There is as yet no explanation to account for this observation.

A further observation of interest is that of the pattern of CEA expression by these xenografts (cellular, stromal, glandular), and the considerable heterogeneity that existed among these sections.

Extraction of CEA was performed from a limited number of tumors only to illustrate the range of CEA content of xenografts with different CEA expression. High CEA content was found for the high expressors LS174T and BENN, and reduced content for A549. It appears therefore that a range of CEA expression and production exists among cell lines and

their xenografts, and that even though CEA may not be detected in certain cell lines by immunocytochemical techniques, the antigen may be produced in vivo, as evidenced by the extraction data. Serum CEA was not evaluated in these studies.

IV 4.2 Preliminary Experiments

Results obtained from preliminary experiments are summarised by figures 15 and 16, where it is clear that growth of HT29 and LS174T xenografts was suppressed by free vindesine at this dosage and schedule. In view of the limited numbers of mice used in these experiments, no further conclusions are made. These experiments confirmed the feasibility of this model system to assess therapeutic response.

IV 5.0 TARGETING STUDIES

Having established a suitable model with which to evaluate the efficacy of targeted chemotherapy, human tumor cell lines, on the basis of their earlier characterisation, were selected for assessment in individual experiments.

Statistical advice had suggested that treatment groups, each containing no less than six mice, would be an adequate population size from which conclusions regarding therapeutic efficacy could confidently be made. Therefore, for each experiment, sufficient mice were inoculated simultaneously so that not less than six tumors would be included into each of three treatment groups at the start of the experiment.

In experiment 1, mice were inoculated bilaterally, whereas in the remaining targeting experiments, unilateral inoculation was performed.

It was initially proposed that the use of two tumors per animal would be advantageous, in that a saving of animals could be made, and this was based on the experience of others (Rowland, personal communication) and from the literature (Varenius et al, 1980). The latter proposed that statistical results were similar regardless of whether the animal supported one or two tumors, if each tumor was assessed individually.

Upon the completion of experiment 1 it was apparent that the use of two tumors per animal had several disadvantages, including earlier disability of the host as a result of two tumor masses, requiring sacrifice earlier than if only one tumor had been present. Furthermore, the interpretation of results posed additional questions, as will be discussed. As a result of experience gained from experiment 1, unilateral inoculation of mice was performed for all subsequent experiments.

Three treatment groups were included in each experiment. Control mice received an intraperitoneal (ip) injection of 1.0 ml sterile phosphate buffered saline, every two weeks for three doses. Mice treated with conjugates of 11-285-14 VDS, were treated on a similar schedule, at doses of 10 mg kg⁻¹ with respect to drug concentration. The volume of conjugate injected varied between 0.5 and 3.0 ml, depending on the weight of the mouse and the batch of conjugate used.

This regimen was selected on the advice of Rowland (personal communication) who had used a similar regimen to treat mice bearing the MAWI colorectal xenograft, maintained by serial passage. However, the only published regimen in the literature was that of a dose of 6 mg kg⁻¹ (with respect to drug concentration) given twice weekly for 5 weeks

(Rowland et al, 1985). An important difference regarding scheduling used in these studies related to the time of the initial injection. It was elected for these studies to wait until tumors were visible (tumor volumes between 75 and 150 mm³) before starting treatment to ensure 100% "take" on tumors, whereas Rowland began treatment at the time of tumor initiation, when tumor burden was lowest. The only exception to this being three mice in experiment 1, where treatment was initiated with impalpable tumors, two of which subsequently grew (see section IV 5.1).

Mice receiving free vindesine were treated with 1.0 ml of the drug at concentration of 2 mg kg⁻¹, given on a similar schedule.

The intraperitoneal route was chosen over the intravenous route for its ease of access and reliability, and no adverse physical effects were noted with this volume of solution. Repeated injections of up to 3.0 ml of a solution, via the small tail vein of the mouse was not a suitable alternative, although in general the intravenous route is to be preferred. The toxicity study, section II 10.4, with results III 6.3, was performed in view of the much higher doses of conjugate over free drug to be injected, with respect to vindesine concentration. It confirmed that using doses of free drug at 10 and 6 mg kg⁻¹ toxicity and mortality was exhibited within days of injection, and was not tolerated by the mice.

