PRODUCTION OF BISPECIFIC MONOCLONAL ANTIBODIES RECOGNISING BOTH CARCINOEMBRYONIC ANTIGEN (CEA) AND DOXORUBICIN

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By

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ABSTRACT

The objective of this project was to produce bispecific monoclonal antibodies (BsMabs) which recognise both the tumour associated carcinoembryonic antigen (CEA) and the chemotherapeutic agent doxorubicin, as a complementary approach to the use of immunoconjugates for site specific drug delivery. A monoclonal anti-CEA hybridoma (11-285-14) was made sensitive to hypoxanthine, aminopterin and thymidine (HAT), by back selecting it in increasing concentrations of 8-azaguanine. Eight 8-azaguanine resistant fusion partners were selected based on growth characteristics and continued anti-CEA production. As doxorubicin (Dox) is a hapten, it was conjugated to carrier proteins keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) using 1-ethyl-3- (dimethyl-aminopropyl) carbodiimide. Dox-KLH and Dox-BSA conjugates were employed to immunize mice and spleen cells were used for fusions with the HAT sensitive anti-CEA 11-285-14 using standard hybridoma procedures. Enzyme linked immunosorbent assays (ELISAs) were developed to test the hybrids obtained for anti-CEA, anti-Dox, anti-BSA and dual bispecific activity. Sixteen fusions from Dox-KLH immunized mice yielded 621 hybrids of which 47 showed low level bispecificity. Eight fusions with Dox-BSA immunized mice yielded 297 hybrids. Hybrids showing dual activities were cloned and 7 out of 286 of the positive clones have been selected for expansion.

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Having faced a personal tragedy with cancer, I wish to dedicate this thesis to my family, whose encouragement and moral support reinforced my motivation towards cancer research.

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ABTS	2,2-azino'-di- (3-ethyl benzthiazoline sulphonic acid)
AMT	Antibody mediated targeting
BsMabs	Bispecific monoclonal antibodies
β-gal	β-galactosidase
BSA	Bovine serum albumin
CEA	Carcinoembryonic antigen
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dox	Doxorubicin
DW	Distilled water
ECDI	1-ethyl-3-(dimethylaminopropyl) carbodiimide
ELISA	Enzyme linked immunosorbent assay
FCS	Fetal calf serum
GAR-HRP	Goat anti-rabbit Horseradish peroxidase immunoglobulins
GLN	Glutamine
HAMA	Human anti-mouse antibody response
HAT	Hypoxanthine-aminopterine-thymidine
HGPRT	Hypoxanthine guanosyl phosphoribosyl transferase
HRP	Horseradish peroxidase
HSA	Human serum albumin
ICs	Immunoconjugates

i.m.	Intramuscular
i.p.	Intraperitoneal
ITs	Immunotoxins
i.v.	Intravenous
KLH	Keyhole limpet hemocyamin
Mabs	Monoclonal antibodies
M	Mean
NCA	Nonspecific cross-reacting antigen
NRS	Normal rabbit serum
NT	Not tested
OD	Optical density (absorbance)
Pabs	Polyclonal antibodies
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
RAID	Radioimmunodetection
RAIT	Radioimmunotherapy
RAM-HRP	Rabbit anti-mouse Horseradish peroxidase immunoglobulins
RT	Room temperature
s.c.	Subcutaneous
SD	Standard deviation
TAA	Tumour associated antigen
6-TG	6-thioguanine
THF	Tetrahydrofuran
TSA	Tumour specific antigens

CHAPTER I

INTRODUCTION

I. 1.0 INTRODUCTION

The initial pages of this chapter deal with the cellular aspects of cancer as related to therapy. This is followed by a discussion of antibody mediated targeting (AMT) as a background to bispecific monoclonal antibodies.

I. 1.1 Cancer Cell Biology

The growth rate of a population of normal or abnormal (cancer) cells depends on three properties: the cell cycle time, the growth fraction and the rate of cell loss (Baserga, 1981). The shorter the cell cycle time (interval between mitoses), the faster cells are produced. The growth fraction refers to the fraction of cycling cells. The rate of cell loss refers to the fraction of cells that die or migrate to other tissues. The number of cells produced is determined by the cell cycle time and growth fraction whereas the number of cells lost is determined by rate of cell loss (Baserga,1981). Normally in an adult where growth has ceased, the number of cells produced per unit time equals the number that die. In cancer, this balance has gone awry, resulting in an increase in cell number. This is not necessarily due to a shorter cell cycle time, as it has been observed that certain normal tissues, such as jejunal mucosa of mice, proliferate faster than the fastest growing mouse tumour (Baserga, 1981; Tannock,

1978 & 1989). Therefore, chemotherapy aimed at killing rapidly proliferating cells also destroys such normal cells. This is one of the major limitations of chemotherapy.

I. 1.2 Cancer Cell Kinetics

Tumour invasion and metastases are additional limitations in the treatment of cancer. Approximately 30% of newly diagnosed solid tumours (excluding carcinoma in situ and skin cancers, except melanoma) already have clinically detectable metastases at the time of diagnosis. Of the remaining 70% of cancer patients who are clinically free of metastases, only approximately half can be cured by currently available forms of therapy. Therefore, over 60% of patients have either microscopic or clinically evident metastases at the time of diagnosis (Liotta & Stevenson, 1989).

The reason for this may be better understood by the cell kinetics of cancer. The smallest tumour size clinically detectable by physical or radiologic examination has a diameter of about 1 cm, containing approximately 10^8 to 10^9 cells and weighing 1 g (DeVita, 1989). Considering this to be clonally derived, it involves 30 doublings in cell number. From this minimal detectable limit to a potentially lethal mass of 1 kg (10^{12} cells) involves only 10 additional doublings in cell number and hence the high probability of metastases at the time of presentation by the patient (DeVita, 1989).

I. 1.3 Limitations of Cancer Chemotherapy

The preceding paragraphs illustrate the challenge of treating cancer from the cellular aspect and is reflected by the data from NCI's Surveillance, Epidemiology and End Results [SEER] program, 1984-1986 (Devita, 1989; Boring, Squires & Tong, 1991). In the U.S.A., cancer is second only to cardiovascular disease as a cause of death and accounts for 22% of all deaths. Out of a total of 930,000 cases with serious cancer (excluding cases of skin and in situ cancers), 332,000 already have clinically evident metastasis or are considered inoperable at the time of presentation. 225,000 will recur after local treatment. Thus around 557,000 patients are potential candidates for chemotherapy, confirming the 60% metastases rate mentioned above. The chemotherapy of cancer may thus be considered as the treatment of metastasis, (DeVita, 1989). The impact of chemotherapy since its advent in the late 1950's, has been significant, resulting in over 30% improvement in survival rates in the past two decades, with the use of chemotherapy alone or in combination with surgery and radiotherapy (DeVita, 1989). However, most of the success has been in the curative treatment of lymphomas, ovarian cancer, leukemias and several other childhood cancers, which, although impressive, comprise only about 12% of advanced human tumours (DeVita, 1989).

The most common cancers are, the solid tumours of lung (15%), colorectal (14%), breast and prostate (27%), and account for 56% of the total cancer cases and 55% of cancer deaths (Boring et al, 1991). We have reached a plateau in the treatment of these tumours with the currently available modalities of treatment and new approaches are being evaluated to decrease this mortality rate.

While the advantage of chemotherapy is its use in both localised and disseminated cancer, the toxicity limits the therapeutic index obtained, particularly for the refractory solid tumours. The avenues explored include optimization of drug scheduling, development of new cancer chemopreventive agents with enhanced activity and/or reduced toxicity (Boone, Kelloff & Malone, 1990) and better evaluation of regional therapy (Chabner Fine, Allegra, Yeh & Curt, 1984). With the advances in molecular biology, recombinant haematopoietic growth factors are playing an increasing role in reducing the bone marrow toxicity associated with chemotherapy (Groopman, Molina & Scadden, 1989).

Another reason for decreased efficacy of drugs is the appearance of multidrug resistance (MDR) mediated by the presence of a 170,000 dalton plasma membrane-associated p-glycoprotein (Kartner & Ling, 1989). The expression of p-glycoprotein correlates with decreased intracellular accumulation of drugs (Gerlach, Kartnor, Bell & Ling, 1986).

Calcium channel blockers such as verapamil have been shown to be able to reverse multidrug resistance by competing with the drugs for the p-glycoprotein pump and are being further evaluated (Yin, Bankusli & Rustum, 1989).

Considering this limited success of chemotherapy, a fourth modality of treatment called Biologic therapy has rapidly emerged in the last 15 years. Biologic therapy (immunotherapy) refers to the use of natural host defence mechanisms or natural mammalian substances in the treatment of cancer (Rosenberg, Longo & Lotze 1989). The important milestones in biologic therapy are the advent of monoclonal antibody (Mab) technology in 1975 (Kohler & Milstein, 1975) and of the recombinant DNA technology that could lead to an unlimited supply of Mabs and biological modifiers.

I. 1.4 Cancer Immmunotherapy

Immunotherapy can be classified into active and passive approaches. Examples of each are given in table 1.

Table 1: Classification of Cancer Immunotherapies

Classification	Examples
I. Active Immunotherapy	Immune adjuvants such as BCG, C-Parvum, Levamisole. Biological response modifiers such as Interleukin-2 (IL-2), Interferon
1. Non Specific	
2. Specific	Immunisation with tumor cell vaccines or mabs (eg, anti-idiotypic in lymphomas)
II. Passive Immunotherapy	Mabs or polyclonal antibodies (Pabs) either alone or conjugated with toxins or radiolabels
1. Antibodies	
2. Cells	Cytotoxic T cells. Lymphokine Activated Killer cells (LAK cells), Tumor infiltrating lymphocytes (TILs)
III.Indirect	Removal of blocking factors or suppressor factors Inhibition of growth factors or angiogenic factors

(Adapted from Rosenberg, et al, 1989)

Active immunotherapy is analogous to immunization for infections diseases, referring to immunization of a tumour bearing host with substances that elicit an immune response capable of retarding or eliminating the tumour. Attempts using nonspecific adjuvants such as Bacille Calmette Guerin (BCG), Corynebacterim Parvum (C. Parvum) and Levamisole have been

disappointing, as were specific immunization attempts using tumour cells or tumour-cell extracts either alone or as vaccines (Rosenberg et al, 1989). Further, active immunotherapy may be impeded by a pre-existing immunosuppressed status of the cancer patient. Despite the early promise of interferons in many cancers, after extensive analysis in clinical trials, interferon-alpha is currently the treatment of choice only for the uncommon Hairy cell leukaemia, with a possible effect on cutaneous T-cell lymphoma (Krown, 1988; Galvani, Griffiths & Cawley, 1988). In addition, its therapeutic potential has been restricted due to its extensive list of toxic side effects, which has also been a limiting factor of high doses of interleukin-2 (IL-2) therapy.

Currently, Rosenberg's group has shown meaningful response rates in phase I/II trials, with a combination of low dose interleukin-2, LAK (lymphokine activated killer) cells or TILs (tumour infiltrating lymphocytes) and cyclophosphamide (chemotherapy) in selected malignancies such as colonic adenocarcinoma and malignant melanoma (Rosenberg, Spiess & Lafreniere,1986; Cameron,Spiess & Rosenberg,1990).

I. 2.0 ANTIBODY MEDIATED TARGETED THERAPY

I. 2.1 Objective

In antibody mediated targeted (AMT) therapy, antibodies are evaluated as carriers of toxic agents such as drugs, toxins and radioisotopes directly to the cancer site. The objectives are two-fold: (1) selective delivery to cancer cells and (2) reduced toxicity to normal cells. (Ford & Casson, 1986; Dillman, 1989; Ford, Richardson, and Reddy, 1990).

II. 2.2 History

The concept of using antibodies as carriers of toxic agents dates back almost a century, first postulated by the Nobel Prize laureate Paul Ehrlich (1854-1915). The term antibodies, coined by Ehrlich, originated from his famous side-chain theory (Dale 1957). As proposed by Ehrlich, in 1897, each cell in the body carried on its surface specific side-chains (receptors) (Ehrlich 1897). When encountered by toxins (represented by toxic foodstuffs) for which the side chains have specific affinity, the receptors (also called anti-toxins) are produced in excess and liberated from the cells. Appearing in the body fluids, they unite with the toxins and thus protect the cells from damage. Such anti-toxins, later called antibodies can be induced following a single immunization with suitable bacteria (eg. cholera) or toxin (eg. diphtheria). He referred to antibodies as exclusively 'parasitotrophic' and so

"... it is not surprising that they seek out their targets like magic bullets" explaining the miraculous cures sometimes obtained (Ehrlich, 1897).

Ehrlich coined the word 'chemotherapy' and is considered the father of modern chemotherapy (Ehrlich, 1908). Realizing the nonspecific toxicity of pharmaceutical agents with treatment of disease on normal tissues of the host, in Ehrlich's own words, *"We have no other choice than to learn to shoot better"* (Ehrlich, 1908). This concept is the basis of antibody mediated targeted therapy.

I. 2.3 Components in targeted therapy

Targeted therapy comprises broadly the following components: (i) Target, (ii) Carrier, (iii) Toxic agents. These are further elaborated in subsequent pages with examples.

I. 3.0 TARGET

I. 3.1 Does tumour specificity exist?

An ideal target is a cell surface antigen that is specific to cancer cells and not present on normal cells. The search for tumour specific antigens (TSA) was begun by Ehrlich, and almost a century later, despite innumerable efforts by cancer immunologists, their existence remains to be proven (Old 1981; Schreiber, Ward, Rowley & Strauss, 1988). Preliminary experiments involved immunization of outbred mice or rats with tumour tissue, which, on subsequent challenge rejected the tumours (Woglom, 1929). Although initial interpretations suggested tumour immunity, later experiments revealed that these mice rejected normal tissues from donors as well and led to the discovery of the major histocompatibility complex dampening the enthusiasm for the existence of TSA (Schreiber et al 1988).

I. 3.2 Tumour specific antigens

The only evidence suggesting the existence of TSA comes from transplantation studies using highly inbred mice carrying tumours induced by chemical or physical carcinogens, eg. methylcholanthrene-induced carcinoma meth A, and the ultraviolet light-induced skin tumour 1591 (Schreiber et al 1988). However, such studies may not necessarily correlate with tumours occurring in humans who are extremely outbred,

despite a high incidence of tumours induced by physical or chemical carcinogens.

Therefore, after extensive searching for TSA for nearly a century and despite recent technological advances, unique TSA are yet to be discovered in humans. Perhaps the only exceptions are the presence of the idiotype marker on certain B and T cell lymphomas and leukemias (Stevenson George & Glennie, 1990) and, more recently, the product of a p53 mutated gene, under investigation (Harris, 1990).

I. 3.3 Tumour associated antigens (TAA)

The best targets available for the commonly occurring solid tumours are the TAAs which show greater expression on cancer cells relative to their expression on normal cells. In addition, their lower expression on key normal tissues such as bone marrow and intestinal mucosa further emphasizes their role in reducing chemotherapy associated toxicity in antibody mediated targeting.

Several TAAs have been identified to date (Bates & Longo, 1987) and evaluated for their use as tumour markers in cancer diagnosis and management (Table 2). An ideal tumour marker should possess the following characteristics (Bates & Longo, 1987): i) be produced by tumour cells and easily detectable in body fluids; ii) should be present only in malignancy and not in health or benign diseases; iii) should be useful for

screening and detecting early cancer levels; iv) should be detectable in the absence of clinical evidence of tumours and v) its decrease should correlate with efficacy of anti-cancer therapy. None of the tumour markers discovered to date meet all of the above criteria.

Table 2: Classification of tumour markers

TAA	Cancers
Oncofetal antigens Carcinoembryonic antigen (CEA)	Colorectal (80%), lung, pancreas, breast, gynaecologic (all 30-70%)
Alphafetoprotein (AFP)	Hepatocellular carcinoma (72%) or embryonal cell cancer
Ca-19-9	Colorectal, pancreatic
Ca-125	Ovarian
Placental proteins Human chorionic gonadotropin (HCG)	Trophoblastic tumours (100%) Testicular germ cell tumours
Human placental lactogen Regan isoenzyme (of alkaline phosphatase)	Trophoblastic
Enzymes Acid Phosphatase	Prostatic cancer

Adapted from Bates & Longo, 1987

Three well characterised TAAs for solid tumours are carcinoembryonic antigen (CEA), alphafetoprotein (AFP) and human chorionic gonadotrophin (HCG). AFP, although a valuable marker for hepatomas and testicular cancers is not as

widespread as CEA. Furthermore, the cure rate for testicular cancer has risen from 10% in 1973 to 70% in 1983 (Chabner et al, 1984) due to a combination of surgery and chemotherapy. HCG is limited to choriocarcinomas which have a cure rate of over 90%. Since the discovery of CEA by Gold and Freedman (Gold and Freedman, 1965) progress has been rapid particularly in the last five years in the dissection of the antigen both at the cellular and molecular level. Structural and functional properties of CEA are presented in section I.12.0.

I.3.4 Characteristics of target

The choice of a target, apart from tumour specificity, depends on several characteristics which are discussed below.

I. 3.4 (a) Antigenic heterogeneity

Tumour cell heterogeneity is a frequent problem experienced in both diagnosis (phenotypic variation) and management of cancer (Fidler & Poste, 1985; Schnipper 1986). This heterogeneity is illustrated by the differences between patients bearing the same histological class of tumour leading to survival times ranging from seven months to seventeen years (Oldham, 1987). In addition, heterogeneity exists between individual cells of each tumour in each patient and has proved to be a feature of most TAAs (Greiner, 1986). Approaches to overcome this problem include the use of "cocktails" of Mabs

recognizing different antigens and/or different epitopes on the same antigen. In a recent report, Durrant and his colleagues have demonstrated that a combination of Mabs recognizing different TAAs such as CEA and 791T/36 recognized 100% of all the 50 individual human primary colorectal cancers studied (Durrant, Robins, Ballantyne, Marksman, Hardcaastle & Baldwin, 1989). The Mabs were selected because of their preferential binding to tumour cells compared to a panel of normal tissues when assayed by immunocytochemical staining of cryopreserved tissues.

Other approaches include the use of interferon (IFN) to enhance TAA expression resulting in increased localization of ^{125}I-labelled Mab in human colonic xenografts (Greiner, Guadagni, Noguchi, Pestka, Colcher, Fisher & Schlom, 1987) and of a ^{111}In-labelled Mab 96.5 in patients with melanoma (Rosenblum, Lamki, Murray, Carlo & Gutterman, 1988). However, in a phase II trial using recombinant IFN potentiated antibody dependent cellular cytotoxicity (ADCC) in patients with advanced colorectal cancers, the delivery of Mabs to the tumour site remained a major obstacle (Weiner, Moldofsky, Gatenby, O'Dwyer, O'Brien, Litwin & Comis, 1988).

Another approach is to use radionuclides as the toxic moiety in order to eradicate 'bystander' non-antigen expressing tumour cells (Order, Sleeper, Stillwagon, Klein & Leichner,

1990). However, this may lead to nonspecific toxicity to normal cells.

I. 3.4 (b) Antigen expression / secretion

The antigen may be membrane bound, intracellular or secreted into the extracellular fluids. Although, theoretically intracellular location of antigens may impede recognition by antibodies, their accessibility has been demonstrated in breast cancer, with Mabs to keratin polypeptides as the target antigen (Dairkee & Hackett, 1988). In addition, ^{125}I-labelled Mabs to an intracellular melanoma glycoprotein could be localized in xenografts up to 10 weeks post injection (Welt, Mattes, Grando, Thomson, Leonard, Zanzonico et al, 1987). However, although Mabs to intracellular oncogene products have not been found to be suitable targets (Embleton, Habib, Garnett & Wood, 1986) inhibition of tumour growth *in vivo* has been demonstrated by a Mab reactive with transmembrane glycoprotein encoded by the neu oncogene (Drebin, Link, Weinberg & Greene, 1986).

Most TAAs evaluated in targeting studies are both membrane associated and secreted into the extracellular fluid (ECF). The secretion of antigen does not usually prevent antibody localization, as demonstrated by a number of studies, unless the plasma antigen concentration is very high (Begent, Searle,

Stanway, Jewkes, Jones, Vernon & Bagshawe, 1980; Searle, Boden, Lewis & Bagshawe, 1981) perhaps leading to formation of circulating antigen/antibody complexes and rapid removal by the reticuloendothelial system.

I. 3.4 (c) Antigen Density

It has been clearly demonstrated that the degree of tumour reduction with Mab therapy is proportional to the cell surface antigen density in solid tumours and hence the expression of antigen on the surface may be a crucial factor in immunotherapy (Capone, Papsidero & Chu, 1984). Several agents are being evaluated for their capability of enhancing expression of TAAs such as recombinant IFN (Rosenblum et al, 1988), butyrate, glucocorticoids and cytotoxic drugs (Bagshawe, 1989). Importantly, transforming growth factor β (TGF-β) has been shown to augment CEA secretion/expression and modulation in human colon carcinoma cells (Chakrabarty, Tobon, Varani & Brattain, 1988).

A low level of antigen expression may lead to subtherapeutic delivery of Mabs, but is overcome in tumour cells that show regeneration and re-expression of the antigen on the cell surface within a short period of time, thus trapping the Mabs and resulting in greater cytotoxicity (Wang, Lumanglas, Silva, Ruszala-Mallan & Durr, 1987).

I. 3.4 (d) Antigenic modulation

The phenomenon of antigenic modulation involves the redistribution of cell surface antigens in the presence of bivalent antibody (Cobbold & Waldmann, 1984). Modulation occurs within minutes and is reversible in the absence of antibody (Schroff, Farrell, Klein, Oldham & Foon, 1984). However, it may still be feasible to obtain cytotoxicity with Mabs if conjugated to drugs/toxins and are rapidly internalized, in contrast to unconjugated Mabs, as demonstrated using anti-human T-cell Mab T101 *in vitro* and *in vivo* in patients with chronic lymphocytic leukaemia and cutaneous T cell lymphoma (Shawler, Miceli, Wormsley, Royston & Dillman, 1984; Schroff et al. 1984). Thus, antigenic modulation can be a limiting factor in Mab therapy and attempts to overcome it include the use of univalent antibodies, like bispecific Mabs. Antigens may also be nonmodulating such as campath-1 antigen, found on T and B cells, but not on stem cells, which can facilitate the targeting Mab to mediate ADCC or complement mediated lysis (Riechman, Clark, Waldmann & Winter, 1988).

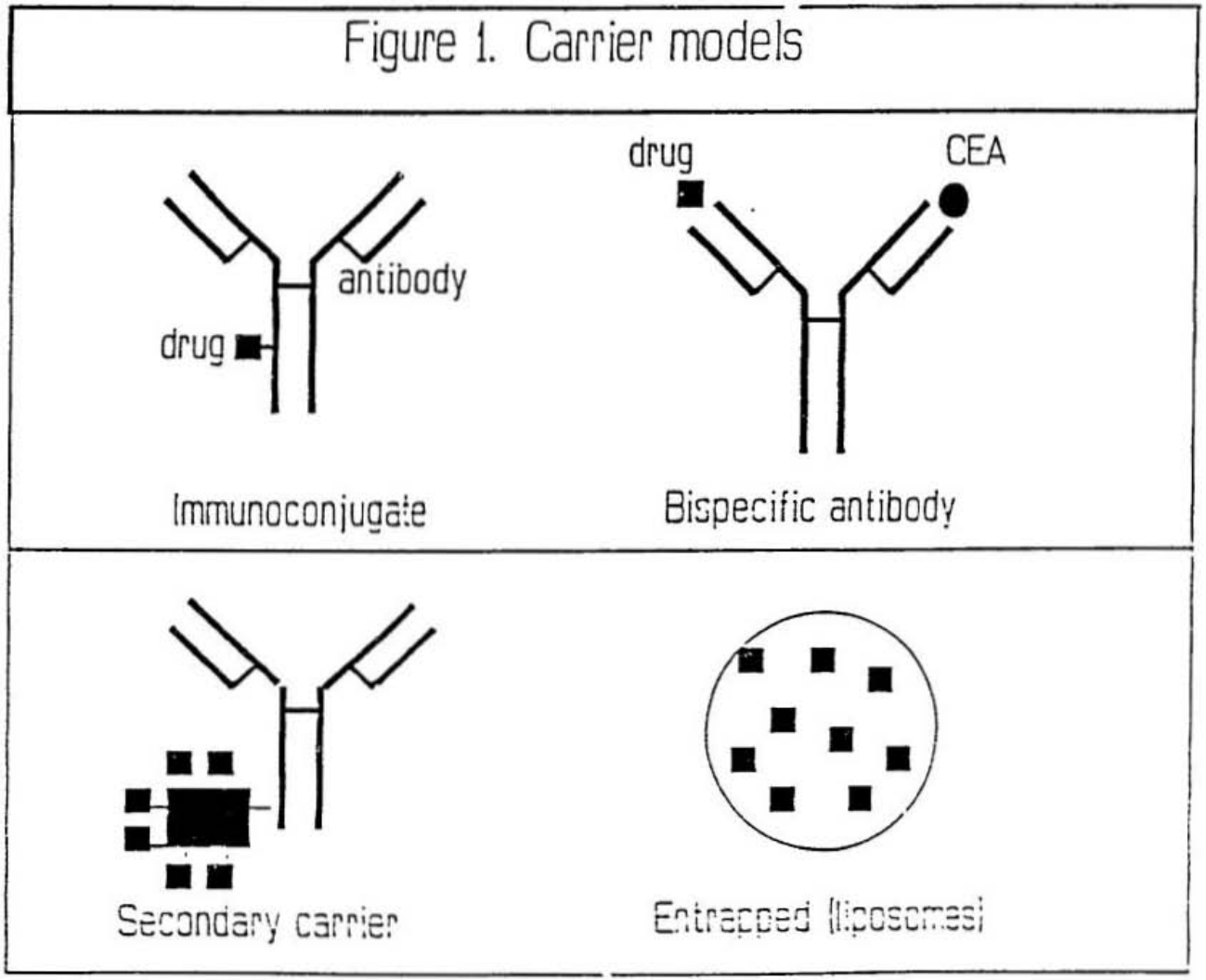
I. 4.0 CARRIERS

I. 4.1 Introduction:

An ideal carrier is one that has the following properties. Ease of chemical linkage of the toxic agent to the carrier with both the carrier and agent retaining their function. The carrier should deliver the toxin to the cancer site, releasing the agent to act at the specific site. The carrier should have a reasonable half-life to reach the tumour, but evade the host's immune defence mechanisms (Ford & Casson, 1986).

Several carriers have been evaluated for the above properties and the two outstanding candidates are antibodies and liposomes (Figure 1). The specificity, monoclonality and unlimited supply of Mabs makes them more suitable vehicles for carrying toxic agents to tumour cells and will be discussed in detail in the following sections. Liposomes are small spheres consisting of concentric phospholipid bilayers separated by an aqueous phase. A variety of substances can be incorporated into liposomes including drugs, hormones and enzymes (Weinstein, 1984). The problems with the use of liposomes include: nonspecific uptake by the reticuloendothelial system, poor permeability out of the blood stream, degradation, toxicity and antigenecitiy (Weinstein, 1984; Gregoriadis, 1990).

Figure 1. Carrier models



I. 4.2 Antibodies as carriers

The concept of using antibodies as carriers of toxic agents, although simple and attractive (frequently referred to as magic bullets) did not gain momentum until the early 1970s, since investigators were faced by the following obstacles: (1) apprehension regarding administration of large quantities of foreign protein to patients; (2) immune response to administration of these proteins; (3) lack of relevant pre-clinical models for *in vivo* testing; (4) heterogeneity of the antibodies with respect to their class, antigen specificity and affinity; and (5) poorly defined targets (Ford and Casson, 1986). Many of these obstacles have been partially or completely overcome. For example, it has been demonstrated in several studies that xenogenic antibodies can be safely administered to patients (Newman, Ford, Davies & O'Neill, 1977; Hamblin, Abdull-Ahad, Gordon, Stevenson & Stevenson, 1980) and can localise the tumours in patients (Goldenberg, Leland, Kim, Bennett, Primus, Van Nagell, Estes, DeSimone & Rayburn, 1978). The development of the human tumour xenograft model has been a major step towards elucidating the targeting potential of antibodies (Rygaard & Poulsen, 1969).

A milestone in Immunology has been the development of the hybridoma technique in 1975 by George Kohler and Cesar Milstein (Kohler & Milstein, 1975) leading to the continuous supply of antibodies of predefined specificity termed monoclonal

antibodies for antibody mediated targeting (AMT). Prior to this innovation, all attempts at AMT involved polyclonal antibodies (Pabs). Pabs are not entirely without advantages, as exploited by Stanley Order's group at John's Hopkins centre (Lenhard, Order, Spunberg, Asbell & Leibel, 1985). Pabs may be produced in a wide range of species avoiding repeated presentation from one species to the patient's immune system. Additionally, polyclonality can result in the recognition of different epitopes on a given target, thereby increasing the capacity for drug/toxin delivery. This can also be achieved by 'cocktails' of Mabs as mentioned in the above section on targets. Polyclonal antibodies suffer from the disadvantages of being heterogeneous mixtures and the lack of reproducibility from one polyclonal serum to another. A homogeneous unlimited supply of monoclonal antibodies of predefined specificities, therefore has advantages over Pabs or other carrier systems, and the rest of this chapter will be restricted to monoclonal antibodies, the present status and the future with reference to targeting.

I. 5.0 MONOCLONAL ANTIBODIES IN THE DIAGNOSIS OF CANCER

The main uses of Mabs in the diagnosis of cancer are in (1) Immunohistopathological diagnosis of cancer, (2) Serum tests for assaying various tumour markers, and (3) in radioimmunodetection (RAID) (Ghee Teh, Stacker, Thompson & McKenzie, 1985; Larson, 1986). Principles of RAID are similar to radiolabelled Mab therapy and are presented in section I. 7.1).

I. 6.0 MONOCLONAL ANTIBODIES IN THE TREATMENT OF CANCER

As the main focus of this thesis is on the production of bispecific monoclonal antibodies for targeted drug delivery, the current status of Mabs in therapy shall be first reviewed briefly, summarised from the enormous literature and several recent reviews and books on this subject (Ford & Casson 1986; Levy 1987; Embleton 1987; Houghton & Scheinberg 1986; Vitetta, Fulton, May, Till & Uhr, 1987; Byers & Baldwin 1988; Dillman 1989; Hertler & Frankel 1989; Goldenberg 1989; Bagshawe 1989; Rosenberg et al, 1989; Ranada 1989; Kosmas, Kolofonos & Epenetos, 1989; Ford et al, 1990)

The basic mechanisms by which Mabs could be used in cancer therapy are outlined in Table 3. Broadly, Mabs may be used *in vivo*, either alone or as carriers of cytotoxic agents; *in vitro* to purge tumour cells before autologous bone marrow transplantation after high dose systemic cytotoxic therapy or

to purge marrow of T cells to prevent graft-versus-host disease for allogenic bone marrow transplantation (Kernan, Byers, Scannon, Mischak, Brochstein, Flomenberg, Dupont, O'Reilly, 1988).

Table 3: Monoclodonal Antibodies for Cancer Therapy

Antibody Alone Indirect Cytotoxicity Complement - mediated lysis Antibody - dependent cellular cytotoxicity (ADCC) polymorphonuclear leukocytes Direct Cytotoxicity Catalytic antibodies Regulatory antibodies Immunization Anti-idotypic antibodies
Antibody Immunoconjugates Radiolabelled antibodies Immunotoxins Chemotherapy immunoconjugates Immunobiologicals
Bone Marrow Transplantation *In vitro* Anti-T cell purging to prevent graft-versus-host disease (GVHD) in allogeneic transplants Anti-tumour purging before autologous transplantation *In vivo* Anti-T cell antibodies to abrogate GVHD or rejection in allogeneic transplants

Adapted from Dillman, 1989; Rosenberg, 1990

I. 6.1 Monoclonal antibody alone: Indirect cytotoxicity

Antibodies constitute the main humoral immune defense mechanism in man, their primary role being to eliminate microorganisms by causing activation of complement or by interacting with phagocytic cells. Due to practical and economical reasons, most of the monoclonal antibodies evaluated have been of murine origin. As in humans, murine antibodies exist in different classes and subclasses, but a problem in therapy is that mouse or rat Mabs may not interact with human effector mechanisms. While this may not hinder targeting cytotoxic agents, it could be a limiting factor in re-directing human effector cells against cancer, a promising approach pioneered by Rosenberg's group at the NIH.

Based on data collected from *in vitro* studies, *in vivo* animal model systems and on a systematic approach by constructing panels of chimeric antibodies using recombinant DNA technology, different isotypes of murine and human origin have been evaluated (Bruggemann, Williams, Bindon, Clark, Walker, Jefferis, Waldman & Neuberger, 1987; Riechmann, Clarke, Waldmann & Winter, 1988; Dillman, 1989; Morrison & Vernon, 1989; Clark 1989). The most useful isotypes for interaction with human complement are mouse IgM, followed by IgG_3; rat IgM and IgG_{2b}. For ADCC, the results are complicated by the three different Fc receptors on different effector cells. In

general, the best isotypes for ADCC are mouse IgG_{2a} and IgG_3 and rat IgG_{2b}. For human antibodies and mouse-human chimeric antibodies, the best results of interaction with human complement are obtained with IgM > IgG_1 > IgG_3 > IgG_2 > IgG_4 and for ADCC with human effectors they are human IgG_1 and IgG_3 (Dillman 1989). IgM antibodies, however, have technical disadvantages due to their large size, which may impede penetration into the tumour and additionally, could produce a hyperviscosity syndrome.

I. 6.2 Monoclonal antibody alone: Direct effects

A novel approach involves raising Mabs against the transition state of substances. On binding, antibodies act as catalysts to induce changes on cell membranes, cellular proteins or nucleic acids (Iverson & Lerner, 1989). The use of such catalytic antibodies, specific for antigens on cancer cells, may prove to be an alternative avenue for treating cancer.

Mabs may prove to be directly cytotoxic when directed against receptors for growth factors that promote proliferation of cancer cells. Mabs directed against interleukin-2 and epidermal growth factor receptors are being evaluated in bone marrow transplantation and cancer, respectively (Queen,

Schneider, Selick, Payne, Landolfi, Duncan, Avdaloric, Levitt, Junghans, & Waldmann, 1989).

Clinically, the most successful therapeutic use of unconjugated antibody has been in the use of OKT_3, a murine Mab directed against CD_3 (T Cell Receptor, TCR) on mature human T cells, in renal allograft rejection. This is now an approved therapy in patients experiencing acute rejection, with a reversal of 94% of the rejections and significant improvement of one year graft survival up to 62% (Ortho Multicenter transplant study group, 1985; Byers & Baldwin 1988).

I. 6.3 Mabs alone: Immunization

In 1984, Neils Jerne received the Nobel prize in Medicine for his idiotype "network hypothesis" (Jerne NK 1974). According to this hypothesis, murine antibody (AB_1) directed against a tumour associated antigen or a B cell lymphoma idiotype, would induce in addition to an anti-F_c antibody response, an anti-idiotype antibody response (AB_2). AB_2 would be a mirror image of AB_1 and thus resemble the original antigen. AB_2 may be more immunogenic and could be used to immunize humans to induce AB_3 production that would react with the tumour antigen, similar to AB_1, except that AB_3 would be a human antibody produced endogenously (Traub, Dejager, Primus, Losman / Goldenberg, 1988). Although such active immunization

with idiotype Ig led to the emergence of surface Ig-negative variants, the tumours showed a slower growth than the original tumour, which may prove beneficial (George, Spellerberg & Stevenson, 1988).

In addition, anti-idiotypic therapy of leukemias and lymphomas with or without cytotoxic agents linked to the Mabs, is an attractive approach due to the tumour specificity of the B cell idiotype (Stevenson, George & Glennie, 1990).

I. 7.0 CONJUGATED ANTIBODY TARGETING

Due to the aforementioned reasons, antibodies are generally not efficient by themselves in eradicating cancer. In addition, certain privileged sites such as the central nervous system lack access to the effector mechanisms. To circumvent this problem, antibodies have been conjugated to radioisotopes, cytotoxic toxins and drugs. The antibody then does not contribute directly to the cytotoxic effect, but acts as a carrier of the cytotoxic agent providing appropriate target specificity. For a detailed list of cytotoxic agents used for conjugation to Mabs, the reader is referred to Houghton & Scheinberg (1986).

I. 7.1 Radiolabelled antibodies

In addition to their value in diagnosis and therapy, radiolabelled antibodies have contributed much in quantitating and studying the pharmacokinetics of Mabs *in vivo*.

I. 7.1 (a) Radioimmunodetection (RAID) for Diagnosis of Cancer

Tumour localization by radiolabelled antibodies may be considered as two eras; the first was pioneered by Pressman in the 1950s in animal models and the second by Goldenberg and others in the 1970s onward (Presmann & Korngold, 1953; Goldenberg et al. 1978). A decade of clinical RAID trials from 1978 to 1988 involving 1831 patients, using 61 antibody preparations (52 being monoclonal) against twenty different tumour types has been summarised by Steven M. Larson of the Memorial Sloan Kettering Cancer Centre (Larson, 1990). Despite this diversity, several parameters have been evaluated and the future includes a standardised, systematic approach. The problems included the limited fraction (in many cases, less than 1%) of injected radioactivity localized in the tumour. The problem of HAMA (human antimouse antibody response) limiting repeated administration may be overcome by the superiority of Fab fragments over intact Igs in tumour localization and the use of chimeric Mabs. HAMA is an impediment to repeated administration of murine mabs and will be addressed in a separate section (I 8.5).

^{131}I has been most frequently used due to its ease of conjugation, wide availability and a half-life of eight days, but is not an ideal radiolabel due to its β-emission. Newer isotopes evaluated include ^{111}In with good imaging qualities, but with a half-life of 68 hrs, it is expensive, emits gamma energy and accumulates in the reticuloendothelial system (RES). Technetium-99, has superior imaging capabilities, and is relatively inexpensive and widely available. Additional promise for RAID has been demonstrated with improved imaging techniques such as Single Photon Emission Computerised Tomography (SPECT) over conventional gamma camera scanning and with more recent technology such as hand held probes during surgery, enhancing tumour localisation (Larson, 1990). In conclusion, since an early diagnosis of clinically silent micrometastasis may be the key to successful therapy, investigators have reasons to be optimistic about a significant role of RAID in the future. The problems involved in RAID and methods of overcoming them (Sands, 1990) are common to targeted therapy and are considered in the following sections.

I. 7.1 (b) Radioimmunotherapy (RAIT)

The role of radiolabelled antibodies as therapeutic agents has been recently reviewed in comparison with conventional approaches in radiation therapy. RAIT was compared with geometric isotopic implants, external irradiation, tumour dose response and summarised with the energy of various isotopes used (Order, Sleeper, Stillwagon, Klein & Leichner, 1990). The goal of RAIT in comparison with RAID is to increase the uptake by the tumour, preferably by all the cells, and for the radiotherapeutic effect to be long lived. ^{131}I has been used widely due to its β emission and long half-life (8 days) and has shown partial anti-tumour effects with anti-ferritin Pabs in hepatomas (Order et al, 1985) and Hodgkins disease (Lenhard et al, 1985). ^{131}I Mabs have shown partial responses in a phase I/II trial of cutaneous T cell lymphomas (Rosen, Zimmer, Goldman-Leiken, Gordon, Kzikiewicz, Kaplan, Variakojis, Marder, Dykewicz, Piergies, Silverstein, Roenigk & Spies, 1987) and in B cell lymphomas (DeNardo, DeNardo, O'Grady, Levy, Adams & Mills, 1990). The route of administration is important depending on the tumour site, as demonstrated by partial to complete responses ranging from 7 to 24 months with no toxicity in 4/5 patients with leptomeningeal tumours injected intrathecally with ^{131}I labelled Mabs (Lashford, Davies, Richardson, Bourne, Bullimore, Eckert, Kemshead & Coakham,

1988). The intraperitoneal (i.p.) approach for advanced ovarian cancer has been evaluated (Kalofonos, Stewart & Epenetos, 1988). Interestingly, in a double antibody study, with radiolabelled antibodies administered both intravenously (i.v.) and i.p. in patients with advanced ovarian cancer, i.v. was better than i.p. when the tumour was subserosal whereas i.p. was more effective in ovarian cancer with ascites (Britton 1990).

The properties of the isotope are important to avoid total body irradiation, a limiting toxicity of RAIT. Alpha particles have a very high linear energy (5 to 8 Mev) and a short path length (40 to 80 μm) limiting cytotoxicity to several cell diameters, thus reducing non-specific irradiation of distant tissues. ^{212}Bi, an α emitting radionuclude, despite a short half-life (approximately 1 hr), has been demonstrated to be valuable in localised i.p. malignancy in an animal model, where tumour cells are easily accessible (Macklis, Kinsey, Kassis, Ferrara, Atcher, Hines, Coleman, Aldelstein & Burakoff, 1988). Trials in progress include imaging with technetium or indium labelled Mabs to calculate dosimetry, followed by RAID with β-emitters rhenium-188 or yttrium-90 labelled Mabs, respectively.