At the termination of each experiment, the xenograft tumors were characterised in the usual manner to confirm that the model was still relevant and to attempt to detect any differences between the treatment groups of each experiment. Table 26 correlates the immunocytochemical characteristics of xenografts from each targeting experiment with antigen

expression of the inoculating cell line. Consistent with previous findings are the results that expression of CEA by the xenografts, from whatever treatment group studied, is less than that of the cells used for inoculation. The absence of antigen expression by the SKCO1 xenografts (experiment 4) reflects the absence of sufficient material available for testing.

IV 5.1 Experiment 1

No conjugate effect upon the growth of CQLO320DM xenografts was seen in this experiment, as shown by the similarity of growth curves in figure 18. Figure 28 also contrasts the format in which these curves may be presented and this, too, shows no conjugate effect.

This would be the predicted result for a non-CEA expressing tumor, and immunoperoxidase of xenograft sections prepared following termination of the experiment confirmed the lack of antigen expression.

When mean tumor volume of conjugate treated mice was expressed as a percentage of control, section III 9.4, it can be seen that although these tumors reached about 80% of control values, this was not statistically significant when evaluated with a Student's t test. These findings were also reflected by the tumor weights at sacrifice, table 25, where the weight of conjugate treated tumors reached 77% of control.

An effect of vindesine was seen in this experiment, and this is most clearly seen in the lower graph of figure 28. The tumor weights of the vindesine treated tumors were also noted to be less than 50% of control tumors, although no histological differences between the groups

were noted. As no deaths of mice occurred during this experiment, analysis of variance could be applied to these results as outlined in III 9.6, revealing no statistically significant difference between groups.

Several questions arose as a result of the bilateral inoculation of mice in this experiment, and in the statistical interpretation of these results. For calculation of mean tumor volumes, each tumor was considered as a discrete entity and was included in the calculation, unless of course the tumor was physically missing as may have occurred for example through the death of an animal. Such tumors were denoted by 'M' in the tables of results, whereas tumors that became unmeasurable during an experiment, such as that on the right flank of mouse 1 in the control group of experiment 1, table 20, were still subject to the same experimental conditions as the measurable tumors and were therefore included in calculations. This reasoning was also applied to the tumors of mice 5 and 6 of the same group, whose tumors, although not measurable at day 3 post-inoculation at the start of treatment, were included in the calculation of mean tumor volume because they were still subject to treatment (ie. an injection of sterile PBS) and indeed all did eventually grow by 10 days.

One exception to this was a tumor that did not establish itself at all, the left tumor of mouse number 3 of the group receiving vindeine in experiment 1, table 22. This tumor volume of 0 was not included in the estimation of mean tumor volumes for this group as it never appeared during the course of the experiment and must be assumed (reasonably) to have not "taken". However, the tumor volumes of 0 that were recorded at days 31, 35 and 38 for mouse number 1 were included in calculations, as

previously explained.

These problems were not encountered in subsequent experiments as all tumors were present at the start of treatment and were included in the calculation of mean tumor volume, unless death of a mouse occurred.

One criticism of using one animal to support two tumors was regarding the exposure of each tumor to the same amount of drug or conjugate. It assumed that at such concentrations, both tumors were equally exposed to either drug or conjugate, and to evaluate this, curves were constructed of tumor burden of mice in each group, ie. the sum of the two tumor volumes carried by each mouse. When graphs of mean tumor burden of mice in each group were constructed, they were seen to be almost identical to that of figure 18. In addition, further graphs were constructed comparing mean tumor volumes of all right-sided tumors (excluding the left tumors totally) between groups, and vice versa. Essentially the final graphical form was similar to figure 18: that the absence of conjugate effect was found no matter how the results were presented.

However, ideally it would be necessary to repeat this experiment using a unilateral tumor inoculum, to maintain consistency with the other experiments.

IV 5.2 Experiment 2

The graphical representation of the results obtained from experiment 2, figures 20 and 29, convincingly show that the conjugate is producing a reduction in the growth of LS174T xenografts when compared to controls. It is personal preference as to which method of graphical

presentation is the better for presenting such results, and these are contrasted in figure 29. However, the latter method of presentation has the advantage of enabling standard errors to be plotted.

Mean tumor volume of mice treated with conjugate reached 30% of control during the second week of treatment (ie. 21 days post-inoculation), which was significant ($p < 0.005$) when evaluated by Student's t test.