I. 7.2 Immunotoxins (ITs)

Toxins are attractive cytotoxic candidates for targeting on a molar basis, as they are more potent than chemotherapeutic drugs. A single molecule of ricin or diptheria A chain in the cytosol may be sufficient to kill a cell (Yamaizumi & Mekada, 1978; Vitetta, Fulton, May, Till & Uhr, 1987). As a result, several toxins have been conjugated to antibodies, and evaluated as immunotoxins including ricin, abrin, gelonin, pseudomonas exotoxin A, diptheria toxin. Most toxins are proteins which share in common their mode of action by inhibiting the elongation step of protein synthesis, elicited by the toxic moiety of the molecule. The cell binding moiety of the toxin (B-chain) is removed before the toxic part is linked covalently to the antibodies, thus reducing nonspecific binding. Progress has been rapid in the construction of ITs leading to the production of second and third generation ITs using recombinant DNA technology. Their efficacy and pharmacokinetics, have been reviewed, *in vitro* and *in vivo* in pre-clinical and clinical studies (Vitetta et al, 1987; Byers & Baldwin, 1988; Hertler & Frankel, 1989). Numerous clinical studies have been performed using ITs in the treatment of diseases such as chronic lymphocytic leukemia (CLL), B cell leukemia (BCL) and selected solid tumours, including a systematic-phase I/II trial in malignant melanoma (Hertler & Frankel, 1989; Spitler, Rio, Khentigan, Wedel, Brophy, Miller,

Harkonen, Rosendorf, Lee, Mischak, Kawahata, Stoudemire, Fradkin, Bautista & Scannon, 1987). A successful application has been in the prevention and treatment of steroid resistant graft-versus-host disease (GVHD) by depleting the T cells, using an anti-CD_5 IT, from allogenic bone marrow transplants. Following the dramatic response in an 8 year old girl with severe grade III-IV, steroid resistant GVHD (Kernan et al. 1988), phase I/II trials are underway on a larger group of patients, with promising early results (Byers V: M.D. Anderson Cancer Centre, Personal communication). Equally promising is the potential in the treatment of AIDS with ITs that can neutralise HIV virions and kill T cells infected with diverse strains of HIV-1 (Kim, Fund, Sun, Sun, Chang, Chang, 1990)

I. 7.3 Antibody-drug immunoconjugates (ICs) (Immunochemotherapy)

Chemotherapeutic drugs as toxic agents have the advantages of familiarity due to their wide use clinically, with their pharmacokinetics, mode of action, tumour susceptibility and toxicity well elucidated. The report by Mathé (1958) with prolongation of survival of mice with L1210 leukemia treated with antibody targeted methotrexate, first demonstrated the feasibility of targeted chemotherapy. It was also shown that antibody and drug mixed noncovalently were synergistic in their

action but it was necessary for drugs to be covalently linked to the antibodies for maximum targeted effect (Davies & O'Neill, 1973; Newman et al, 1977).

Typically, after chemical coupling of drugs and antibodies, the ICs go through a systematic process of *in vitro* testing using cultured human cancer cell lines, *in vivo* pre-clinical studies with animal models (the most useful model being the nude athymic mouse with human tumour xenografts) and finally clinical trials. Due to this elaborate production and testing process, most clinical trials to date have been preliminary phase I/II trials. These trials have demonstrated the efficacy and feasibility of this approach, as well as the problems involved and suggestions for overcoming these problems (Ranada 1989). Preclinical and clinical studies have been reported with ICs of different chemotherapeutic agents. Human studies using ICs were pioneered by Ghose's group from Halifax in the early 1970s, using chlorambucil and the folic acid antagonist methotrexate (Ghose, Norvell, Guclu & Macdonald, 1972 & 1975). A few recent examples are with the anthracyclines, doxorubicin (Dox) (Pietersz, Smyth & Mckenzie, 1988; Pietersz, Smyth, Kanellos, Cunningham & Mckenzie,1989; Yang & Reisfeld 1988) and daunomycin (Dillman et al, 1988; Diener, Diner, Sinha, Xie & Vergidis, 1986; Pietersz et al, 1988; Diener, Xie, Yu, Longenecker & Sinha, 1988). In addition, recent studies have also included methotrexate

(Ghose, Blair, Kralovec, Mammen, Vadia, 1988; Baldwin & Byers, 1989), alkylating agents (Smyth, Pietersz & McKenzie, 1988a; Pietersz et al, 1989) and the work from this laboratory with vinca alkaloids (Ford, Bartlett, Casson, Marsden & Gallant, 1987a). Preclinical studies of Mabs specific for CEA (Mabs 11-285-14 & 14-95-55) linked to vindesine have clearly demonstrated efficacy, both *in vitro* and *in vivo*, which correlated with the degree of expression of target antigen CEA (Rowland, Simmonds, Core, Marsden & Smith, 1986; Ford et al, 1987; Casson, Ford, Marsden, Gallant & Bartlett, 1987a). The sensitivity of the cell line to the therapeutic agent plays an important role as demonstrated by the efficacy of anti-CEA Pabs or Mabs linked to vindesine or doxorubicin (Ford et al. 1987a; Richardson, Ford, Tsaltas & Gallant, 1989).

Although immunoconjugates may often be less effective *in vitro* than the free drug, their *in vivo* efficacy and therapeutic index may be increased as demonstrated by vindesine-anti CEA ICs (Rowland et al, 1986; Casson *et al*. 1987) and Doxorubicin-T101 Mab ICs (Dillman et al, 1986). In addition, anti-Ly-2.1 chlorambucil ICs have shown an increased efficacy compared to the free drug, both *in vitro* and *in vivo* against a murine thymoma cell line (Smyth, Pietersz, Classon & McKenzie, 1986).

I. 8.0 PROBLEMS INVOLVED WITH IMMUNOCONJUGATES

The problems of ICs in therapy may be related to their production, delivery or the target antigen itself. Heterogeneity of antigen expression and antigen modulation were presented in section I.3.0. The following sections deal with ICs production and delivery.

I. 8.1 Production of immunoconjugates:

The number of active drug molecules that may be directly linked depends on the number of modifiable sites on the Mab molecule with a potential for loss of immunoreactivity (Durrant, Robins, Armitage, Brown, Baldwin & Hardcastle, 1986). This is a major problem because most drugs are hydrophobic compounds and have to be attached to hydrophilic antibodies, with the retention of both Mab and drug activity. Most coupling procedures, like the commonly used glutaraldehyde or carbodiimide reagents, result in a loss of drug activity and/or antibody activity (Pietersz et al, 1989). Efforts have been directed at improving the methods of conjugation.

In addition, secondary carrier molecules may be used, to which a larger number of drug molecules can be attached, followed by conjugation to Mabs. ICs produced by Doxorubicin linked via a cis-aconityl spacer molecule to a Mab (9.2.27) recognising a melanoma antigen have been demonstrated to be more effective than the free drug *in vivo* (Yang & Reisfeld,

1988). Simiarly, greater efficacy has been demonstrated for anti-alpha protein Pabs or Mabs linked to daunomycin via a dextran bridge (Tsukada, Ohkawa & Hibi, 1987). Another study demonstrated that up to 38 moles of methotrexate could be attached per mole of human serum albumin (HSA) with subsequent linkage to Mab 79IT/36 directed against an osteogenic sarcoma cell line. However, despite an increase in cytotoxicity compared with the free drug, the antigen binding activity of the ICs was reduced by nearly 70% (Garnett & Baldwin 1986). More promising is the report of conjugation of up to 30-50 molecules of MTX by an intermediate amino-dextran carrier system to anti-CEA Mab with retention of antigen binding activity (Shih, Sharkey, Primus & Goldenberg, 1988). A disadvantage with this approach is the larger molecular weight of these secondary carrier ICs, which, although effective *in vitro*, are eliminated quickly *in vivo* by the reticuloendothelial system.

A complementary approach to better methods of conjugation is to use new or more potent derivatives. An example is bromo-idarubicin, an analogue of idarubicin. Two to five residues of bromo-idarubicin have been coupled to antibody via an ester link, with minimal loss of antibody activity. Furthermore, the conjugation resulted in only a fourfold loss of drug activity compared to a 40-fold loss with iodacetyl adriamycin and the

ICs were more toxic both *in vitro* and *in vivo* in a murine thymoma model (Pietersz et al, 1989).

A new two stage approach called antibody directed enzyme prodrug therapy (ADEPT) involves first targeting an enzyme which has no human analogue. This is followed by administration of the prodrug that is activated to those sites at which the enzyme is distributed by the Mab (Bagshawe, 1989). Preliminary results from Bagshawe's group in London, have demonstrated the localisation of an anti-human chorionic gonadotropin (hCG) - carboxypeptidase G2 in choriocarcinoma xenografts. This was followed by administration of an inert alkylating agent bis-chloromustard that was activated at the tumour site eradicating small tumours (Bagshawe, 1989).

I. 8.2 Pharmacokinetics

Few clinical studies have been performed with ICs and the little that is known of the pharmacokinetics of ICs has come from studies in *in vivo* xenograft models. For example, VDS-anti-CEA (11.285.14) ICs showed a prolonged serum survival and increased tumour localisation of VDS compared with the free drug (Rowland et al, 1986). Similarly, a specific increased uptake of MTX was obtained from MTX-Mab (79IT/36) ICs compared with unconjugated MTX (Pimm, Clegg, Garnett & Baldwin, 1988).

Most biodistribution and pharmacokinetic studies have been performed with radiolabelled Mabs. Few studies with Mab-drug

ICs have been reported due to the difficulty in producing radiolabelled or suitably tagged drug, and, therefore, the difficulty in the measurement of *in vitro* and *in vivo* dissociation of drug from the IC. However, studies performed recently with tritium or carbon-14 labelled MTX (Pimm et al, 1988), doxorubicin (Yang & Reisfeld, 1988) and vindesine (Rowland et al, 1986) clearly demonstrate that antibodies can target drugs to tumours and lead to a higher accumulation of drug in the tumour than elsewhere.

In addition, a systematic approach has recently been reported comparing the pharmacokinetics and tissue distribution of tritium labelled (^{3}H) N-acetyl melphalan, free anti ly-2.1 Mab and the Mab-drug ICs *in vivo* in mice bearing murine thymomas (Pietersz, Krauer, Toohey, Smyth & McKenzie, 1990). The results clearly demonstrated that while the free drug [^{3}H]-N-AcMel was rapidly eliminated from the circulation ($T\frac{1}{2}$ α of 0.5 hrs and $T\frac{1}{2}$ β of 60 hrs), an accumulation of 2-5 times more drug was found in the tumour with the ICs than with the free drug. Indeed, the immunoconjugates were superior in their antitumour activity and a greater therapeutic effect was obtained than with either N-acetyl melphalan or melphalan itself. Interestingly, increasing the dose of IC from 330 μg up to 1650 μg did not increase the percentage of injected

dose/gram tumour, indicating saturation in the tumour (Pietersz et al, 1990).

The distribution of the unconjugated anti ly-2.1 Mab was similar to the IC, while a control anti-CEA antibody conjugate had no selective accumulation in the tumour. Pietersz and colleagues further extended their study to the effect of route of administration of the IC on the localization to tumour and showed greater efficacy of the IC given i.p. compared to the i.v. route. The slower absorption from the i.p. route probably led to the greater accumulation in the tumour of 15% and 20% of injected dose/gram tumour compared to 11% and 9% when given i.v., after 24 hrs and 48 hrs respectively. However, in patients a prolonged i.v. infusion may be given to achieve a steady state level and higher concentration of ICs in the tumour.

These studies indicate that Mab-drug ICs are finally being systematically evaluated in a similar fashion to conventional chemotherapeutic drugs. However, although in most studies up to 20% of the Mab may reach the tumour in experimental animals, in humans this amount falls to as low as 0.01% of the administered dose (Epenetos, Snook, Durbin, Johnson & Papadimitriou, 1986; Pietersz et al, 1989). Therefore, once appropriate conjugates have been produced, the next obstacle is their optimal delivery to the cancer site and penetration, as will be discussed in the following section.

I. 8.3 Physiological barriers to delivery and penetration

Many investigators consider that the major problem with AMT is the delivery of Mabs to the tumour site and further penetration into the tumour. However, it should be reinforced that this is not restricted to delivery of Mabs but is universal to all therapeutic molecules used in cancer (Sands, 1990). While delivery of Mabs may not be a problem for radioimmunodetection or treatment of leukemias, lymphomas and small tumours (micrometastases), heterogeneity of antigen expression alone has failed to explain the poor localisation of Mabs in solid tumours and three physiological barriers have been identified (Jain, 1990):

(a) heterogenous blood supply (poor vascularity), which limits the delivery of blood borne molecules to well-perfused areas of a tumour,

(b) elevated interstitial pressure, which opposes the inward diffusion, and

(c) large transport distances in the interstitium which increases the time required for diffusion of Mabs to reach distal regions of a tumour.

Approaches to overcome these barriers include the use of physical (radiation, heat) and/or chemical (vasoactive) agents to increase tumour blood flow (Jain, 1990). For example, a threefold increase in tumour localisation of Mabs was

demonstrated with combined therapy with β-adrenergic blocking agents like propranolol (Pietersz et al, 1989). Furthermore, tumour necrosis factor (TNF), by virtue of its properties of eliciting a local tumour inflammatory response leading to vasodilatation, increased permeability, leakiness and blood flow, acts synergistically with immunoconjugates and has resulted in the eradication of a number of tumours (Smyth, Pietersz, McKenzie, 1988b).

Another approach is to use smaller molecular weight fragments like $F(ab)_2$ to improve penetration into tumours, but their advantage may be outweighted by their shorter half-life and rapid clearance as demonstrated by several studies (Piertsz et al, 1989; Jain, 1990). However, monovalent antibodies such as bispecific Mabs, due to their reduced avidity, may be able to percolate deeper into tumours and this is one of the potential advantages of such antibodies. Bispecific antibodies are presented in section I.9.2.

I. 8.4 Internalization

Few studies have addressed the mechanism of internalization of antibodies, which plays an important role in the mode of action of Mabs (Matzku, Brocker, Bruggen, Dippold & Tilgen, 1986; Mariani, Kassis & Adelsstein, 1990). Several factors are associated with internalization of Mabs

including the Mab itself (conjugated vs unconjugated) and the dynamics of the antigen (integral membrane protein vs secretory product). In addition, the fate and action of the antigen-antibody complex depends on internalization. This may be ineffective with modulating antigens and may result in shedding of the complex (Matzku, Bruggen, Brocker & Sorg, 1987).

The transferrin receptor is a frequently used model for internalization of Mabs in tumour cells due to its abdundance and well characterised endocytic pathway (Sutherland, Delia, Schneider, Newman, Cornohoad & Creaves, 1981; Taetle & Honeysett, 1989). Other studies are focusing on the uptake of unconjugated antibodies against tumour associated antigens (Tsaltas, Ford and Gallant, 1992) and on the uptake of ICs in hematological and solid malignancies (Press, Farr, Borroz, Anderson & Martin, 1989; Wargalla & Reisfeld, 1989). These studies will be beneficial in the selection of appropriate Mab and target antigen for complete cytotoxicity with AMT.

I. 8.5 Toxicities and human antimouse antibody response

The most frequently questioned and criticised aspect, especially from the clinical point of view, remains the human antimouse antibody (HAMA) response, which limits the repeated administration of Mabs. Ideally, while human Mabs are most desirable, many technical problems remain in their production (Larrick & Bourla, 1986; Borrebäck, 1988). The administration

of nonhuman antibodies could theoretically lead to IgE mediated hypersensitivity reactions or IgG/IgM mediated blocking antibodies and even delayed hypersensitivity reactions. A summary of clinical trials and associated HAMA has been reviewed by Dillman (1990). Acute hypersensitivity reactions have been rare with a frequency of less than 1%. Other adverse effects were minor and included febrile reactions, chills and pruritic skin rashes, seen in 10-15% of patients. These effects correlated with HAMA responses. Although most clinical trials have been preliminary and more than 50% of patients developed HAMA, there have been few reports of renal disease or similar complications associated with immune complex deposition.

Several strategies have been attempted to abrogate HAMA and these include: (1) the use of immunosuppressants, cyclophosphamide, cyclosporine A or azathioprine, of which cyclosporine A seems the most promising (Dillman, 1990). (2) Certain substances such as polyethylene glycol (PEG) (Maiti, Lang & Sehon, 1988) and low weight dextran (Fagnani, Hagan, Bartholomew, 1990) when linked to Mabs induce tolerance and immunosuppression specific to the Mabs. While human studies are yet to be reported, this approach may be the future answer to HAMA and even benefit other disorders including allergies. (3) With recent advances in recombinant DNA technology, chimeric antibodies have been produced (Morrison, 1985;

Morrison & Vernon, 1989). Since the Fc portion is considered the most immunogenic, such recombinant antibodies contain human Fc portions and mouse variable regions. The production of anti-idiotypic antibodies can still be a problem, and chimeric humanized antibodies which are entirely human except for the antigen binding hypervariable regions should further reduce anti-idiotypic response (Verhoeyen & Riechmann, 1988). Such reshaped antibodies have entered the clinic and the preliminary results reveal diminished HAMA and longer circulation times (Lobuglio, Wheeler, Trang, Haynes, Rogers, Harrey, Sun, Ghrayeb & Khazaeli, 1989). Finally, the proof that Mabs are being seriously considered as an alternative therapeutic measure in cancer can be realised from the FDA's (Food and Drug Administration) approval in the document "*Points to consider in the manufacture and testing of monoclonal antibody products for human use*" (Hoffman, 1990).

Some of the problems associated with antibody targeted therapy and potential solutions to overcome these problems are summarised in table 4.

Table 4: Problems and Possible Solutions Associated with AMT

Problem	Potential Solutions
Antigenic modulation	Use bispecific Mabs (univalent antibodies). Choose nonmodulating antigen.
Antigenic heterogeneity	Treat with cocktail of Mabs that react with different antigens.
Lack of *in vivo* expression of antigen	Treat with cytokines that induce antigen expression (IFN, TNF).
Circulating free antigen blocks antibody localization after forming immune complexes	Increase dose of Mab to saturate the blood antigen, so that remaining dose can localize to larger antigen pool in the tumor; plasmapheresis.
Incomplete penetration into tumors	Use vasoactive agents (e.g. Propranolol). Produce capillary leak with IL-2, TNF; Fab fragments; BsMabs.
HAMA	Immunosuppressive drugs; induce tolerance (with PEG, Dextran, etc.); human chimeric Mabs; Fab or Fv fragments.
Non-specific uptake of Mabs by liver and other normal tissues	Choose Mabs that do not cross react with liver; alternatively block hepatic uptake with 'cold' antibody before injecting immunoconjugate.
Bone marrow toxicity from toxin-labelled Mabs	Use in association with autologous bone marrow transplantation.

Adapted and modified from: Larson, 1986; Rosenberg, Longo & Lotze, 1989.

I. 9.0 BISPECIFIC MONOCLONAL ANTIBODIES

I.9.1 History

The idea of using bivalency of antibodies to cross-link two antigenic substances is not new and chemical recombination of univalent Fab fragments of different specificities was attempted in 1961 by Nisonoff and Rivers (1961). The first use of bispecific antibodies prepared from polyclonal rabbit antimouse IgG against anti-ferritin or anti-southern bean mosaic virus (SBMV) antibodies, was as markers for locating cell surface antigens by electron microscopy (Hammerling, Aoki, DeHarven, Boyse & Old, 1968; Hammerling, Aoki,Wood, Old, Boyse & DeHarven, 1969). With the realisation that such antibodies avoided the various problems involved in chemically linking different markers to the antibodies, early attempts were made at coupling two different intact immunoglobulin molecules or half molecules (Nisonoff & Mandy, 1962; Nisonoff & Palmer, 1964; Ghetie & Mota, 1980). However, these early bispecific antibodies were polyclonal, heterogeneous mixtures. With the advent of Mabs it is now feasible to produce bispecific monoclonal antibodies (Milstein & Cuello, 1983).

I. 9.2 Advantages of bispecific monoclonal antibodies (BsMabs)

As was discussed under the immunoconjugate section, a major problem in the production of ICs is the chemical conjugation of highly hydrophobic drugs to hydrophilic antibodies. These procedures can lead to a loss of up to 70% of antibody activity and up to 90% of the drug activity. Particularly relevant to the anthracyclines is that the commonly used conjugation procedures involve chemical linkage of the amino group of doxorubicin and daunorubicin to the antibody which may result in inactivity of the drug (Hurwitz, Ronald, Maron, Wilchke, Arnon, Sela, 1975). In addition, the activity of the ICs varies from batch to batch.

Apart from the advantage that no chemical manipulations are necessary to link the drugs to the BsMabs, these antibodies although structurally bivalent, are functionally monovalent. Monovalency has been shown to prevent antigenic modulation, a mechanism by which tumour cells escape antibody mediated destruction by redistributing the surface antigens, thus shedding the antigen antibody complexes (Cobbold & Waldmann, 1984). An added potential advantage is that the amount of monovalent antibody (BsMabs) bound to the cell is increased, compared to conventional Mabs. This may be critically important if there is a paucity of antigen expression on the tumour cell, leading to delivery of more antibody molecules to the cell. Binding may also be more efficient with BsMabs in

cases of low antigen density, as bivalent Mabs require antigens sufficiently close to each other for the two antibody sites to attach (Milstein & Cuello, 1984; Suresh, Cuello & Milstein, 1986a).

Although the reduced avidity of BsMabs due to possession of only a single antigen binding site may be a disadvantage, conversely, BsMabs could penetrate deeper into the tumour in a similar way to the percolation of Fab fragments. This would be an advantage *in vivo* leading to delivery of more of the cytotoxic agent into the normally inaccessable portions in the centre of the tumour (Vitetta et al, 1987; Jain 1990).

I. 10. PRODUCTION OF BISPECIFIC ANTIBODIES

Different approaches to producing bispecific antibodies are presented below.

I. 10.1 Heteroconjugated antibodies

Two different intact Mabs may be heteroconjugated by chemical means using protein A or the cross linking reagent N-Succinimydyl 3-(2-pyridyldithiol) proprionate (SPDP) (Ghetie & Mota, 1980; Paulus, 1985; Lansdorp, Aalberse, Bos, Schutter, Van Bruggen, 1986). Heteroconjugates of Mabs directed against the T cell receptor and tumour cell antigen have been demonstrated to focus cytotoxicity on to the target cells (Staerz, Kanagawa, Bevan 1985; Staerz & Bevan, 1986).

A further refinement of the above is to recombine monovalent fragments from Mabs after reduction of $F(ab)_2$ in the presence of a specific dithiol complexing agent such as arsenite (which prevents the formation of internal disulphides) and effecting disulphide formation with a thiol activating agent such as 5,5′-dithiobis(2-nitrobenzioc acid) (Brennen, Davison, Paulus, 1985; Paulus, 1985). An example is the production of two bispecific antibodies, both recognising avidin with one arm and either horseradish peroxidase or β-galactosidase with the other. These antibodies act as linkers

for immobilisation of the enzymes on a biotin-substituted matrix in the presence of avidin (Paulus, 1985).

However, a limitation of the above methods is that chemical manipulations are required for chain separation and recombination leading to some protein denaturation, loss of antibody activity and further, considerable waste of Mabs. In addition, molecular and functional homogeneity may not be always assured (Milstein & Cuello, 1983).

I. 10.2 Hybridoma technology: theoretical considerations

Normally, individual antibody producing cells express only one heavy and one light chain allele by a phenomenon called allelic exclusion. This is facilitated by correct rearrangement of the variable and constant DNA segments in only one of the two alleles (Reth, Ammirati, Jackson, Alt, 1985; Alt, Blackwell, Yancopoulos, 1987). However, when two such committed B cells are fused, the derived hybrid codominantly expresses both parental heavy and light chain genes. The four chains are then free to recombine in the cysternal space, resulting in the formation of both parental and hybrid immunoglobulins (Milstein & Cuello, 1984; Suresh et al, 1986b).

Since, theoretically, any light chain can associate with any of the heavy chains, the main molecular species resulting from such combinations are as follows. Type 1: Total random

association of the two heavy (H_1 & H_2) and two light chains (L_1 & L_2) results in ten different species. However, only *cis* associations (HL pairs derived from the genes of a single parent) can form functional Fab arms. Type 2: Random heavy chain association with fully restricted chain association, results in preferential parental Mabs or BsMab formation. Type 3: Random heavy chain association with partly restricted light chain assembly, resulting in one functional arm (Suresh et al, 1986b; Milstein & cuello, 1984; Songsivilai & Lachman, 1990).

In reality, the intracellular assembly of chains shows a preferential association of homologous vs heterologous pairs. In addition, depending on the differential rate of chain synthesis, up to 30% to 50% of the secreted immunoglobulins may be the desired BsMabs (Milstein & Cuello, 1984; Suresh et al, 1986a & b). This has been demonstrated with a antisomatostatin-antiperoxidase hybridoma. The crude antibody mixture separated into three peaks following ion exchange chromatography. Peaks 1 and 3 on SDS-polyacrylamide gel electrophoresis analysis indicated restricted homologous light and heavy chain association resulting in parental antibodies. Peak 2 consisted mainly of BsMabs. The activities were confirmed by immunoassays. Peak 1 had antisomatostatin activity and composition similar to the parental antisomatostatin IgG with a single light chain band. While

peak 3 demonstrated antiperoxidase activity, containing parental heavy chains, the composition also revealed both light chains, suggesting a type 3 chain association mentioned earlier. As the authors suggest, this problem may be overcome by screening for a more suitable clone yielding a Type 2 pattern of chain association.

Theoretically, if there is a random association of heavy chains, then upto 50% of yield could be of BsMabs formation, with the ratio of the three immunoglobulin peaks being 1:2:1 (Milstein & Cuello, 1984; Suresh et al, 1986a). As evidence, a cellulose acetate electrophoretic pattern of ascites of one of the hybridomas demonstrated a higher intensity of the middle band (Suresh et al, 1986b).

I. 10.3 Production of hybrid-hybridomas

Two general methods are described below.

I.10.3 (a) Hybridoma x spleen cell fusions

The principle involves fusion of a hypoxanthine/ aminopterin/thymidine (HAT) sensitive hybridoma (secreting monoclonal antibodies) with spleen cells from animals immunized with the second antigen of choice. The hybridoma is made HAT sensitive by growing in increasing concentrations of the purine analogue, 8-azaguanine. The enzyme hypoxanthine-guanosyl-phosphoribosyl-transferase (HGPRT) catalyses the incorporation of 8-azaguanine into DNA, which interferes with normal protein

synthesis and the cells die (Hudson & Hay, 1980). Resistant cells are obtained by mutation or loss of the HGPRT gene. Such HGPRT deficient cells when placed in hypoxanthine, aminopterin and thymidine (HAT) medium are unable to utilise hypoxanthine to synthesise purines. The alternative pathway of DNA synthesis is *de novo* synthesis. However, aminopterin is an analogue of folic acid and binds folic acid reductase, thus inhibiting the *de novo* synthesis of DNA (Littlefield, 1964). 8-azaguanine resistant (HGPRT deficient) hybridomas thus die in the HAT selection medium. These cells, are fused with normal lymphoid cells and the resulting hybrid-hybrids survive HAT, as the lymphoid cells provide the HGPRT and the parental hybrid provides the immortality.

I. 10.3 (b) Hybridoma x Hybridoma fusions

The principle involves fusion of two established and well characterised hybridomas by (a) a chemical inactivation method or (b) a combination of the chemical inactivation method and HAT selection.

The method of chemical inactivation involves the use of two distinct site specific irreversible inhibitors of macromolecular biosynthesis (Suresh et al, 1986b), thereby inhibiting independent metabolic pathways of the two cell lines. Fused cells survive by complementing each other. An example is the fusion between an antiperoxidase YP4 hybridoma with antisubstance P NCI/34 hybridoma. The NCI/34 was made

sensitive to emetine (an inhibitor of protein synthesis blocking the translocation step) and the YP4 was made sensitive to actinomycin D (an inhibitor of RNA synthesis). When grown in medium supplemented by critical concentrations of these two drugs, the fused cells survive by complementing each other (Suresh et al, 1986b).

I. 10.4 Recombinant DNA technology.

Chimeric BsMabs have been produced by transfecting immunoglobulin genes into myeloma cells (Songsivilai, Clissold & Lachmann, 1989; Songsivilai & Lachmann, 1990). The limitation of this approach, at present, is the availability of cloned immunoglobulin genes of the desired specificities.

I. 11.0 OBJECTIVES

The objective of this project was to produce novel BsMabs that simultaneously recognize the target carcinoembryonic antigen (CEA) and the chemotherapeutic drug doxorubicin (adriamycin).

I. 12.0 Why CEA?

In order to evaluate the role of BsMabs in cancer treatment, the appropriate choice of target was crucial and we required: (a) a target present on major solid tumours for which improvement in therapy was urgently needed; (b) one whose structure was familiar, (c) one that could be assayed in the laboratory, (d) one to which Mabs were available, and (e) a proven track record in targeting.

Amongst the TAAs available, CEA stands out as the most promising and its properties are detailed below.

I. 12.1 History and clinical relevance

CEA is an oncofetal antigen normally found in embyronic and fetal gut and was first discovered to be expressed on colorectal cancers by Gold and Freedman in 1965. CEA is the most well studied and widely known tumour associated marker, and is found to be elevated in >65% of colorectal (up to 100% in metastatic disease), >50% of lung, >60% of pancreas, >30% of ovary and breast cancers, i.e. most of the common solid

tumours with the highest mortality rates (Bates & Longo, 1987). Monitoring CEA levels is an important parameter in assessing the response to therapy and in the post-operative surveillance of cancer patients (Bates & Longo, 1987).

I. 12.2 Molecular and genetic organization

CEA is a glycoprotein of molecular weight 180,000. The antigenic structure of CEA has been dissected into its various epitopes using over 52 well characterized Mabs (Price 1988; Hammarstrom et al, 1989).

The CEA gene family, its molecular structure, evolution and functional significance have been elucidated and extensively reviewed (Rogers, 1983; Shively & Beatty, 1985; Thompson & Zimmermann, 1988).

I. 12.3 Evolution and functional significance

Despite CEA being the oldest tumour marker studied, its function remained a mystery to investigators until recenlty, when its role as an intracellular adhesion molecule was suggested (Benchimol, Fuks, Jothy, Beauchemin, Shirota & Stanners, 1989). CEA has therefore joined the immunoglobulin (Ig) superfamily, a group of molecules with a common Ig domain like structure which are involved in basic cell surface recognition events (Williams, 1987). Not surprisingly, increased homotypic intercellular adhesion has been

demonstrated to favour the metastatic process, as cell aggregates that break away from the primary tumour may have a greater chance than single cells in surviving the circulation and lodging in secondary sites. A similar metastatic role has been postulated for CEA, based on the clinical data that up to 100% of metastatic colorectal cancers have elevated CEA levels and, in addition, colonic liver secondaries produce high levels of CEA mRNA and protein (Benchimol et al, 1989). Additional evidence is the apical localisation of CEA in the normal colonic epithelium versus the basolateral localisation in embryonic and cancerous tissue, leading to disruption of normal architecture and invasion. This hypothesis is currently under investigation.

I. 12.4 CEA as a model for AMT

CEA has proven to be a promising target for both antibody mediated diagnosis and treatment. Initially, the efficacy of a Pab sheep anti-CEA-vindesine IC was demonstrated *in vitro*, with retention of drug and Ab activity and carrier specificity (Johnson, Ford, Newman, Woodhouse, Rowland & Simmonds, 1981; Rowland, Simmonds, Corvalan, Marsden, Johnson, Woodhouse, Ford & Newman, 1982). Subsequently, an ^{131}I labelled IC could be radioimmunolocalised (RIL) in patients with advanced metastatic adenocarcinomas (Ford, Newman, Johnson, Woodhouse, Reeder,

Rowland & Simmonds, 1983). In addition, improvements in RIL have proven the technique to be safe and to detect CEA expressing primary and metastatic lesions in patients with colorectal or breast cancer using ^{123}I, ^{99m}Tc or ^{111}In labelled Mabs or Fabs (Beatty, Duda, Williams, Sheibani, Paxton, Beatty, Philben, Werner, Shively, Vlahos, Kokal, Riihimaki, Terz & Wagman, 1986; Goldenberg, Goldenberg, Sharkey, Higginbotham, Ford, Lee, Swayne, Burger, Tsai, Horowitz, Hall, Pinsky & Hansen, 1990).

For the better evaluation of ICs both *in vitro,* for selection of binding and cytotoxicity properties, and *in vivo* for therapeutic potential a model has been established in our laboratory as follows. Human cell lines of colorectal, lung, cervical and breast cancer origin have been characterised by immunocytochemical, ·adiolabelled Ab binding and competitive inhibition studies with four Mabs recognising different CEA epitopes (Ford et al, 1985, 1986, 1987a). One of the Mabs (11-285-14) specific for CEA, was confirmed to be non-reactive with nonspecific cross reacting antigens (NCA) by further studies comparing fifteen anti-CEA Mabs (Price, 1988). NCA is a cytoplasmic component of granulocytes which infiltrate into inflamed parts of the colon. Although CEA and NCA have similar polypeptide chains, they differ in their degree of glycosylation and are also antigenically distinct (Shively &

Beatty, 1985). Apart from specificity for CEA, 11-285-14 has also been extensively characterised immunocytochemically (Gatter et al, 1982; Hockey, Stokes, Thompson, Woodhouse, Macdonald, Fielding & Ford, 1984; Ford, Gallant & Ali, 1985b). 11-285-14 has been shown to localise in xenografts (MacDonald, Crowson, Allum, Life & Fielding, 1986) and in patients with gastrointestinal cancer (Allum, Macdonald, Anderson & Fielding, 1986), and was therefore selected for targeting cytotoxic drugs. 11-285-14-Vindesine (VDS) conjugates demonstrated (a) efficacy *in vitro*, (b) correlation with CEA density and (c) efficacy *in vivo* over the free drug with xenografts using a variety of dosage schedules (Casson et al, 1987). Carrying this success a step further, 11-285-14-Dox conjugates have demonstrated considerable efficacy (Richardson et al, 1989) and are currently being investigated.

In summary, the rationale for selecting CEA is:

(a) There is an increased expression of CEA on the cell membrane and in the cytoplasm of cancer cells. These cancers represent the common solid tumour group including colonic, lung and breast cancers, that are presently refractory to therapy.

(b) CEA is the most well studied and extensively characterised of the TAAs. In addition, its expression from the gene to the cellular level has been well elucidated.

(c) Our group and others have a range of anti-CEA Mabs which have proven the value of CEA in histopathology (Corvalan, Axton, Brandon, Smith & Woodhouse, 1984; Hockey et al, 1984; Ford et al, 1985b; MacDonald et al, 1986; Price et al, 1988). Furthermore, CEA has been demonstrated to be bound and internalized by antigen expressing cells in culture (Rosenthal, Tompkins & Rawls, 1980: Tsaltas et al, 1992).

(d) Our Mabs have been shown to localise *in vivo* in colorectal xenografts (MacDonald et al, 1986), in patients with GI malignancies (Allum et al, 1986), and to be non-reactive with cross reacting antigens (Corvalan et al, 1984).

(e) A CEA model has been developed and the efficacy of anti-CEA 11-285-14-VDS (Ford et al, 1987a) and Dox (Richardson et al, 1989) have been demonstrated both *in vitro* and *in vivo* in a nude mouse xenograft system (Casson, Ford, Marsden, Gallant & Bartlett, 1987).

(f) Furthermore, producing BsMabs recognising both CEA and Dox would be complementary to the ongoing work in this laboratory using 11-285-14-Dox ICs and would contribute to the evaluation of the potential of BsMabs in comparison with conventional ICs.

No other tumour associated antigen meets these criteria.

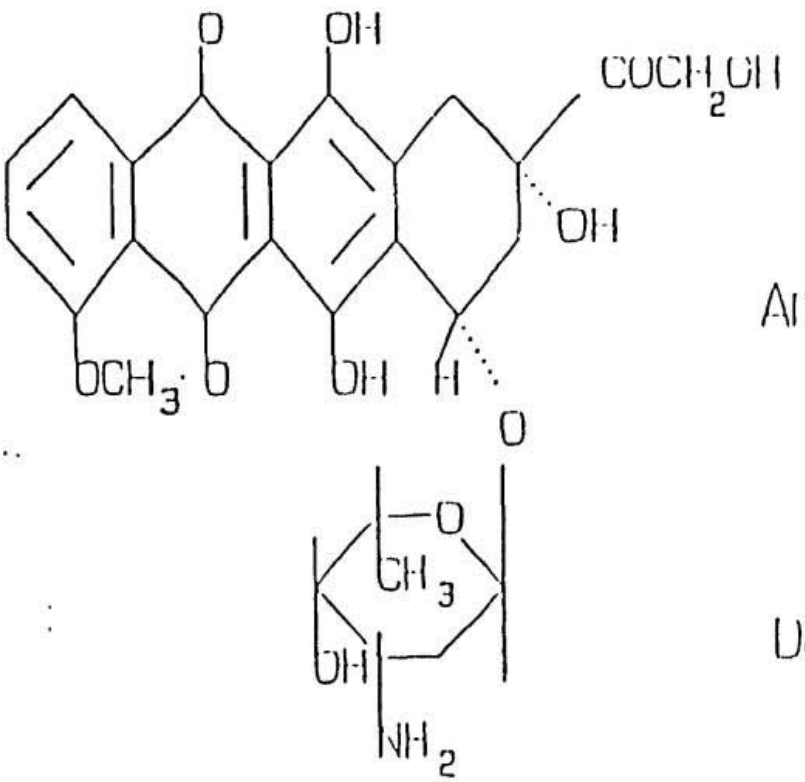
I. 13.0 Why Doxorubicin?

Doxorubicin (Dox) is an anthracycline antineoplastic agent originally isolated from the fungus *Streptomyces peucitius* and is perhaps the most widely used chemotherapeutic drug worldwide (Weiss, Sarosy, Clagett-Carr, Russo & Leyland-Jones, 1986). Dox (Adriamycin) has an extraordinarily broad spectrum of antitumour activity against many human cancers, in particular the solid tumours such as breast, lung, ovary, thyroid and soft tissue sarcomas.

I. 13.1 Structure and mechanism of action

The Dox molecule contains an amino sugar, daunosomine, linked through a glycosidic bond to a naphthacene-quinone nucleus (Figure 2). Although Dox differs from the other commonly used anthracycline, daunorubicin (DNR), by only a single hydroxyl group on carbon 14, it is much more potent than DNR. The three mechanisms of antitumour effect of Dox (Young, Ozols & Myers, 1981) are: (a) by DNA intercalation, thereby inhibiting DNA and RNA synthesis, (b) free radical formation (responsible more for cardiotoxicity than antitumour effect; Myers, 1988), and (c) Dox has been reported to have a cytotoxic effect by acting directly on the cell membrane (Tokes, Rogers & Rembaum, 1982; Tritton & Yee, 1982) without having to enter the cells.

Figure 2. Structure of Doxorubicin



Anthracycline

Daunosamine

I. 13.2 Potential in targeting

Despite its efficacy, Dox has been hampered by both conventional (bone marrow suppression, nausea and vomiting, and alopecia) and unique (cardiomyopathy) toxicities (Young et al, 1981). Furthermore, although many CEA expressing cell lines are sensitive to Dox, a limitation of Dox in colorectal cancers, may be its poor penetration beyond the outer 4-6 layers, which could be overcome by Mab-mediated targeting. Supporting this, preclinical evaluation of ICs of Dox linked via a cis-aconityl spacer to a Mab (9.2.27) recognising a melanoma antigen, have been shown *in vivo* to be more effective than the free drug (Yang & Reisfeld, 1988).

In summary, the rationale for producing BsMabs against Dox includes:

(1) Widespread application

(2) The potential of delivering more drug to the cancer site and less to the cardiac tissue, thus limiting cardiotoxicity.

(3) BsMabs may also be used to target more potent analogues of Dox under evaluation, such as cyanomorphilino-doxorubicin, which is up to 1000 times more potent than Dox (Beckman, McFall, Sikik & Smith, 1988), thereby increasing BsMab efficacy.

(4) In addition, although the feasibility of using Dox-ICs has been shown in a phase I trial involving forty-two

patients with refractory solid tumours, the major technical obstacle continues to be the effective chemical linkage of drug to Mabs (Pietersz et al, 1989). BsMabs would, therefore, be a novel way to overcome this problem.

I. 14.0. OUTLINE OF EXPERIMENTAL PROTOCOL

The steps involved in the production of BsMabs using the spleen cell x hybrid fusion method (Suresh et. al., 1986b) are outlined in Figure 3.

(1) Backselection of 11-285-14 in 8-azaguanine to produce suitable clones based on (a) HAT sensitivity; (b) growth characteristics; and, (c) continued anti-CEA production.

(2) Production of Dox-protein carrier conjugates.

(3) Immunization of mice with (2) and use of spleens for fusions with mutant 11-285-14.

(4) Fusions using hybridoma technology.

(5) Development of assays to detect (a) anti-CEA; (b) anti-Dox; and, (c) BsMabs.

(6, 7, 8) Selection of dual positive hybrids and subcloning.

(9) Expansion in culture.

Figure 3. Experimental Outline
(1) 11-288-14
Back select in 8-aza
HAT-sensitive hybrids
(4)
Spleen cells
Fusion
HAT selection (4)
Development of Assays (5)
Selection for dual specificity (6)
Cloning (7) (8)
Expansion (9).

CHAPTER II

MATERIALS AND METHODS

II. 1.0 11-285-14 ANTI-CEA HYBRIDOMA

The maintenance cell culture techniques for 11-285-14 are given in the following subsections. The techniques are similar for all cell lines used in this project. Differences exist in the media used and are dealt with under the appropriate sections.

II. 1.1 History of 11-285-14

11-285-14, a hybridoma secreting monoclonal anti-CEA antibody, was provided by the Oncology Research Laboratory and was produced by Ford and Woodhouse (Woodhouse, 1982a) in collaboration with Corvalan et al, of Eli Lilly & Co., (Corvalan et al, 1984). 11-285-14 resulted from the fusion of the NS-1 myeloma cell line and spleen cells from a mouse immunized with CEA. The 11-285-14 fusion protocol, described in the Ph.D. thesis of C. Woodhouse, has been the guideline for the fusions in this thesis. 11-285-14 is an IgG_1 Mab and has been extensively characterised and evaluated for *in vitro* and *in vivo* targeting as described in the introductory chapter (Section I. 12.0).

II. 1.2 Tissue culture maintenance

11-285-14 was grown as a suspension culture in 75 cm^2 polypropylene tissue culture flasks (Falcon, Becton-Dickinson) and maintained at 37° C with a 5% carbon dioxide atmosphere in a humidified incubator.

II. 1.3 Medium for 11-285-14 (RPMI-GLN-FCS)

(1) RPMI-1640 500 ml (#15-040-LV, Cellgro, Mediatech, Washington)

(2) Fetal calf serum (FCS) 50 ml (#29-161-54, Silver, Cellect Flow Labs Inc, Mclean Virginia, 22102)

(3) L-Glutamine (GLN) 6 ml (#25-005-L1, Cellgro, Mediatech)

(4) Penicillin (10,000 units/ml) Streptomycin (10,000 µg/ml) in 12 ml (#30-001-L1, Cellgro, Mediatech)

II. 1.4 Cell growth

Cells were grown as suspension culture and fresh medium was topped up daily to maintain optimal growth and viability

II. 1.5 Cell count

A Neubauer Hemocytometer was used for calculating the concentration of cells in suspension. The viability of cells was assessed by using the dyes acridine orange (AO) and

ethidium bromide (EB). Acridine orange stains viable cells brilliant green and ethidium bromide stains dead cells brown, when viewed under a fluorescence microscope.

II.1.5 (a) Materials

(1) Hemocytometer (Neubauer) with cover slip.

(2) Acridine orange, AO (#A-6014, Sigma Chemical Co, St. Louis, Missouri).

(3) Ethidium Bromide, EB (#E-8751, Sigma Chemical Co). AO and EB used as a 0.001% solution and mixed together, stored in 1 ml aliquots at -20°C.

(4) Fluorescent microscope (Ortholux II, Lietz), with a 50 watt mercury vapour lamp.

II.1.5 (b) Method

Cells from the culture flask were transferred to 15 ml or 50 ml sterile conical tubes and centrifuged at 1000 RPM (175 x g). The supernatant was discarded and the pellet of cells in the bottom resuspended in the appropriate dilution of medium (usually 5 to 10 ml). One drop of the cell suspension was added to one drop of AO/EB and the mixture placed under the coverslip of a hemocytometer. Viable and non-viable cells were counted under the fluorescent microscope and the percentage of viable cells calculated.

% Viability = (Total AO cells/Total cell count) x 100%

II. 1.6 Cryopreservation of cells

II. 1.6 (a) Materials

Media for freezing cells:

10% (1:10) DMSO Dimethyl Sulphoxide (#10323, BDH Chemicals, Toronto) with Fetal calf serum (FCS), was filtered through a 0.22 μm millipore filter (Millipore Products Division, Bedford MA 01730) and stored in a Revco freezer (-70°C) in 5 to 10 ml aliquots.

II.1.6 (b) Method

(1) The number of cells to be frozen was counted.

(2) The cell suspension was centrifuged at 1000 rpm (175 x g) for five minutes and the supernatant discarded. The pellet was shaken to mix well.

(3) Depending on the number of cells, the appropriate quantity of cold medium for freezing (kept in a bucket of ice) was added to the pellet of cells. 1 ml of the medium was used to freeze 4 to 6 x 10^6 cells.

(4) 1 ml of the cell suspension was aliquoted into each vial appropriately marked.

(5) The vials were transported in a bucket of ice to the Revco, and transferred to the -70°C.

(6) A day later, usually, the vials were submerged in a liquid nitrogen tank.

II.1.7 Thawing cells

(1) Using a sterile syringe, 9 ml of RPMI-GLN-FCS medium was placed in a 15 ml centrifuge tube.

(2) The vial of cells was removed from liquid nitrogen and thawed quickly in a 37°C waterbath.

(3) With a few frozen cells remaining in the vial, the vial was transported in ice to the sterile hood.

(4) The cells were removed with a syringe and added to the tube containing medium.

(5) This was immediately centrifuged at 1000 rpm (175 x g) for 5 min.

(6) The supernatant was discarded.

(7) Using a syringe, 5 ml fresh medium was added and the suspension poured into a sterile 50 ml flask reserving a little for the cell count.

(8) After performing the cell count, the percentage yield was calculated.

II. 2.0 SELECTION OF HYBRIDOMA FUSION PARTNER

II. 2.1 Production of HAT Sensitive 11-285-14

Azaguanine-resistant Hybrioma lines were propagated as follows to obtain HAT sensitive 11-285-14 cell lines. The procedure followed is adapted from Suresh et al, (1986b).

II. 2.1 (a) Materials

(1) 8-azaguanine (#A-8526, Sigma Chemical Co.), 300 mg.

(2) Distilled water

(3) 1 N Sodium hydroxide

(4) 10 N Sodium hydroxide

(5) RPMI-GLN-FCS (Section II. 1.3)

II. 2.1 (b) Method

(1) 20 mM stock of 8-azaguanine was prepared by dissolving 300 mg 8-azaguanine in 99 ml of distilled water. Since 1 ml of 1 N NaOH did not result in the 8-azaguanine dissolving, (as per Suresh et al 1986b method) 8-10 drops of 10 N NaOH was used instead of 1 N NaOH.

(2) The stock was filter sterilized and stored in aliquots at -20°C.

(3) Serial dilutions of 8-azaguanine in doubling dilutions of 30, 15, 7.5, 3.75, 1.88, 0.94 µg/ml were prepared in 10% RPM1-GLN-FCS and equilibrated in a 5% CO_2 incubator.

(4) 24 subconfluent wells (6 columns x 4 rows) of vigorously growing hybidoma cells were prepared.

(5) The various azaguanine concentrations were added in triplicate to the subconfluent hybridoma plate, with the last row being kept as a control.

(6) The medium was drained and cells removed every two days if excess growth was seen.

(7) Cultures growing at the highest drug concentration were selected and the above procedure repeated with these cells.

(8) Cells that appeared to have adapted to the 30 ug/ml drug level, were then cloned by limiting dilution (section II 9.7) in RPM1-GLN-FCS containing 30 mg/ml azaguanine.

II. 2.2 Growth characteristics of 11-285-14

II. 2.2 (a) Materials

50 ml sterile tissue culture flasks

RPMI-GLN-FCS with 30 ug/ml 8-azaguanine.

II. 2.2 (b) Method

5 to 10 cells/ml of rapidly growing clones were picked as below:

(1) 11-285-14 mutant clones obtained by limiting dilution were aliquoted into five flasks with a fixed

concentration of cells i.e. 0.05 x 10^6 cells in 10 ml medium and the number of viable cells counted on each day.

(2) The supernatants were assayed for anti-CEA production by enzyme linked immunosorbent assay (ELISA) (section II 8.3).

II. 2.3 Maintenance of NS-1 fusion partner

The NS-1 plasmacytoma cell line is a common fusion partner used in hybridoma production (Hudson and Hay, 1980), since it is a non-secretor of immunoglobulins.

II. 2.3 (a) Materials

(1) NS-1 (Surgical Immunology Unit, Birmingham, England).

(2) Medium: RPM1-GLN-FCS (Section II 1.3)

1250 μl of 1 x 10^{-2} of 6 thioguanine (6-TG) (Sigma Chemical Co)

0.167 gm of 6-thioguanine was dissolved with few drops of 10 M NaOH. The solution was adjusted to 100 ml with distilled water and filter sterilised through a 0.22μm filter. The final 6-TG concentration was 2 x 10^{-5} M.

10 ml of 4.5 g/litre glucose (# G-5000 Sigma Chemical Co).

(3) Sterile 75 cm^2 polypropylene flasks (Falcon, Becton-Dickinson).

(4) Incubator at 37°C, humidified and gassed with 5% CO_2.

II. 2.3 (b) Method

(1) The maintenance of the NS-1 cell line was similar to the maintenance of 11-285-14 (Section II.1.0).

(2) Cells for fusion were used during the exponential phase of growth.

II. 3.0 PRODUCTION OF DOX-KLH CONJUGATES

II. 3.1 Introduction

Doxorubicin was initially conjugated to keyhole limpet hemocyanin (KLH) using ECDI [1-ethyl-3-(dimethylaminopropyl) carbodiimide] as the heterobifunctional crosslinker. ECDI links the amino group of doxorubicin to the carboxyl group of hemocyanin forming an amide bond (Vunakis, Langone, Riceberg & Levine, 1974; Hurwitz, Levy, Maron, Wilchek, Arnon & Sela, 1975). The other heterobifunctional linkers that are routinely used to link haptens and proteins are glutaraldehyde and periodate.

II. 3.1 (a) Materials

(1) Doxorubicin Hydrochloride (Adriamycin HCl; Adria Laboratories Inc, Columbus, Ohio)

(2) ECDI (Sigma Chemical Co.)

(3) Keyhole Limpet Hemocyanin (H-2133, Sigma Chemical Co.)

(4) Phosphate Buffered Saline (PBS) - 7.2g NaCl (#ACS783, BDH Chemicals, Toronto),

(5) 14.3g Na_2HPO_4 anhydrous (#5274B-500, Fisher Scientific, Ontario),

(6) 4.3 g of KH_2PO_4 (Fisher Scientific, #P-382) dissolved in 10 litres of distilled water, pH 7.2.

(7) Phosphate buffered saline tablets (Oxoid, Unipath Ltd., England).

(8) Sephadex G-25 (#CD00470, Pharmacia Fine Chemicals, Upsala, Sweden).

(9) Gel filtration column (BioRad Econo column Length 30 cms). The preparation of the gel filtration collumn is described in the next section (II. 3.2).

II. 3.1 (b) Method:

Modification of method from Vunakis *et al.* (1974).

(1) 5 to 10 mg of Dox was dissolved in 2 ml PBS (made from tablets). As 4/5 of the weight is due to lactose, the dry weight of the drug ranged from 25 to 50 mg. This was found to be the optimal weight of the drug that resulted in a conjugate.

(2) 15 mg of KLH was dissolved in 2 ml PBS (made from tablets, Oxoid).

(3) The above two solutions were kept separately at room temperature (RT) for half an hour to dissolve.

(4) After being centrifuged separately (175 x g), the supernatants were removed and the undissolved pellets were discarded.

(5) (a) The two supernatants were mixed together.

(b) The absorbance of the supernatants was assessed spectrophotometrically at 280 nm (for KLH and Dox) and 495

nm (Dox alone). Since only 1 to 2 ml of each supernatant was present, a 1/100 dilution was made and the absorbance read. This resulted in a more precise estimate of the amount of the drug and KLH dissolved prior to each conjugation.

(6) 10 to 15 mg of ECDI was dissolved in 1 ml PBS and added to the supernatant mixture.

(7) During initial conjugation experiments, a variety of pH ranges were evaluated, but pH 7.2 yielded the best results (Tables 9a, b & c under Results).

(8) The solution containing Dox, KLH and ECDI was kept at room temperature (RT) for 4 hours with occasional stirring (every half hour).

(9) The solution was passed down a Sephadex G-25 gel filtration column (see section below) and collected at the rate of 5 minutes for each fraction, eluted with PBS. This was set up in the cold room at a temperature of 4°C.

(10) Approximately 50 fractions were collected per conjugation and the fractions were read by spectrophotometry at 280 nm and 495 nm.

(11) The molar ratios were calculated based on the following information. Doxorubicin (Adria Laboratories): Molecular weight 580, Molar extinction coefficient = $\frac{OD_{495}}{OD_{280}} = \frac{13,000}{8,000}$

KLH (Sigma Chemicals): Molecular weight (mol. wt.)range 9 to 15 x 10^6. For calculating the molar ratio, mol. wt. of 10 x 10^6 was used. Molar extinction coefficient of KLH 1% solution (1 g/100 ml) = 16 at $OD_{280\,nm}$.

II. 3.2 Gel Filtration

II. 3.2 (a) Materials

(1) G-25 Sephadex gel superfine medium (# 75104 Pharmacia Fine Chemicals, Uppsala, Sweden).

(2) Gel filtration column (BioRad Econocolumn, 30 cm).

(3) Fraction collector with glass test tubes. (Pharmacia programmable fraction collector FAPC-30U).

(4) Phosphate buffered saline (PBS)

II.3.2.(b) Method

(1) The volume of the column to be used was measured.

(2) The amount of Sephadex G-25 to be used was calculated as 1 g of Sephadex for 5 ml volume. The total column volume was 80 ml, therefore approximately 16 to 18 g of Sephadex G-25 was used.

(3) The gel was boiled in an excess of PBS for 1 hour in a water bath. This gel was then poured into the column and the column placed in cold room at a temperature of 4°C.

II. 4.0 PRODUCTION OF DOX-BSA CONJUGATES

Dox was linked to bovine serum albumin (BSA) in an identical procedure as for Dox-KLH conjugates, except that a higherer concentration of carrier (50 to 80 mg of BSA) was used. Dox-BSA was separated form the free drug by gel filtration, as described above for Dox-KLH conjugation.

II. 5.0 PRODUCTION OF DOX-ENZYME CONJUGATES

In order to develop a dual assay that detects BsMabs directly, several attempts were made to link Dox to the enzymes horseradish peroxidase (HRP) or β-galactosidase.

II. 5.1 Dox-HRP conjugations

II. 5.1 (a) Materials

(1) Horseradish peroxidase (HRP: #P8375 type VI and #P8125 type I Sigma Chemical Co. Missouri).
has a molecular weight of 40,000 and is available in six types (I to VI) with a RZ of 1.1 to 3 (RZ: Reinzethal ratio is the optical density ratio at 280 nm:495nm). The RZ does not correlate with the activity of the enzyme and represents the carbohydrate moiety (Sigma Chemical Co).

(2) Glycerol (Sigma Chemical Co).

(3) Sodium peridoate, $NaIO_4$ (#1867070, Analar, BDH Chemicals Ltd., Poole, England).

(4) Sodium carbonate, Na_2CO_3 (#AC8290, Anachemia Ltd. LTEE, Montreal).

(5) Sodium bicarbonate, $NaHCO_3$ (#S233, Fisher Scientific Co, New Jersey).

(6) Sodium borohydrate, $NaBH_4$ (#3-V023, JT Baker Chemical Co., Phillipsburg, NJ).

(7) Ethylenediamine Tetraacetic acid Disodium salt, EDTA (S-311, Fisher Scientific Co.).

(8) Glutaraldehyde (G-5882, Sigma Chemical Co.)

(9) 2,4,Dichloromethane CH_2Cl_2 (Aldrich Chemicals).

(10) Dimethyl Sulphoxide (DMSO) (BDH Chemicals).

(11) 1-ethyl-3-(dimethylaminopropyl) carbodiimide (ECDI) (Sigma Chemical Co.)

II. 5.1 (b) Method

Several methods were evaluated to conjugate Dox to HRP as given in the following subsections:

II. 5.1 (c) Periodate conjugation

Periodate oxidation of the drug cleaves the bond between C-3 and C-4 of the amino sugar, producing carbonyl groups capable of reacting with free amino groups on the protein. The resulting Schiff base linkages were reduced with

sodium borohydride ($NaBH_4$) (Nakane & Kawaoi, 1974; Hurwitz et al, 1975; Boorsma, 1983; Varga, 1985).

(1) 10 mg of Dox was dissolved in 1 ml PBS, and mixed with 0.1M $NaIO_4$ (42.8 mg in 1 ml PBS), followed by incubation for 1 hour at room temperature in the dark.

(2) 536.8 μl of glycerol (1 M) was then added to consume the excess periodate.

(3) The resulting solution of oxidised drug was mixed with 1 ml of sodium carbonate bicarbonate buffer (0.2 M, pH 9.5) containing 4 mg of HRP, followed by incubation at RT for one hour.

(4) 3 mg of $NaBH_4$ was added to give a final concentration of 0.3 mg/ml, and the mixture was kept at 4°C for 2 hours.

Since the above method was unsuccessful, the procedure was slightly modified using 0.5 M $NaIO_4$, 268 μl of 1 M glycerol, 2 mg HRP in 1 ml carbonate and the resulting 5 ml of solution was separated by gel filtration (Sephadex G-25).

II. 5.1 (d) Carbodiimide conjugation

ECDI was used to link the drug via its amino group to the carboxyl group of the HRP similar to Dox-KLH production (Hurwitz et al, 1975; Vunakis et al, 1974; Goodfriend, Levine & Fasman, 1964).

(1) 5 mg of Dox in 1 ml PBS was mixed with 3 mg of HRP (RZ 0.6) or 17 mg HRP (RZ 1.1 type I) in 1 ml PBS.

(2) 8 mg of ECDI dissolved in 1 ml PBS was added to the above mixture and kept at RT for 4 hrs in the dark.

(3) Since Dox can produce superoxide radicals in solution which could periodate the enzyme resulting in self coupling (Brian Hasinoff, Dept. of Chemistry, Memorial University of Newfoundland, personal communication), sodium cyanide (1 μmol) or flouride (1 mmol) was added in an attempt to prevent such an unwanted reaction.

(4) As the presence of metallic ions also may hinder the conjugation, 1 mmol of EDTA was used to prevent their interaction in the reaction.

(5) The enzyme fractions were dialysed against PBS to remove the free fluoride.

II. 5.1 (e) Glutaraldehyde conjugation I

Glutaraldehyde cross links the amino groups of Dox and the enzymes (Vunakis et al, 1974; Hurwitz et al, 1975). 200 μl of glutaraldehyde (0.1%) was added to 1 ml of PBS containing 6 mg

of HRP and 2 mg Dox in 1 ml solution. The reaction was allowed to proceed at RT for 4 hours.

II. 5.1 (f) Glutaraldehyde conjugation II

Since the procedure used by Pagé & Thibeault (1987) was reportedly successful in linking Dox to proteins without significant loss of drug or antibody activity, attempts were made to link Dox with HRP using this method as follows.

(1) 150 μl of 25% aqueous glutaraldehyde was added to 0.5 mg/ml of Dox in 4 ml PBS and the mixture stirred at RT for 20 min.

(2) 2 ml of distilled water was then added and the excess glutaraldehyde was extracted with dichloromethane using a separating funnel.

(3) The activated Dox was washed with 5% $NaHCO_3$, dried with Na_2SO_4 and evaporated to dryness under nitrogen.

(4) The mixture was dissolved in 1 ml of DMSO and the amount of drug measured spectrophotometrically at 495 nm.

(5) HRP dissolved in PBS was then added to yield a 13:1 ratio of Dox:HRP (type I and type VI HRP were utilised).

II. 5.2 Dox-β galactosidase conjugations

II. 5.2 (a) Materials

(1) β-galactosidase (#G-6008, EC 3.2.1.23, Sigma Chemical Co), molecular weight 540,000.

(2) M-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS; Sigma Chemical Co).

(3) Tetrahydrofuran (#ET01316HP, Aldrich Chemical Co., Milwaukee, Wisconsin).

(4) 0.05 M Tris buffer: 7.9 g of Trizma base (#T-1503, Sigma Chemical Co) in 800 ml distilled water. Concentrated HCl was used to adjust the pH to 7.6, and then the volume was made up to 1 litre.

(5) Phosphate buffer (0.1 M, pH 6.0) sodium dihydrogen orthophosphate (monobasic) NaH_2PO_4 - 15.6 g (#ACS795, BDH Chemicals, Toronto), sodium phosphate anhydrous Na_2HPO_4 - 14.2 g (#S374B-500, Fisher Scientific Co, Ontario) dissolved in 1 litre of distilled water.

(6) 0.02M Phosphate buffer (pH 7.0): NaCl 11.688 g (0.1 M, #ACS783, BDH Chemicals, Toronto), $MgCl_2$ 0.406 g (1 mM, #M-0250, Sigma Chemical Co), Albumin, bovine serum 2.0 g (0.1%; #A-7888, Sigma Chemical Co), sodium azide (NaN_3) 2.0 g (0.1%, #B30111, BDH Chemicals), NaH_2PO_4 6.24 g, made up to 2 litres.

(7) Substrate for β-galactosidase:

a) 0.1 M Sodium phosphate buffer pH 7.3, Na_2HPO_4 1.141 g (Fisher Scientific), NaH_2PH_4 1.37 g (BDH Chemicals) dissolved in 100 ml of distilled water (DW);

b) Mercaptoethanol (#M6250, Sigma Chemical Co) 1 ml added to 4.25 ml DW;

c) $MgCl_2$ (#M-0250, Sigma Chemical co) 122 mg in 20 ml DW;

d) β-galactosidase (G-6008, Sigma Chemical Co);

e) 0-nitrophenyl-β-D-galactopyranoside (ONPG, #N-1127, Sigma) 20.5 mg in 1 ml of a. The above was mixed in the following proportions, 10.4 ml of a, 0.4 ml of b, 0.4 ml of c and 0.4 ml of e. 100 µl of the mixture was used as substrate for Dox-β-gal conjugate.

II. 5.2 (b) Method (Fujiwara, Yasuno & Kitagawa, 1981).

(1) 300 µg of MBS in 10ml 0.1 M Phosphate buffer (pH 6) was added to a solution containing 3 mg of Dox in 10 ml buffer.

(2) To 2 mls of the above mixture, 300 µl of THF was next added followed by incubation at RT for 30 min with vigorous stirring. 460 µl of this solution was

mixed with 0.5 mg β-gal in 1 ml buffer and stirred at RT for 30 min.

(3) The mixture was separated on a Sephadex G-25 column equilibrated with 0.02 M phosphate buffer (pH 7.0).

(4) The conjugates were tested with ONPG substrate and read by spectrophotometer (405 nm).

(5) Conjugation was also attempted using ECDI as the linker in an identical procedure to Dox-KLH conjugation.

II. 5.3 Dox-Avidin/Biotin conjugations.

An indirect method of labelling Dox was attempted by first conjugating Dox to avidin or biotin, using biotin or avidin-HRP as the label. Avidin-biotin complex is a well studied system in molecular biology and immunology (Bayer & Wilchek, 1980; Wood & Warnke, 1981; Wilchek & Bayer, 1984). Biotin is a water soluble vitamin present in egg white. Avidin is a biotin binding protein with a molecular weight of 60,000. Experiments were adapted to conjugate doxorubicin to avidin or biotin either directly or indirectly via albumin as a bridge (Goding, 1986; Boorsma et al, 1986).

II.5.3 (a) Materials

(1) Avidin (#A-9275, Sigma Chemical Co).

(2) Peroxidase-Biotin labelled l(#P-9272, Sigma Chemical Co).

(3) Dox-human albumin-succinyl (Dox-HSA; supplied by Dr. V. Richardson, Oncology Research)

(4) N-hydroxy succinimidobiotin (#H-1759, Sigma Chemical Co).

(5) 0.1 M sodium bicarbonate $NaHCO_3$ (pH 8.3, #1-3506, Baker Chem. Co.) Mol. wt. 84.01, 8.401 g in 1 litre distilled water.

(6) Succinic anhydride (# S7626 Sigma Chemical Co).

II. 5.3 (b) Method 1: Dox-Avidin conjugation

(1) 10 mg Dox was dissolved in 2 ml PBS and centrifuged (175 x g, 5 min) after ½ hr at RT to remove the precipitated drug.

(2) 3 mg avidin dissolved in 2 ml PBS was added to the Dox solution.

(3) This was followed by addition of the 11 mg/ml of ECDI.

(4) The mixture was kept in the dark at RT for 4 hrs and separated on a sephadex G-25 column.

II. 5.3 (c) Method 2: Dox-Avidin-succinyl conjugation

Since the above method was unsuccessful, avidin was first succinylated (Klapper & Klotz, 1971) and subsequently it was attempted to conjugate this with Dox using ECDI as follows:

(1) 1 mg of Succinic anhydride was added to 7 mg of avidin dissolved in 2 ml DW (Means & Feeney, 1971).

(2) The above was mixed with Dox and ECDI (similar to the Dox-KLH procedure).

II. 5.3 (d) Method 3: Dox-BSA-Avidin conjugation

Dox-BSA conjugates produced (Section II 4.0) were utilised for conjugation with avidin using ECDI as the cross linker. The method utilised was similar to the production of Dox-KLH conjugates using ECDI as given in Section II 3.0.

II. 5.4 Dox-Biotin conjugation.

Alternatively, doxorubicin-human serum albumin (HSA) succinyl (kindly supplied by Dr. V. Richardson, Oncology Research Laboratory) was linked directly to biotin as follows (Goding 1986; Ford et al, 1987a).

(1) Dox-HSA conjugates were dialysed at 4°C with 0.1 molar $NaHCO_3$ buffer. The final concentration of Dox-HSA was adjusted to give 1 mg (HSA) per ml aliquots.

(2) 1mg/ml of N-hydroxysuccinimido biotin was prepared in DMSO and 200 μl of the solution was added immediately to each aliquot of Dox-HSA and vortexed.

(3) The mixtures were then incubated at RT for 4 hrs with vortexing every 15 min.

(4) The aliquots were finally dialysed extensively at 4°C with PBS, the last two buffer changes containing 0.01 azide for some of the aliquots.

The procedure was repeated in an attempt to biotinylate Dox-BSA conjugates instead of Dox-HSA.

II. 6.0 CEA-HRP CONJUGATE PRODUCTION

II. 6.1 Materials

(1) Carcinoembryonic antigen (supplied by Oncology Research Laboratory and purified from liver metastases as published (Ford et al, 1987b).

(2) 0.01 M sodium carbonate bicarbonate buffer (pH 9.5)

(a) sodium carbonate 5.3 g in 500 ml distilled water (1 M solution)

(b) sodium bicarbonate 4.2 g in 500 ml distilled water (1 M solution)

25.4 ml of (a) was added to 74.4 ml of (b) and made up to 1 litre.

(3) Dinitrofluorobenzene (1%) (#D-6879, Sigma Chemical Co.)

100 µl in 10 ml absolute ethanol

(4) Sodium periodate $NaIO_4$ (0.08 M)(# 1867070, BDH Chemicals, England)

17.12 mg dissolved in 1 ml of distilled water.

(5) Ethylene glycol monoethylether (Fisher Scientific Co., New Jersey)

16 µl Ethylene glycol in 10 ml water.

II. 6.2 Method

The procedure was originally developed to label antibodies with HRP (Boorsma, 1983; Nakane & Kawaoi, 1984) and has previously been used to produce a 11-285-14 HRP conjugate (Woodhouse, 1982a). A modification of the method was used to enzyme label CEA as follows:

(1) To 6.2 mg HRP (type VI, RZ = 3) in 1 ml carbonate buffer (0.01 M), 100 µl of 1% dinitrofluorobenzene in absolute ethanol was added and stirred gently for 2 hrs at RT.

(2) 1 ml of 0.08 M sodium periodate was added to the solution followed by gentle mixing for 30 min at RT.

(3) This was followed by adding 1 ml of 0.16 M ethylene glycol.

(4) The mixture was then stirred for 1 hr at RT and dialysed against three 1 litre changes of carbonate buffer overnight at 4°C.

(5) CEA 1 mg/ml in carbonate buffer was added to the above solution and mixed gently for 3 hours at RT.

(6) Subsequently, 4.1 ml of sodium borohydride (2 mg/ml) was added and the solution dialysed against PBS overnight at 4°C.

(7) The CEA-HRP conjugate was separated from free HRP using a Sephadex G-75 gel filtration column.

II. 7.0 IMMUNIZATION OF RABBIT

II. 7.1 Materials

(1) Dox-KLH conjugates (Section II 3.0)

(2) Complete Freund's Adjuvant (CFA; # 660-5721, Gibco Labs, Ohio)

(3) Incomplete Freund's Adjuvant (IFA; # 660-5720, Gibco Labs)

(4) Syringes and needles (18 G 1 1/2; Becton Dickinson, Rutherford, N.J.)

II. 7.2 Method

Oil in water emulsions of Dox-KLH and CFA (for primary immunization) and IFA (for secondary boosters) were prepared. 1 ml of emulsion was injected intramuscularly and subcutaneously. The immunization dosage of doxorubicin and the intervals at which serum samples were drawn is given in Table 5.

Table 5: Immunization and bleeding schedule for rabbit

Day	Immunizing agent	Quantity of Dox μg	Adjuvant	Route	Comments
0	Dox-KLH	14.4	CFA	i.m & s.c.	Pre-immune blood drawn
22	Dox-KLH	11.5	IFA	i.m.& s.c.	--
29	--				Post-immune blood sample #1 drawn
50	Dox-KLH	8.6	CFA	i.m.	Post-immune blood sample #2 drawn
84	Dox-KLH	16	CFA	i.m.	--
93	--				Post-immune blood sample #3 drawn

Dox: Doxorubicin
KLH: Key hole limpet hemocyanin
CFA: Complete Freund's adjuvant
IFA: Incomplete Freund's adjuvant
i.m: Intramuscular
s.c: Subcutaneous

II. 8.0 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

II. 8.1 Introduction

Radioimmunoassay (RIA) was first developed (Yalow & Berson, 1959) recognising the specificity of antigen antibody reactions. In addition, because of its high sensitivity in the range of a few nanograms per millilitre, RIA has found widespread applications in medical research and clinical diagnosis. However, radioisotopes are inconvenient due to their high cost, risk of radioactivity and technical limitations (Messeyeff 1979). Enzyme labels have therefore replaced radiolabels for many applications and have the added advantage of stable storage for periods in excess of one year (Hudson & Hay, 1980). Horseradish peroxidase (HRP) and alkaline phosphatase are the commonly used enzyme labels for most ELISAs, the others include β-galactosidase and glucose oxidase.

An anti-CEA semiautomated ELISA (Woodhouse, Ford & Newman. 1982b) using micro cuvettes, was modified and adapted to be used as a 96 well microtiter plate ELISA. The microtitre plates are convenient for testing large numbers of hybrids simultaneously. ELISAs were also developed for the detection of anti-Dox antibodies, anti-BSA and BsMabs directly.

II. 8.2 Materials

The materials that are common to the different ELISAs are given in this section. Additional materials are given under the appropriate ELISAs in the sections below (II 8.3 to 8.9).

(1) Microtitre ELISA plates (Linbro)

(2) Titertek digital multichannel pipette and disposable tips (Flow Labs).

(3) Buffers for ELISA:

a) Carbonate buffer (pH 9.2), sodium carbonate Na_2Co_3 0.795 g, sodium bicarbonate $NaHCO_3$ 1.465 g dissolved in distilled water. If pH over or below 9.2, 6 M HCl or 10 M NaOH was used, respectively, to adjust the pH. The volume was made up to 500 ml.

b) 1% BSA in carbonate buffer (pH 9.2). BSA (# A-7888 Sigma Chemical Co) 1g, Buffer 100 ml.

c) 1% BSA in PBS-tween. BSA 1g dissolved in 100 ml PBS pH 7.2. 100 μl of tween detergent (BDH Chemicals Cat # RO6435-74) was added.

d) Citrate phosphate buffer, pH 4.0. Sodium hydrogen phosphate (#5374B-500, Fisher Scientific) Na_2HPO_4 4.08 g, citric acid crystals 4.53 g (#B27780, BDH), dissolved in distilled water. 6M HCl was used to adjust pH to 4.0. It was made up to a final volume of 500 ml.

e) 0.15 M NaCl with tween (0.1%). Sodium chloride NaCl 35.4g. Dissolved in 4 litres distilled water. Tween detergent (BDH Cat # R06435-74) 4 ml.

f) 2,2-azino-di-[3-ethyl benzthiazoline sulphonic acid] (ABTS) (#A-1888, Sigma Chemical Co). Stock: 27.8 mg/ml dissolved in distilled water and stored as 100 µl aliquots at -20°C. Freshly prepared 0.2224g/litre of ABTS as substrate for each ELISA contained:

100 µl stock

12.5 ml citrate phosphate buffer

1 µl hydrogen peroxide (30%; #B45202 BDH Chemicals).

II 8.3 ANTI-CEA ELISA

II 8.3 (a) Materials

(1) CEA (supplied by Oncology Research Laboratory) purified as published (Ford et al, 1978b) in carbonate bicarbonate buffer (Section II 8.2).

(2) 11-285-14 anti-CEA monoclonal antibody (positive control).

(3) Ag-8 (P3X63Ag8; IgG_1 as negative control; American Type Culture Collection, Rockville, Maryland).

(4) Rabbit anti-mouse immunoglobulins HRP conjugate (p 161, Dako, Denmark).

(5) ABTS (Substrate prepared as in section II 8.2).

(6) Normal rabbit serum (NRS) 1:1000 dilution in PBS-tween.

II 8.3 (b) Coating

After standardisation for optimum CEA coating (see under results) ELISA plates were coated with 100 µl per well of 5 µg/ml CEA in carbonate buffer (pH 9.2) and incubated at 37°C for 3 hours followed by incubation in a humidified box at 4°C overnight. In later assays, the coated plates were directly incubated at 4°C without significant reduction in sensitivity.

II. 8.3 (c) Assay

(1) The coating solution was discarded by inverting and shaking the plates well.

(2) The plates were washed six times with NaCl-tween solution using a wash bottle, ensuring that each well was filled.

(3) 200 µl of 1% BSA in carbonate buffer was added to each well, as the blocking solution. For Dox-BSA hybrids, 10% normal rabbit serum (NRS) was used for blocking instead of 1% BSA in order to avoid non-specific "sticking" to BSA.

(4) The plates were incubated at 37°C for 1 hour.

(5) The plates were washed as before (step #2).

(6) The test supernatants were added, 100 µl per well. The controls used for anti-CEA assay were: Background - RPMI-GLN-FCS (Medium as for supernatants); Positive control, 11-285-14 Mab 2.5 µg/ml in RPMI; Negative control, Ag-8, 2.5 µg/ml in RPMI. All these were added in triplicate.

(7) The plates were then incubated at 37°C for 3 hours.

(8) This was followed by x 6 washes as before.

(9) Rabbit anti-mouse immunoglobulins - HRP conjugate (RAM) 1:1000 dilution in 1% BSA PBS-tween was added, 100 µl per well. In the case of DOX-BSA fusion hybrid supernatants RAM was made up in 1% normal rabbit serum in PBS-tween to avoid the possibility of reactivity with BSA by anti-BSA mabs.

(10) The plate was then incubated at 37°C for 3 hours.

(11) The plate was washed x 6 as before.

(12) Freshly prepared ABTS substrate, 100 µl was added per well and the plates were read after 1 hour at room temperature (RT) at 405 nm single wave length, by a Bio-Tek EL 310 EIA plate reader (Mandel Scientific, Rockwood, Ontario).

II. 8.4 Anti-Doxorubicin ELISAs

II. 8.4 (a) Materials

(1) ELISA buffers (Section # II.8.2.)

(2) Doxorubicin 2 µg/ml (as Doxorubicin or Doxorubicin-KLH or Doxorubicin-BSA) in carbonate bicarbonate buffer, confirmed by spectrophotometry at 495 nm optical density.

(3) Test antibody supernatants.

(4) Goat anti-rabbit immunoglobulins HRP (GAR-HRP) (# E961 ICN-Immune biologicals Lisle, IL).

(5) Rabbit anti-mouse immunoglobulins HRP (RAM-HRP).

(6) Substrate ABTS (Section II 8.2.)

(7) Bio-Tek EIA plate reader.

(8) Positive control: Rabbit post-immune serum containing anti-Dox antibodies.

(9) Negative control: Rabbit pre-immune serum (no anti-Dox antibodies).

(10) Background control: RPMI-GLN-FCS and 11-285-14 (2.5µg/ml) and/or BSA-PBS-tween.

II. 8.4 (b) Coating

The coating depended on the two different types of assays performed (i) anti-Dox and (ii) anti-Dox BSA. After standardisation for Dox coating, the optimum coating concentration for Dox was found to be 2 µg/ml (see under Results). Therefore, 2 µg/ml of Dox as Dox alone or as Dox-BSA

in carbonate buffer was coated at 100 μl per well and incubated overnight at 4°C.

II. 8.4 (c) Assays

The procedure was similar to that for the anti-CEA assay. Pertinent differences are mentioned below for individual assays.

(i) Testing rabbit serum:

(1) 2 μg/ml of doxorubicin coating.

(2) Post-immune sera (with anti-Dox antibodies) tested along with pre-immune (no anti-Dox antibodies) in dilutions 1/100 to 1/1,000,000.

(3) 1% BSA in PBS-tween used as control.

(4) Goat anti-rabbit immunoglobulin-HRP (ICN - Immune. Biologicals) (1:4000 dilution) used as second antibody.

(ii) Dox-KLH immunized mice sera:

(1) Dox-BSA (2μg/ml Dox) used as coating for testing mice sera of fusions 11, 13, 14 and 16. Doxorubicin alone at 2μg/ml used for the rest of fusions.

(2) 1% BSA in PBS-tween as background control.

(3) Non-immunized mice sera used as pre-immune control.

(iii) Dox-BSA immunized mice sera:

(1) Fusions 2, 5, 7 and 8 were tested with Dox-KLH coating at Dox 2μg/ml.

(2) Fusions 1, 3 and 4 tested with Dox-BSA coating at Dox 2μg/ml.

(3) 10% normal (non immunized) rabbit serum (NRS) in PBS-tween employed as blocking agent instead of 1% BSA in order to avoid reactivity with antibodies against BSA.

(iv) Testing hybrid supernatants:

(1) Dox alone used as coating for all Dox-KLH fusions except fusion # 16. Dox BSA at 2 μg/ml Dox used as coating for testing hybrids from Dox-KLH fusion 16.

(2) Dox-BSA coating used for Dox-BSA fusion hybrids with simultaneous BSA coated ELISAs (see below, Section II.8.5).

(3) 11-285-14 at 2.5 μg/ml and RPMI-GLN-FCS medium were each employed in triplicate wells as background controls.

(4) Sera from mice immunized with Dox-KLH or Dox-BSA used as positive control.

(5) Non-immunized mice sera used as negative control.

(6) For Dox-BSA fusions, the test mouse serum was diluted in normal (non-immunized) rabbit serum (1%, NRS)) in PBS-tween, instead of 1% BSA in PBS-tween.

II. 8.5 Anti-BSA ELISA

II. 8.5 (a) Coating

For testing hybrids obtained from Dox-BSA fusions, anti-BSA ELISAs were performed simultaneously with anti-Dox-BSA assays. The coating of Dox-BSA was first prepared at 2 μg/ml of Dox and the concentration of BSA was determined by spectrophotometry (1% BSA at 280 nm = 6.6). This BSA concentration was then utilized as coating for the parallel anti-BSA ELISA.

II. 8.5 (b) Assay

The procedure was identical to the anti-Dox BSA ELISA with the same controls.

II. 8.6 ELISA for Dox-HRP conjugates

II. 8.6 (a) Coating

The post immune rabbit serum # 3 (Table 5) containing anti-Dox antibodies was used as coating to detect Dox-HRP conjugates. 100 μl per well of serum in dilutions of 1/100, 1/1000 and 1/10,000 in carbonate buffer were coated in triplicate in the wells of microtitre ELISA plates, incubated

at 37°C for 3 hours and then kept overnight at 4°C. Rabbit pre-immune serum (without anti-Dox antibodies) was coated simultaneously as a control.

II. 8.6 (b) Assay

(1) Coating solution discarded, followed by washing as in anti-CEA ELISA.

(2) 1% BSA in carbonate buffer was used at 200 µl/well for blocking, and incubated for 1 hour at 37°C.

(3) After washing, the test conjugate was added 100 µl/well in triplicate as 1:1, 1:10 and 1:100 dilutions.

(4) The plate was incubated at 37°C for 3 hours.

(5) After washing, freshly prepared substrate ABTS was added 100 µl/well and the reaction read at 405 nm single wavelength after 1 hour at RT.

II. 8.7 ELISA for Dox-β-galactosidase conjugates

Similar to the ELISA testing of Dox-HRP conjugates described in the previous section, except that the substrate ONPG was used (see Dox-β-gal conjugation Section II 5.2). Furthermore, the enzyme substrate reaction was read after incubation at room temperature (RT) after 3 minutes and after 1 hour.

II. 8.8 ELISA for Dox-biotin or Dox-avidin conjugates.

II. 8.8 (a) Materials

(1) Rabbit pre-immune serum

(2) Rabbit post-immune serum

(3) Streptavidin peroxidase (# 43-4323, Zymed Labs, San Francisco, Ca).

(4) Peroxidase-biotin labeled (P-9272 Sigma Chemical Co).

(5) ELISA buffers (II 8.2).

(6) Goat anti-rabbit immunoglobulins HRP.

II. 8.8 (b) Assay

(1) Two separate ELISAs were performed. Rabbit pre-immune (no anti-Dox) and post-immune (with anti-Dox) sera in serial dilutions of 1:10, 1:100, 1:1000 and 1:10,000 in carbonate buffer were coated at 100µl/well in triplicate. Alternatively, Dox-avidin or Dox-biotin conjugates were used to coat plates. The plates were incubated overnight at 4°C after 3 hours at 37°C.

(2) After blocking with 200 µl of 1% BSA for 1 hour, the test conjugate (Dox-avidin or Dox-biotin) was added in 1/10, 1/25 and 1/50 dilutions in PBS. For Dox-Avidin/Biotin coated wells, rabbit pre-and post-immune sera were added at 1/10, 1/100 and 1/1000 dilutions.

(3) After a 3 hour incubation at 37°C, 100µl of the corresponding indicator, either streptavidin-peroxidase

(1:5000 dilution) or Biotin-peroxidase (10 μg/ml) was added per well and incubated for a further 3 hours at 37°C. For Dox-Avidin or Biotin coated plates, goat anti-rabbit immunoglobulin HRP (1:4000 dilution) was used.

(4) Freshly prepared ABTS substrate was added and the colour reaction read at 405 nm, after one hour at RT.

II. 8.9 ELISA for CEA-HRP conjugates

II. 8.9 (a) Coating

ELISA microtitre plates were coated with 11-285-14 anti-CEA antibody or Ag-8 control antibody at 5μg/ml in 100μl per well in triplicate. The plates were incubated for 2 hours at 37°C and overnight at 4°C.

II. 8.9 (b) Assay

(1) Blocking was with either 1% BSA or 1% NRS 200μL/well followed by 1 hour incubation at 37°C.

(2) CEA-HRP in dilutions of 1/25, 1/50, 1/75 and 1/100 in 1% BSA-PBS (see ELISA buffers) was added and incubated for 3 hours at 37°C.

(3) Freshly prepared ABTS 100μl/well was added and the reaction read at 405 nm on the EIA Biotech plate reader.

II. 9.0 PRODUCTION OF BISPECIFIC MONOCLONAL ANTIBODIES

II. 9.1 Immunization of spleen cell donors

II. 9.1 (a) Materials

(1) Complete Freunds adjuvant (CFA; Gibco)

(2) Incomplete Freunds adjuvant (IFA; Gibco)

(3) Dox-KLH or Dox-BSA conjugates (Section II 3.0 & 4.0)

(4) Glass syringes and needles (Becton Dickinson)

(5) PBS (Oxoid);autoclaved sterile.

II. 9.1 (b) Method

The concentration of doxorubicin in conjugates was calculated by spectrophotometry. An oil in water emulsion was prepared with Dox-KLH and CFA or IFA (table 6). 1 ml of the emulsion was injected intraperitoneally or subcutaneously as indicated in tables 6a to 6d. The immunization protocol for mice with Dox-BSA conjugates is given in tables 7a & b.