Characterisation of tumors from each treatment group confirmed CEA expression by these xenografts, table 26, and the only difference between groups when examined histologically was that of increased tumor necrosis in the sections of tumor obtained from mice treated with conjugate.

Experiment 2 confirmed the predicted efficacy of targeted chemotherapy using this in vivo model. When taken with experiment 1, selectivity of this approach may be concluded also.

IV 5.3 Experiment 3

The results obtained when experiments were performed using the high CEA expressor ~~REN~~ also confirmed the efficacy of this approach, figures 22 and 30.

However, it was also demonstrated that survival of mice bearing this particular tumor was prolonged following treatment with conjugate, as shown by the survival chart, figure 23. The observation that mice bearing this tumor generally became cachectic was initially made during

the course of the xenograft characterisation studies, and was confirmed by this experiment. The increased survival resulting from conjugate treatment is likely a temporary effect, as mice in this group too, at the termination of the experiment were beginning to show early signs of cachexia.

When growth delay was compared between the first three experiments, it appeared to correlate to the degree of target antigen expression, as outlined in section III 9.2.

IV 5.4 Experiment 4

The SKOOL cell line was the only cell line not previously characterised by earlier in vivo studies. Despite a no growth state having been established, comparison of growth curves between different treatment groups suggested that there was still a conjugate effect operating in this system, figure 25, which presumably would have occurred early after the start of the experiment before the tumor 'died' as happened in the control group.

Histology of tumor remnants obtained following sacrifice of these mice revealed only non-specific findings and was unhelpful in attempting to determine the reasons for this "growth pattern".

IV 5.5 Experiment 5

This experiment is ongoing and it can be seen from figure 26 that a different trend of tumor growth is occurring. Minimal tumor growth of the control and conjugate treated tumors is occurring, whereas a steady

progressive increase in growth is seen for the xenografts of the vindesine treated group.

This experiment is particularly interesting as SW1116 is a high CEA expressor (90%, figure 1) and one might have expected a targeting effect. However, in vitro results (not shown in this thesis), clearly indicated that there was no effect of free drug on this cell line, ie. it was 'resistant' to vindesine in the uridine assay. In view of this, it was not surprising that conjugated drug was ineffective in vitro. With the in vivo experiment the lack of conjugate effect could be predicted from the in vitro assay, confirming its value as a pre-in vivo targeting system. However, while lack of response to free drug would similarly have been predicted from the in vitro data, the increased rate of growth in the vindesine treated animals was surprising. There is no obvious explanation to account for this, other than retardation of growth in the control and conjugate groups, or possibly, a stimulatory effect of free drug on the resistant SW1116 cell line when grown in vivo.

The relative efficacy of free drug upon three other cell lines (COLO320DM, LS174T, BENN) in vivo is also quite different as seen by contrasting figures 28, 29 and 30. Vindesine efficacy in vivo is noted to be greater than that of the conjugate against cell lines COLO320DM (figure 28) and LS174T (Figure 29) whereas for BENN (figure 30), it has intermediate efficacy between control and conjugate treated groups. These observations illustrate that there is a "relative sensitivity" of cell lines to vindesine, and although not the ideal control, allows results obtained with conjugates to be interpreted on the basis of sensitivity to the vinca alkaloids.

In vivo efficacy has also been reported for conjugates comprising

warheads of daunomycin (Arnon et al, 1982), adriamycin (Pimm et al, 1982), and for vindesine conjugated to monoclonal anti-CEA, anti-melanoma and anti-osteogenic sarcoma antibodies (Rowland et al, 1985). Important differences in methodology employed in testing the latter have been addressed in section IV 5.0, and the studies outlined in this thesis have not only demonstrated efficacy using a different model CEA targeting system, but in addition, conjugate selectivity by employing cell lines of different target antigen expression.

IV 6.0 IMMUNOCHEMOTHERAPY: PROSPECTS

The testing of any new therapeutic approach aimed at eventual clinical use should logically progress through several stages designed to assess its efficacy, toxicity etc., and include testing in vitro and in vivo using animal models, proceeding through Phase I, II and III Clinical Trials.

The studies in this thesis report preliminary results confirming the efficacy and selectivity of immunochemotherapy, in vitro and in vivo using a relevant pre-clinical model, in keeping with the theoretical predictions regarding this approach. Although these results support the hypothesis of a targeting mechanism, they do not prove it.