Table 6a : Dox-KLH immunization schedules

Fusion #	Days	Quantity of Dox μg	Adjuvant	Route	Comments
1, 2 & 3	0	4.0	CFA	IP	One mouse used for all three fusions. Total cells obtained = 10.57×10^7
	21	4.0	CFA	IP	
	29	Fusion			
4	0	4.0	CFA	IP	Total spleen cells obtained = 4.0×10^7
	21	4.0	CFA	IP	
	32	4.0	CFA	IP	
	42	8.0	CFA	IP	
	48	Fusion			
5	0	2.0	CFA	IP	Total spleen cells obtained = 16.7×10^7
	21	4.0	CFA	IP	
	32	4.0	CFA	IP	
	42	8.0	CFA	IP	
	57	2.0	PBS	IV	
	63	Fusion			
6	0	2.0	CFA	IP	Two mice used. Total spleen cells obtained from both mice = 23×10^7 Fusion performed on 97th day.
	21	4.0	CFA	IP	
	32	4.0	CFA	IP	
	42	8.0	CFA	IP	
	69	15.0	CFA	IP	

continued.....

Table 6b : Dox-KLH immunization schedules

Fusion #	Days	Quantity of Dox μg	Adjuvant	Route	Comments
6 (cont'd)	92	8.0	CFA	IP	see Table 6a
7	0	8.0	CFA	IP	Total spleen cells obtained = 14.3×10^7
	90	1.1	CFA	IP	
	107	2.5	PBS	SC	
	116	1.2	PBS	SC	
	125	0.5	PBS	IV	
		1.5	PBS	SC	
	130	5.0	PBS	SC	
	132	Fusion			
8	0	8.0	CFA	IP	Total spleen cells obtained = 8.34×10^7
	90	1.1	CFA	IP	
	107	2.5	PBS	SC	
	116	1.2	PBS	SC	
	195	7.0	PBS	IP	
	204	0.7	PBS	IV	
		6.0	PBS	SC	
	209	Fusion			
9	0	8.0	CFA	IP	Total spleen cells obtained = 4.56×10^7
	90	1.1	CFA	IP	
	107	2.5	PBS	SC	
	116	1.2	PBS	SC	
	220	0.5	PBS	IV	
		4.0	PBS	SC	
	224	Fusion			

continued.....

Table 6c : Dox-KLH Immunization Schedules

Fusion #	Days	Quantity of Dox μg	Adjuvant	Route	Comments
10	0	8.0	CFA	IP	Total spleen cells obtained = 6.72×10^7
	90	1.1	CFA	IP	
	107	2.5	PBS	SC	
	116	1.2	PBS	SC	
	230	0.75	IFA	IP	
	237	1.3	PBS	IV	
	240	1.0	PBS	SC	
	243	Fusion			
11	0	8.0	CFA	IP	Total spleen cells obtained = 9.06×10^7
	92	1.1	CFA	IP	
	107	2.5	PBS	SC	
	116	2.5	PBS	SC	
	230	0.75	IFA	IP	
	263	7.5	IFA	IP	
	293	3.2	PBS	IV & SC	
	295	Fusion			
12 a & b	0	4	CFA	IP	Total = 8×10^7 spleen cells used for 2 fusions with 11-285-14 and NS_1
	19	2.5	CVA	IP	
	84	7.0	in PBS	IP	
	119	0.75	IFA	IP	
	126	0.9	IFA	IP	
	152	7.5	IFA	SC	
	168	15	in PBS	IV	
	171	Fusion			
13	0	4.5	CFA	IP	Total spleen cells obtained = 10.6×10^7
	35	4.0	CFA	IP	
	231	3.65	IFA	IP	
	239	3.2	in PBS	IV & SC	

continued .....

Table 6d : Dox-KLH Immunization Schedules

Fusion #	Days	Quantity of Dox μg	Adjuvant	Route	Comments
	243	Fusion			
14	0	4.5	CFA	IP	Total spleen cells obtained = 7.94×10^7
	35	4.0	CFA	IP	
	229	3.65	IFA	IP	
	240	0.73	PBS	IV & SC	
	243	Fusion			
15 a & b	0	4.5	CFA	IP	Total spleen cells obtained = 7.14×10^7. Used for 2 fusions Aza-2 & NS_1
	35	4.0	CFA	IP	
	229	3.65	IFA	IP	
	240	0.73	in PBS	IV & SC	
	244	Fusion			
16 a & b	0	19.5	CFA	IP	Total spleen cells obtained per mouse = 11.1×10^7. Two mouse spleens pooled and used for two fusions
	19	Mice sera tested			
	20	9	in PBS	IV & IP	
	22	Fusion			
17	0	17	CFA	SC	Total spleen cells = 7.64×10^7
	30	2	IFA	SC	
	78	7.5	PBS	IP	
	83	Fusion			

CFA: Complete Freund's adjuvant
IFA: Incomplete Freund's adjuvant
PBS: Phosphate buffered saline
IP: Intra-peritoneal
IV: Intra-venous
SC: Subcutaneous

Table 7a : Dox-BSA Immunization Schedules

Fusion #	Days	Quantity of Dox μg	Adjuvant	Route	Comments
1	0	50	CFA	SC	1 month old mouse. Total viable spleen cells = 8.16×10^7
	13	50	IFA	SC	
	28	50	in PBS	IP	
	31	fusion			
2	0	50	CFA	SC	Mouse from same litter as above and identical immunization. Total viable spleen cells = 12.16×10^7
	13	50	IFA	SC	
	28	50	in PBS	IP	
	31	fusion			
3	0	50	CFA	SC	2 month old mouse. Total viable spleen cells = 6.88×10^7
	16	50	IFA	SC	
	41	50	in PBS	IP	
	45	fusion			
4	0	50	CFA	SC	Mouse details identical to Fusion # 3. Total viable spleen cells = 7.72×10^7
	16	50	IFA	SC	
	41	50	in PBS	IP	
	45	fusion			

Legend as for Table 6

continued .....

Table 7 b: Dox-BSA Immunization Schedules

Fusion #	Days	Quantity of Dox μg	Adjuvant	Route	Comments
5	0	50	CFA	SC	2 month old mouse. 0.5 ml blood drawn on day 14. Total spleen cells = 14.76 x 10^7
	14	50	IFA	SC	
	28	55	in PBS	IP	
	31	fusion			
6	0	50	CFA	SC	Mouse from same litter as in Fusion 5. 0.5 ml pre-immune blood drawn on Day 0. Total viable spleen cells = 11.96 x 10^7
	14	50	IFA	SC	
	28	55	in PBS	IP	
	32	fusion			
7	0	50	CFA	SC	3 month old mouse. 0.5 ml mouse blood drawn on day 15. Total viable spleen cells = 5.03 x 10^7
	15	55	CFA	SC	
	29	50	in PBS	IP	
	32	fusion			
8	0	50	CFA	SC	3 month old mouse. 0.5 ml blood drawn on day 16. Total viable spleen cells = 5.43 x 10^7 CFA used throughout in Fusion # 7a and b similar to Balsari et al, 1988
	16	50	CFA	SC	
	30	55	in PBS	IP	
	34	fusion			

Legend as for Table 6

II. 9.2 Preparation of spleen cell suspension

This procedure and the fusion protocol was adapted from Woodhouse (1982a), Galfre & Milstein (1981) and Suresh *et al.* (1986b).

II. 9.2 (a) Materials

(1) CO_2 flowing from incubator
(2) Absolute ethanol
(3) PBS tablets (Oxoid); autoclaved sterile
(4) Nylon mesh as sieve
(5) Sterile pasteur pipettes
(6) Laminar flow cabinet
(7) Petri dishes (100 x 15 mm)
(8) Scissors 2 pairs, sterile
(9) Forceps 2 pairs, sterile
(10) Disposable plastic syringes 10 ml capacity
(11) 50 ml sterile conical tubes
(12) Bench top centrifuge

II. 9.2 (b) Method

(1) The immunized mouse was killed by placing it in a jar containing CO_2.
(2) The mouse was dabbed with alcohol and using a sterile pair of scissors and forceps the abdomen was opened with a midline incision and the spleen was exposed.

(3) The spleen was removed and, after a quick spray with alcohol, was placed in a petri dish containing sterile PBS.

(4) The petri dish with the spleen was transferred to a laminar flow cabinet.

(5) The rubber end of the disposable syringe plunger was used to prepare a cell suspension by pressing the spleen through the nylon sieve into the PBS.

(6) The spleen cell suspension was left to stand in a conical tube for a few seconds and the cells in the clear suspension were removed with a pasteur pipette, the large clumps at the bottom being discarded.

(7) The cell suspension was washed in fresh PBS by centrifuging at 1000 rpm (175g) for 5 min and the pellet was resuspended in 10 ml PBS.

II. 9.3 Buffers and media for Fusions

(1) HT x 100 and x 50 Stock:

Thymidine (T-9250 Sigma) 0.0387g; hypoxanthine (H 9377, Sigma Chemical Co). 0.1361 g; distilled water 100 ml. The water was warmed to 60 - 70°C and the components dissolved in it to give x 100 HT. An equal volume of distilled water was added to x 100 HT to give x 50HT. Both were sterilised by passing through a 0.22 μm filter (Millipore products division, Bedford, Ma. 01730) and then stored at -20°C in aliquots.

(2) HAT x 50 Stock:

Aminopterin (A-1784, Sigma) 0.0176 g; distilled water 100 ml; sodium hydroxide 0.1 M; HT x 100 stock 100 ml. The aminopterin was dissolved in about 80 ml of distilled water. NaOH was added to aid in dissolution. The minimum amount necessary to dissolve the reagents was used. The volume was made up to 100 ml with distilled water. 10 ml of aminopterin solution was added to 100 ml HT x 100 stock and 90 ml distilled water to give HAT x 50 stock. This was sterilised by filtration through a 0.22 μm filter (Millipore products division), and stored at -20°C.

(3) RPMI-GLN (culture medium):

RPMI 1640 (320-1875 AJ Gibco) 100 ml.

L-glutamine, 200nM (16-801-49 Flow labs) 1 ml.

Penicillin (10000 units/ml) (Flow Labs)

Streptomycin (10000 μg/ml) solution (# 16-700-49 Flow Labs) 1 ml.

Obtained sterile and mixed aseptically.

(4) RPMI-GLN-FCS (culture medium):

RPMI-GLN (see above) 500 ml.

Fetal calf (bovine) serum (FCS) 50 ml (10%) or 100ml (20%).

(FCS cellect^{R} Gold (# 29-167-54) or FCS cellect^{R} Silver (# 29-161-54, Flow Labs Inc. Mclean, Va 22102).

(5) PEG-DMSO-RPMI:

Polyethylene glycol (PEG) (BDH Chemicals) 1500 av. MW. 8.0g

Dimethyl sulphoxide (DMSO) (# B10323 BDH Chemicals) 1.5ml

RPMI-GLN (see above # 3) 8.5 ml

Polyethylene glycol was steam autoclaved in a glass bottle and allowed to cool. Dimethyl sulphoxide was sterile-filtered into the RPMI-GLN through a 0.22 μm filter and added to the polyethylene glycol before it solidified.

(6) RPMI-HAT (fusion medium for hybridomas):

RPMI-GLN 100 ml

HAT x 50 stock 2 ml

Mixed aseptically.

(7) RPMI-HT-FCS:

RPMI-GLN-FCS 100 ml

HT x 50 stock 2 ml

Mixed aseptically.

II. 9.4. Bispecific fusions: Spleen cells with 11-285-14

II. 9.4 (a) Materials

(1) Azaguanine resistant hybridoma 11-285-14 secreting monoclonal anti-CEA antibody.

(2) Spleen cell suspension (previous section).

(3) Phosphate buffered saline (PBS),(Oxoid), sterile.

(4) Sterile 50 ml & 15 ml conical tubes (Falcon, Becton Dickinson, N.J.).

(5) Glass beakers, 250 ml and 1 ml.

(6) 37°C waterbath.

(7) PEG-10% DMSO (no FCS,see Buffers and Media, above section).

(8) RPMI-HAT (without FCS) (see Buffers & Media, above section).

(9) Pipettes, 1 ml, 5 ml and 10 ml, sterile.

(10) 2 stop clocks (Cat # 40005, The West Bend Company, West Bend, WI, 53095).

(11) Bench top centrifuge.

(12) Incubator at 37°C with 5% CO_2 and humidification.

(13) Linbro 96 well (1 x 0.7 cm, 0.35 ml well capacity) flat bottomed tissue culture plates (# 76-003-05 Flow Labs).

II. 9.4 (b) Method

(1) The parental cells, 8-azaguanine resistant 11-285-14, in exponential growth and spleen cells (section above) were washed separately in sterile PBS by centrifugation (5 min at 175 x g) and resuspended at 10^7 cells/ml.

(2) Spleen cells and 11-285-14 were mixed in a ratio of 5:1 in a 50 ml sterile conical tube and centrifuged at 800 x g for 5 minutes.

(3) After removing the supernatant, the cell pellet was warmed by standing the tube in a beaker containing water at 37°C.

(4) One ml of PEG-DMSO-RPMI (without FCS) at 37°C was added to the cell pellet drop by drop over a period of 1 minute and the cells were gently stirred very cautiously with the tip of the pipette.

(5) The gentle stirring was continued for an additional minute.

(6) Two ml of RPMI-HAT (without FCS) at 37°C was added over 2 minutes with gentle stirring.

(7) Eight ml of RPMI-HAT (without FCS) at 37°C was added over 3 minutes with gentle stirring.

(8) The cells were centrifuged at 400 x g at RT for 5 minutes and the supernatant discarded.

(9) The cells were resuspended in 25 ml of RPMI-HAT-FCS as gently as possible to avoid mechanical damage to the cells.

(10) Fifty μl of the cell suspension was added into each well of a tissue culture plate already containing 100 μl of RPMI-HAT-FCS (see step 11 below). Approximately 480 wells (5 plates) were used for each fusion.

(11) For most of the fusions, 100 μl of RPMI-HAT-FCS was added into each well for the five plates, preferably the day before or early on the day of the fusion and incubated at 37°C in 5% Co_2.

(12) On addition of the fused cell suspension, the plates were incubated at 37°C in 5% CO_2.

II. 9.5 Growth of bispecific hybridoma cultures

II. 9.5 (a) Materials

(1) RPMI-HAT-FCS (10% FCS Silver^R)

(2) RPMI-HT-FCS (10% FCS Silver^R)

(3) RPMI-GLN-FCS (10% FCS Silver^R)

(4) Hypodermic needles, 18½ gauge sterile (Becton Dickinson)

(5) Pipettes, 10 ml, sterile

(6) Suction bottle (vacuum)

(7) Javex

(8) Linbro 96 well plates (Flow)

(9) Linbro 24 well plates (3.5 ml well capacity, Area 2.0 cm^2, # 76-033-05, Flow Labs)

(11) Titertek multichannel pipetter (Flow Labs)

(12) Sterile tips 200μl capacity (Flow Labs).

II. 9.5 (b) Method

(1) The plates containing the fused cells were left undisturbed for five days at 37°C in 5% CO_2.

(2) On the fifth day, a sterile 18½ G needle attached to a vacuum suction line was used to remove half the medium without disturbing the cells at the bottom. This was replaced with 100 μl of fresh RPMI-HAT-FCS using a multichannel pipetter.

(3) The above procedure was repeated every three days for two weeks.

(4) After two weeks, half the medium was replaced with RPMI-HT-FCS.

(5) Medium changes with RPMI-HT-FCS were repeated every three days for at least three changes.

(6) For the initial (Dox-KLH) fusions, all further changes of medium were done with RPMI-GLN-FCS. Since the hybrids were unstable during this transition period, for the Dox-KLH (fusion # 16) and all Dox-BSA fusions, the hybrids

were continued to be grown in RPMI-HT-FCS. This has been shown to improve their stability in culture as demonstrated in some studies (Goding, 1986).

(7) When the hybrid colonies covered about 25% of each well, the supernatants were tested for anti-CEA, anti-Dox and/or dual activity.

(8) Positive hybrids were either cloned immediately and/or transferred to 24 well culture plates containing 500 μl of medium in each well.

(9) Supernatants of confluent colonies were rescreened by ELISAs after a few days depending on growth.

(10) Positive clones and subclones were either transferred to 50 ml sterile flasks or frozen in liquid N_2.

II. 9.6 Preparation of feeder layers for cloning.

Feeder cells consisting of splenocytes and/or thymocytes were prepared on the day prior to fusion and maintained at 37°C in 5% CO_2.

II. 9.6 (a) Materials

(1) BALC/c mice

(2) Linbro flat bottomed 96 well tissue culture plates (Flow Labs)

(3) RPMI-HAT-FCS (see buffers and media) with 20% FCS cellect^R Gold

(4) RPMI-HT-FCS (see buffers and media) with 20% FCS cellect^{R} Gold

(5) Disposable 5 ml and 10 ml syringes, sterile

(6) 18½ G hypodermic needles, sterile

(7) Nylon sieve, sterile

(8) Petri dishes, sterile (Polar Plastic Ltd., St.Laurent, Quebec H4R 2B9).

(9) Absolute alcohol

(10) 50 ml conical tubes, sterile

II. 9.6 (b) Method

(1) Mice were terminated as described in removal of spleens for fusion (Section II 9.2).

(2) The spleens were removed in an identical manner.

(3) The skin over the upper part of the chest was incised in the middle and the subcutaneous thymus was removed aseptically.

(4) The thymuses were transferred to a petri dish with sterile PBS and then into the laminar cabinet.

(5) A sterile syringe plunger was used to express the thymus cells through the nylon sieve into a petri dish with sterile PBS.

(6) After allowing the large clumps to settle to the bottom, the supernatant was transferred to a 50 ml conical tube and washed twice with PBS.

(7) The cells were resuspended in medium, either RPMI-HAT-FCS or RPMI-HT-FCS depending on the number of days after fusion (Section II 9.5).

(8) The spleen cells were prepared as described under the fusion and were adjusted to a final concentration of 1 x 10^6 cells/ml to yield a cell density of 1 x 10^5 cells contained in 100 μl per well.

(9) The thymocytes were adjusted to a concentration ranging from 5 x 10^6 cells/ml (Woodhouse, 1982a) to 1 x 10^7 cells/ml (Goding, 1986; Eshhar, 1985). The final density of thymocytes was in most cases 1 x 10^6 cells in 100 μl per well (Goding 1986, Eshhar, 1985).

(10) In some cases, a combination of spleen cells and thymocytes was used as feeders at the above concentrations resulting in a cell density of 5 x 10^4 splenocytes with 5 x 10^5 thymus cells in each well.

II. 9.7 Cloning of positive hybrids by limiting dilutions

While cloning is an essential step in the preparation of Mabs, its outstanding importance in the selection of hybrid-hybrids has been demonstrated by previous studies (Suresh et al, 1986 a & b). This is due to the increased polyploidy of the hybrids and their higher propensity to lose chromosomes than conventional hybridomas. The method of limiting dilution

was used for cloning (Hudson & Hay, 1980; Galfre & Milstein, 1981; Woodhouse 1982).

II. 9.7 (a) Materials

(1) Positive hybrid cells to be cloned.

(2) Sterile 96 well tissue culture plates, 0.35 ml well capacity (Linbro # 76-003-05, Flow Labs).

(3) Incubator 37°C, humidified and gassed with 5% CO_2.

(4) Feeder layers (Section II 9.6).

(5) RPMI-HT-FCS or RPMI-HAT-FCS (20% Gold FCS used for selection and cloning of Dox-BSA fusion hybrids).

(6) Titertek multichannel pipetter (Flow Labs).

(7) Sterile 200 µl tips (autoclaved).

(8) Sterile petri dishes (100 x 15 mm).

II. 9.7 (b) Method

For each positive hybrid well:

(1) The cells were pipetted into suspension and counted as in Section II.1.

(2) The suspension was adjusted to 10 cells/ml and 5 cells/ml in medium.

(3) 100 µl of the 10 cells/ml suspension was aliquoted into each of the 48 wells of half a microtitre plate. Similarly, 100 µl of the 5 cells/ml suspension was aliquoted into the remaining half of the plate.

(4) The cells were incubated in a humid 37°C incubator gassed with 5% CO_2.

(5) Colonies were usually visible after 1-2 weeks and then the supernatants were tested for antibody activity.

Note: The distribution of cells per well follows Poisson statistics, with about 60% of the wells receiving only one cell, resulting in true clones. Many of the remainder wells will receive 2 or more cells following aliquots of 10 cells/ml suspension (Hudson & Hay, 1980).

Cloning was repeated at least 2 or 3 times to ensure clonality of positive subclones.

II. 10.0 FUSION OF SPLEEN CELLS WITH NS-1 MYELOMA.

Materials and methods identical to fusions of spleen cells with 11-285-14 (Section II 9.4). The NS-1 myeloma growing in 6-thioguanine (Section II 2.3) was used with spleen cells from mice immunized with Dox-KLH or Dox-BSA. The selection and cloning procedure was similar to the section on bispecific fusions (Sections II 9.4 to II 9.7).

CHAPTER III

RESULTS

III. 1.0 GENERATION OF 11-285-14 FUSION PARTNERS

The 11-285-14 hybridoma was first back selected in increasing concentrations of 8-azaguanine. 11 batches, growing well at the 30 µg/ ml concentration were produced. These 11 batches were cloned by limiting dilution and of the 72 clones of 11-285-14 isolated, 27 were positive for anti-CEA production by ELISA. Rapidly growing clones were evaluated for (1) growth characteristics, (2) maintained anti-CEA production, (3) HAT sensitivity, (4) fusion efficiency.

III. 1.1 Growth Characteristics

Three of eight rapidly growing, anti-CEA producing clones termed Aza 1, 2 and 3, were compared with the parental non-azaguanine resistant 11-285-14 hybridoma in conventional (RPMI-GLN-FCS) medium. The cells were inoculated at an initial concentration of 0.05×10^6 cells in 10 ml medium. The number of cells was counted daily and this has been plotted in Figure 4. The mutant clones appeared to be slow growing compared to their parental 11-285-14 cell lines during the five days they were counted (Figure 4).

Figure 4. Growth of 11-285-14 clones

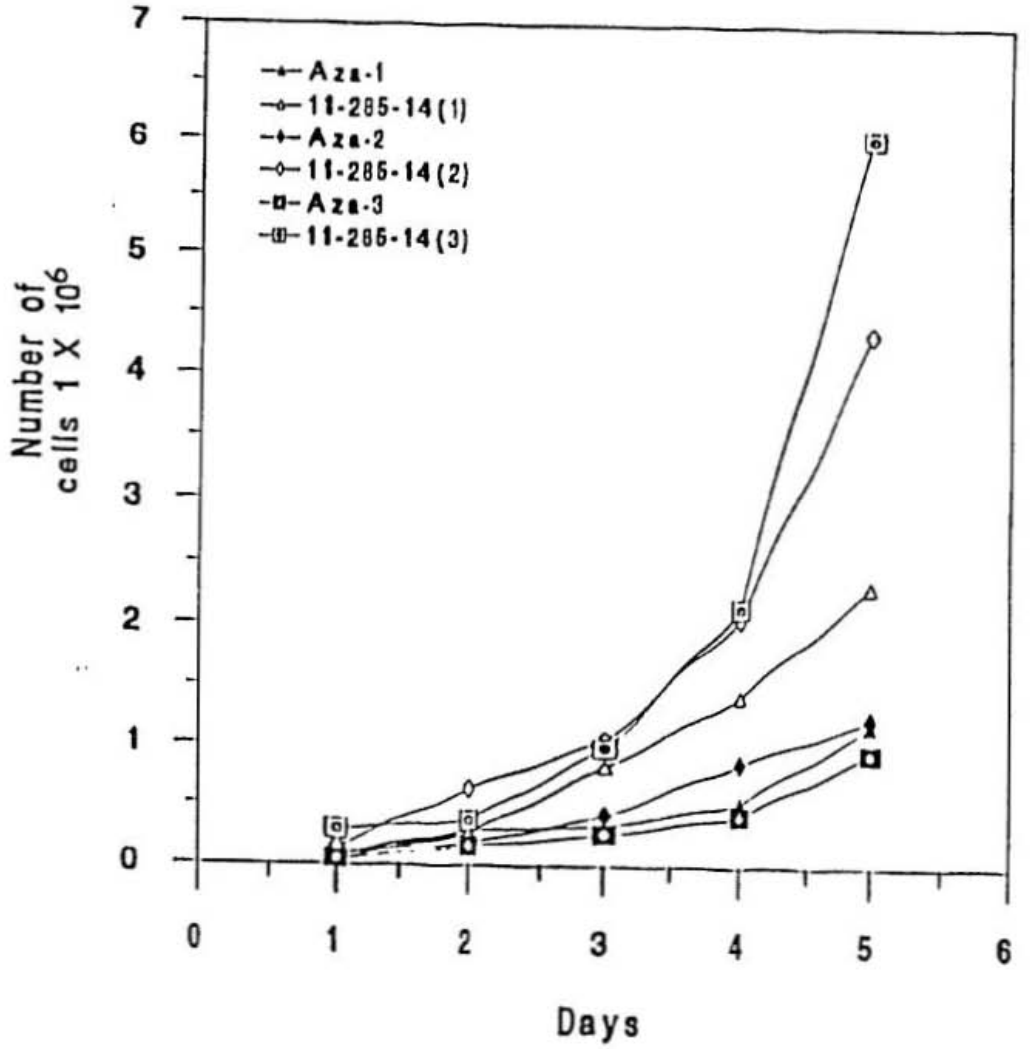


Figure 4. Three azaguanine resistant 11-285-14 clones (Aza-1, 2 & 3; closed symbols) were compared with their parental 11-285-14 (open symbols) growing in RPMI-GLN-FCS. The number of cells started was 5 X 10^4/ml and was counted daily.

Figure 5. Comparison of anti-CEA[128] production

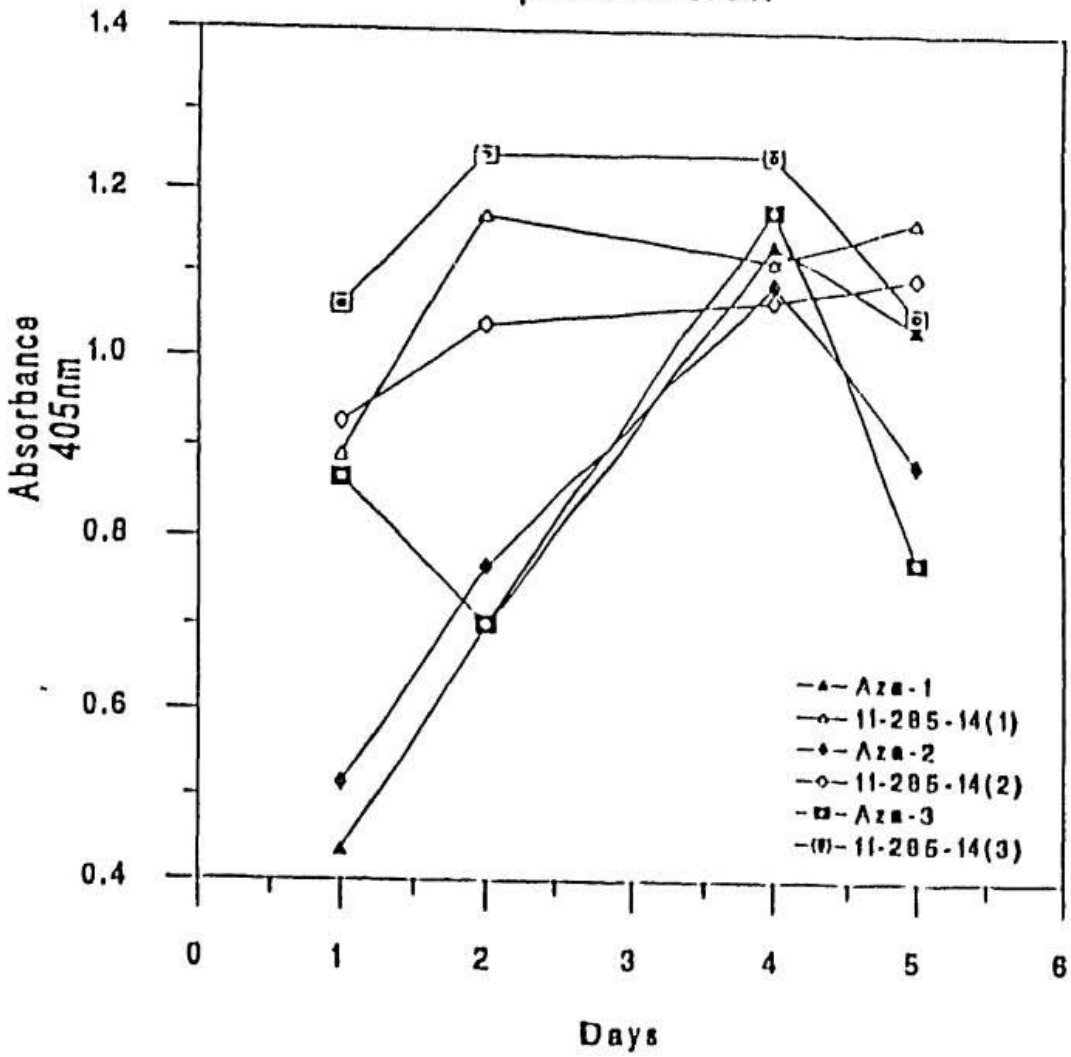


Figure 5. The supernatants of cell lines shown in Figure 4 were tested for anti-CEA production daily by ELISA. The open symbols represent the parental 11-285-14 (1) (2) and (3) compared with the azaguanine resistant Aza-1, 2 and 3. Each point represents the mean value of absorbance readings in triplicate. The standard deviation has been omitted for clarity.

III. 1.2 Anti-CEA production

The three mutant clones were also compared with the parental lines for their anti-CEA production (Figure 5). There was continuous anti-CEA production in the medium up to 5 days of testing. In addition, the anti-CEA activity of all the eight hybridoma fusion partners was tested prior to fusions to confirm anti-CEA production.

III. 1.3 HAT sensitivity

Prior to each fusion, aliquots of the 11-285-14 fusion partner were transferred to HAT selection medium as controls and were found to cease growth and to die confirming their HAT sensitivity.

III. 1.4 Fusion efficiency

Fusion efficiency indicates the success in producing hybrids (Galfre Milstein, and Wright, 1979) and is defined here as the number of hybrids resulting from each fusion. Fusions were performed to assess the eight 11-285-14 azaguanine resistant clones as fusion partners and these are summarised in Table 8a, b & c.

Table 8a: Fusion efficiency of 11-285-14 clones

11-285-14 Fusion Partner/Comments	Fusion #	Total hybrids	Antibody activity of hybrids	
			Anti-CEA	Anti-Dox
Clone A: Poor to moderate growth.	1	0	0	0
	5	14	14	2
	6	2	1	0
	8	0	0	0
	Total = 4	16	15	2
Clone III Good growth and viability.	2	0	0	0
	5	7	7	2
	6	25	1	18
	9	1	0	0
	Total = 4	43	8	20

All were fusions with spleen cells from Dox-KLH immunized mice

Table 8b: Fusion efficiency of 11-285-14 clones

11-285-14 Fusion Partners / Comments	Fusion #	Total Hybrids	Antibody activity of hybrids	
			anti-CEA	anti-Dox
Clone IV: Good growth and viability.	6	6	1	3
	10	0	0	0
	Total = 2	6	1	3
Clone V: Poor growth and viability. Although initially anti-CEA positive, became anti-CEA negative subsequently and was therefore discarded.	3	0	0	0
	5	10	2	1
	6	11	1	6
	Total = 3	21	3	7
Clone VI: Good growth and viability.	4	48	48	4
	5	2	0	1
	6	41	2	11
	7	147	34	74
	Total = 4	238	84	90

All were fusions with spleen cells from Dox-KLH immunized mice

Table 8c: Fusion efficiency of 11-285-14 clones					
11-285-14 Fusion Partner/comments	Fusion #	Total Hybrids	Antibody activity of hybrids		
			anti-CEA	anti-Dox	Dual
Aza-1: Good growth and viability.	14	0	0	0	0
	Total = 1	0	0	0	0
Aza-2: Good growth and viability.	11	56	22	6	1
	12	0	0	0	0
	15	0	0	0	0
	Total = 3	56	22	6	1
Aza-3: Good growth and viability.	13	46	46	19	0
	1-8 (Dox-BSA)*	297	297	126	8
	Total = 9	343	343	142	8

* Fusions 1 - 8 with Aza-3 were performed with spleens from Dox-BSA immunized mice. Rest of the fusions were performed with spleens from Dox-KLH immunized mice.

Although clone VI gave the highest fusion frequency, yielding 59.5 hybrids per fusion, many of the hybrids were negative for anti-CEA and for anti-Dox activity. However, one of the clones, Aza-3, resulted in hybrids that were consistently positive for anti-CEA activity (Table 8c). Aza-3 also had suitable growth and anti-CEA production (Figures 4 & 5) and was therefore selected as the fusion partner for subsequent fusions.

III. 2.0 PRODUCTION OF DOXORUBICIN-PROTEIN CONJUGATES

Doxorubicin was made immunogenic by conjugation to the carrier proteins keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA).

III 2.1 Doxorubicin-KLH conjugates

The initial concentration of doxorubicin (Tables 9a, b & c) is given after the deduction of weight of lactose in doxorubicin hydrochloride (4/5th of the dry weight is lactose). Phosphate buffered saline (PBS) prepared as described in the Materials and Methods was used as solvent in conjugates # 1 and 3 to 15. Distilled water was used for conjugate # 2. However, the results were inconsistent, with the drug protein complex precipitating out of solution prior to gel filtration on the Sephadex column. In addition, there was a poor yield of conjugate. Modification of pH to dissolve the precipitate, although initially successful, could not be reproduced (conjugates 11 & 12, Table 9b).

Table 9A : Doxorubicin-KLH conjugates

#	Initial concentration			Conditions	Conjugates		
	Dox mg	KLH mg	ECDI mg		Dox:KLH molar ratio	Concentrations Dox ug/ml	KLH mg/ml
1	5	15	10	PBS pH 7.2	No conjugate obtained		
2	5	15	10	Distilled Water pH 7.0	No conjugate obtained		
3	5	15	10	KLH dialysed in PBS pH 7.0	130 : 1	5.35	0.45
4	5	20	11.4	KLH dialysed in PBS pH 7.5	190 : 1	7.7	0.6
5	5	15	10	PBS pH 7.4	186 : 1	0.9	0.7
6	5	15	10	PBS pH 7.2	160 : 1	2.4	0.2
7	5	15	10	PBS pH 7.2	290 : 1	4.32	0.18
8	5	15	10	PBS pH 7.2	66 : 1	8	1.9
9	5	20	9	PBS pH 7.35	132 : 1	13.4	1.4
10	5	17	10	PBS pH 7.2	No conjugate		

continued .....

Table 9 b : Doxorubicin-KLH conjugates

#	Initial concentration			Conditions	Conjugates		
	Dox mg	KLH mg	ECDI mg		Dox:KLH molar ratio	Concentrations Dox ug/ml	KLH mg/ml
11	5	14	10	PBS pH 8.0 precipi-tate dissolved	320 : 1	14	0.78
12	5	20	10	PBS pH 8.4	No conjugate		
13	3.85	10.5	10	PBS pH 7.3	No conjugate		
14	2	14	10	PBS pH 7.3	83 : 1	1.65	0.28
15	8.1	21	10	PBS pH 7.7 precipi-tated	No conjugate		
16	0.5	8.1	10	PBS from tablets pH 7.4	57 : 1	2.5	0.5
17	2.26	9	10	PBS from tablets pH 7.4	143 : 1	9	0.7
18	1.8	15	10	PBS from tablets pH 7.2	160 : 1	7	0.5
19	2.6	13	11	PBS from tablets pH 7.2	270 : 1	15	1.0
20	1.6	15	10	(precipitated even before addition of ECDI)			

continued .....

Table 9 $_C$: Doxorubicin-KLH conjugates

#	Initial concentration			Conditions	Conjugates		
	Dox mg	KLH mg	ECDI mg		Dox:KLH molar ratio	Concentrations Dox ug/ml	KLH mg/ml
21	2.6	13	10	PBS from tablets pH 7.2	330 : 1	38.2	1.8
22	3.4 (6.6)	8.4 (16)	10	No conjugate, precipitated immediately			
23	1.6 (5)	11 (15)	10	PBS pH 7.2	323 : 1	18.7	1.2
24	3.4 (5)	4 (15)	10	PBS pH 7.2	305 : 1	6.2	0.3
25	0.7 (5)	15 (17)	11	PBS pH 7.2	568 : 1	25.0	0.7
26	4.2	16	10	PBS pH 7.2	238 : 1	12.7	0.9
27	12	18	10	PBS pH 7.2	163 : 1	8.2	0.8

Legend:
1. Conjugations 1 - 15, 26 & 27: Initial concentration of Dox given as dry weight.
2. Conjugations 16 - 25: Dox concentration based on absorbance at 495 nm.
3. For conjugates # 22 to 25: Parenthesis indicates the dry weight for Dox and KLH.

From conjugate # 16 onwards, PBS made from tablets (Oxoid, England) proved to be a more reliable solvent and the results were more consistent (Tables 9b & 9c). In addition, from conjugate # 16, the precipitated drug was removed by centrifugation, and the dissolved drug concentration was calculated by spectrophotometry. This, more accurate concentration of the drug, is given from conjugate # 16 onwards in Tables 9b and 9c. As illustrated for conjugates 22 to 25, when compared with the dry weight of approximately 5 mg, there was a loss from 50 to 80% of the drug due to precipitation even prior to the conjugation step. The number in parentheses for conjugates 22 to 25 indicates the dry weight of doxorubicin and hemocyanin prior to dissolving in PBS. For conjugates 1 to 15 and for 26, 27, only the initial dry weight of doxorubicin is given.

The Dox-KLH-ECDI mixture was eluted from a Sephadex G-25 column. The timer for eluent flow was set at 5 to 10 minutes per fraction, The volume obtained was approximately 4 ml per fraction and 50 to 60 fractions were collected from each conjugation experiment. Approximately five of these fractions contained the conjugate. This was ascertained by reading each of the fractions at 495 nm and 280 nm. The protein fractions were identified by the readings at 280 nm and the amount of drug calculated from the readings at 495 nm.

Figure 6. Dox-KLH conjugate separation

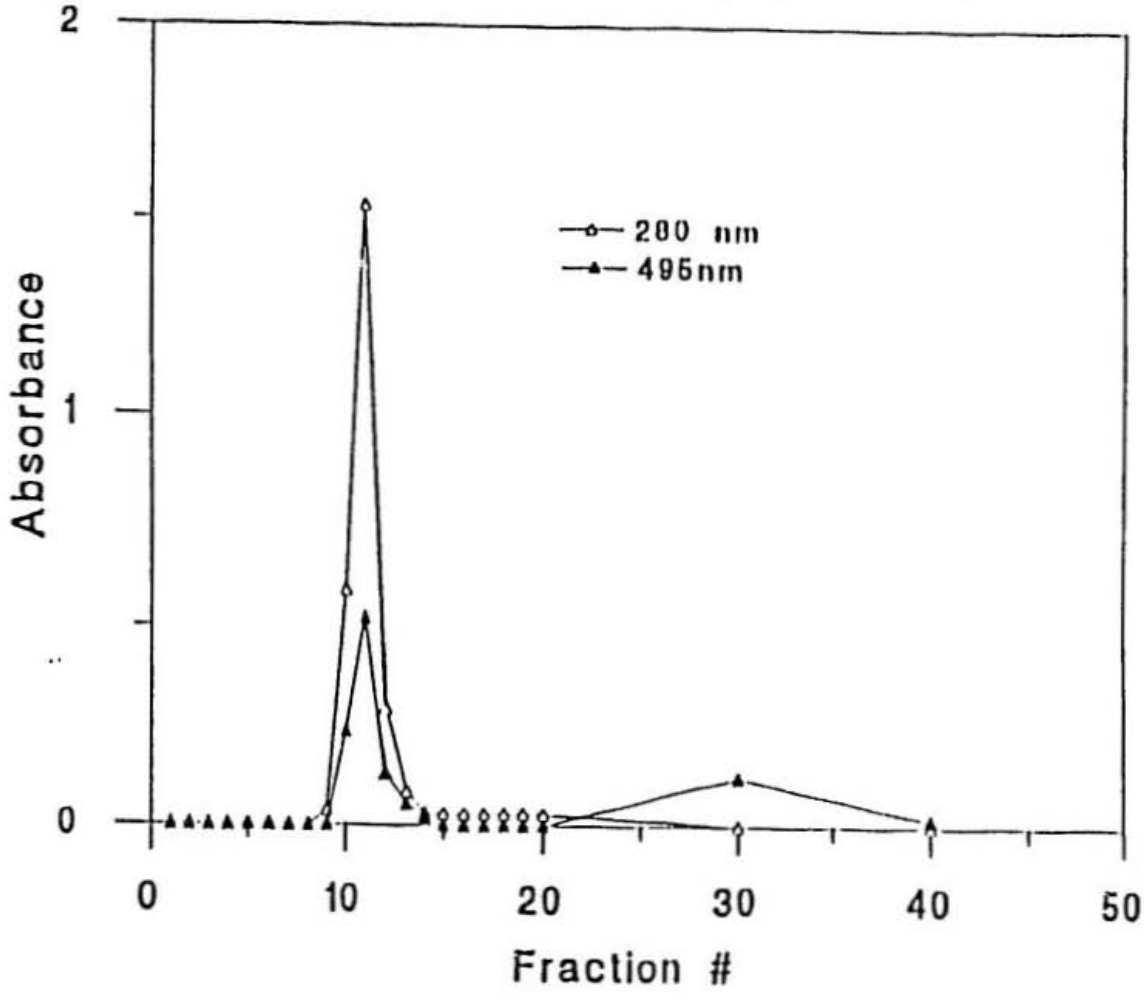


Figure 6. Dox-KLH-ECDI mixture was eluted down a sephadex G-25 gel filtration column. The fractions collected were determined by spectrophotometry for Doxorubicin and KLH. KLH with its higher molecular weight separates first as confirmed by a high 280 nm protein peak. The reading of 495nm in the protein peak indicates the amount of drug conjugated to KLH. The free drug is the last to elute as indicated by a later 495nm peak.