The use of CEA as a target in this targeting system underlies the belief that this approach has potential clinical application, and this is supported by parallel studies where CEA has been used as a target for immunodiagnosis (Goldenberg et al, 1978; Mach et al, 1980). Indeed such studies have progressed well beyond the level reached by immunochemotherapy and the administration of antibody radioisotope conjugates to

patients is well documented for both diagnosis and therapy (International Symposium on Labelled and Unlabelled Antibody in Cancer Diagnosis and Therapy, Baltimore, 1985).

In addition, collaborative studies, performed in Birmingham, U.K., evaluating the use of the monoclonal anti-CEA antibody 11-285-14, as a carrier of radionuclides for immunodiagnosis have confirmed the feasibility of administering such conjugates to patients (Allum et al, 1985a, b), and are proceeding with further clinical evaluation.

Such studies, in addition to being of importance in their own right diagnostically, provide confirmation of the rationale for using such an approach, as evidenced by the selective uptake of antibody-isotope conjugates at target sites, and provide additional confidence with the administration of other immunoconjugates to patients.

The use of anti-neoplastic drugs as warheads underlies the conviction that this approach has clinical applicability as most drugs have already been administered to patients and have received extensive clinical evaluation. Although toxins have not seen use clinically, it has recently been reported that approval has been granted for clinical trials of immunotoxins to proceed (International Symposium on Labelled and Unlabelled Antibodies in Cancer Diagnosis and Therapy, Baltimore, 1985), and preliminary results are to be expected in the following year. However, the use of vinca alkaloids as warheads may receive criticism if these antibody drug conjugates are used clinically, for example, to treat colorectal cancers, which have been shown to be unresponsive to this class of agent (or indeed any other), despite convincing efficacy against colorectal cell lines demonstrated in this model. It should be emphasized however that the conjugated form of these agents may well eventually

be shown to be effective against a range of hitherto unlikely tumors (by today's standards) in view of the selective nature of this therapy. The coupling of many other anti-cancer drugs to a variety of antibody carriers (section I 6.4) is encouraging in this expanding area.

Several fundamental questions regarding this approach are outstanding, and although extensive further investigation is required, it does not necessarily preclude the initiation of clinical trials. Indeed, approval for the administration of antibody drug conjugates to patients has recently been granted to some workers in this area by the Food and Drug Administration (F.D.A.) and preliminary results are to be expected in coming years (International Symposium on Labelled and Unlabelled Antibody in Cancer Diagnosis and Therapy, Baltimore, 1985). In this regard, it is worth noting that a preliminary feasibility and toxicity clinical study with polyclonal anti-CEA antibody vindesine conjugate demonstrated that such conjugates could be administered to patients with colorectal and ovarian cancers without adverse effects (Ford et al, 1983).

Relatively little is known of the physico-chemical characteristics of the antibody-drug conjugates tested in this thesis, even though much information has been generated on their two components, the monoclonal antibody carrier and the drug warhead. The exact details of preparation of these conjugates have been retained by the drug company involved in their manufacture (Simmonds, personal communication), for reasons regarding patents etc. although the method has been outlined in recent publications (Rowland et al, 1985). Studies to fully document the physico-chemical characteristics of these conjugates are underway. It

has been suspected however that stability of the conjugate is light-dependent (through an effect upon the vinca alkaloid component) and it is for this reason that conjugates are stored in the dark at 4°C (Appendix A). As little is known about the long-term stability of conjugates, every effort was made to use them as soon as possible after preparation, although these studies have shown that their efficacy and anti-CEA binding activity is maintained for at least several months following preparation (Appendix A).

The early criticism of conjugate dissociation in vivo (Davies et al, 1973) appears to be less valid with the use of covalent linkage, although the fate of these complexes following systemic administration is still not known with certainty. Localisation studies, for example, indirectly support the assumption that the complex reaches its target site intact, although this is not proven and in addition, evidence of monoclonal antibody localisation in solid tumor has now been recently documented (Oldham et al, 1984). The fate of the complex following its arrival at the target site is also open to speculation and how such complexes achieve their therapeutic effect requires urgent investigation. It is still not known with certainty whether the complex is internalised intact by the cancer cell, or whether dissociation occurs following antigen-antibody interaction, with subsequent local release and build up of the warhead, followed by local cell killing. Also, the amount of conjugated drug required for cell kill is not known with certainty as the effect of conjugation upon the drug component requires further study. However, from the in vitro studies of this thesis, estimates suggest that a 50 % cell kill is achieved when the conjugate is in the 1-10 $\mu\text{g ml}^{-1}$ concentration range (with respect to vindesine

concentration).