This separation of the conjugate from the free drug for conjugate # 25 is shown in Figure 6.

The results of the various conjugation experiments have been summarised in Tables 9a, 9b & 9c delineating the conjugation conditions and protein drug concentrations. The highest molar ratios for individual fractions before pooling the individual conjugate fractions is indicated in the tables.

III. 2.2 Doxorubicin-BSA conjugates

A list of Dox-BSA conjugates produced with ECDI, the initial drug and protein concentrations and the experimental conditions are shown in Table 10. Since a major portion of the drug precipitates out of solution, the accurate amount of drug used for conjugation prior to loading the column was determined by spectrophotometry and is given in parentheses for some conjugates. This was not checked for all the conjugates, since it did not have a direct bearing on the amount of conjugate produced. PBS made from tablets (Oxoid, U.K.) was used for all conjugations and Sephadex G-25 was used for separation of free and conjugated drug similar to Dox-KLH separations. The individual fractions were of 2 to 4 ml volume and of the greater than 50 fractions collected, about 10 ml of conjugate resulted from each experiment. The molar ratio and concentrations are shown for the individual fractions showing the highest reading after each conjugation. Conjugate #10 separation is illustrated in figure 7 as an example.

Table 10: Doxorubicin-BSA conjugates

#	Initial concentrations in mg			Conditions	Conjugates		
	Dox	BSA	ECDI		Dox:BSA Molar ratio	Concentrations	
						Dox $\mu g\ ml^{-1}$	BSA $mg\ ml^{-1}$
1	10 (2.3)	319.40 (19.6)	19.4	PBS, pH 7.4 Sephadex G-25	0.76 : 1	17.6	2.62
2	5 (2.15)	28 (15.6)	10	"	0.5 : 1	11.6	2.65
3	7	38	15	"	1.6 : 1	18.6	3.39
4	10	68.2	17.5	pH increased to 7.5 using 0.1 M NaOH	0.9 : 1	84.7	10.8
5	6	120	30	PBS, pH 7.2	3 : 1	91.0	4.5
6	9 (2.07)	160 (161)	20	PBS, pH 7.2	2.8 : 1	88.8	3.59
7	17 (2.8)	200 (170)	47	PBS, pH 7.2	5.4 : 1	135	2.8
8	16	183	40	"	5 : 1	116	2.66
9	15	180	40	"	4.1 : 1	106	2.89
10	12.6	197	40	"	5 : 1	128	2.93
11	16	195	39.7	"	5.5 : 1	135	2.81

Parenthesis indicate the amount of drug or protein determined by spectrophotometry.

The weight of Dox used has been corrected to exclude lactose.

Figure 7. Dox-BSA conjugate separation

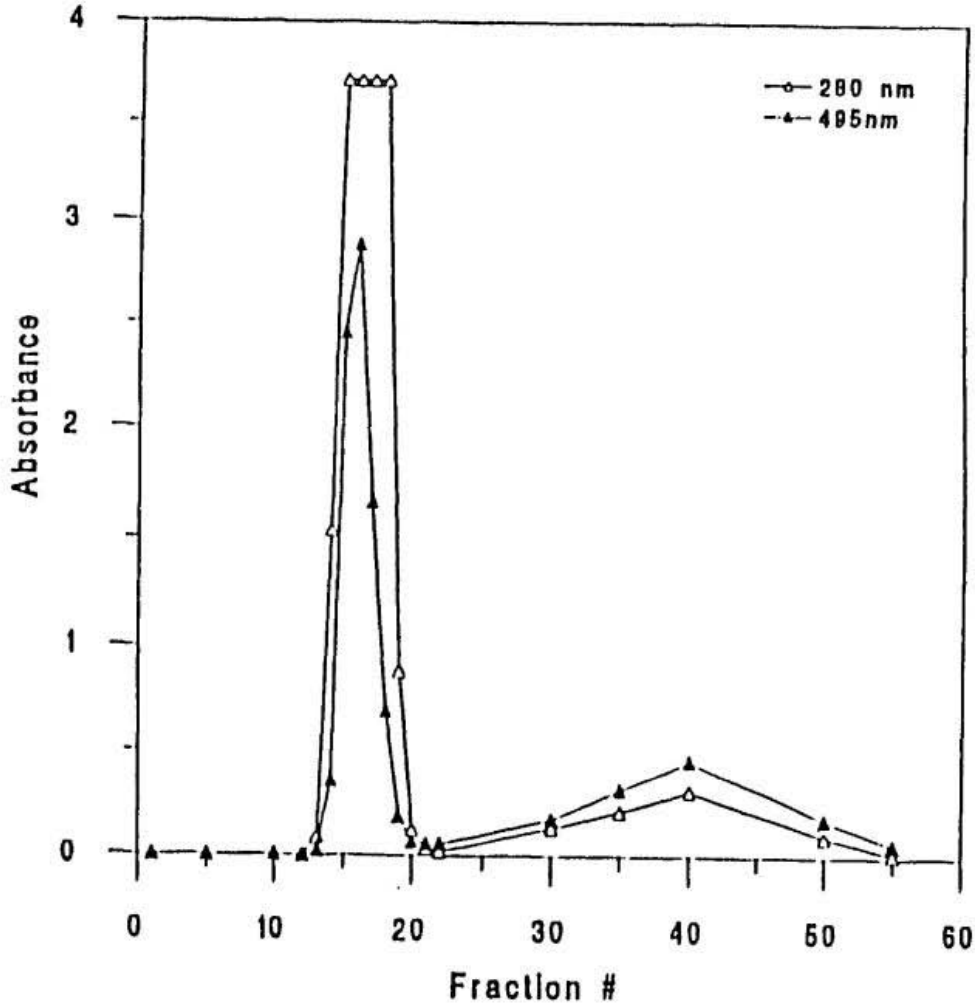


Figure 7. Dox-BSA-ECDI mixture was eluted down a sephadex G-25 gel filtration column. The fractions collected were determined by spectrophotometry for Doxorubicin and BSA. BSA with its higher molecular weight separates first as confirmed by a high 280 nm protein peak. The reading of 495nm in the protein peak indicates the amount of drug conjugated to KLH. The free drug is the last to elute as indicated by a later 495nm peak.

III. 3.0 PRODUCTION OF DOXORUBICIN-ENZYME CONJUGATES

Attempts were made to link doxorubicin to enzymes either directly with heterobifunctional linkers or indirectly through an avidin/biotin bond.

III. 3.1 Doxorubicin-horseradish peroxidase conjugates

Since the anti-CEA and anti-Dox ELISA were standardised using horseradish peroxidase (HRP) labelled detector second antibody, HRP was the enzyme of choice to be conjugated with doxorubicin. A summary of the methods attempted in chemical coupling of the drug to HRP is given in Table 11. The principles behind each approach have been dealt with in the Materials and Methods section II 4.1.

The conjugates were separated from the free drug by Sephadex G-25 gel filtration. The presence of HRP was confirmed by addition of 100 ul of ABTS substrate to 50 μl of each fraction aliquoted into a 96 well microtitre ELISA plate. The plate was read by the E1A Biotech reader at 405 nm.

Table 11: Doxorubicin-HRP conjugates

Method, HRP type and conditions	Highest optical density readings for conjugate fractions obtained			Results and Comments
	280 nm	403 nm	495 nm	
I. Periodate Method				
1. HRP Type VI (RZ = 3.0) Buffer pH 7.4	0.590	0.774	0.346	Fractions # 15 to 26 were positive for enzyme activity and were extensively dialysed. Negative for presence of Dox by ELISA
2. HPR Type VI (RZ = 3.0) Buffer pH 7.3	0.171	0.258	0.098	Similar to above results
3. HRP Type VI (RZ = 0.6) Buffer pH 9.5	3.165	2.346	1.428	Similar to above results
II. Carbodiimide Method				
4. HRP Type VI (RZ = 3.0) Buffer pH 7.3	0.640	2.0	0.235	Similar to above results
5. HRP Type I (RZ = 1.1)	NR	1.779	0.366	Doxorubicin not detected by ELISA
6. ECDI, HRP Type I (RZ = 1.1) NaF 0.001 M	1.081	1.312	0.435	Fractions # 9 to 14 showing highest HRP activity were dialysed to remove excess NaF and tested for Dox by ELISA.

continued .....

Table 11: Doxorubicin-HRP conjugates (continued)

Method, HRP type and conditions	Highest optical density readings for conjugate fractions obtained			Results and Comments
	280 nm	403 nm	495 nm	
7. HRP Type I (RZ = 1.1) NaF, 0.1M and EDTA 0.001 M	1.584	1.750	0.506	Dialysed fractions 9 to 19 showing strong enzyme activity. Negative by ELISA for doxorubicin
III. Glutaraldehyde method				
8. 0.1% glutaraldehyde HRP type I (RZ = 1.1)				Preciptated. No conjugate.
9. HRP Type VI (RZ = 3) Pagé 1987	0.370	0.647	0.150	Negative by ELISA for doxorubicin
10. HRP Type I (RZ = 1.2) Pagé 1987	1.026	0.560	0.561	Same results as above
Dox-β-galactosidase conjugates				
11-13. MBS as the hetero bifunctional linker	Procedure repeated three times. Negative readings by Spectrophotometry and by ELISA			
14. ECDI	No conjugate detected			

The main problems that were associated with coupling Dox to HRP are discussed below.

Firstly, both Dox and HRP are visible at optical density (OD) 280, 403, 495 and 510 nm leading to difficulty in determining the precise molecular concentrations of the drug and enzyme separately. Although the molar extinction coefficient (MEC) of 1% HRP at OD_{510} nm is 6.58 (molecular weight of HRP=40,000; Sigma Chemicals), the difference in readings between OD_{510} nm and OD_{495} nm was negligible and therefore, OD_{495} nm was determined instead of OD_{510} nm. Single fractions with the highest readings obtained for each conjugation are given in Table 11.

Conjugate #7 is illustrated in the following example to show evidence for Dox-HRP conjugation. This conjugate was produced incorporating the modifications suggested by Dr B. Hasinoff, Chemistry Department, Memorial University (Personal communication, also see Materials & Methods). The elution profile of the fraction from this conjugation is given in Figure 8. If we assume OD_{403} nm reading (1.750) to be entirely due to HRP, OD_{495} nm should read 0.218 based on the control readings for HRP as calculated below. For a given control solution of HRP (type I, RZ 1.1) $\frac{OD_{403}}{OD_{280}} = \frac{1.056}{0.926} = 1.1$;

$\frac{OD_{403}}{OD_{495}} = \frac{1.056}{0.132} = 8$; and $\frac{OD_{280}}{OD_{495}} = \frac{0.926}{0.132} = 7$. Extrapolating these values to the conjugate, instead of the expected 0.218 at OD_{495} ($\frac{1.750}{8}$) a reading of 0.506 was obtained at OD_{495} (conjugate #7; Table 11). The difference (0.506 - 0.218) of 0.288 would be accounted for by the drug conjugated to HRP and was calculated to be approximately 13 µg/ml. This figure is a slight underestimation of the drug concentration, since the OD403 reading of 1.75 includes 'contamination' by the drug absorbance as well (usually about a third of the OD_{495} nm of the drug absorbs at OD_{403} nm also).

Having confirmed the presence of doxorubicin as explained above and the presence of HRP by addition of ABTS to the conjugate fractions (Figure 8 and Table 11), the definitive evidence for Dox-HRP conjugate would be recognition by anti-Dox antibodies. The presence of doxorubicin was tested by ELISA. Rabbit serum containing anti-Dox antibodies was used to coat plates and the Dox-HRP employed as the detector. However, the ELISA failed to detect the presence of Doxorubicin in conjugate #7, indicating either absence of Dox or the presence of Dox in such a modified form that it was not recognised by the

Figure 8. Dox-HRP conjugate separation

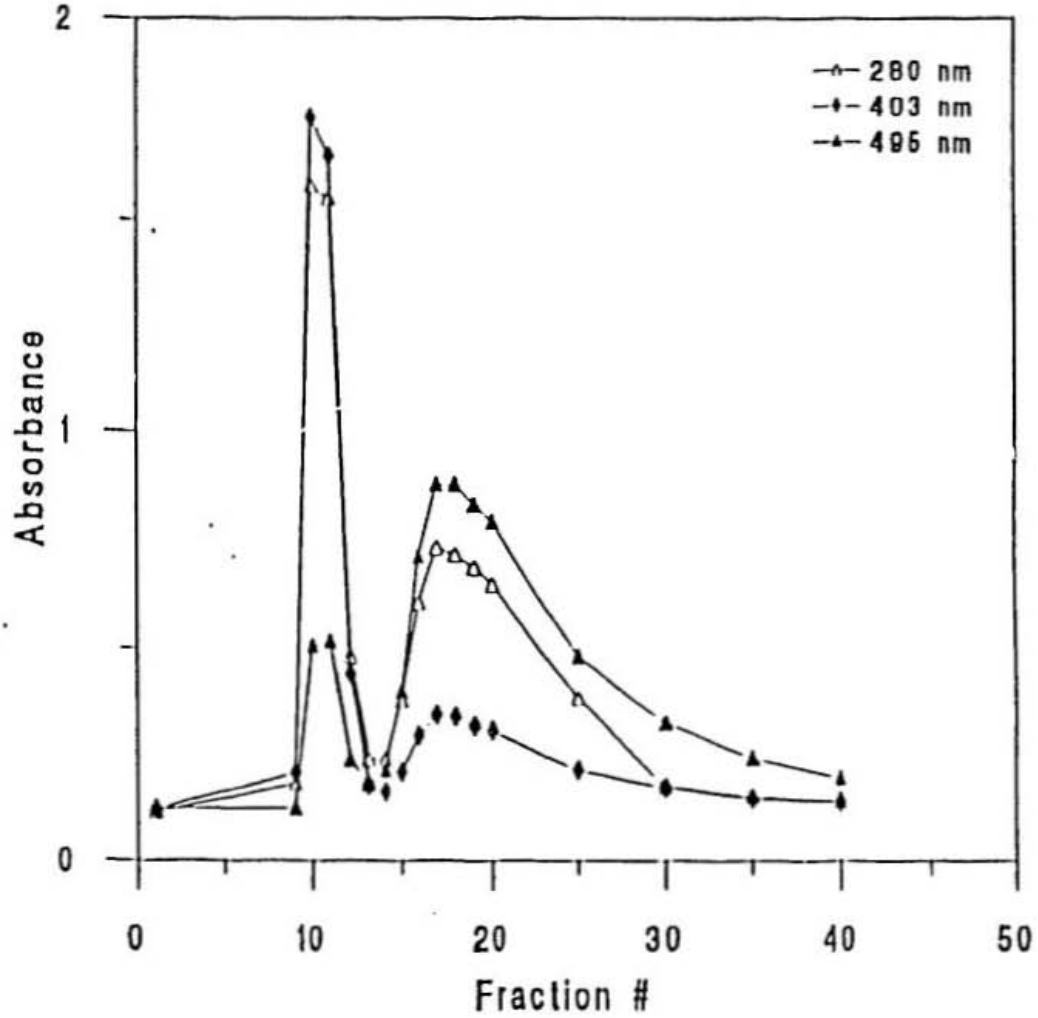


Figure 8. Dox-HRP conjugate fractions are separated from the free drug in a similar fashion to Dox-KLH and Dox-BSA as explained under Figures 6 and 7. However, HRP is determined by both 280nm and 403nm and is shown to be eluted in the initial peak fractions. A simultaneous reading at 495nm of this initial peak indicates the amount of Dox conjugated to HRP. The free drug is the last to elute and the later fractions show predominently Doxorubicin at 495nm.

anti-Dox antibodies. Similar negative results were obtained with Dox (conjugate # 7) used as ELISA coating (data not shown).

The probable reasons for the failure of Dox-HRP conjugation are discussed in detail in the final chapter and include the formation of free superoxide radicals by Dox in solution resulting in self-coupling of the enzyme (Dr. Brian Hasinoff, Chemistry department, Memorial University; personal communication). In addition, the presence of metallic ions in solution could hinder the chemical procedure (Hasinoff, Davey & O'Brien, 1989).

Although ten conjugations were performed to link doxorubicin to HRP, conjugation # 7 has been illustrated above to indicate the final outcome of these experiments. Table 11 summarises results of these direct linkage attempts using periodate, carbodiimide or glutaraldehyde.

III. 3.2 Doxorubicin-β-galactosidase conjugates

The only reported enzyme labelling of Dox in the literature was that of Dox-β-galactosidase by Fujiwara et al, (1981) using the heterobifunctional reagent M-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS). This procedure was attempted three times without success (Table 11). An attempt was also made to link Dox and β-galactosidase with ECDI but was unsuccessful (Table 11).

III 3.3 Doxorubicin-avidin and doxorubicin-biotin conjugates

Due to the failure of the above methods, indirect methods were attempted to link doxorubicin through an avidin/biotin bridge. The conjugates obtained were tested with biotin peroxidase or streptavidin-peroxidase as the indicator in ELISAs. A summary of the various conjugation experiments is shown in Table 12.

Table 12: Doxorubicin-Avidin/Biotin conjugates

#	Initial Material	Cross-linker	Results
(1)	Dox + Avidin	ECDI	Separated by gel filtration. No conjugate.
(2)	Dox-BSA-succinyl	N-hydroxy succinimide biotin ester	The mixture was extensively dialysed and up to 1/10 dilution containing 2μg ml^{-1} of Dox was tested by ELISA. Negative results.
(3)	Dox-BSA	N-hydroxy succinimide biotin ester	Similar negative results as above. ELISA #453 Dox-BSA-Biotin recognised by Rabbit αDox abs.
(4)	Dox + Avidin succinic anhydride	ECDI	Definite conjugate on gel filtration up to 24 μg/ml of Dox at 495nm. Dox was not recognised by rabbit anti-Dox antibodies, both as coating and indicator.
(5)	Dox-BSA + Avidin	ECDI	Separated by Sephadex G-75. Negative by ELISA.
(6)	Dox-BSA-Biotin	Biotin-hydroxy succinimide ester	Extensive dialysis and negative by ELISA.
(7)	Dox-BSA with Biotin and DMSO	Biotin-hydroxy succinimide ester	Extensive dialysis and tested by ELISA. No significant difference between pre and post-immune rabbit anti-Dox sera.

Dox-avidin conjugate (# 4, Table 12) appeared to be the most promising, the separation from the free drug is shown in Figure 9. The conjugate yield contained up to 24 μg of doxorubicin per ml, when assessed by spectrophotometry. ELISAs were then performed with Dox-avidin as the coating antigen. The presence of doxorubicin was not detected by rabbit post immune serum containing anti-Dox antibodies (Figure 10). To confirm the activity of the rabbit anti-Dox antibodies, the same solutions of pre-immune and post immune sera were tested in the ELISA using comparable quantity of Dox-BSA as the coating. As shown in Figure 10, there was a greater than six fold positive difference with the post-immune serum, supporting the presence of anti-Dox antibodies and appropriate coating. Alternatively, the rabbit serum was used to coat the ELISA plates and Dox-Avidin conjugate (# 4, Table 12) was next added. Biotinylated HRP as the indicator did not indicate the presence of conjugate by ELISA (results not shown). These results suggest that although doxorubicin appears to be linked to avidin by spectrophotometry, it is probably present in a modified form not recognised by the anti-Dox antibodies.

Attempts were also made to link doxorubicin to avidin or biotin using Dox-BSA or Dox-HSA (human serum albumin) conjugates in order to promote protein-protein conjugation. These experiments were unsuccessful, and are summarised in Table 12.

Figure 9. Dox-avidin conjugate separation

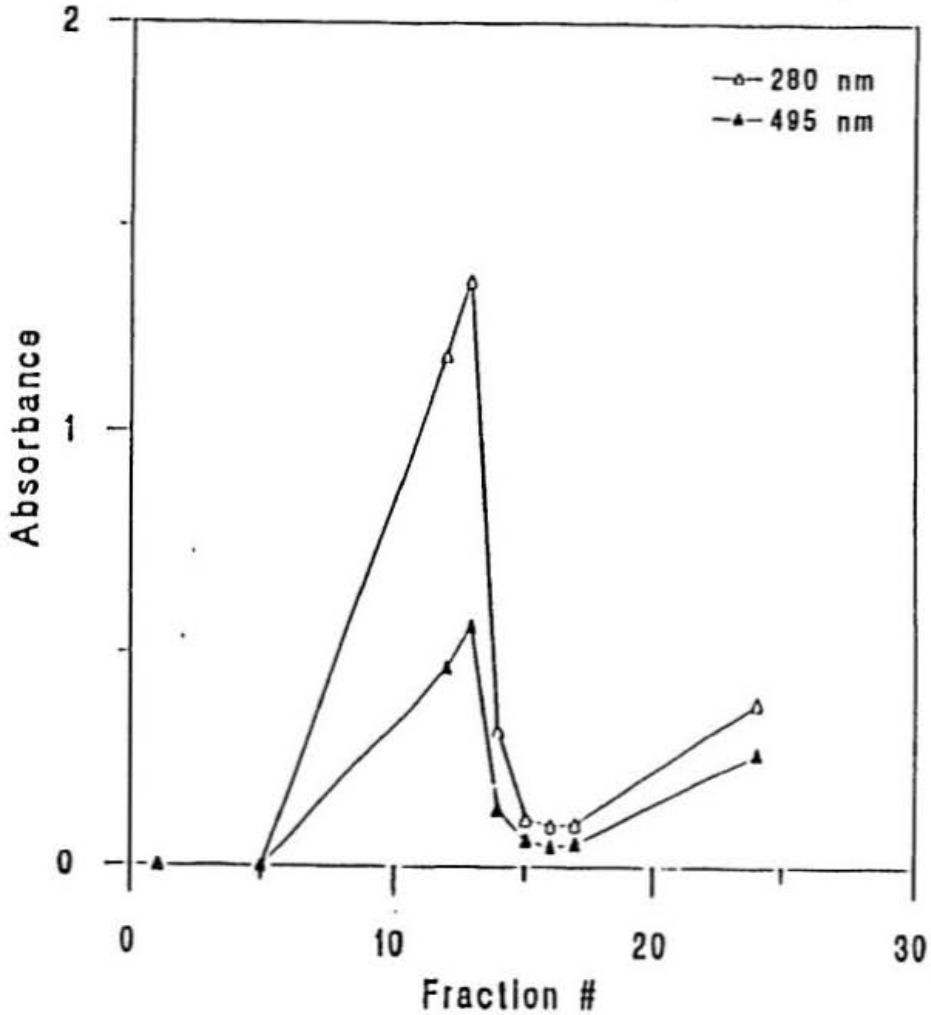


Figure 9. Dox-avidin mixture is eluted down a sephadex G-25 gel filtration column. The initial 280nm peak in the fractions indicates the presence of the protein (avidin). The reading of 495nm in this initial peak indicates the conjugation of Dox to avidin. The smaller molecular weight Dox elutes at the later peak, calculated from the reading at 495nm.

Figure 10. Dox-avidin conjugate

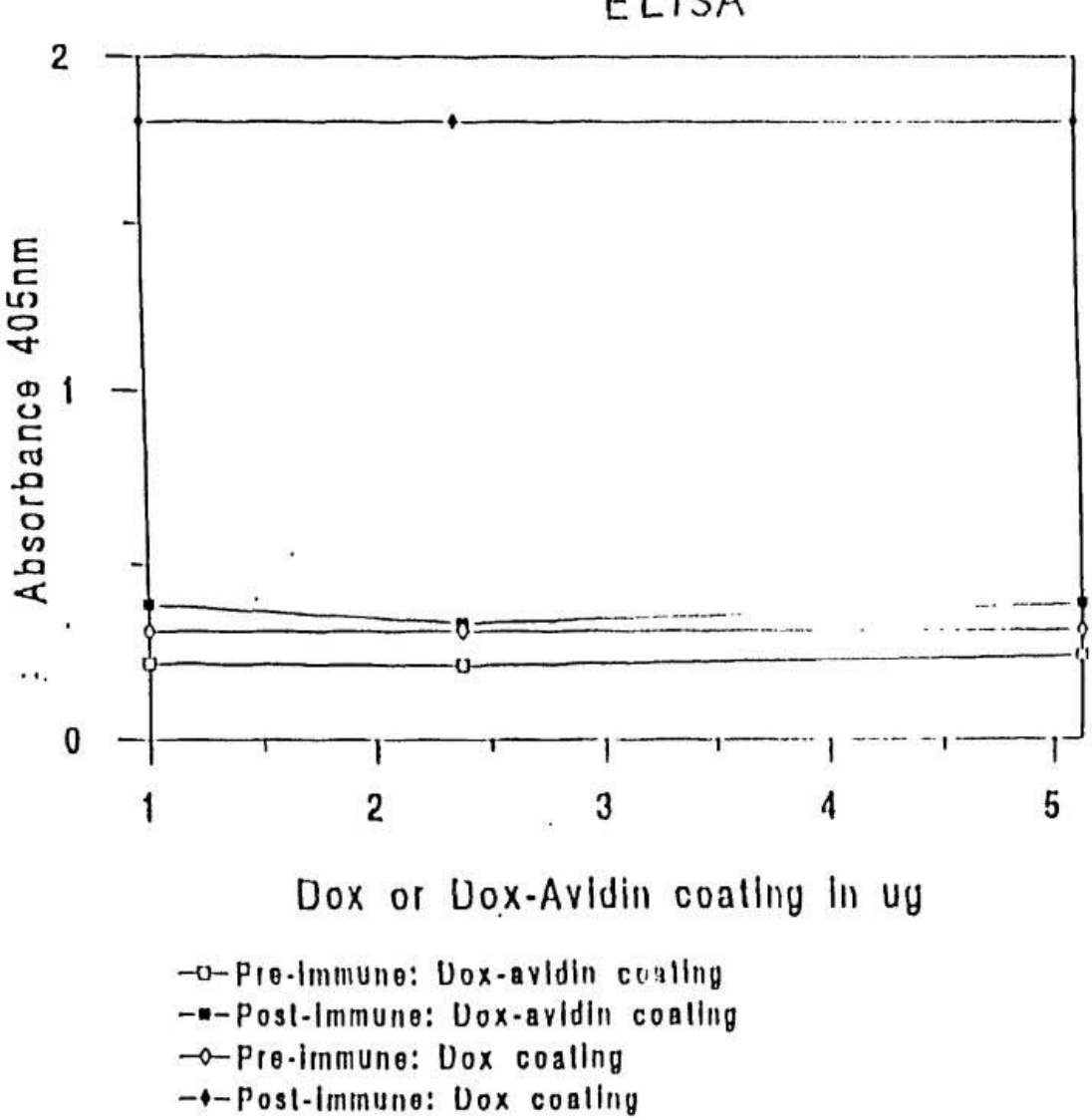


Figure 10. Dox-avidin fractions eluted as the initial peak in Figure 9, were evaluated by ELISA for the presence of Doxorubicin. The concentration of Dox in Dox-avidin fractions was calculated by spectrophotometry and used an ELISA coating as shown.

III 4.0 PRODUCTION OF CEA-HRP CONJUGATE

The unsatisfactory results of enzyme labelling Dox led to the exploration of an alternative approach in developing a bispecific antibody assay. The periodate method was used to label CEA with HRP (type VI, RZ 3) and the CEA-HRP was separated by Sephadex G-75 (Mol. wt. of CEA = 180,000 and HRP = 40,000). All the HRP and CEA in solution appeared to be conjugated since there was no free enzyme or CEA peaks visualised after the appearance of the CEA/HRP peak (Figure 11). 50 μl of each fraction was aliquoted into each well of an ELISA plate. On addition of the substrate ABTS, the CEA-HRP fractions (Figure 11) correlated with strong HRP activity. To confirm that (a) CEA was conjugated to HRP and (b) CEA-HRP could be recognized by anti-CEA Mab (11-285-14), ELISAs were performed as outlined under Materials and Methods. Figure 12 illustrates these results. CEA-HRP thus produced was used to develop an ELISA to detect BsMabs directly.

Figure 11. CEA-HRP conjugate separation

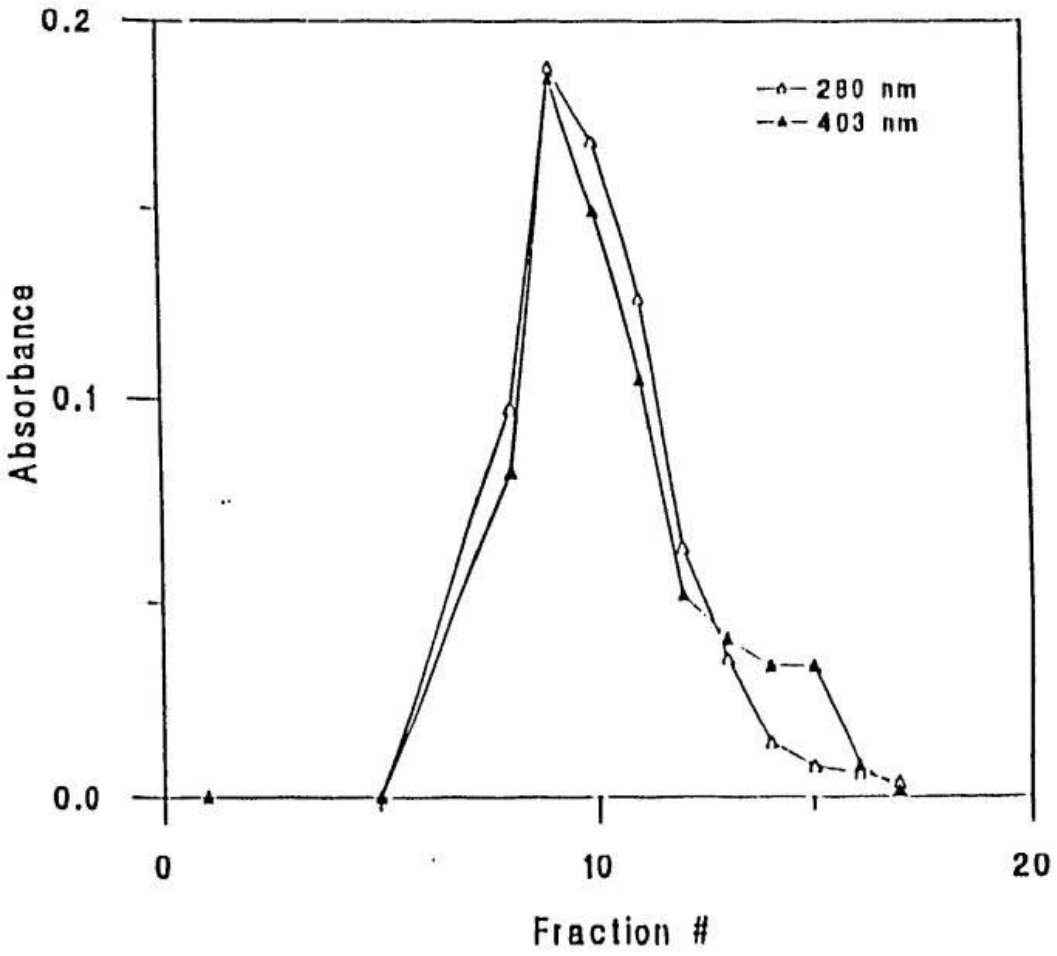


Figure 11. CEA-HRP mixture was eluted down a sephadex G-75 gel filtration column. The fractions collected were determined by spectrophotometry for CEA (280nm) and HRP (403nm).

Figure 12. CEA-HRP ELISA

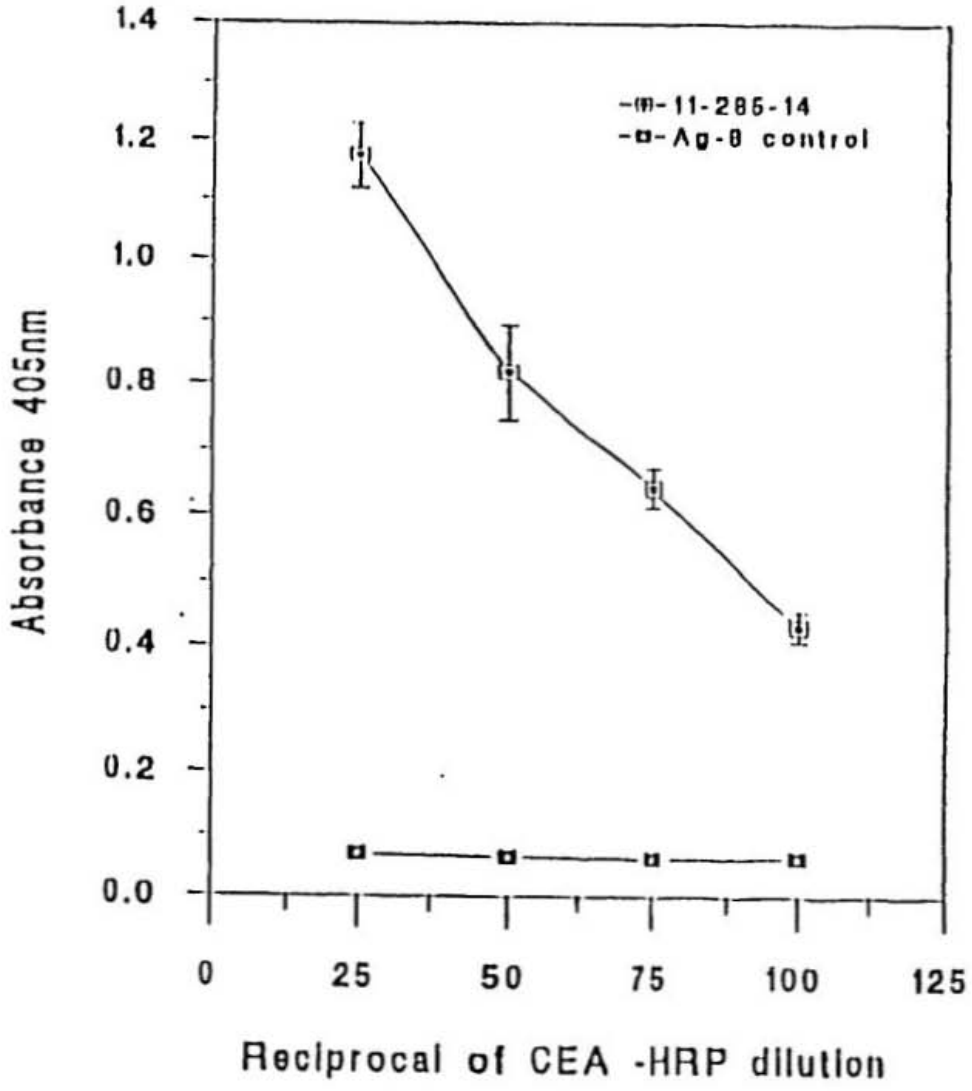


Figure 12. The CEA-HRP conjugate peak fractions separated as shown in Figure 11 were pooled and tested by ELISA. ELISA plate was coated with 5ug/ml of 11-285-14 (anti-CEA). Ag-8, (non specific antibody that does not recognise CEA) was used as a coating control. Reciprocal of CEA-HRP dilutions added is shown here. Each point represents the mean value of absorbance readings in triplicate +/- twice the standard deviation.

III. 5.0 DEVELOPMENT OF ENZYME LINKED IMMUNOSORBENT ASSAYS

III. 5.1 Development of anti-CEA ELISA

The appropriate CEA coating was determined by evaluating CEA coating concentrations ranging from 0.625μg to 20μg per ml. The coating was tested with anti-CEA 11-285-14 at 0.1 to 1000ng per ml concentrations. CEA coating at 5μg per ml was found to be twice as sensitive as 2.5μg/ml (Figure 13). The results were identical for 5 and 10μg/ml CEA coating and therefore CEA was employed at a coating concentration of 5μg/ml in anti-CEA ELISAs.

The optimal dilution of the second antibody indicator, rabbit anti-mouse immunoglobulin horseradish peroxidase (RAM-HRP) was determined using CEA coating at 5μg/ml. 11-285-14 at a concentration rang. of 0.1 to 1000 ng/ml was used as the first antibody. 1/1000 dilution of RAM-HRP was selected for future ELISAs, after comparison with dilutions ranging from 1/250 to 1/4000 (Figure 14).

Figure 13. CEA coating standardisation

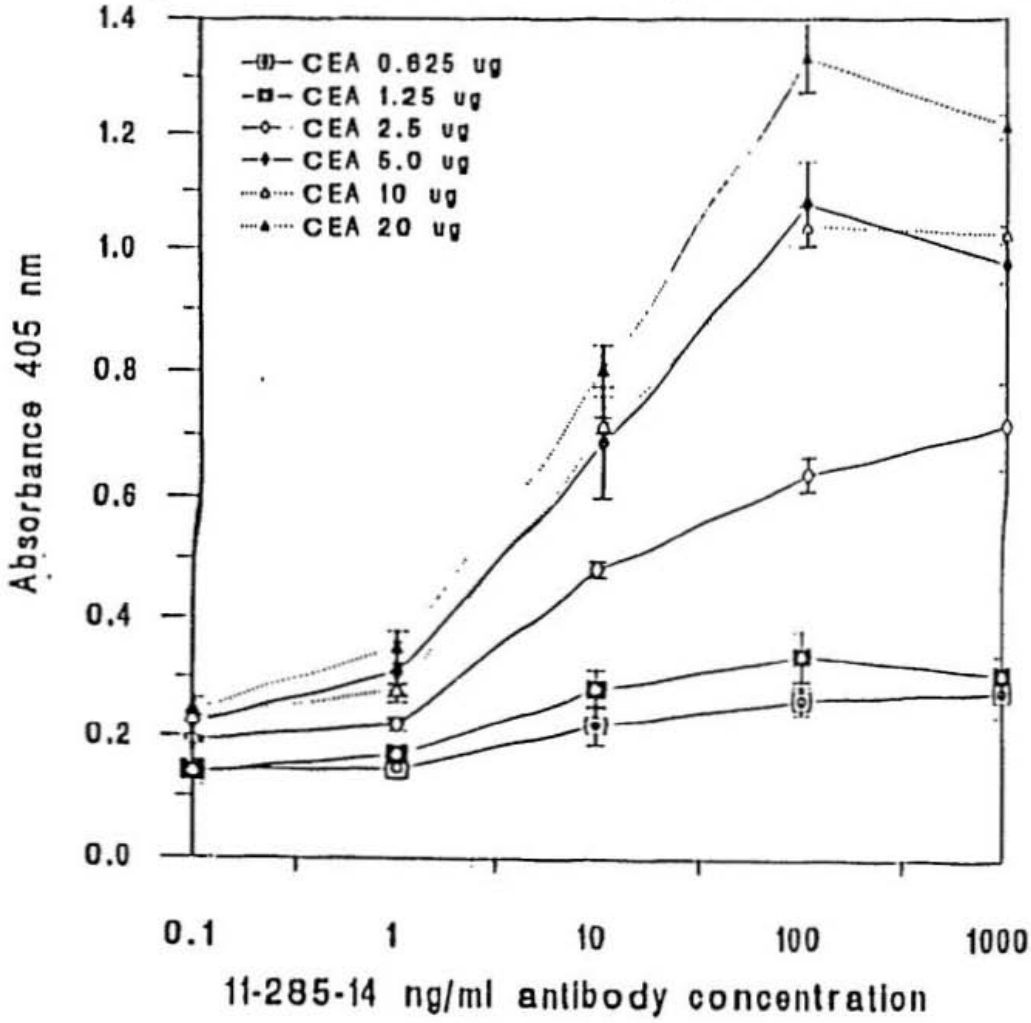


Figure 13. ELISA performed with CEA coating range from 0.625ug to 20 ug/ml. 11-285-14 (anti-CEA) antibody was added in varying concentrations as shown. 5ug/ml CEA coating was selected for future ELISAs. Each point represents the mean value of absorbance readings in triplicate +/- twice the standard deviation.

Figure 14. RAM-HRP standardisation

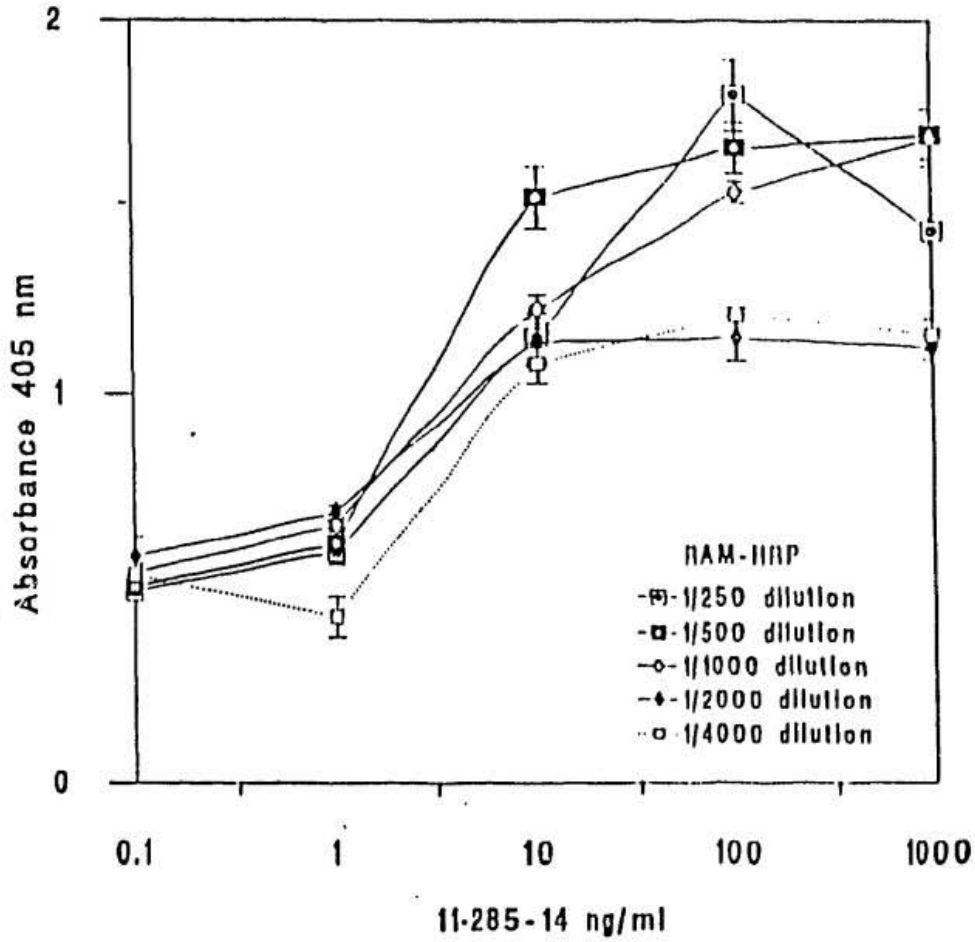


Figure 14. ELISA performed with CEA coating at 5ug/ml. Varying concentration of 11-285-14 Mab added as shown. Varying range of rabbit anti-mouse immunoglobulins labelled with HRP (RAM-HRP) dilutions, were evaluated as the indicator. Each point represents the mean value of absorbance readings in triplicate +/- twice the standard deviation.

III. 5.2 Development of anti-doxorubicin ELISAs

Following immunization of a rabbit with the Dox-KLH conjugates (see below: Section III.6.0), the rabbit serum was used to develop an anti-doxorubicin ELISA. The optimal doxorubicin coating was selected by comparing doubling concentrations of doxorubicin from 1μg/ml to 8μg/ml (Figure 15). The rabbit post immune serum (containing anti-Dox antibodies) was the first antibody. The second antibody detector was goat anti-rabbit immunoglobulins linked to HRP used at a dilution of 1 in 500.

The results (Figure 15) are illustrated using varying range of doxorubicin as the coating. Since there was no significant difference between the readings obtained with various coating concentrations, 2μg/ml Dox was selected as the coating concentration for future assays. The results are shown in Figure 15. Due to the questionable stability of Dox (Section IV 2.2) and the reproducibility of the assay, Dox-BSA coating at a concentration of 2ug Dox/ml was employed for testing hybrids obtained from Dox-BSA fusions. The results with Dox-BSA coating were similar to assays using Dox alone (data not shown).

Figure 15. Dox coating standardisation

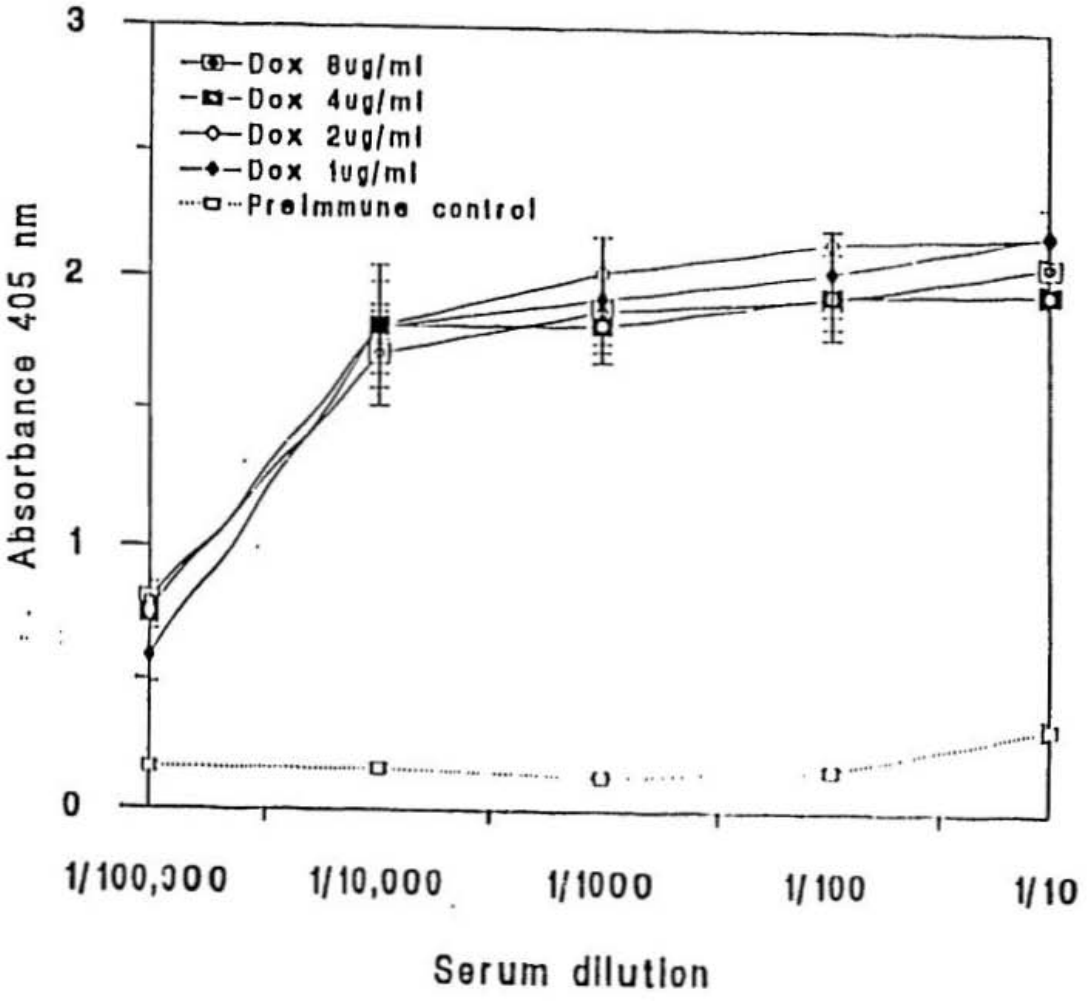


Figure 15. ELISA performed with doubling concentrations of Dox coating ranging from 1ug/ml to 8ug/ml. Rabbit post-immune serum (containing anti-Dox antibodies) was added in varying dilutions as shown. Pre-immune serum (no anti-Dox) was added in similar dilutions as control. Each point represents the mean value of absorbance readings in triplicate +/- twice the standard deviation.

III. 5.3 Development of anti-BSA ELISAs.

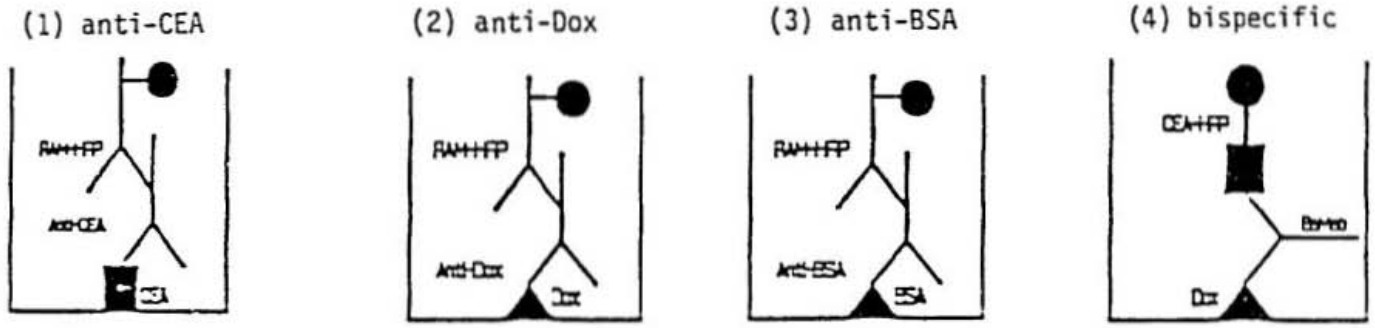
Subtraction assays were performed to select hybrids from Dox-BSA fusions. Hybrids were tested for both anti-Dox BSA activity (as above), and anti-BSA activity by ELISAs. Hybrids that were positive in anti-Dox-BSA and negative in anti-BSA ELISAs, were selected for further expansion. The coating for anti-BSA assays was selected for individual assays, based on the parallel anti-Dox-BSA ELISAs. While the doxorubicin coating in the Dox-BSA assays were constant (2μg/ml), the BSA quantity varied, depending on the batch of Dox-BSA conjugate produced (Section III 2.2., table 10). The accurate quantity of BSA was determined by spectrophotometry (see Materials and Methods) prior to each Dox-BSA assay and a similar concentration used in the corresponding anti-BSA ELISA. This eliminated any possible discrepancy between the anti-Dox BSA and anti-BSA ELISA results due to a difference in the BSA coating. The remaining steps in the anti-BSA ELISA were identical to the anti-Dox BSA assay.

III 5.4 Development of bispecific antibody Assay

Since HRP was the enzyme utilised in anti-CEA and anti-Dox ELISAs, several attempts were made to enzyme label Dox with HRP. Dox-HRP would then be used to develop an ELISA to detect dual activity directly. However, enzyme labelling experiments of Dox with HRP and β-galactosidase were unsuccessful (Sections

III 3.1, 3.2 and 3.3). A CEA-HRP conjugate was then produced (Section III 4.0) and was found to be recognised by anti-CEA antibodies (Figure 12). A direct ELISA for detection of bispecific antibodies was developed using the CEA-HRP conjugate. Hybrids that were positive in both anti-Dox and anti-CEA ELISAs, were then selected for dual activity ELISA testing directly. ELISA plates were coated with doxorubicin (for Dox-KLH fusions) or Dox-BSA (for Dox-BSA fusions). Test hybrid supernatarts were added next for assessing anti-doxorubicin binding. CEA-HRP was then added, which, if recognised by the other arm of the bispecific antibody yielded a positive result on addition of the substrate (ABTS). The various ELISAs developed are illustrated diagrammatically in Figure 16.

Figure 16. Diagrammatic illustration of ELISAs



Each figure represents a single well of an ELISA plate

(1) anti-CEA assay

(2) anti-doxorubicin assay

(3) anti-BSA assay

(4) bispecific assay

RAM-HRP: rabbit anti-mouse immunoglobulins HRP

III. 6.0 EVALUATION OF IMMUNOGENICITY OF DOX-PROTEIN CONJUGATES

III. 6.1 Immunogenicity in a rabbit

The immunogenicity of Dox-KLH conjugates was evaluated by immunizing a rabbit according to the schedule in Table 5 (Materials and Methods). The rabbit serum drawn on different days (Table 5) was tested for anti-Dox antibodies by ELISA. The serum drawn prior to immunization was used as control (pre-immune serum). Doxorubicin by itself was used for ELISA coating. Results show the presence of high titre anti-Dox antibodies up to a 1/100,000 dilution of the sera (Figure 17), indicating successful immunogenicity of Dox-KLH.

III 6.2 Immunogenicity in mice

On confirming the immunogenicity of Dox-KLH in the rabbit, mice were immunized and the spleens of these mice were used for fusions. The immunization schedules, along with the amount of Dox in Dox-KLH, adjuvant and the route of administration have been summarised in Tables 6a, 6b and 6c (Materials and Methods). The length of the immunization varied for each fusion and the reasons for this are discussed in Chapter IV.

On the day of the fusion, the mice were terminated and the spleens used for fusion. The sera from these mice were tested for anti-Dox antibodies by ELISA. As in the rabbit, the sera of the mice indicated successful immunization with an anti-Dox titre of up to 1/100 to 1/1000. These results are presented under the appropriate fusions (Figures 18, 19 & 20).

Figure17. Rabbit anti-Dox antibodies

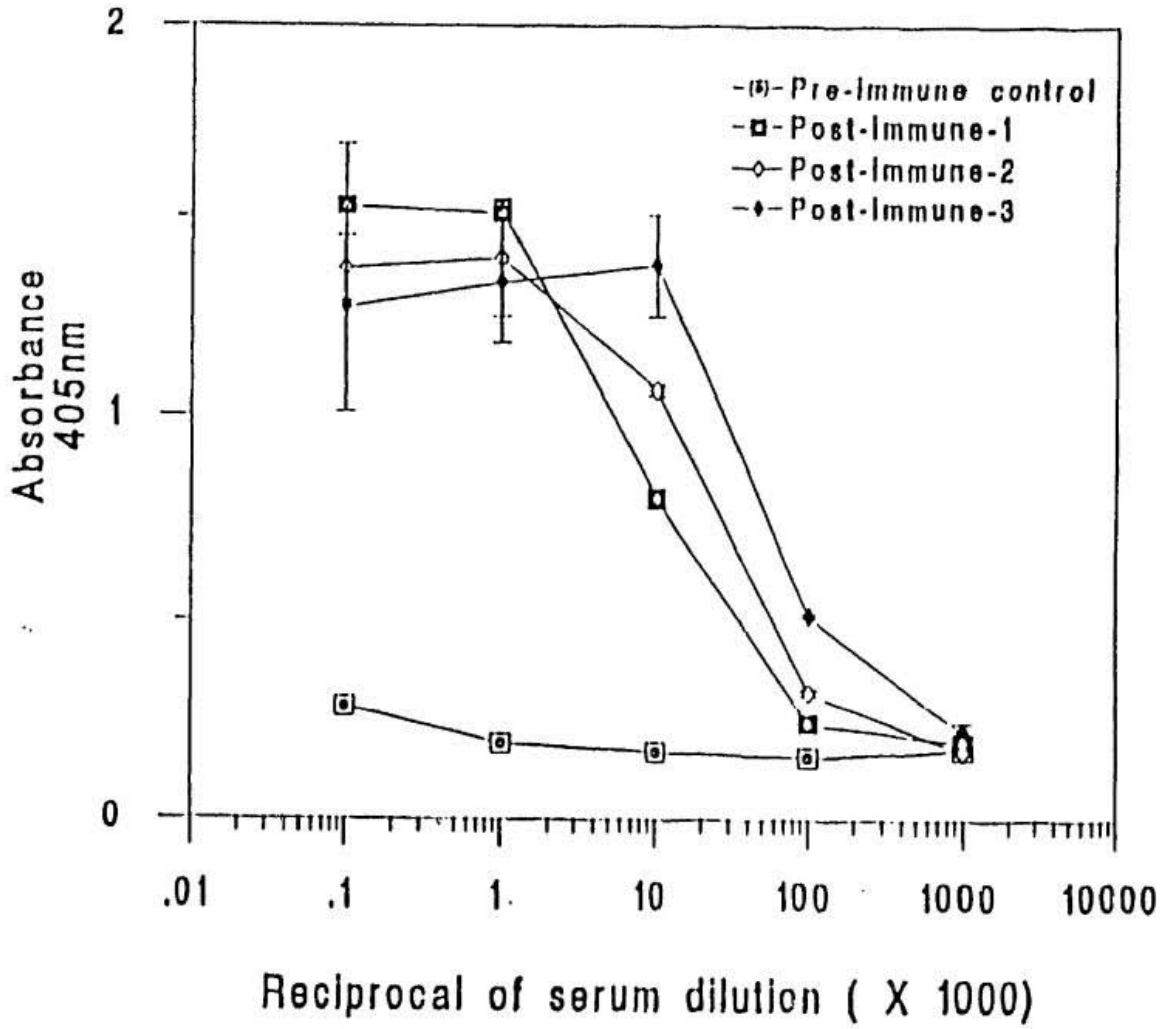


Figure 17. ELISA performed with Dox coated plates. The post-immune rabbit serum drawn at different intervals 1,2 and 3 (Table 5, section II. 7.0) was evaluated in varying range of dilutions. The reciprocal of dilutions is shown. Rabbit serum drawn prior to immunization was employed as control. Each point represents the mean value of absorbance readings in triplicate +/- twice the standard deviation.
Titre in this thesis is defined as the highest dilution giving an absorbance higher than the control value + 2 standard deviations.

III. 7.0 DOX-KLH (11-285-14) BISPECIFIC FUSIONS

III. 7.1 Introduction

Fusions were performed using the methodology described in Materials and Methods. The immunization schedules for each fusion has been tabulated in Tables 6a, 6b & 6c (Materials and Methods). The number of spleen cells used for each fusion and the total number of 11-285-14 hybridoma cells used, including their viability, spleen cell:11-285-14 ratio and the cell density per well are summarised in Table 13. Individual fusions are described below according to the total number of hybrid colonies obtained and subsequent clones, and their reactivity in anti-CEA and anti-Dox ELISAs. BsMab ELISAs using CEA-HRP conjugate were performed only for those hybrids and clones positive in both anti-CEA and anti-Dox ELISAs. The hybrids obtained from Dox-KLH immunised mice fusions are summarised at the end of the Dox-KLH individual fusions (Section III 7.12) (Table 21).

III 7.2 Dox-KLH Fusions 1, 2 and 3

These were trial fusions performed to become familiar with the procedure and to evaluate three different batches of azaguanine resistant 11-285-14 cells as fusion partners. A spleen from a single mouse was divided and used for the three fusions (Tables 6a & 13). No hybrids resulted from these fusions.

Table 13 : Dox-KLH fusion details

Fusion #	Total Viable spleen cells x10^6	% viability of spleen cells	11-285-14 clone used	Total viable 11-285-14 cells x10^6	% viability of 11-285-14	Spleen cell : 11-285-14 ratio	Cell density per well
1	42	88%	A	7.0	87%	6 : 1	1.0 x10^5
2	28	88%	III	5.6	80%	5 : 1	0.7 x10^5
3	35.7	88%	V	7.3	84%	5 : 1	0.8 x10^5
4	40	90%	VI	8.8	82%	5 : 1	1 x10^5
5	41.75	88%	A	8.28	93%	5 : 1	5.2 x10^5
	41.75	88%	III	8.236	99%	5 : 1	5.2 x10^5
	41.75	88%	V	6.4	85%	6 : 1	5.0 x10^5
	41.75	88%	VI	0.6	66%	69 : 1	4.4x 10^5
6	46	92%	A	9.2	89%	5 : 1	2.8 x10^5
	46	92%	III	8.0	72%	5.75 : 1	2.8 x10^5
	46	92%	IV	9.2	91%	5 : 1	2.8 x10^5
	46	92%	V	9.2	82%	5 : 1	2.8 x10^5

continued .....

Table 13 : Dox-KLH fusion details

Fusion #	Total Viable spleen cells $x10^6$	% viability of spleen cells	11-285-14 clone used	Total viable 11-285-14 cells $x10^6$	% viability of 11-285-14	Spleen cell : 11-285-14 ratio	Cell density per well
6	46	92%	VI	9.2	88%	5 : 1	2.8×10^5
7	143	99%	VI	29.4	96%	4.9 : 1	3.6×10^5
8	83.4	94%	clone A	16.68	98%	5 : 1	2×10^5
9	45.6	95%	III	9.18	97%	4.9 : 1	1.14×10^5
10	67.2	96%	VI	13.4	97%	5 : 1	1.6×10^5
11	90.6	95%	Aza-2	19.34	96%	4.7 : 1	2.2×10^5
12a	40	95%	Aza-2	8.525	97%	4.7 : 1	2.5×10^5
13	106	94%	Aza-3	23.1	96%	4.6 : 1	2.6×10^5
14	79.4	96%	Aza-1	16.1	97%	4.9 : 1	1.9×10^5
15a	35.7	94%	Aza-2	8.36	95%	4.3 : 1	2.2×10^5
16a	111	95%	Aza-3	20	91%	5.2 : 1	2.2×10^5

III 7.3 Dox-KLH Fusion 4

The immunization schedule is given in Table 6a. The mouse serum yielded 1/1000 anti-Dox antibody titre (Figure 18). The ratio of spleen cells and 11-285-14 (batch # VI) cells used in this fusion along with their viability and density per well are summarised in Table 13a. The hybrids obtained were tested by ELISA. The code number of the ELISAs performed, the number of hybrids tested and the number that were positive in anti-CEA and anti-Dox assays is shown in Table 14a. A total of 48 hybrids were obtained from this fusion. 45 of these hybrids were positive for anti-CEA activity and 11 for anti-Dox activity as well. Bispecific assays using CEA-HRP were performed, but the results were negative and they are not shown in this table.

Hybrids were selected based on ELISA readings when compared to background control readings. RPMI-GLN-FCS medium was used as negative control. Nine individual hybrids which had the highest absolute ELISA readings (OD_{405nm}) in both anti-CEA and anti-Dox assays are depicted in table 14b. These hybrids were further expanded into larger wells or 50 ml flasks.

Two of the positive hybrids (# 4 A6 and 3 C7, Table 14b) were cloned. The 4 clones obtained were tested for anti-Dox activity, but were found to be negative (ELISA 345; data not shown). The hybrids that were initially positive for anti-CEA

Figure 18. Dox-KLH Fusions 1 to 5 sera

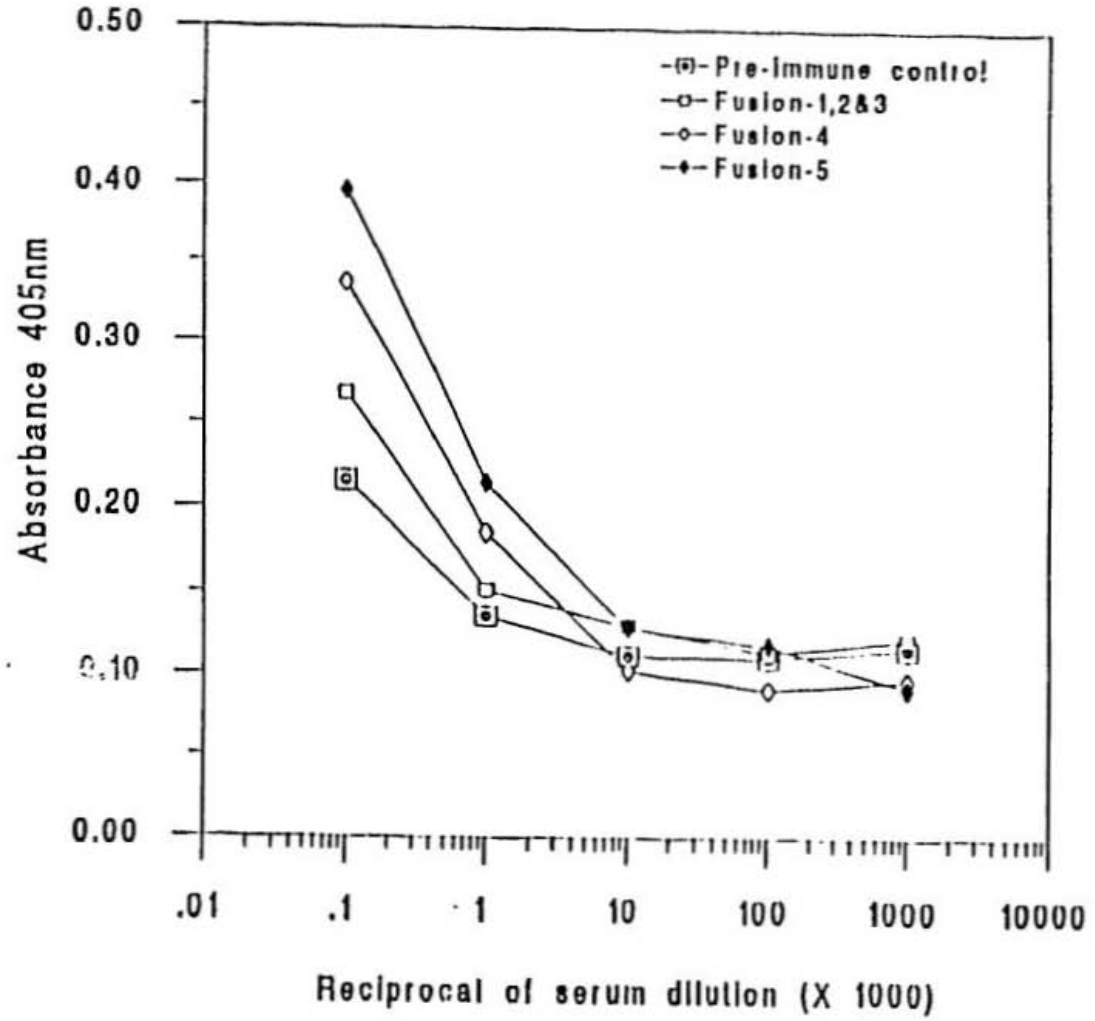


Figure 18. ELISA performed with Dox (2ug/ml) coated plates. The sera of mice drawn on the day of fusion was tested for anti-Dox acitivity, in dilutions as shown. Pre-immune control was from a non immunised mouse (no anti-Dox). Each point represents mean of the absorbance values read in triplicate. Standard deviations have been omitted for clarity.

Table 14a: Dox-KLH Fusion 4 results			
ELISA #	Number of wells tested	Anti-CEA activity	Anti-DOX activity
294	39	39	1
297	32	32	4
303	41	39	0
306	43	3	0
310	44	41	6
312	43	40	11
315	13	NT	6
320	23	NT	5
326	13	NT	8
330	10	NT	1
331	28	NT	4
343	10	NT	0
345	8 (4 clones)	NT	0
346	8	6	NT
351	5	4	NT
352	5	NT	4
353	4	NT	NT
354	4	NT	NT
359	2	NT	2
367	1	1	NT
Total hybrids	48*	45	11

NT: Not tested simultaneously in both anti-CEA and anti-Dox assays due to limited quantity of supernatants

Legend for Table 14a: As the hybrid colonies appeared in wells, the supernatants were tested by repetitive ELISAs. Total hybrids (*) represent the hybrid colonies obtained in the fusion and not the sum total tested. Clones are not counted as original hybrid colonies and therfore not included in the total count. For example, as the first 39 wells containing hybrid colonies appeared, the supernatants were tested in serial ELISAs starting from ELISA #294. In ELISA #310, the same 39 wells were retested in addition to 5 new colonies. However most of these colonies ceased to proliferate or were negative for anti-Dox activity.

Table 14b: Dox-KLH fusion 4 ELISA results

ELISA #	Anti-CEA reading			Anti-DOX reading	
	Hybrid code	OD_{405}	Mean + 2SD	OD_{405}	Mean+ 2SD
297	III B_2	1.002	0.135	0.262	0.107
	IV A_2	0.514		0.309	
	V F_1	0.487		0.197	
	V G_2	0.501		0.265	
312	4A6	0.442	0.077	0.186	0.080
	3C7	1.180		0.108	
320	4A6	Not tested (NT)		0.389	0.135
	3D8			0.494	
	5F6			0.582	
351 & 352	3G11	0.711	0.050	0.129	0.086
	5C9	0.732		0.153	
	4A6	0.040		0.082	

NT= Not tested due to limited quantity of supernatant available.
M + 2SD = Mean + standard deviation (x2) of background control values.
OD= Absorbance at 405 nm

and anti-Dox activities, including hybrid #4A6, subsequently turned negative for both activities (Table 14b).

III. 7.4 Dox-KLH Fusion 5

Immunization details are given in Table 6a (Materials and Methods). The spleen cells were divided into four and fusions were performed with four different azaguanine resistant 11-285-14 batches (Table 13). Due to an error in calculation 1 x 10^6 cells of clone VI were used instead of 6 x 10^6 (table 13). Therefore, the ratio between spleen cells and 11-285-14 was 69:1. The mouse serum revealed 1/1000 titre anti-Dox antibodies by ELISA (figure 18).

Fusion # 5 resulted in 33 hybrids. 14 were from clone A as fusion partner. 7 hybrids resulted from clone III, 10 hybrids from clone V, and 2 from clone VI as 11-285-14 fusion partners. This individual breakdown for these clones has been compared in Tables 8a & 8b as part of their fusion efficiency. The number of ELISAs performed and their serial numbers are given in Table 15. However, these hybrids could not be propagated in culture.

Table 15: Dox-KLH Fusion 5 results			
ELISA #	Wells tested (including subclones)	Anti-CEA activity	Anti-DOX activity
306	17	17	0
311	18	18	0
313	22	15	0
320	31	NT	2
331	27	NT	4
346	2	1	NT
352	3	NT	2
367	4	2	NT
370	2	2	NT
Total hybrids	33	18	6

NT = Not tested
Legend as for Table 14a

III 7.5 Dox-KLH Fusion 6

Two mice were utilised for this fusion. Six injections of Dox-KLH were given, the total dose of doxorubicin being 41 µg per mouse (Table 6a). Spleen cells from both mice were pooled yielding 2.3 x 10^8 cells. In order to compare the fusion efficiency of the different 11-285-14 fusion partners, the spleen cells were divided and five separate fusions were performed (Table 13). Two 96 well culture plates were used for each batch of 11-285-14. Although a smaller number of clone III was available, with poorer viability (72%, Table 13), the viable cell density in each well was comparable around 2.8 x 10^5. The mouse sera obtained prior to fusion were tested by anti-Dox ELISA and showed up to 1/1000 titre anti-Dox antibodies (Figure 19). Since the spleen cells were divided, and five different 11-285-14 fusion partners were used, the fusion efficiency of these five 11-285-14 clones could be compared. The individual breakdown has been given previously in Tables 8a & 8b and is as follows. Clone VI yielded the greatest number of hybrids, 41. Clones III, IV, V and A, resulted in 25, 6, 11 and 2 hybrids respectively. The ELISAs performed and the serial numbers are given in Table 16. In order to simplify the selection process, it was decided to perform anti-Dox and/or bispecific ELISAs as the initial assays. Hybrids shown to be positive in these ELISAs were then

Figure 19. Dox-KLH Fusions 6 to 10 sera

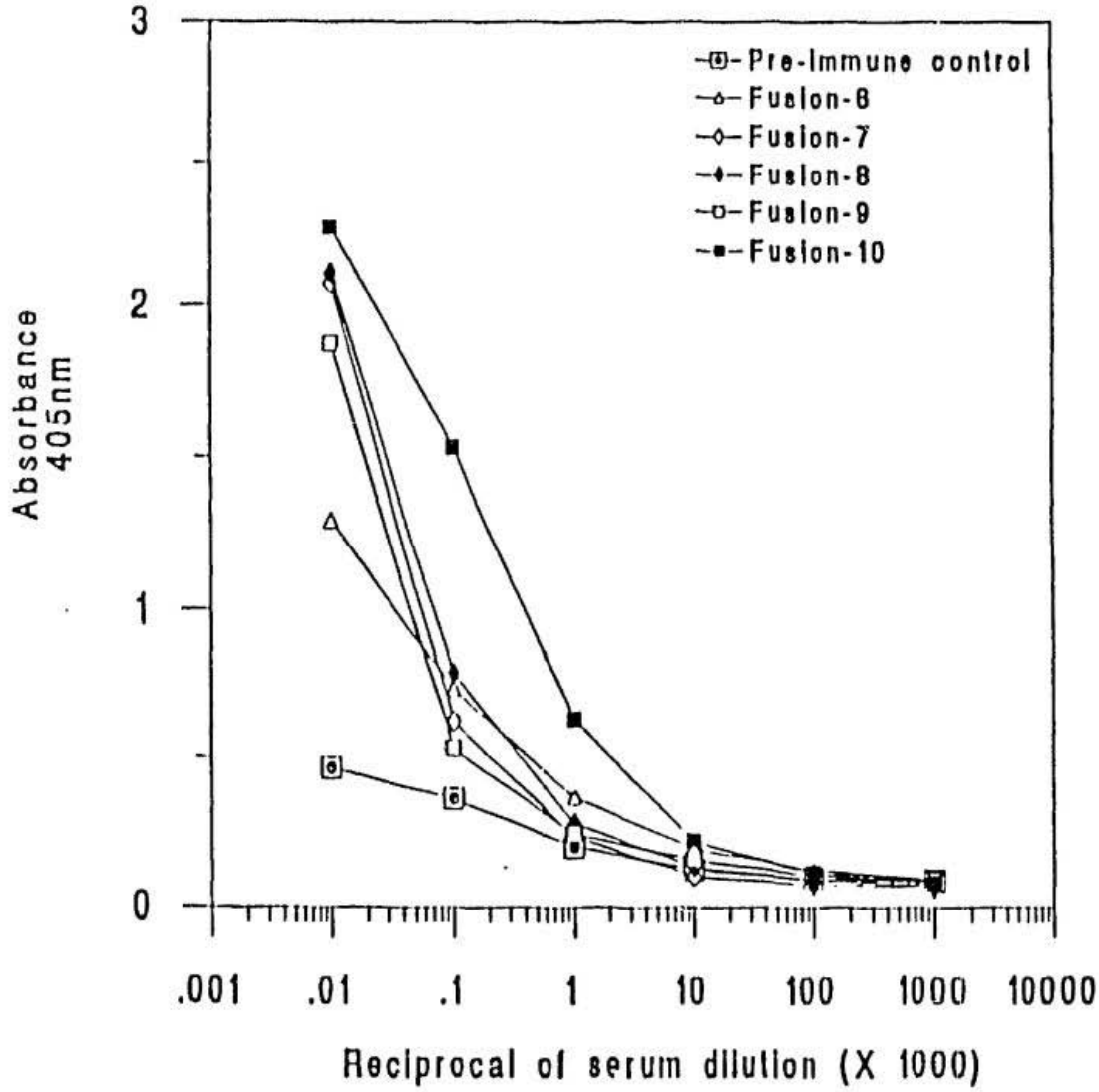


Figure 19. Legend as for Figure 18. The serum was collected from the mice on the day of fusions and tested for anti-CEA activity.

considered for anti-CEA ELISA. However, although 44 hybrids (out of the 95 total) were anti-Dox positive initially, on subsequent testing they were found to be negative in both anti-Dox as well as bispecific assays. 13 out of 23 hybrids tested showed anti-CEA activity.

Table 16 : Dox-KLH Fusion 6 results

ELISA #	Wells tested	anti-CEA activity	anti-DOX activity	BsMab activity
326	40	NT	19	NT
330	86	NT	31	NT
331	8	NT	0	NT
337	38	NT	NT	0
343	24	NT	0	0
344 & 345	20	NT	0	0
346	20	12	NT	NT
351	2	2	NT	NT
352	9	NT	6	NT
353	8	NT	NT	0
354	2	NT	NT	0
367	1	0	NT	NT
Total hybrids	95	13	44	0

NT = Not tested
Legend as for Table 14a

III. 7.6 Dox-KLH Fusion 7

Fusion 7 was performed 132 days following the primary immunization (Table 6b). 19.8 μg of doxorubicin was the total immunization dose. The number of spleen cells obtained was 14.3 x 10^7. Since clone VI gave the highest fusion frequency in the previous fusion (43% of the total hybrids), compared to four other 11-285-14 fusion partners (Fusion 6 & Table 8b), clone VI was selected for this fusion. The mouse serum obtained prior to the fusion contained up to 1/100 anti-Dox antibody titre (Figure 19). Five plates were used in the fusion with a cell density of 3.6 x 10^5 per well (Table 13).

At this stage, some technical problems involved in the selection process of hybrids will be mentioned. A total of 146 hybrids were obtained in the fusion, the highest number up to that time. Some of these hybrids were seen as early as two weeks after the fusion while others appeared as late as one month after the fusion. Sequential assays were necessary to test new hybrids as well as to retest hybrids that were positive in earlier ELISAs. Approximately 100μl of hybrid supernatant was available for ELISA testing. Taking any more supernatant would disturb the hybrid colony at the bottom of the well. Given this limited amount of supernatant, only one ELISA could be performed at a given time. Although the supernatant could be diluted and used for all three ELISAs,

this was avoided. The dilution would diminish the antibody concentration increasing the chance of false negative ELISAs.

It was therefore elected to perform the bispecific antibody ELISA directly, as an initial screening assay. However, 104 out of the total 146 hybrids obtained from Fusion 7, when tested in the bispecific assay were found to be negative (ELISA 354 & 355; Table 17a). Surprisingly, when many of these hybrids were tested for anti-Dox activity in ELISAs 359 and 360, they were found to be positive (Table 17b). Hence, suspecting the early negative bispecific ELISA results were due to loss of anti-CEA activity, an anti-CEA ELISA was performed (ELISA 363 a & b; Table 17a). Only 14 out of the 148 hybrids tested (9%) were anti-CEA positive, confirming that the negative bispecific ELISA results were likely due to loss of anti-CEA antibody production, rather than loss of anti-Dox activity. The weak ELISA readings of bispecific assays of some hybrids is given in table 17b. Three of the hybrids that were positive in anti-doxorubicin ELISAs 359 and 360 were cloned. All of the 51 clones obtained were found to be negative in anti-CEA assay (# 367, Table 17a), thus eliminating the possibility of bispecific antibodies.

Table 17a: Dox-KLH Fusion 7 results				
ELISA #	Wells tested (including clones)	Anti-CEA activity	Anti-DOX activity	BsMab activity
353	45	NT	NT	0
354 & 355	104	NT	NT	0
359 & 360	155	NT	74	NT
361	92	NT	NT	10
362	73	NT	NT	11
363a,b	148	14	NT	NT
364	58	NT	NT	6
366	14	1	NT	NT
367	51 (all subclones)	0	NT	NT
370	1	0	NT	NT
Total Hybrids	146	14	74	21

Legend for Table 17a as for Table 14a

Table 17b: Dox-KLH Fusion 7 ELISA results							
ELISA #	Hybrid Code	Anti-CEA readings		Anti-Dox readings		BsMab readings	
		OD405	M+2SD	OD405	OD 405	OD 405	M+2 SD
359/360 & 361	1-14	0.103	0.078	0.766	0.100	0.098	0.11
	1-1	0.082		0.104		0.138	
363/364	3-27	0.298	0.178	NT		0.107	0.09
	4-32	0.348	0.151	NT		0.099	

BsMab = Bispecific Monoclonal Antibody
OD = Optical density at 405 nm
M + 2SD = Mean + 2 x Standard deviation of control in triplicate
NT = Not tested

III. 7.7 Dox-KLH Fusions 8, 9 and 10

Due to the loss of anti-CEA activity of hybrids resulting from B VI as 11-285-14 fusion partner (fusion 7), fusions 8, 9 and 10 were performed using clones A, III and VI as fusion partners (Table 13). However, no hybrids resulted from these fusions, despite a 1/1000 anti-Dox antibody titre detected in the serum of the mouse used for Fusion 10 by ELISA (Figure 19).

III. 7.8 Dox-KLH Fusion 11

The immunization protocol used is shown in Table 6c. Seven injections of Dox-KLH were given and the fusion performed 294 days after the primary immunization. A total of 24.5μg of doxorubicin was used. The serum of the mouse obtained prior to the fusion yielded a 1/1000 antibody titre (Figure 20). Clone aza-2 was utilised as fusion partner in this fusion (Table 13).

59 hybrids resulted from this fusion. In keeping with the preceeding paragraphs, hybrids were first tested in anti-CEA and anti-doxorubicin ELISAs. Those positive in both ELISAs were then evaluated for dual activity using CEA-HRP conjugate. The ELISAs and the number of hybrids tested, including 16 subclones are given in Table 18a. The highest ELISA readings with the corresponding hybrid codes is given in table 18b.

The problems in selecting hybrids in this fusion were similar to fusion 7. As illustrated in Table 18b (ELISAs 413

Figure 20. Dox-KLH Fusions 11 to 15 sera

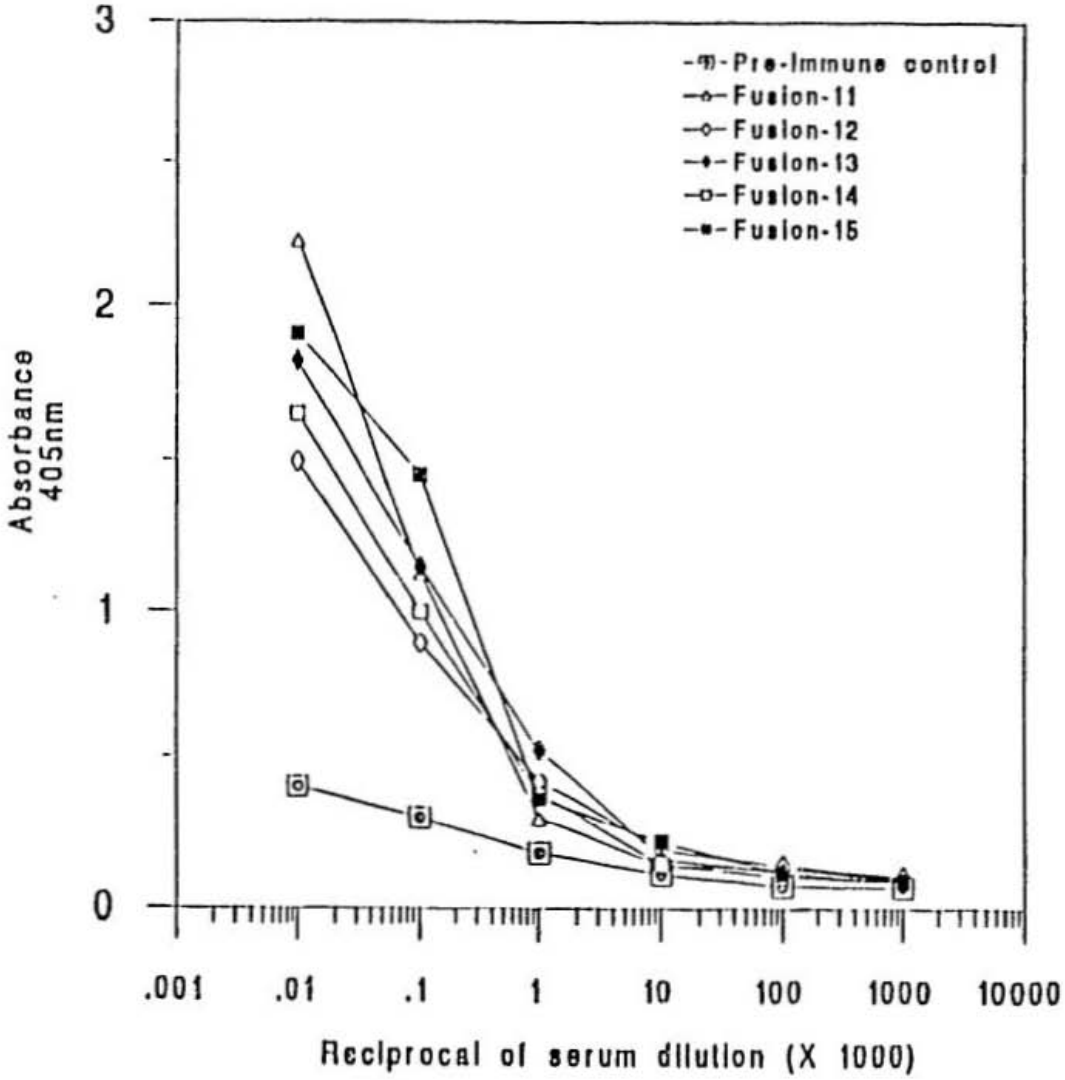


Figure 20. Legend as for Figure 18. The sera of mice were drawn on the day of Dox-KLH fusions 11 to 15 and tested for anti-Dox activity.