The fate of the conjugate following systemic administration, too, is uncertain. The complexing of conjugates with circulating CEA en route to the target site was a problem encountered in early attempts at tumor imaging using antibody radioisotope conjugates, and may have relevance to therapy also. The concurrent administration of free, blocking antibody may become a necessary manoeuvre in patients with high circulating target antigen levels. In addition, the threat of renal toxicity exists if such complexes become deposited in the glomerulus, and such aspects will require evaluation. Similarly the extent of the host's own immune response to these murine antibodies also requires consideration, but currently available evidence suggests that this may be of less importance than previously supposed (Newman, 1978; Oldham et al, 1984; Dillman et al, 1984).

One key area of further investigation is that of the effect of antigenic heterogeneity of the tumor upon the efficacy of the conjugate. Such heterogeneity of CEA expression was demonstrated in these studies by immunocytochemistry and is a widely reported phenomenon, being dependent on the epitope recognised by the antigen and possibly on the tumor cell cycle. The selective killing of target cells within the tumor may well account for the initial response seen to conjugate therapy, while continued growth of cells not expressing the antigen may be responsible for continued tumor growth and relapse.

Although the highly specific monoclonal antibodies offer the greatest advantages as carriers, it is conceivable that the use of

mixtures of conjugates may offer further therapeutic advantage, especially if they are capable of recognising additional epitopes of the target antigen (therefore delivering more warhead), or recognising other antigens expressed by the tumor (therefore theoretically recognising a greater proportion of cells). The latter approach has been explored in immunoradiodiagnosis, where enhanced imaging of tumors expressing CEA and colon specific antigen-p (CSAp) has been obtained (Gaffar, Pant, Shochat, Bennett & Goldenberg, 1981; Nelson, DeLand, Shochat, Bennett & Goldenberg, 1983). The use of conjugates with other types of warhead, including different drugs, may allow a "combination immunochemotherapy" approach to be developed.

Antibody on its own has also been demonstrated to inhibit the growth of colorectal cancer xenografts (Herlyn, Steplewski, Herlyn & Koprowski, 1980). However, the evaluation of antibody alone was not an objective of this thesis, as experience with MAWI colorectal xenografts had indicated that while some suppression of tumor growth could occur late in the experiment with 11-285-14 alone, it was in contrast to the statistically significant suppression obtained with 11-285-14 vindesine conjugate (Rowland et al, 1985). As noted previously, the major aims of this thesis were to evaluate conjugate efficacy and selectivity in relation to target antigen density. Further study will have to be undertaken to evaluate the role, if any, of antibody alone in vitro and in vivo with the cell lines used in this thesis.

Of equal importance is the requirement of a proper evaluation of the setting in which immunoconjugates should be used. It is possible

that with other tumor targets immunochemotherapy may achieve the status as a primary therapeutic modality in the treatment of selected haematological malignancies, where access to disseminated target tumor cell populations would allow a proportionally greater cell kill. An important potential application may be in the ex vivo elimination of leukaemic or cancer cells from human bone marrow prior to autologous marrow rescue in patients receiving chemotherapy. Studies leading to such a goal include the in vitro elimination of cancer cells from human bone marrow with a pokeweed anti-viral protein immunotoxin (Uckun, Ramakrishnan & Houston, 1985) and the ex-vivo treatment of human bone marrow with anti-T cell immunotoxin for the prevention of graft-versus-host disease following bone marrow re-infusion (Filipovich, Vallera, Youle, Quinones, Neville & Kersey, 1984). Its role in the treatment of solid tumors (addressed principally by these studies), and in the systemic treatment of metastases (especially micrometastases) requires an even more careful evaluation, as indeed does conventional chemotherapy.