& 414), some hybrids like 3-11 were positive for anti-CEA and negative for anti-Dox and vice versa for hybrid # 3-6. Furthermore, hybrids like 1-1 were initially positive in all three ELISAs (# 401-403), but on cloning (eg. 1-1-3) became negative for anti-CEA and only weakly positive for anti-Dox activity (Table 18b).

Table 18a: Dox-KLH fusion 11 results

ELISA #	Wells tested	Anti-CEA activity	Anti-Dox activity	BsMab activity
401	26	26	NT	NT
402	28	NT	14	NT
403	42	NT	13	NT
404a&b	26	6	0	0
405	19	15	NT	NT
406, 407 & 408	28	20	2	2
409	5	0	0	0
411	30	NT	7	NT
413	6	0	1	NT
414	17	5	7	NT
415a	16	0	9	NT
b	11	7	3	NT
417a	16	1	4	NT
b	4	2	0	NT
420	2	1	1	NT
424	4	NT	NT	1
Total Hybrids	59	26	23	3

NT = Not tested due to limited quantity of supernatant.
Legend as for Table 14a.

Table 18b: Dox-KLH fusion 11 ELISA results

ELISA #	Hybrid Code	Anti-CEA reading		Anti-Dox reading		BsMabs reading	
		OD_{405}	M+2SD	OD_{405}	M+2SD	OD_{405}	M+2SD
401, 402 403	1-1	0.185	0.070	0.146	0.100	0.11	0.100
	1-3	0.283		0.089		0.11	
	1-5	0.174		0.092		0.09	
	2-9	0.153		0.092		0.09	
406 407 408	1-1	0.099	0.072	0.087	0.08	0.09	0.08
	3-11	0.997		0.065		0.07	
	3-25	1.026		0.073		0.08	
413 & 414	1-1	0.095	0.148	0.112	0.093	Not tested	
	3-11a	0.841		0.080			
	3-11b	0.944		0.081			
	3-25	0.757		0.088			
	3-6	0.078		0.316			
417 & 420	1-1-3	0.068	0.072	0.079	0.071	Not tested	
	3-11a	0.283		0.072			
	3-11b	0.299		0.066			

M + 2SD = Mean + standard deviation (x 2) of background control values
OD = Optical density at 405nm

III. 7.9 Dox-KLH Fusion 12a

Two fusions were performed using the spleen from a mouse immunized with the protocol shown in Table 6c. Aza-2 was the 11-285-14 fusion partner (fusion 12a). The second fusion was performed with 6-thioguanine resistant NS-1 myeloma cells (fusion 12b). No hybrids were obtained with the 11-285-14 fusion (12a). However, 31 hybrids were obtained with the NS-1 fusion (12b). Results of the NS-1 fusion are given later (Section III.8.0).

III. 7.10 Dox-KLH Fusion 13

Fusion 13 was performed 243 days after primary immunization. 15.35μg of doxorubicin was given as Dox-KLH in divided doses (Table 6c). 10.6×10^7 spleen cells were obtained and clone Aza-3 (11-285-14) was used as the fusion partner at a ratio of 4.6:1 (Table 13). The mouse serum showed an anti-Dox titre of 1/1000 by ELISA (Figure 20).

Of the 45 hybrids resulting from this fusion all tested positive for anti-CEA and 30 for anti-Dox. The ELISAs and the number of hybrids tested are summarised in Table 19a. Two of the strongly positive anti-Dox hybrids, # 17 and # 23 (Table 19b) were cloned. 61 clones were obtained from these two hybrids, with 7 demonstrating weak bispecific activity. The highest ELISA readings are given in Table 19b.

Table 19a: Dox-KLH Fusion 13 results

ELISA #	Wells tested	anti-CEA activity	anti-DOX activity	BsMabs activity
420a b	16 24	16 NT	NT 21	NT NT
421	32	NT	19	NT
424 & 425	35	35	30	23
430 & 431a	46 24 (clones)	46 NT	27 8	NT NT
431b*	35	NT	7	NT
433 & 434	59	59	36	NT
435	44	44	1	NT
437	43	43	6	0
438 & 439	16 5	16 5	0 2	0 0
441, 442 & 443	61	57	7	7
447, 448 & 449	61	57	0	NT
453	12	NT	0	NT
458	12	12	0	NT
Total hybrids	106 (45 + 61 clones)	103	55	23 + 9 clones

NT = Not tested
*ELISA 431b was performed with Dox-KLH coated plates
Legend as for Table 14a

Given the weak reactivity in anti-Dox ELISAs compared to anti-CEA, the hybrids were tested for activity against Dox-KLH coated plates (ELISA 431b). Since the mice were immunised with Dox-KLH, it was anticipated that many of the hybrids would be strongly reactive against Dox-KLH. However, only 7 (20%) out of 35 tested showed anti-Dox-KLH activity (Table 19a). The ELISA readings for two of these hybrids (25 and 30) are given in Table 19b (ELISA 431b). Hybrids # 17 and 23, which were previously positive for anti-Dox in ELISA 421 turned out to be negative for anti-Dox-KLH, in ELISA 431b. This illustrates the instability of antibody production by the hybrids.

III. 7. 11 Dox-KLH Fusions 14 and 15

These two fusions were performed with Aza-1 and Aza-2 clones of 11-285-14, respectively. Half of the spleen cells from fusion 15 were also utilised for fusion with NS-1 myeloma cell line. The immunization protocol and fusion details are summarised in Tables 6c and 13. Sera from the immunized mice yielded a 1/1000 anti-Dox antibody titre (Figure 20). No hybrids resulted from these fusions.

Table 19b: Dox-KLH fusion 13 ELISA results

ELISA #	Hybrid Code	Anti-CEA reading		Anti-Dox reading		BsMab reading	
		OD_{405}	M+2SD	OD_{405}	M+2SD	OD_{405}	M+2SD
420	1	1.663	0.087	0.130	0.07	Not tested	
	2	1.563		0.117			
421	17	Not tested		0.179	0.081		
	23			0.158			
424 & 425	1	1.861	0.10	0.105	0.068	0.150	0.104
	12	1.499		0.102		0.105	
	17	1.224		0.082		0.106	
	23	1.087		0.095		0.099	
	7	1.690		0.083		0.138	
	13	1.571		0.067		0.137	
430 & 431a	1	0.319	0.065	0.108	0.076	Not tested	
	17	0.314		0.090			
	23	0.377		0.109			
431b	17	Not tested		0.092	0.172	Not tested	
	23			0.123			
	25			0.278			
	30			0.265			
441, 442 & 443	17-a	0.233	0.072	0.105	0.105	0.107	0.082
	17G	0.319		0.133		0.068	
	23-1	0.123		0.150		0.08	
	23-13	0.241		0.208		0.074	
	23-2	0.303		0.104		0.088	
447, 448 & 449	17-a	0.669	0.100	0.091	0.071	Not tested	
	17-G	0.817		0.073	0.070		
	17-K	0.716		0.069	0.065		
	23-1	0.795		0.079			

Note: ELISA 431b performed with Dox-KLH coated plates.

III. 7. 12 Dox-KLH Fusion 16a

Of the several clones of 11-285-14 used in the fusions described above, Aza-3 (fusion 13) yielded hybrids that were consistently anti-CEA secretors. Aza-3 was therefore selected as the fusion partner for fusion 16. A shorter immunization protocol of 22 days was used. The selection of mice used in this fusion was as follows. 5 mice from a litter were immunized with a total of 28.5μg of doxorubicin in the form of Dox-KLH (Table 6c). Prior to fusion, the sera of the 5 mice (drawn from the tail vein) were tested by ELISA. Although immunized in an identical fashion, two mice (# 2 and 3) demonstrated a higher anti-Dox titre up to 1/100 and 1/1000, respectively (Figure 21). The spleen cells from these two mice were pooled and were divided for two fusions. Fusion 16a was performed with Aza-3 and Fusion 16b with 6-thiaguanine resistant NS-1 myeloma cell line. It was anticipated that this approach, if successful, would result in BsMabs in fusion 16a and/or hybrids secreting anti-Dox monoclonal antibodies in fusion 16b.

Fusion 16a resulted in 195 hybrids, the maximum number up to that time. The initial 60 hybrids obtained were tested in both anti-CEA and anti-doxorubicin ELISA (Table 20a). All of the 60 hybrids tested were positive for anti-CEA activity confirming the anti-CEA stability of hybrids resulting from Aza-3. However, only 3 (5%) were positive for anti-Dox

Figure 21. Selection of mice for Dox-KLH fusion number 16

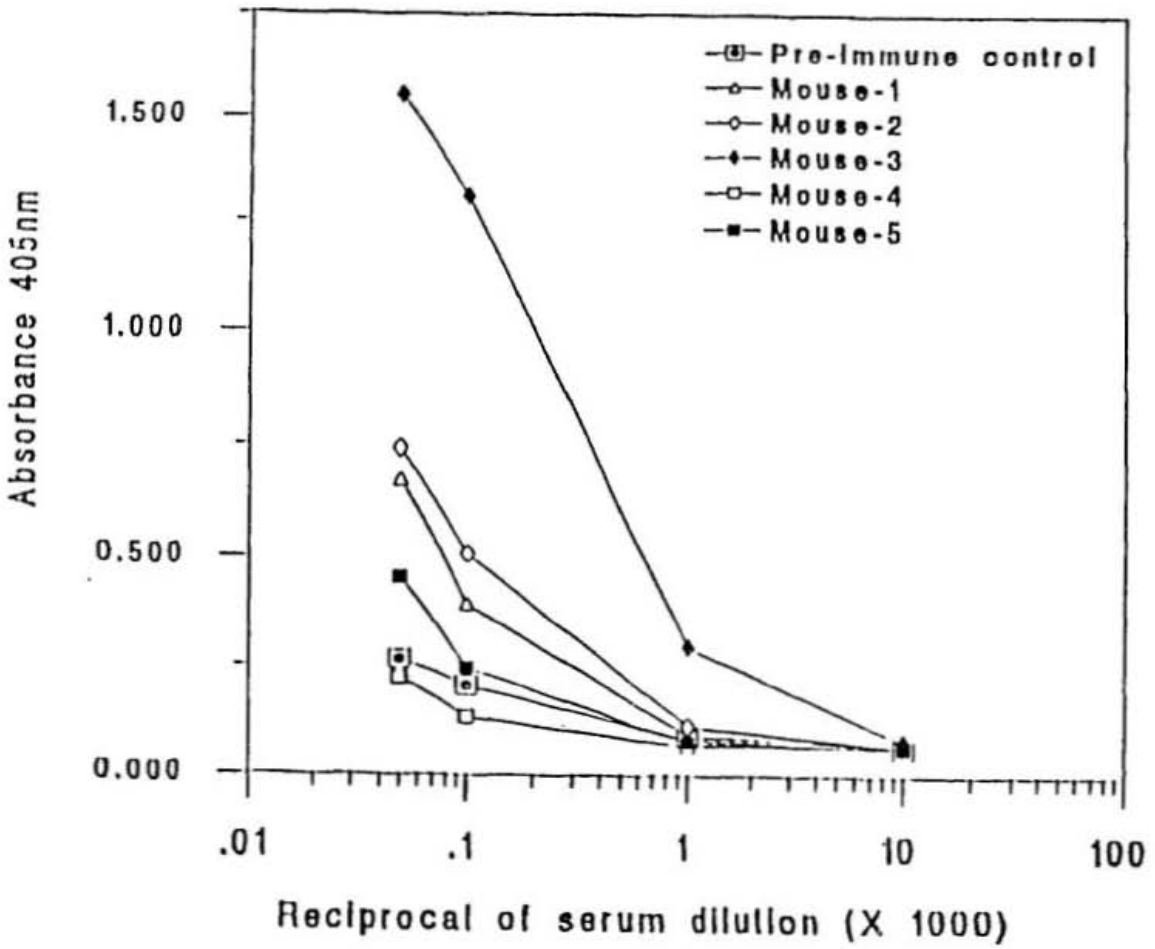


Figure 21. ELISA performed with Dox-BSA coating (2ug/ml Dox). Five mice were immunized in an identical fashion (Table 6d, Section II. 9.1). The sera was drawn prior to the fusion date and evaluated by ELISA for anti-Dox activity. The control was serum from a non immunized mouse (pre-immune). Each point represents the mean value of absorbance readings in triplicate. The standard deviation has been omitted for clarity.

activity. It was therefore decided to concentrate on anti-Dox ELISAs. Hybrids positive for anti-Dox activity were selected for cloning and further expansion. The numbers of ELISAs performed and the results are given in Table 20a.

Table 20a: Dox-KLH Fusion 16 results

ELISA #	Wells tested	Anti-CEA activity	Anti-DOX activity
484 & 485	60	60	3
486	82	NT	17
487 & 488	164	NT	34
489	82	NT	2
490	82	NT	9
491	71	NT	8
492	56	NT	4
493	36	NT	1
494	67	NT	1
495, 496, 497 & 498	272	NT	0
Total hybrids	195 hybrids + 249 clones	60*	51

NT = Not tested
* All hybrids obtained were not tested for anti-CEA activity. However, the 60 hybrids that were tested were anti-CEA reactive.
Legend as for Table 14a.

Table 20b: Dox-KLH Fusion 16 ELISA results

ELISA #	Hybrid Code	Anti-CEA		Anti-DOX	
		OD_{405}	Mean +2SD	OD_{405}	Mean +2SD
484 & 485	5-4	0.225	0.138	0.114	0.077
	6-7	0.461		0.107	
	6-32	0.256		0.139	
486	3-2	Not tested		0.154	0.092
	3-5			0.144	
	3-14			0.162	
	5-4			0.070	
	5-12			0.105	
	6-7			0.068	
	6-9			0.282	
	6-32			0.068	
488	3-14-1	Not tested		0.372	0.098
	3-14-2			0.556	
	5-12-2			0.206	
	6-32-2			0.770	
489, 490 & 491	3-14-1	Not tested		0.070	0.124
	3-14-2			0.062	
	5-12-2			0.068	
	6-32-2			0.111	
494	6-32-2	Not tested		0.104	0.094

ELISAs 486,487,488 and 491 performed with maxisorp ELISA plates (Gibco # 4-42404) due to lack of conventional ELISA plates mentioned in Materials and Methods.

M + 2SD = Mean + standard deviation (x2) of background control values.

A total of 195 hybrids resulted from Fusion 16a. 51 were anti-Dox positive. Only those hybrids showing the highest anti-Dox absolute ELISA readings were considered for cloning. For example, although several hybrids were above the 0.092 (mean + 2 x standard deviation) control background, only 17 were over 0.1 ELISA reading at 405 nm (ELISA 486; Table 20b) and were cloned by limiting dilution. 249 clones resulted from this procedure. ELISA readings for 4 of the positive clones are shown in Table 20b (ELISA 488). However, these clones became non reactive in subsequent ELISAs (Table 20b). Direct BsMab ELISAs were not performed, since the hybrids became negative for anti-Dox activity.

The outcome of fusion 16 is best illustrated by hybrid # 6-32. Hybrid 6-32 was positive for both anti-CEA and anti-Dox ELISAs 484 and 485 (Table 20b) and was immediately cloned. Although the original hybrid lost its anti-Dox activity (ELISA 486), the subclone 6-32-2 was highly positive (ELISA 488), with an absolute ELISA reading of 0.770 (Table 20b), the highest up to that date. However, on subsequent growth and testing, 6-32-2 lost its anti-Dox activity (ELISA 491). Similarly, all the initially positive hybrids including their clones became negative for anti-Dox activity (ELISAs 495-498, Table 20a), within 3 to 4 weeks of the fusion.

The results of all the Dox-KLH fusions are summarised in Table 21.

Table 21: Summary of Dox-KLH fusions hybrids

Fusion #	Hybrids (wells)	Clones	Total= Hybrids+ Clones	Anti-CEA	Anti-Dox	BsMabs
1,2,3	0	0	0	0	0	0
4	48	4	52	48	11	NT
5	33	0	33	18	6	NT
6	95	0	95	13	44	0
7	146	51	197	14	74	21
8,9,10	0	0	0	0	0	0
11	59	16	75	26	23	3
12	0	0	0	0	0	0
13	45	61	106	103*	55	23
14,15	0	0	0	0	0	0
16a	195	249	444	60*	51	0
Total= 16	621	380	1001	282*	264	47

* = Not all hybrids obtained were tested for anti-CEA activity.

III.8.0 NS-1 FUSIONS

As mentioned in the introductory chapter, an alternative method of generating bispecific antibodies is by fusing two different hybridomas (Section I.10.0). The resulting hybrid-hybrid would then secrete antibodies with dual parental specificities.

In an attempt to produce a hybridoma secreting monoclonal antibodies against doxorubicin, fusions were performed between NS-1 myeloma cells (6-thioguanine resistant) and spleen cells from doxorubicin immunized mice. A hybridoma thus produced, could be used as a fusion partner with the 8-azaguanine resistant anti-CEA 11-285-14 hybridoma.

III. 8.1 Dox-KLH (NS-1) Fusions 12b, 15b, 16b and 17

Four fusions were performed with the first three corresponding to fusions 12a, 15a and 16a of Dox-KLH bispecific fusions discussed in section III 7.0. (sections 7.9, 7.10, 7-11 and 7.12). A fourth fusion (# 17) was performed separately. The immunization protocol with Dox-KLH and the number of spleen cells used for the fusions is shown in table 6c along with the other Dox-KLH fusions, in the Materials and Methods section. The number of NS-1 cells used and other fusion details are summarised in Table 22.

Table 22: Dox-KLH (NS-1) fusion details

Fusion #	Spleen cells total viable x 10^6	% viability	NS-1 total viable cells x 10^6	% viability	Spleen : NS-1 ratio	Cell density per well
12b	40	95%	8.04	97%	5 : 1	2.5 x 10^5
15b	35.7	94%	7.47	82%	4.8 : 1	2.2 x 10^5
16b	111.1	95%	14.05	90%	8 : 1	2.17 x 10^5 2.59 x 10^5
17	76.4	92%	6.9	84%	11: 1	1.73 x 10^5

Despite identical fusion conditions, fusion 12b (NS-1 fusion partner) yielded 31 hybrids compared to fusion 12a (11-285-14 fusion partner) which did not result in any hybrids. However, only a single anti-Dox hybrid resulted from 12b, which failed to grow in culture.

Fusions 15a and b did not yield any hybrids. Fusion 16 was performed under identical conditions using half of the pooled spleen cells each for fusions 16a (with 11-285-14) and 16b (with NS-1). While 16a resulted in a large number (195) of hybrids, 16b resulted in a single, anti-Dox negative hybrid. Fusion # 17 resulted in 11 hybrids that died in culture following contamination by yeast.

III. 8.2 Dox-BSA (NS-1) Fusions

Three NS-1 myeloma fusions were performed with spleen cells from Dox-BSA (bovine serum albumin) immunized mice. The reason for selecting BSA as the carrier protein is discussed in the following section (III 8.3). The immunization protocol is given in Table 23.

Table 23: Dox-BSA Immunization Schedules for NS-1 Fusions

Fusion #	Days	Quantity of Dox μg	Adjuvant	Route	Comments
1	0	60μg	CFA	SC	40 day old mouse at time of immunisation. Total viable spleen cells obtained = 7.12×10^7.
	14	60μg	IFA	SC	
	28	60μg	PBS	IP	
	31	Fusion			
2	0	50μg	CFA	SC	2 month old mouse. Total viable spleen cells obtained = 9.54×10^7.
	16	50μg	IFA	SC	
	52	50μg	PBS	IP	
	56	Fusion			
3	0	50μg	CFA	SC	2 month old mouse. Total viable spleen cells obtained = 12.2×10^7.
	16	50μg	IFA	SC	
	58	50μg	PBS	IP	
	62	Fusion			

CFA & IFA: Complete and incomplete Freund's adjuvant
SC: subcutaneous
IP: intraperitoneal
PBS: phosphate buffered saline

The spleen cells and NS-1 cells used and other fusion details are given in Table 24.

Table 24: Dox-BSA (NS-1) fusion details

Fusion #	Spleen Cells		NS1		Spleen cell:NS 1 ratio	Cell density per well
	Total viable cells	% viability	Total viable cells	% viability		
1	71.2×10^7	82	1.424×10^7	95	5 : 1	2.2×10^5
2	9.54×10^7	97	1.908×10^7	96	5 : 1	3.9×10^5
3	12.2×10^7	78	2.22×10^7	95	5.5 : 1	5.0×10^5

Most significant of the NS-1 (Dox-BSA) fusions are the results of fusion # 1 resulting in 227 hybrids. The ELISA results are summarised in table 25a. 26 of these were strongly positive for anti-Dox- BSA over 5 to 6 times the anti-BSA ELISA readings and 10 times the control background. The highest ELISA readings are shown in Table 25b. However, these strongly positive hybrids either became negative in later ELISAs and/or ceased to proliferate in culture.

Table 25a: Dox-BSA NS-1 fusion 1			
ELISA #	Total hybrids	Anti-Dox-BSA activity	Anti-BSA activity
510 a,b	10	0	10
511 a,b	45	21	24
514 & 515	86	8	0
517 & 518	47	0	12
517c & 518c	37	2	0
519 & 520	79	10	12
Total hybrids	227	26	58

Legend as explained for Table 14a

Table 25b: Dox-BSA NS-1 fusion 1 ELISA results					
ELISA #	Code	Anti-Dox-BSA		Anti-BSA	
		OD_{405}	M+2SD	OD_{405}	M+2SD
511 a & b	2-9	1.287	0.095	0.200	0.111
	2-12	1.001		0.164	
	2-16	1.041		0.141	
	2-20	1.164		0.166	

M + 2SD = Mean + standard deviation (x 2) of background control values.

The NS-1 fusion results are summarised in Table 26.

Table 26: Summary of NS-1 fusions (Dox-KLH AND Dox-BSA) results

Fusion #	Immunogen	Hybrids Total	Anti-Dox/ Dox-BSA	Anti-BSA
12b	Dox-KLH	31	1	0
15b	Dox-KLH	0	0	0
16b	Dox-KLH	1	0	0
17	Dox-KLH	11	0	0
1	Dox-BSA	227	26*	86
2	Dox-BSA	29	4	0
3	Dox-BSA	4	2	0

NT = Not tested
* These hybrids were negative in the anti-BSA ELISAs

III. 9.0 DOX-BSA (11-285-14) BISPECIFIC FUSIONS

III. 9.1 Introduction

Analysing the poor results of Dox-KLH fusions, hybrids from the initial fusions showed loss of anti-CEA activity, thus diminishing the chance of obtaining BsMabs. To overcome this problem, Aza-3 was used as the 11-285-14 fusion partner which yielded consistent anti-CEA secretors. The main problem in subsequent Dox-KLH fusions appeared to be the instability of hybrids for anti-Dox secretion in culture. Since even the anti-Dox-KLH activity appeared weak, as demonstrated in fusion 13, it was decided to switch the carrier protein to bovine serum albumin (BSA). Dox-BSA was used as the immunogen in an attempt to improve the success rate. Evidence for Dox-BSA as a suitable immunogen came from the discussion with Dr. S. Menard (personal communication, European Immunology Meeting, 1988, Rome, Italy). This was further supported by a report from Dr. Menard's group of the production of anti-doxorubicin monoclonal antibodies using Dox-BSA immunized mice (Balsari, Alzani, Parrello, Morelli, Tagliabue, Gianni, Isetta, Menard, Colnaghi & Ghione M, 1988).

An approximate four week immunization duration was followed as shown in the Materials and Methods (Tables 7a & 7b). It was possible to link a much greater quantity of doxorubicin to BSA than it was to KLH (Tables 6 & 7). 150μg of doxorubicin in the form of Dox-BSA could be given as antigen

per mouse prior to fusion (Tables 7a & 7b). The sera of mice drawn prior to the fusions revealed high titre antibodies (1/1000 up to 1/1,000,000) in ELISAs performed with Dox-KLH coating indicating successful immunization (Figures 22 & 23). These results are presented under the individual fusions. Since hybrids produced from Aza-3 as the fusion partner appeared to be consistent anti-CEA secretors, Aza-3 was used for all Dox-BSA fusions. Eight fusions were performed.

III. 9.2 Dox-BSA Fusion 1

Fusion 1 was performed 31 days following the primary immunization. The serum contained anti-Dox antibodies up to 1/1000 titre (Figure 22). The number of spleen cells and Aza-3 cells used in this fusion are given in Table 27. Only a single hybrid resulted from this fusion which was an anti-BSA secretor.

Figure 22. Dox-BSA Fusions 1 to 4 sera

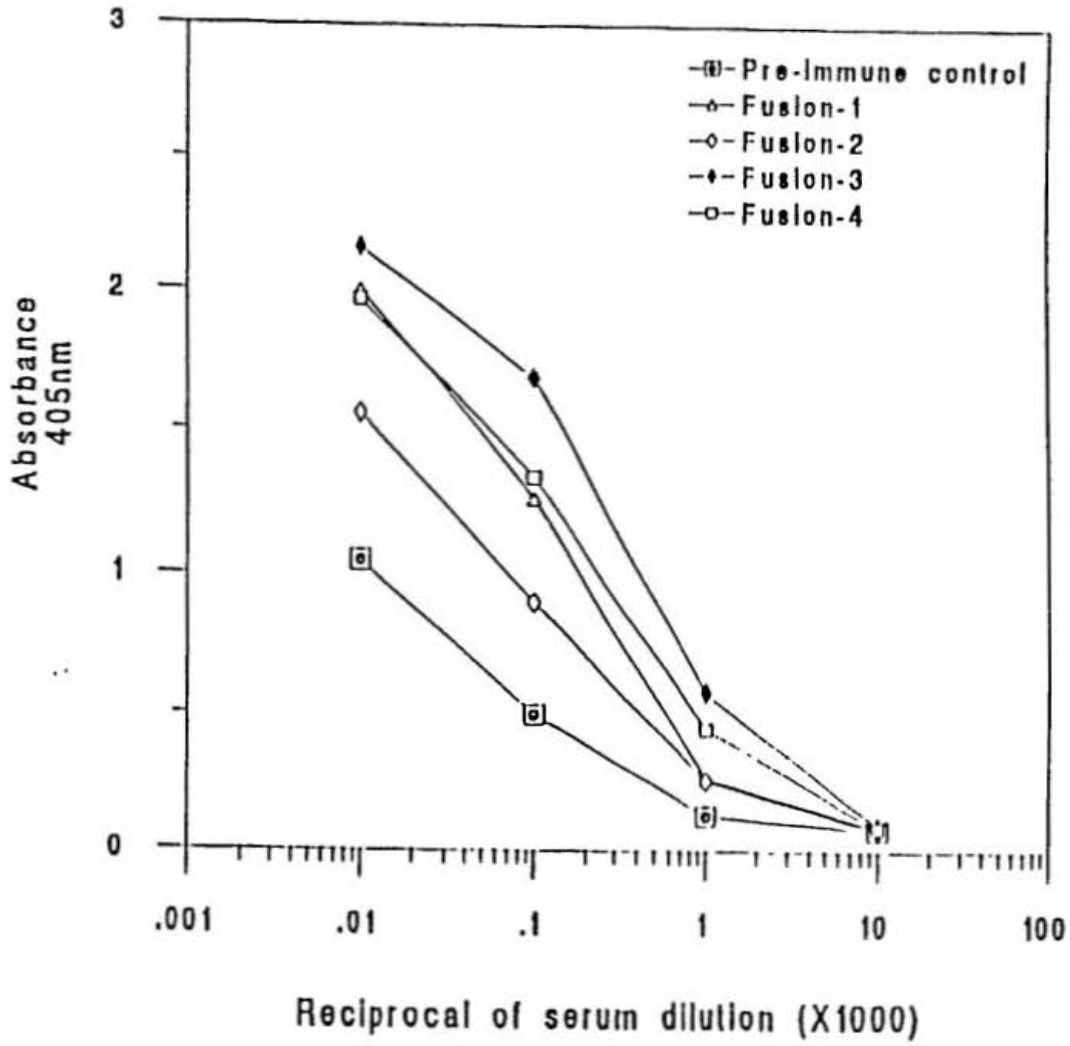


Figure 22. ELISA performed to evaluate sera drawn from mice on the day of Dox-BSA fusions # 1 to 4. 2ug/ml of Dox in the form of Dox-KLH was employed as coating. The pre-immune control was sera from a non immunised mouse. Each point represents mean values of absorbance readings in triplicate. The standard deviation has been eliminated for clarity.

Table 27: Dox-BSA fusion details

Fusion #	Spleen cells		11-285-14			Spleen cells : 11-285-14	Cell Density per well x 10^5
	Total viable cells x 10^6	% viability	Batch	Total viable cells x 10^6	% viability		
1	81.6	96	Aza-3	16.3	92	5 : 1	3.4
2	121.6	95	Aza-3	21.68	92	5.6 : 1	3.7
3	68.8	96	Aza-3	5.32	96	13 : 1	2.5
4	77.2	92	Aza-3	17.5	88	4.4 : 1	2.4
5	147.6	94	Aza-3	30.17	95	4.9 : 1	4.6
6	119.6	99	Aza-3	20.93	92	5.7 : 1	4.2
7	50.3	95	Aza-3	10.06	93	5 : 1	2.09
8	54.3	99	Aza-3	10.86	97	5 : 1	3.4

III.9.3 Dox-BSA Fusion 2

A male mouse from the same litter as in fusion 1 (Table 7a) was used for this fusion with an identical immunization protocol. The serum revealed up to 1/1000 titre anti-Dox antibodies (Figure 22). 12.6×10^7 spleen cells were obtained and the fusion details are given in Table 27.

77 hybrids resulted from this fusion. The ELISAs, number of hybrids tested and results are given in Table 28a. Some of the ELISA readings are given in Table 28b. Hybrids that were initially positive in Dox-BSA and negative in BSA coated ELISAs (# 508) were considered anti-Dox secretors. However, these subsequently became negative, including the clone 2-2 that was obtained from hybrid # 2 (Table 28b; ELISA # 513).

Table 28a: Dox-BSA fusion 2 results

ELISA #	Total hybrids	anti-Dox activity	anti-BSA activity	anti-CEA activity
508 & 509	25	12	3	NT
510	36	14	13	NT
511 & 512	25	NT	NT	25
513 to 516	45	1	13	NT
517a & 517b	35	16	11	NT
528	8	0	0	NT
Total hybrids	77	14*	34	25

*Anti-Dox positive hybrids were negative in anti-BSA ELISAs.
NT = Not tested
Legend as explained for Table 14a

Table 28b: Dox-BSA fusion 2 ELISA results

ELISA #	Code	Anti-Dox-BSA		Anti-BSA		Anti-CEA	
		OD_{405}	M+2SD	OD_{405}	M+2SD	OD_{405}	M+2SD
508 & 509	1	0.383	0.071	0.087	0.071	Not tested	
	2	0.183		0.071			
	3	0.215		0.091			
510a 511 & 512	1	0.698	0.084	0.104	0.089	0.464	0.06
	2	0.146		0.055		0.298	
	3	0.199		0.111		0.334	
	5	0.160		0.094		0.302	
	15	0.242		0.076		0.248	
	27	0.187		0.057		Not tested	
513 & 516	2-2	0.196	0.055	0.087	0.090	Not tested	
	1	0.051		0.089			
	3	0.065		0.109			
	15	0.065		0.099			
	27	0.055		0.075			
	2	0.055		0.102			

III. 9.4 Dox-BSA Fusion 3

Fusion was performed 6 weeks after primary immunization (Table 7a). A lesser number of Aza-3 cells were used due to availability. Therefore, the spleen cell to 11-285-14 ratio was 13:1, higher than for the other fusions (Table 27). The mouse serum showed up to a 1/1000 anti-Dox titre by ELISA (Figure 22). Twelve hybrids resulted from this fusion. The ELISAs performed and the ELISA readings are given in Tables 29a and 29b.

Table 29a: Dox-BSA fusion 3 results			
ELISA #	total hybrids	anti-Dox-BSA	anti-BSA
528	6	0	1
529	10	1	0
Total hybrids	12	1	1

Table 29b: Dox-BSA fusion 3 ELISA results					
		Anti-Dox-BSA		Anti-BSA	
ELISA #	Code	OD_{405}	M+2SD	OD_{405}	M+2SD
528	1-2	0.162	0.056	0.09	0.051
	1-5	0.103		0.102	
529	1-5	0.105	0.080	0.059	0.068
	1-2	0.074		0.051	

Legends as explained for Table 14a and 14b

III. 9.5 Dox-BSA Fusion 4

The results were similar to fusion 3. The immunization protocol is shown in Table 7a. The post-immune mouse serum demonstrated up to a 1/1000 anti-Dox titre by ELISA (Figure 22). The fusion details are summarised in Table 27. Of the 10 hybrids obtained, there was only one hybrid recognising doxorubicin. ELISA results and absolute readings are given in Tables 30a and 30b.

Table 30a: Dox-BSA fusion 4 results			
ELISA #	total hybrids	anti-Dox-BSA	anti-BSA
537	8	0	0
538	1	1	0
541	3	0	1
Total hybrids	10	1	1

Table 30b: Dox-BSA fusion 4 ELISA results

ELISA #	Code	Anti-Dox-BSA		Anti-BSA	
		OD_{405}	M+2SD	OD_{405}	M+2SD
538	4-8	0.164	0.116	0.080	0.095
541	4-5	0.091		0.290	

Legend as for Tables 14a and 14b

III. 9.6 Dox-BSA Fusions 6, 7 and 8

Although fusion 5 was performed prior to fusions 6,7 & 8, due to the repeated cloning and subcloning involved, the results of fusion 5 were obtained later and therefore given in the next section. Fusions 6, 7 and 8 were performed following a four week immunization protocol with 155μg of doxorubicin total dosage for each mouse (Table 7b). The primary booster for fusion 8 mouse was given in complete Freund's adjuvant instead of the incomplete Freund's adjuvant utilised for other Dox-BSA fusions (Balsari et al, 1988). The sera of these mice tested in anti-Dox ELISAs revealed high titre (1/1,000,000) antibodies (Figure 23). Accidentally, the serum from the mouse used for fusion 6 was discarded, and therefore not tested for anti-Dox antibodies.

Figure 23. Dox-BSA Fusions 5 to 8 sera

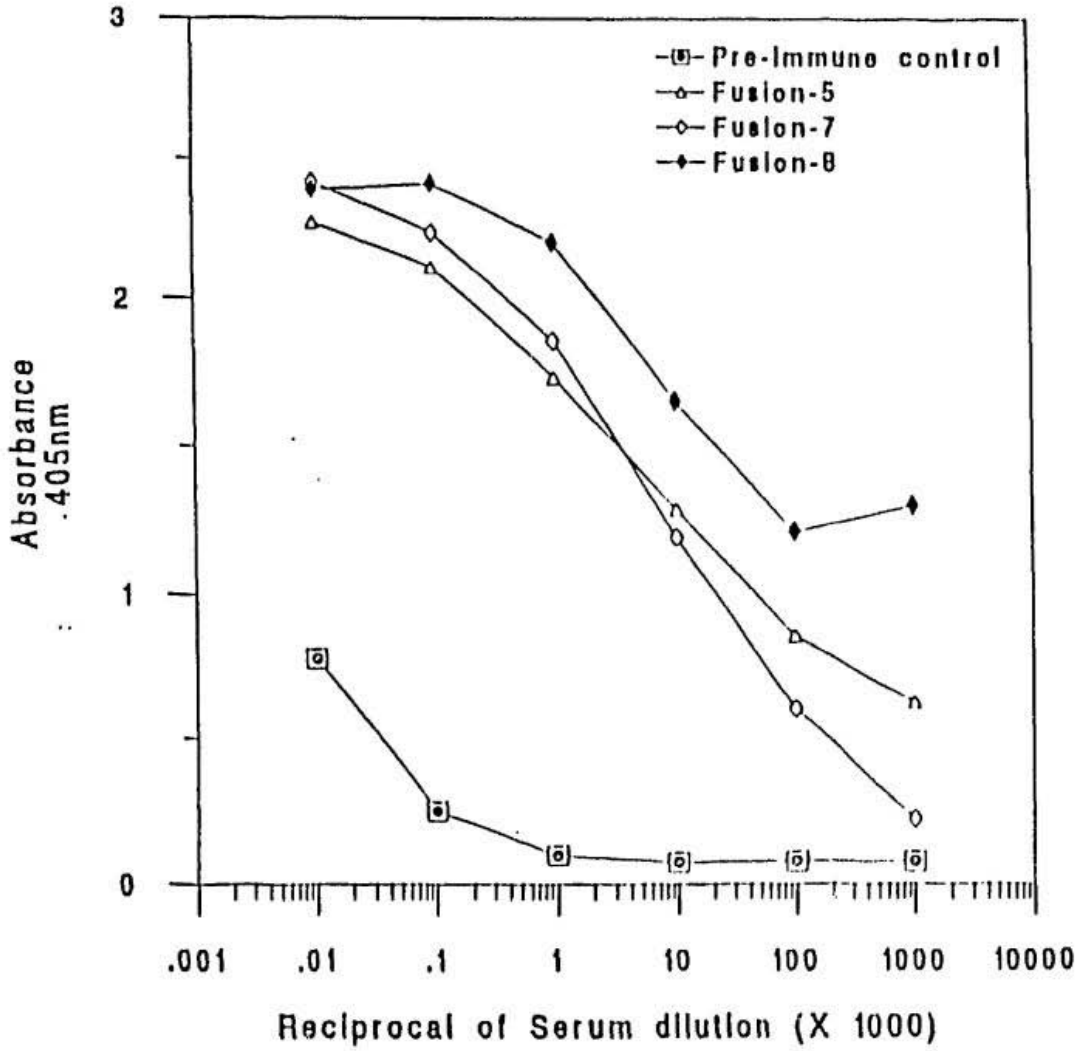


Figure 23. Legend as for Figure 22. The serum was drawn on the days of Fusions # 5,7 and 8.

The number of spleen cells obtained and fusion details are summarised in Table 27. Nineteen hybrids resulted from these three fusions. Only 3 were anti-Dox positive, all resulting from fusion 7. The ELISAs performed and the ELISA readings for a few of the hybrids from fusion 7 are given in Tables 31a & 31b. No stable bispecific hybridoma lines were obtained from fusions 6, 7 and 8.

Table 31a: Dox-BSA fusion 7 results

ELISA #	Total hybrids tested	Anti-Dox	Anti-BSA
543	2	1	0
546	1	1	0
547	1	0	0
549	3	1	0
550	4	0	0
551	6	0	0
Total hybrids	5	3	0

Table 31b: Dox-BSA fusion 7 ELISA results

ELISA #	Code	anti-Dox-BSA		anti-BSA	
		OD_{405}	M+2SD	OD_{405}	M+2SD
543	3-1	0.157	0.100	0.079	0.093
546	3-1	0.104	0.079	0.062	0.080
547	3-1	0.059	0.093	0.068	0.080
549	3-3	0.114	0.068	0.077	0.092

Legend as for Table 14a and b.

III. 9.7 Dox-BSA Fusion 5.

A 4 week immunization protocol was used (Table 7b). 155μg of doxorubicin in the form of Dox-BSA was the total immunization dosage. The mouse serum contained a 1/1,000,000 anti-Dox antibody titre when tested against Dox-KLH coated plates (Figure 23), the highest antibody titre at that time. The spleen of the mouse yielded the maximum number of spleen cells obtained up to that time (14.76×10^7). The fusion details are summarised in Table 27. The cell density per well was the highest amongst the Dox-BSA fusions.

A large number of hybrids (178) resulted from this fusion. A list of the ELISAs performed and the number of hybrids tested serially is given in Table 32. Given the stability of anti-CEA secretion by hybrids produced from Aza-3, the focus was on assessing the anti-doxorubicin secretion. Hybrids positive in anti Dox-BSA ELISAs and negative in anti-BSA were selected for cloning. The ELISA readings for some of the hybrids selected are given in Tables 32 and 33. Although, initial ELISAs from # 537 to 546 revealed hybrids positive for BSA, several readings for anti-Dox-BSA were at least twice that of anti-BSA. Such hybrids were still considered for further evaluation, since the original hybrid colony could have been a mixture of cells secreting antibodies against Dox or BSA individually and/or BsMabs.

The hybrid colonies 1-14a and 1-14b were selected for cloning based on ELISA readings 5 to 6 times over the background for anti-Dox BSA compared to equivocal anti-BSA readings. They were also positive for anti-CEA activity (ELISAs 551 & 552, Table 32). Hybrids were initially cloned at 10 cells per ml by limiting dilution (1 cell per well) and subsequently at 5 cells per ml (0.5 cells per well). Clones of 1-14a/b and their second and third generation subclones were tested for anti-Dox activity.

Of the 178 hybrids obtained from this fusion, 107 were anti-Dox positive. 20 of these hybrids showing the highest ELISA readings were cloned. 245 clones thus obtained were further subcloned resulting in 1325 second and third generation clones. 286 of the 1325 potential bispecific hybrids with the highest absolute ELISA readings have been cryopreserved in liquid nitrogen for future evaluation. Seven third generation clones of 1-14a and 1-14b (listed under ELISAs 584 & 587; Table 33) were expanded in culture and retested for anti-Dox-BSA, anti-BSA, anti-CEA as well as in the bispecific assay directly. ELISA readings for these hybrids are given in Table 33 (ELISAs 581 to 587).

Table 32 : Dox-BSA fusion 5 results				
ELISA #	Hybrid wells tested	anti-Dox	anti-BSA	anti-CEA
537	33	1	32	NT
538	54	42	0	NT
541	65	16	3	NT
542	69	7	2	NT
543a	40	23	5	NT
544	49	10	2	NT
546	41	2	8	NT
547	50	7	1	NT
548	62	4	2	NT
549	50	6	13	NT
550	70	4	9	NT
551	36	6	2	NT
552	36	NT	NT	36
553-556	180	9	162	NT
558-561	296	26 (anti-Dox-KLH)	NT	NT
562	187	140	13	NT
565	64	41	23	NT
566	68	3	6	NT
567	70	17	51	NT
568	70	5	65	NT
569	80	56	6	NT
570	82	27	0	NT
571	82	40	1	NT
572	81	5	0	NT
573	24	NT	NT	24
574	72	36	0	NT
575	83	6	0	NT
576	75	60	3	NT

continued .....

Table 32 : Dox-BSA fusion 5 results (continued)

ELISA #	Hybrid wells tested	anti-Dox	anti-BSA	anti-CEA
577	46	6	0	NT
578	80	29	0	NT
579	81	17	0	NT
580	76	36	10	NT
581	65	39	2	NT
583	20	NT	NT	20
584	38	33	0	NT
587	30	29	21	NT
589	9	0	8	NT
590	9	NT	NT	9
Total hybrids	178 + 1570 clones	107 + 179 clones	65	89*

* All the wells tested were positive for anti-CEA
NT = Not tested
Legend as for Table 14a

Table 33 : Dox-BSA fusion 5 ELISA results

ELISA #	Code of hybrid	Anti-Dox-BSA		Anti-BSA		Anti-CEA	
		OD_{405}	M+2SD	OD_{405}	M+2SD	OD_{405}	M+2SD
537	2-1	0.486	0.114	0.206	0.103	Not tested	
	2-27	0.556		0.226			
	2-28	0.532		0.300			
538	2-1	0.103	0.116	0.057	0.095	Not tested	
	2-23	0.339		0.073			
	2-27	0.248		0.074			
	2-28	0.161		0.065			
	3-5	0.482		0.069			
542	1-14	0.213	0.177	0.149	0.207	Not tested	
	1-27	0.177		0.068			
543	1-14	0.179	0.100	0.130	0.093	Not tested	
	3-56	0.193		0.061			
546	1-14	0.198	0.079	0.191	0.128	Not tested	
	4-30	0.156		0.081			
551	1-14a	0.497	0.099	0.081	0.066	1.610	0.101
552	1-14b	0.582		0.084		1.891	
562	1-14a-26	0.523	0.105	0.103	0.097	Not tested	
567	1-14a-26-10	0.214	0.110	0.062	0.094		
568	1-14a-14-33	1.109	0.173	0.241	0.153	Not tested	
	1-14a-14-35	1.012		0.242			
	1-14b-57-11	0.833		0.261			
	1-14b-57-12	0.987		0.306			
569	1-14a-26-7	1.020	0.135	0.097	0.134	Not tested	
	1-14a-26-6	0.644		0.131			
	1-14a-26-48	1.161		0.179			
570	1-14a-9-1	1.163	0.151	0.072	0.096	Not tested	
	1-14B-57-21	0.576		0.107			
574	1-14a-57-11-25	0.232	0.088	0.088	0.097	NT	
	1-14a-57-11-10	0.329		0.094		NT	

continued .....

Table 33 : Dox-BSA fusion 5 ELISA results (continued)

ELISA #	Code of hybrid	Anti-Dox-BSA		Anti-BSA		Anti-CEA	
		OD_{405}	M+2SD	OD_{405}	M+2SD	OD_{405}	M+2SD
575	1-14a-26-7	0.204	0.106	0.079	0.080	NT	
576	1-14b-57-9-6	0.385	0.164	0.105	0.149	NT	
578	1-14a-57-11-25-17	0.418	0.151	0.123	0.086	NT	
581&583	1-14a-26-61-1	0.543	0.103	0.144	0.111	1.801	0.126
	1-14a-26-61-2	0.508		0.110		1.775	
	1-14a-26-61-4	0.456		0.111		1.801	
	1-14a-26-61-10	0.573		0.122		1.852	
	1-14b-57-9-6-4	0.424		0.088		1.799	
						BsMabs	
						OD_{405}	M+2SD
584 & 587	1-14a-26-61-1	0.390	0.088	0.085	0.073	0.140	0.063
	1-14a-26-61-2	0.400		0.101		0.203	
	1-14a-26-61-4	0.378		0.081		0.130	
	1-14a-26-7-2	0.448		0.105		0.103	
	1-14a-26-7-35	0.186		0.069		0.091	
	1-14b-57-9-64	0.247	0.062	0.079	0.084	0.111	
	37-40-6	0.216		0.098		0.158	

OD = Optical density (absorbance) at 405 nm
M + 2SD = Mean + standard deviation (x 2) of background control values

A recent report has suggested the possibility of immunogenicity induced by the linkers β-alanine methylene malonate ethyl ester (BAMME) and β-alanine pyrrole (BAP) (Johnson DA, Barton RL, Fix DV, Scott WL & Gutowski MC, 1991). The clone 1-14a-26-61-1 was tested by ELISA for reactivity with the ECDI linker used in Dox-BSA conjugates (although such a reaction against ECDI has not been documented in the literature), and was found to be nonreactive. The results in ELISA # 597 for the clone 1-14a-26-61-1 against ECDI was 0.112 (Mean + 2SD) compared to a control background of 0.138 (M + 2SD). This clone has been expanded and the positive results for anti-CEA, anti-Dox, dual assay and negative results in anti-BSA assay are shown in Table 33 (ELISAs # 581, 583, 584, 587).

Dox-BSA fusion results are summarised in Table 34. Fusion 5 resulted in the maximum number of positive hybrids showing dual reactivity for both CEA and doxorubicin in ELISA.

Table 34: Summary of Dox-BSA fusion hybrids

Fusion #	Hybrids			Anti-Dox	Anti-BSA	Anti-CEA	BsMab activity
	Hybrids	Clones	Total				
1	1	0	1	0	1	NT	NT
2	77	0	77	14	34	25	NT
3	12	0	12	1	1	NT	NT
4	10	0	10	1	1	NT	NT
5	178	1570 (245+1325)	1748*	107 hybrids + 179 clones	65	89	7*** (50)
6	11	0	11	0	0	NT	NT
7	5	12	17	3	0	NT	NT
8	3	0	3	0	0	NT	NT
Total= 8	297	1582	1879	126	102	114**	7

* The total number includes 1st generation clones (245) and their 2nd and 3rd generation subclones tested (1325)

Anti-Dox hybrids were exclusively positive in Dox-BSA ELISAs and negative in anti-BSA ELISAs.

** Anti-CEA activity was positive for all hybrids that were tested.

*** Parentheses indicates that at least 50 were positive for dual activity after subcloning twice. However these were cryopreserved without expansion, while 7 clones were expanded prior to preservation.

CHAPTER IV

DISCUSSION

IV 1.0 INTRODUCTION

For the production of BsMabs we chose what initially seemed to be the most straight forward approach ie. hybridoma x spleen cell fusions particularly as an anti-doxorubicin sensitive hybridoma was not available at that time (Suresh et al, 1986b; Corvalan & Smith, 1987). Twenty-four fusions were performed using HAT sensitive 11-285-14 and spleen cells obtained from mice immunized with Dox-KLH or Dox-BSA. The hybrids resulting from Dox-KLH fusions were unstable in culture, in addition to having a low titre antibody activity (by ELISAs). The possible reasons for the failure of these experiments and the successful results of the Dox-BSA bispecific fusions will be discussed in the following sections. In addition, the implications of these results and the future prospects of BsMabs recognising both CEA and doxorubicin will be discussed.

IV 2.0 DOX-KLH FUSIONS

Sixteen fusions were performed using the hybridoma technology with HAT sensitive 11-285-14 and spleen cells from mice immunized with Dox-KLH. A total of 621 hybrids resulted with 47 hybrids demonstrating dual activity (Table 21).

IV 2.1 Selection of 11-285-14 fusion partners

The initial step was to produce HAT sensitive (8-azaguanine resistant) 11-285-14 mutants with suitable growth characteristics, anti CEA production and fusion properties. 11-285-14 itself is a hybridoma produced by the fusion of a HAT sensitive parental NS-1 myeloma, with spleen cells from mice immunized with CEA (Woodhouse, 1982a). This makes 11-285-14 HAT resistant, and adds to the difficulty in selecting HAT sensitive mutants. Although resistance with respect to growth in 8-azaguanine was easily obtained at the recommended concentration of 30 ug/ ml (Suresh et al, 1986b), the 11-285-14 mutants were slow growing in comparison with their parental 11-285-14 growing in RPMI medium (Figure 4). Furthermore, of the 72 HAT sensitive 11-285-14 clones produced, only 27 (37.5%) were positive for anti-CEA Mab secretion by ELISA.

The initial clones used for fusions gave disappointing results due to their poor growth and/or loss of anti-CEA activity either prior to fusions as for clone V or in the resulting hybrids. Clone VI gave the highest fusion

efficiency at 59.5 hybrids per fusion. However, only 21 (35%) of the hybrids were positive for anti-CEA activity, thus diminishing the chance of obtaining hybrids producing BsMabs. Clone Aza-3, despite having a lower fusion efficiency at 38.1 hybrids per fusion, resulted in all hybrids that were tested being positive for anti-CEA activity, thus enhancing the chance of obtaining dual positive hybrids.

In addition to the factors relating to the hybridoma fusion partner, several other variables may play a role in the production of BsMabs, including the drug (antigen) itself, carrier protein, immunization protocol, and fusion procedures which are discussed in the following sections.

IV 2.2 Doxorubicin - protein conjugates

Doxorubicin, being a hapten (molecular weight 580) had to be first conjugated to a carrier protein to induce an immune response. KLH was an attractive choice since it is one of the most immunogenic substances known (Korver, Zeijlemaker, Schellekens & Vossen, 1984). In addition, its molecular structure and antigenicity has been well elucidated (Linzen, Soeter, Riggs, Schneider, Schartau, Moore, Yokota, Behrens, Nakashima, Takagi, Nemeto, Vereijken, Bak, Beintema, Volbeda, Gaykema & Hol, 1985). The mechanism of KLH induced immunogenicity is considered to be a combination of chemical modification and recruitment of carrier specific helper T cells (French, Fischberg, Buhl & Scharff, 1986). Although

several heterobifunctional crosslinking agents are available for the production of hapten-protein or protein-protein conjugates, including glutaraldehyde, N-(γ-maleimidobutyxyloxy) succimimide (GMBS) and N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) (Means & Feeney, 1971; Fujiwara et al, 1981; Pierce Chemical Company: Crosslinking Reagents p333-338; Wold, 1965), carbodiimide (ECDI) was chosen as the crosslinker. The reason for this was that the ECDI reaction had been well documented with the conjugation of doxorubicin via its amino group to form an amide bond with the protein (Vunakis et al, 1974; Goodfriend et al, 1964).

A major problem that was encountered in producing conjugates was the loss of the drug by precipitation out of solution even prior to the conjugation reaction. As demonstrated in the conjugation experiments in Tables 9a, b, c and Table 10, 50 to 80% of the drug pecipitated out of solution. Such losses have also been documented in previous studies (Pietersz et al,1988). The loss of hemocyanin was more variable ranging from 50% (conj #22) to no loss (conj #25) (Table 9c). Molar ratios ranged from 57 to 568 moles Dox per mole KLH when calculated with a molecular weight of 10,000,000 for KLH (Sigma range 9 to 15 x 10^6). This compares with conjugations by other groups that have incorporated 8 to 10 moles of Dox per 100,000g of KLH (Vunakis et al, 1974),

i.e. 800 to 1000 molar ratio assuming a molecular weight of KLH of 10×10^6.

Experiments involving conjugation of Dox to enzymes were much more complicated and yielded disappointing results. HRP was the initial enzyme of choice since the anti-CEA and anti-Dox assays were standardised with HRP as the indicator. In addition, the amino acid structure and biochemical properties of HRP have been well delineated (Welinder, Smillie & Schonbaum, 1972; Welinder & Smillie, 1972; Dolman, Newell, Thurlow and Dunford, 1975). Ten attempts were made to link Dox to HRP using periodate, carbodiimide or glutaraldehyde as the crosslinkers (Table 11). Although the conjugation results indicated Dox was linked to HRP, the rabbit anti-Dox antibodies did not recognise Dox-HRP when tested by ELISA (results similar to Dox-avidin non recognition as shown in Figure 10). The reasons are unclear, but the interpretation includes the possibility of doxorubicin epitopes being altered by the chemical modification. The possible reasons for the failure of Dox-enzyme conjugations are discussed below.

(i) There have been no reports in the literature to date, indicating successful conjugation of Dox to HRP.

(ii) The stability and solubility of Dox has always been poor in comparison to other anthracycline derivatives and extremely variable under identical conditions when used

by different groups (Hoffman, Grossano, Damin & Woodcock, 1979; Benvenuto, Anderson, Kerkof, Smith & Loo, 1981; Bosanquet 1986). In general, doxorubicin has been found to be sensitive to light, adsorbs to membrane filters and containers (except siliconised glass and propylene), can degrade rapidly in medium, as well as chelate metal ions (Bosanquet, 1986).

(iii) The use of the periodate method, which is the most frequently used method in the conjugation of HRP to proteins, involves borohydride for the stabilization of the Schiff bases. However, when doxorubicin was used, this step has been shown to cause loss of activity, for unknown reasons (Ghose, Ramakrishnan, Kulkarni, Blair, Vaughn, Nolido, Norrell & Belitsky, 1981; Pietersz et al, 1988). This loss of activity also applies to Dox-avidin conjugates and may explain the non-recognition of Dox-HRP and Dox-avidin conjugates produced, by the rabbit anti-Dox antibodies in ELISAs (Sections III. 3.1; III. 3.3; and Figure 10). In addition, there are practical difficulties involved in the chemical conjugation and in estimating the quantity of conjugate formed (Section III.3.1).

(iv) Many of the problems in producing antibody-drug immunoconjugates, presented in Chapter I (section I. 8.1) also apply to the conjugation of Dox to enzymes. The

problem is one of linking the hydrophobic drug to hydrophilic protein moieties. In addition, heterobifunctional agents such as carbodiimide and glutaraldehyde, when used for linking anthracyclines to proteins, cause polymerisation, thus hindering the coupling (Pietersz et al, 1988 & 1989). This problem has been circumvented by Pagé's group from Quebec (1987, and Personal Communication ISOBM XV Annual Meeting, Quebec City, Aug. 30-Sept. 3 1987) by activating daunorubicin derivatives with aldehydes and using glutaraldehyde as the linking agent to anti-CEA monoclonal antibodies. The derivatives were found to be pharmacologically active and stable against acid hydrolysis without significant polymerisation of the conjugates. Coupling ratios of up to 12 moles drug per antibody molecule were obtained. However, this procedure was not reproducible when doxorubicin was substituted for daunorubicin, as shown in Section III 3.1.

(v) Finally, this problem was discussed with Dr. Brian Hasinoff, Assistant Professor, Chemistry Department, Memorial University of Newfoundland who has worked extensively with the structure and chemistry of HRP and doxorubicin (Hasinoff, 1970 & Personal Communication). Additional problems appeared to be the formation of free superoxide radicals in solution by HRP and doxorubicin

resulting in self coupling of the enzyme. Further, the presence of metallic ions in the buffer solution interferes with the coupling reaction by binding strongly with Dox forming a complex. (Hasinoff & Davey, 1988; Hasinoff, Davey & O'Brien, 1989). Sodium flouride and EDTA were added to the reactions in an attempt to overcome the above two problems, but did not result in conjugate formation.

At this stage, alternative enzymes were considered, based on a report of β-D-galactosidase conjugation to doxorubicin using the m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) as a coupling agent (Hirano, Nagal, Adachi, Ito & Sugiura, 1983). However, similar problems as with Dox-HRP conjugations discussed above were met, including failure of recognition of Dox-β-galactosidase by rabbit anti-Dox antibodies. Indirect methods were attempted to enzyme label Dox via the avidin-biotin bridge, by first conjugating Dox to either avidin or biotin and then using commercially available biotinylated or streptavidin peroxidase. Procedures were extrapolated from the avidin-biotin system used for labelling antibodies with enzymes or FITC (Goding, 1986; Boorsma, Van Bommel & Vander Raaij-Helmer, 1986; Ford et al,1987a).

Given these problems in producing Dox-enzyme conjugates, a CEA-HRP conjugate was produced by the periodate method and utilised to develop the BsMab dual assay (Section III. 5.4).

IV 2.3 Immunization

Immunogenicity of Dox-KLH was confirmed in a rabbit and shown to induce high titre anti-Dox antibodies up to 1/100,000 dilution of the serum (Figure 17). Dox-KLH was also used to immunize mice in preparation for the fusions. The technique of production of Mabs by Kohler & Milstein (1975), has undergone several modifications and there has been no standardised immunization protocol. Further, immunization varies with the antigen used. In general, particulate and cell surface antigens are given i.p. or i.v. and soluble antigens are given by the s.c., i.m., i.p. or i.v. routes (French et al, 1986; Brown & Ling, 1988). For the initial Dox-KLH fusions, the intraperitoneal approach was used, since this route has been recommended in enhancing Mab production (French et al, 1986). However, the Freund's adjuvant caused increased adhesions and granulomas, making the dissection of spleens difficult. In addition, the length of the immunization may be variable, ranging from weeks to months (Galfre & Milstein, 1981; Brown & Ling, 1988). This justifies the variation in time of the Dox-KLH immunization protocol (Tables 6a, 6b & 6c) which depended upon the availability of Dox-KLH conjugates and ongoing fusions delaying the performance of the next fusion. For better analysis of the fusions, the parameters have been delineated according to the length of the immunization schedule, quantity of Dox given,

adjuvant and route (Table 6a, 6b & 6c). Several of the immunizations resulted in up to 1/1000 titre anti-Dox antibodies (Figure 18 to 21) including fusions 11 and 13 using a prolonged immunization protocol (>200 days; Table 6a, 6b a), indicating successful immunization. However, more hybrids resulted from fusion #16 with a shorter immunization schedule.

IV. 2.4 Enzyme linked immunosorbent assays (ELISAs)

An anti-CEA assay had been standardised in the laboratory using cuvettes as a solid phase support (Woodhouse, Ford & Newman, 1981; Woodhouse et al, 1982b; Ford et.al. 1987a). For BsMab detection, 96 well microtitre plates were chosen instead of the cuvettes, due to their convenience in testing a large number of supernatants simultaneously (Brown & Ling, 1988). In addition, the absorbance readings could be measured using an automated microtitre plate absorbance reader (EIA Bio-Tek) and computer programs were available for rapid analysis of the data (Caulfield & Schaffer, 1984).

The dual assay was standardised using a similar Dox coating as the anti-Dox assay. A 1/50 to 1/75 dilution of CEA-HRP showed significant recognition by 11-285-14 compared to the control (Figure 12). Aliquots of CEA-HRP were frozen in -20°C for future use. For the initial fusions, it was convenient to perform the dual assay alone to evaluate the

hybrids. However, as illustrated by fusion #7 (Table 21), most hybrids (over 90%) were negative for anti-CEA activity (only 14 positive out of 146). This resulted in low titre dual specific antibodies many of which, when later tested for anti-CEA activity, were found to have lost their reactivity with CEA. In addition, since our supply of CEA was limited, it was decided to conserve CEA-HRP by performing the dual assay only on those hybrid supernatants that were found to be positive in both anti-CEA and anti-Dox assays, which would enhance the selection of BsMab positives. Indeed, Milstein's group have recommended simultaneous testing for the individual specificities due to the non reliability of dual specific assays for initial testing (Suresh et al 1986a & b).

IV 2.5 Fusions

The total spleen cells obtained and used with 11-285-14 for each fusion have been summarised with the number of cells aliquoted per well of the fusion plate (Table 13).

Analysing the results, with the number of hybrids as the end point of these fusions, seven of the sixteen fusions yielded hybrids, i.e. #4,5,6,7,11,13 and 16a. However, as seen from Tables 6, 13 and 21 it is difficult to identify a common denominator amongst these. The immunization period varied from as short as 3 weeks for fusion #16a to 42 weeks for #11. The amount of Dox employed ranged from 19 to 41 ug

in total. However, successful fusions resulted from mice which yielded greater than 9.06 x 10^7 spleen cells each. This is above the average of 7.53 x 10^7 spleen cells yield per mouse, utilised for those fusions not resulting in hybrids. Therefore, it may be interpreted that a spleen cell number over 9.06 x 10^7 usually resulted in hybrids, with fusion #4 being the exception, yielding hybrids when only 4.0 x 10^7 spleen cells were obtained from the mouse. This is reflected by a lower cell density per well in this fusion. Excluding fusion #4, it is possible to conclude that a higher number of spleen cells indicate better immunization, and therefore, more positive hybrids. However, when the sera of these mice were analysed for anti-Dox antibodies by ELISA, as an indicator of immunization, sera of all fusions showed an antibody titre of 1/100 to 1/1000 indicating successful immunization.

It has been documented that the viability of the fusion partners is important for successful fusions (Brown & Ling, 1988). The viability prior to Dox-KLH fusions was excellent, being at least over 88% for the spleen cells and mostly over 80% for 11-285-14. The importance of suitable 11-285-14 as the fusion partner is reinforced from fusion 16a using Aza-3, where all the 195 hybrids produced were positive for anti-CEA activity, while only 14 out of 146 were positive in fusion #7 using clone VI. Finally, although fusion #16a resulted in 195

hybrids, fusion #16b performed with NS-1 cells under identical immunization and fusion conditions, resulted in only 1 hybrid which was negative in the anti-Dox ELISA. This highlights the difficulty in identifying a common denominator and the appropriate conditions for fusions that would result in hybrids secreting BsMabs.

IV 2.6 Selection and cloning

The results of Dox-KLH fusions were disappointing, although 621 hybrids resulted from the 16 fusions. 36.5% hybrids were anti-Dox positive and at least 57.8%, anti-CEA positive (Table 21), excluding the hybrids not tested for anti-CEA. 46 hybrids (8%) showed dual activity, but were weakly so and became negative on subsequent cloning and expansion.

At this stage, the data was presented at the Ninth European Immunology meeting in Rome, September 1988 and elicited interest from Dr. Sylvia Menard, Milan, who was part of a group that was working on the production of monoclonal antibodies to Dox. Although their data had not been published at that time, there were similar problems with the production of Mabs, with a long immunization schedule resulting in low titre, poor quality hybrids. This was circumvented by Dr. Menard's group by using a shorter immunization protocol of 2-3 weeks which resulted in high titre stable hybrids. In

addition, BSA was used as the carrier protein which made immunization with up to 50 ug Dox possible at a given time.

Based on this information, fusion #16 was performed using a short immunization cycle of only 3 weeks using Dox-KLH as the immunogen (Table 6c). Surprisingly, although sera from the mice had lower titre anti-Dox antibodies the maximum number of hybrids (195) resulted from this fusion, with a few having highest anti-Dox readings by ELISA (Table 20b) to that date. However, these hybrids were unstable, either dying in culture or turning negative for anti-Dox activity. Furthermore, fusion #16b, performed under identical fusion conditions produced only a single hybrid which was negative in anti-Dox ELISA.

The Dox-KLH fusion results are consistent with the results obtained by Dr. Menard's group (Balsari et al 1988), in the production of anti-Dox Mabs using Dox-BSA conjugates as the immunogen. Fusions performed with low doses of Dox (10 ug), with a prolonged immunization schedule, resulted in a scanty number of unstable non producing hybrids. However, in their study a high serum antibody titre was obtained in response to only larger doses of Dox (50 ug) unlike the good response (up to 1:1,000 titre) in our mice with as little as 4 to 8 ug of Dox given in the form of Dox-KLH conjugate. The results in this thesis confirm the potent immunogenicity of Dox-KLH conjugates, but raises the question as to why this did

not translate into hybrids with a stable production of anti-Dox, despite a high serum antibody titre. The causes could be multifactorial, based on our current knowledge of the molecular and cellular mechanisms of the immune system.

Firstly, these hybrid-hyrids have to retain an aneuploid number of normal chromosomes following the fusion, which adds to their instability (Songsivilai & Lachmann, 1990).

Secondly, as discussed earlier, this polyploidity predisposes to a random loss of chromosomes, with a higher propensity to involve one or more of the immunoglobulin loci which are spread amongst three different chromosomes (Yancopoulos & Alt, 1986, Suresh et al, 1986a).

Thirdly, in addition, the explanation may lie in the immunomodulating properties of doxorubicin. It has been suggested that long term immunization with Dox selects low affinity B lymphocytes resulting in low affinity, unstable hybrids (Balsari et al, 1988). Conversely, it has recently been demonstrated that low doses of Dox can enhance the secretion of immunoglobulin by hybridoma B cells, perhaps associated with differentiation to plasma cells (Teillaud, Fourcade, Huppert, Fridman & Tapiero, 1989). However, our experience has been similar to the Balsari et.al report that long term immunization with low quantities Dox may select poor quality, unstable hybrids (Balsari et al, 1988). Indeed, these results suggest the following hypothesis regarding the

role of doxorubicin as an immunogen. Doxorubicin, itself being a cytotoxic chemotherapeutic agent, when coupled to a carrier protein, acts as a toxic antigen when taken up by antigen presenting cells (APCs). In particular, high affinity B cells, are prone to be destroyed. Low affinity B cells may escape detruction as they contact smaller quantities of the drug, or perhaps, are stimulated indirectly as bystander cells. These surviving low affinity B cells are then available for fusions, resulting in hybrids with low titre anti-Dox activity. The carrier specific immunosuppressive properties have been demonstrated by preliminary experiments recently, where a Dox-BSA conjugate had significantly diminished the primary immune response to BSA in mice (Balsari, Cerofolini & Ghione, 1991). Similarly, the antibody response to Dox-KLH appeared to be diminished in the hybrids produced in our Dox-KLH fusions (Fusion 13, Tables 19a & b).

IV 3.0 DOX-BSA FUSIONS

With the evidence supporting Dox-BSA as a suitable immunogen (section IV.2.6.) eight fusions were performed with Dox-BSA immunized mice. Hybrids were obtained demonstrating dual activity.

IV 3.1 Dox-BSA conjugates

The experiments were similar to Dox-KLH conjugate production, with ECDI as the crosslinker. The details of the conjugates have been summarised in Table 10, and as in Dox-KLH conjugation, there was considerable loss of the drug even prior to the conjugation reaction. For example, for conjugate #7 in Table 10, of the 17 mg of doxorubicin at the start of the experiment, 2.8 mg (16.4%) was available as dissolved doxorubicin, the rest having precipitated out of solution. For the 10 ml of conjugate obtained, the conjugate yield was 1.35 mg Dox as Dox-BSA (Table 10). Thus, less than 8% of the drug resulted in the subsequent conjugate. These results are compatible with the Dox-KLH conjugations and with drug-protein conjugations performed by other groups which have reported 80-90% loss of the drug during conjugation (Pietesz et al, 1988).

The highest molar ratio was up to 5.5 moles of the drug linked per mole of BSA (Table 10) which is within the acceptable range of Dox-protein conjugation reported by others (Hurwitz et al 1975). It is interesting that Dox-KLH

conjugations yielded up to 569 moles Dox per mole KLH. However, KLH is a larger molecule with weight range of 9 to 15 x 10^6 (Sigma Chemical Co.), nearly 200 times that of BSA (66,000 mol. weight). A practical advantage of Dox-BSA over Dox-KLH was the high concentration of the drug (up to 135ug) present per ml of Dox-BSA conjugate, compared to 39 ug of doxorubicin per ml Dox-KLH. This higher concentration facilitated the use of smaller volumes for immunization in the mice, reducing the amount of Freund's adjuvant required.

IV 3.2 Immunization

The immunization protocol (Table 7) was standardised to approximately 4 weeks duration and mice received a higher dose of Dox at 50 ug per injection, compared with the Dox-KLH immunizations. The Dox-BSA conjugates produced an anti-Dox antibody response in mice up to 1/1,000,000 titre as tested against Dox-KLH coated plates in ELISA (Figure 23). It is interesting however, that the spleen cells obtained were variable despite identical immunization conditions. For example, mice used for fusions #1 and 2 yielded 8.16 x 10^7 and 12.16 x 10^7 spleen cells respectively. This may have contributed to the single hybrid resulting from fusion #1 compared to 77 hybrids from fusion #2 (Table 27 & 34).

IV 3.3 Fusions

Unlike the initial Dox-KLH fusions, Dox-BSA fusions and subsequent selection of hybrids was performed with spleen cells and thymus cells as feeder layers. In addition, 20% fetal calf serum was supplemented for the Dox-BSA fusions and for cloning as recommended by Galfre and Milstein (1981).

Of the eight Dox-BSA fusions performed, fusion #5 yielded the maximum number of hybrids (Table 30). Interestingly, the number of spleen cells were the highest for fusions #2 and 5 (table 27), and these two fusions yielded 85.8% of the total hybrids obtained (table 34). This appears to be a common factor amongst the Dox-KLH and Dox-BSA fusions where the highest number of spleen cells resulted in the greatest number of hybrids. However, the anti-Dox titre of the mouse sera did not appear to correlate with the resulting hybrid number. For example, Fusion 8 sera resulted in the highest anti-Dox titre (1/1,000,000), but a poor yield of spleen cells and hybrids was obtained (Figure 23 & Tables 27 & 34).

IV 3.4 Selection and cloning

This has been technically the most demanding part of this project, due to the rapidity of growth of the hybrids and therefore, the urgency of screening positive hybrids for cloning. To enhance the stability of hybrids while cloning, both 20% FCS as well as feeder layers were used. With the

assurance of stable anti-CEA activity of hybrids by using the Aza-3 clone as the 11-285-14 fusion partner, initial selection of hybrids was based on anti-Dox activity. However, anti-CEA assays were intermittently performed to reconfirm anti-CEA activity. Fusion #5 yielded the maximum number of hybrids, 178 in total, 107 of which were positive for anti-Dox activity (Tables 28a & b and 30). Positive clones were recloned down to 1 cell and 0.5 cells per well based on the Poisson distribution (Hudson & Hay 1980). 245 anti-Dox clones obtained were further subcloned to yield 1325 second and third generation subclones. Several of these clones (286 in total) have been cryopreserved in liquid nitrogen directly or after initial expansion. Seven of these subclones have been expanded to maintain adequate stocks for future use. Hybrids # 1-14a-26-61-1, 1-14a-26-61-2, 1-14a-26-61-4, 1-14a-57-9-6-4 have all shown anti-Dox and anti-CEA activity, and have been negative for anti-BSA activity (Table 33). In addition, hybrid 1-14a-26-61-1 has been tested against the Dox-BSA linker ECDI coated plates, by ELISA, in order to eliminate the remote possibility of false positives due to recognition of the linker instead of doxorubicin. All the ELISAs performed used the appropriate positive and negative controls to confirm the validity of the results. The dual assay ELISA readings have been lower when compared to anti-CEA ELISA values. However, these readings depend on the concentration of

antibodies in supernatants tested. In addition, the CEA-HRP conjugate itself may be of too great a molecular weight (CEA + HRP = 224,000) to be held by the univalent antibodies (BsMabs) due to their decreased avidity. This situation would be reversed *in vivo* where the antibodies would bind to a more stable cell membrane CEA molecule, the labile antigen binding site recognising a much smaller doxorubicin (molecular weight 580). In confirmation of this concept, bispecific antibodies recognising CEA have been shown to effectively target vinca alkaloids both *in vitro* and *in vivo* (Corvalan & Smith, 1987). Such poor activity in assays that test for the presence of BsMabs directly has been documented in other studies (Suresh et.al. 1986b). Therefore, though technically demanding, individual assays for the different specificities are recommended (Suresh et al, 1986b).

As presented in the introductory chapter (section I 10.2), and documented by Milstein's group, there is a preferential association of homologous heavy and light chains in hybrids secreting BsMabs (Milstein & Cuello, 1984; Suresh et al, 1986b). This results in three main species of antibodies secreted by the hybrids. These are the BsMab, in addition to the parental antibodies. Furthermore these studies have indicated that the yield of BsMabs may be as high as 30 to 50% of the total secreted immunoglobulins.

In the final results of this thesis, seven stable hybrid

cell lines have been produced, secreting antibodies that demonstrate both anti-Dox and anti-CEA activity by ELISAs (Table 33). While it may be argued that these two specificities may be related to the secretion of parental unispecific antibodies, the seven cell line supernatants also show dual specifity by ELISA, which would not be seen with unispecific antibodies. The weaker reactivity of BsMab supernatants in dual assays are likely due to competetive inhibition by unispecific antibodies (Suresh et al,1986a & b). In addition, these cell lines have been obtained following cloning, at least two or three times by limiting dilution, ensuring clonality and stability of growth and antibody secretion. Whether these BsMabs are efficaceous in targeting would be the subject of an additional research project; however, the objectives set at the begining of this thesis have been achieved with the productio· of these BsMabs demonstrating dual reactivity against CEA and doxorubicin.

IV 4.0 PROSPECTS FOR BISPECIFIC ANTIBODIES

The potential applications of BsMabs, both as investigational and therapeutic reagents, may be realised from the following examples of bispecific antibodies that have been produced by other groups.

IV 4.1 Immunocytochemistry and Immunoassays

Bifunctional antibodies have been produced against enzymes like HRP or to various hormones/antigens, with simultaneous anti-somatostatin, anti-substance P or anti-flourescein isothiocyanate (FITC), activities with potential widespread applications in immunocytochemistry and enzyme immunoassays (Milstein & Cuello, 1983; Suresh et al, 1986b; Karawajew, Behrsing, Kauser & Micheel, 1988). These procedures were made simpler because of the one step shortened incubation time in comparison with the conventional two step assays and may have a potential in routine screening where rapidity rather than maximum sensitivity is required. However, sensitivity is not necessarily sacrificed, as demonstrated by anti-FITC and anti-HRP BsMabs, in the detection of FITC-Mab labelled AFP or HCG bound to solid phase, where the assay was equally sensitive as conventional ELISA systems (Karawajew et al, 1988). Furthermore, BsMabs recognising both HCG and urease have been used in high sensitivity immunoassays for HCG, detecting levels as low as

25 mIU/ml (Takahashi & Fuller, 1988; Takahashi, Fuller & Winston,1991).

IV. 4.2 T cell targeting

BsMabs are currently being evaluated in the targeting of the body's own immune system in the therapy of cancer and viral diseases (Fanger, Segal & Romet-Lemmone, 1991; Waldmann, 1991). Heteroconjugated Mabs of two specificities have been shown to target cytotoxic T-effector cells via the T cell receptor (TcR) against a H-2 antigen on EL-4 murine thymoma (Barr, Macdonald, Buchegger & Fliedner, 1987). Bispecific antibodies recognising the CD3 surface antigen induced lysis of virally infected cells carrying the herpes simplex virus (HSV-1) glycoprotein C (Paya, Mckean, Segal, Schoon, Showalter & Leibson, 1989).

As a further refinement, it has been demonstrated that as little as 2ng/ml of BsMab can target T cells via the TcR inducing lysis *in vitro* of over one third of the murine tumor cells expressing the thy 1.1 antigen (Staerz & Bevan, 1986). Interestingly, the mechanism of lysis of cells by retargeted T-effector cells circumvented the major histocompatibility complex restriction (Clark, Gilliland & Waldmann, 1988 a & b; Qian, Titus, Andrew, Mezzanzanica, Garrido, Wunderlich & Segal, 1991). It has been well established for conventional cell mediated immunity, that the target antigen, in

association with the major histocompatibility complex is a prerequisite for triggering T cells (Lanzavecchia, 1990). Recently a universal BsMab has been produced recognising the T cell CD3 antigen and rat kappa light chain simultaneously, thus indirectly retargeting effector cells to any target coated with rat kappa bearing antibody (Clark et al, 1988; Waldmann, 1989). Bsmabs may also have potential in treatment of certain viral infections, as recently demonstrated by an anti-CD3 bifunctional antibody redirecting cytotoxic T lymphocytes of any specificity to cells that express gp120 of the human immunodeficiency virus (HIV), inducing lysis *in vitro* (Berg, Lotsher, Steimer, Capon, Baenziger, Jack & Wabl, 1991).

Efficacy of BsMabs *in vivo* has been demonstrated in mice bearing BCL-1 lymphoma. 5 ug of BsMabs directed against CD3/TCR complex and surface Id antigen of BCL-1 tumours, when given intravenously induced a cure compared to controls (Brissinck, Demanet, Moser, Leo & Thielemans, 1991). Furthermore, the feasibility of heteroconjugated bispecific antibodies in patients has been demonstrated in a small number of patients with malignant glioma, resulting in effective retargeting of lymphokine activated killer (LAK) cells and regression or early eradication of tumor (Nitta, Sato, Yagita, Okumara & Ishii, 1990). Currently, Phase I-II trials are underway, with BsMab retargeted lymphocytes, for the

intraperitoneal treatment of ovarian carcinoma patients (Bolhuis,Sturm,Gratama & Braakman,1991).

IV. 4.3 Immunochemotherapy

While many studies have focussed on targeting effector T cells, there have been few reports involving BsMabs against chemotherapeutic agents and tumour associated antigens. The reasons may be similar to the poor results obtained in the Dox-KLH fusions, as discussed in section IV 2.6. In particular, as discussed, the concept of the drug acting as a lethal antigen may play a major role in diminishing the immune response by destroying the antigen presenting cells (Balsari et al, 1991). To date there are only three such BsMabs documented in the literature produced by the hybridoma technology. These are BsMabs against vinca alkaloids and CEA (Corvalan et al, 1988), anthracyclines and rat hepatoma antigen (Tsukada, Ohkawa, Hibi, Tsuzuki, Oguma & Satoh, 1989) and methotrexate and gp72 antigen expressing human osteosarcoma (Pimm, Robins, Embleton, Jacobs, Markham, Charleston & Baldwin, 1990). The well studied BsMabs against CEA and vinca alkaloids have been demonstrated to show superior suppression of human colorectal tumour growth *in vivo* in nude mice as compared to the free drug (Corvalan et al, 1988).

Few reports have included chemically heteroconjugated bispecific antibodies, an example being F(ab2) anti-CEA and anti-bleomycin (as a benzoyl derivative). These antibodies were injected into patients with colon cancer, 24 to 120 hours prior to injection of indium labeled drug. There was tumour targeting with low uptake by liver and the bone marrow (Stickney, Slater & Frincke, 1989).

IV.5.0 CONCLUSION AND FUTURE

Reviewing the several modifications of the experimental protocol that eventually led to the production of BsMabs against both CEA and Doxorubicin, the following steps are recommended for those interested in similar production. Selection of suitable carrier protein, shorter immunization protocol, feeder layers with upto 20% FCS supplementation for growth of hybrids, standardization of three ELISAs and urgent cloning of positive hybrids.

Evidence that carcinoembryonic antigen is a suitable antigen for targeting with BsMabs has been provided by recent studies involving lysis of CEA expressing cancer cells *in vitro* with ricin toxin A chain (Embleton, Charleston, Robins, Pimm, and Baldwin, 1991) and *in vivo* with vinca alkaloids (Smith, Gore, Brandon, Lynch, Cranstone & Corvalan, 1990). The *in vivo* studies with anti-vinblastine/anti-CEA BsMabs revealed increased local tumor drug concentration and could

effectively suppress the growth of human tumor xenografts in nude mice, compared with the free drug (Corvalan et al, 1988).

The effectiveness of anthracyclines as suitable chemotherapeutic drugs for targeting has been supported by a recent preliminary report of a BsMab recognising a metastatic hepatoma cell line and daunomycin, also cross reacting with doxorubicin. These BsMabs were more effective in vivo than antibody-drug conjugates, perhaps due to increased local concentration of unmodified drug (Tsukuda, Ohkawa, Hibi, Tsuzuki, Oguma & Satoh, 1989).

With these promising reports of the suitability of CEA as the target and doxorubicin as the therapeutic agent, it is reasonable for an optimistic expectation of the further evaluation of BsMabs produced as the end result of this project. Future progress would involve:

(1) **Production** of larger quantities of antibody *in vitro* and *in vivo* as ascites in Balb/C mice.

(2) **Purification** involving Protein S sepharose as the first stage followed by passage down a CEA affinity column and passage of the eluted material down a doxorubicin affinity column. The eluted material should only contain antibodies with dual specificity.

(3) **Evaluation of the affinity** of the purified BsMabs could be performed by looking at binding assays with radiolabelled or enzyme labelled CEA and then, subsequently, with radiolabelled doxorubicin.

(4) **Competitive inhibition studies** with other anthracyclines should be performed to determine the degree of cross reactivity, if any.

(5) **The targeting potential** of BsMabs would initially be evaluated *in vitro* utilizing CEA expressing human tumour cell lines growing in culture. The cytotoxicity of a mixture of the BsMabs and varying concentrations of doxorubicin would be compared with appropriate quantities of BsMabs, free drug, 11-285-14-Dox immunoconjugates and PBS (control). Subsequently, the efficacy of BsMabs *in vivo*, in the suppression of human tumor xenografts growing in nude mice, would be evaluated. Comparisons would be made with groups of mice treated with appropriate controls as tested *in vitro*. In addition, the half life pharmacokinetics, tissue distribution and toxicity would be studied.

(ii) Targeting Doxorubicin-Carrier protein:

As recalled from the Results (Table 34) while evaluating hybrids for anti-CEA/anti-Dox activity, several hybrids were found to have anti-CEA/anti-BSA activity, since BSA was used as the carrier molecule. Some of these hybrids showing both anti-CEA and anti-BSA activities by ELISAs have been preserved under liquid nitrogen. Such BsMabs could have a role in targeting several molecules of doxorubicin conjugated to a single molecule of BSA as a carrier. This is supported by the recent report by Pimm et al (1990) that a bispecific antibody reactive with methotrexate and a human osteosarcoma associated antigen (gp72) demonstrated augmented cytotoxicity of methotrexate-human serum albumin conjugate. Given the inter-species homology between serum albumins (Meloun, Moravek & Kostka,1975) anti-CEA/anti-BSA BsMabs may be effective in delivering several molecules of doxorubicin linked to human serum albumin, which would be less immunogenic than BSA in patients.

In conclusion, thirty bispecific fusions have been performed. Twenty four fusions were bispecific, sixteen using Dox-KLH and eight using Dox-BSA immunized mice (Tables 22 & 34). In addition, six fusions were performed as an indirect approach in producing a hybridoma secreting anti-Dox Mabs, which did not result in suitable hybrids. 1,192 hybrids and 1,962 clones thereof, have been evaluated for anti-Dox

activity, and positives further selected based on anti-CEA activity. 286 of these hybrids demonstrating both activities have been stored at -70°C and seven of the clones demonstrating the highest absolute ELISA readings and positive reactivity in bispecific, anti-CEA, anti-Dox assays, have been further expanded to provide adequate stocks for future studies.

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