Using a relevant model system it is felt that the results presented in this thesis have contributed as part of the international evaluation of immunochemotherapy. At present, it is unlikely that immunochemotherapy will replace current conventional chemotherapeutic approaches to cancer treatment, but may offer advantage as an additional therapeutic option to the oncologist. It is important that unrealistic claims as to its potential are not made early in its evaluation and that careful, systematic evaluation in vitro, in vivo in pre-clinical models and in carefully conducted clinical studies is performed if this innovative approach to chemotherapy is to be exploited maximally.

IV 7.0 FUTURE STUDIES

Although all of the aspects discussed in the previous section (IV 6.0) require evaluation, immediate future studies, which are presently being pursued in the Oncology Research Laboratory, using the model developed in this thesis, include the following.

1. Further evaluation of a range of cell lines, including those of intermediate CEA expression and of different tissue origin.
2. An evaluation of the optimum schedule and dose for the administration of these conjugates, including the time that treatment is begun. In this regard, schedules utilising smaller doses of conjugate given twice weekly are being investigated with encouraging preliminary results.
3. A full assessment of conjugate toxicity with determination of LD50.
4. Evaluation of the efficacy of mixtures of different conjugates, each recognising different antigens or their epitopes both in vitro and in vivo.
5. The use of additional controls for in vitro and in vivo experiments, when available, including conjugates comprising vindesine and a non specific immunoglobulin, and unconjugated anti-CEA antibody.
6. Studies to indicate the fate of conjugated vindesine. For example, on a cellular level, is the conjugate internalised into the cell or is the drug 'split off' outside the cancer cell?, and in vivo, what is the elimination of conjugated radiolabelled vindesine when administered to mice ?
7. Development of a computer programme specifically to handle the pre-clinical in vivo data.

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APPENDICES

APPENDIX A

11-285-14 Vindesine Conjugates

The monoclonal anti-CEA antibody vindesine conjugates were prepared and supplied by the Lilly Research Centre, Windlesham, Surrey, England.

Details of conjugates used in targeting experiments are shown below. The conjugates were stored in sterile centrifuge tubes wrapped in aluminum foil and refrigerated at 4 degrees Celsius.

Conjugate #9, although prepared from the same batch as conjugate #8 at the Lilly Research Centre, was sent separately. Upon arrival however, it appeared turbid in contrast to the usually clear solution of conjugate. It was therefore filtered under sterile conditions using a sterile 0.22 μ m Millex - GV filter (Millipore, Bedford, MA) and its conjugation ratio (moles vindesine to moles immunoglobulin) recalculated, following the determination of its optical absorbance using a dual beam spectrophotometer and use of simultaneous equations (Lilly Research Centre). Anti-CEA binding activity was confirmed by ELISA and shown to be greater than 90% of unconjugated antibody activity. In addition assessment of its efficacy in vitro using a standard cell line LS174T in a 24 hour exposure uridine assay was performed.

Age of conjugate at the time of use was also noted and varied from two weeks (experiments 1 and 4 in vivo) to three months (experiment 3), at which time efficacy was also demonstrated. Similar results from the in vitro assays confirmed efficacy of conjugates at five months following preparation (data not shown).

appendix A - continued

Conjugate Code	Antibody Concentration mg ml ⁻¹	Vindesine Concentration ug ml ⁻¹	Conjugation Ratio	Date of Conjugation
#				
7	3.862	151	6.9	19-7-84
8	5.980	190	5.6	24-10-84
9	6.210	141	4.0	24-10-84
10	4.810	124	4.5	22-1-85

APPENDIX B

Computer programme (PROG.DAT) for data from Experiment 1 (COLO61184.DAT)
designed for Manova multivariate analysis of variance (SPSSX)

```

title 'repeated measures analysis of colo 6-11-84 data'
file handle mice/name='colo61184.dat'
data list file=mice records=5

  /1 group 1 id 3 sex 5 strtwt,finalwt 6-11 tvoll to tvoll9 12-74
  /2 tvoll10 to tvoll11 1-13

variable labels group 'treatment group'
               strtwt 'starting weight'
               finalwt 'final tumor weight'

value labels
               group 1 'vds' 2 'conjugate' 3 'control'/

missing values strtwt to tvoll11 (-1)

manova tvoll to tvoll11 by group (1,3)/
       wsfactor=time(11)/
       wsdesign=time/
       print=signif(averf)/
       noprint=parameters(estim) signif(multiv,eigen,dimenr,univ)/
       analysis(repeated)/
       method=sstype(unique)/
       design

finish

```

