

PURIFICATION AND CHARACTERIZATION OF MOUSE
BISPECIFIC MONOCLONAL ANTIBODIES RECOGNIZING
CARCINOEMBRYONIC ANTIGEN (CEA) AND DOXORUBICIN

PERRY ALONZO OSBORNE



**Purification and Characterization of Mouse Bispecific Monoclonal Antibodies
Recognizing Carcinoembryonic Antigen (CEA) and Doxorubicin**

By

Perry Alonzo Osborne B.Sc (Honours)

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Department of Surgery
Faculty of Medicine
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ABSTRACT

Bispecific hybrid-hybridomas have been developed previously from an anti-carcinoembryonic antigen (CEA) hybridoma (11-285-14) and spleen cells from mice inoculated with doxorubicin-bovine serum albumin (DOX-BSA) conjugate. Seven hybrid-hybridomas were selected on the basis of binding to both CEA and DOX. The purpose of this study was to purify and characterize these antibodies.

Each hybridoma was separately injected into Balb/c mice intraperitoneally and ascitic fluid collected. The pooled fluid was then Protein A affinity purified and then further purified using an hydroxylapatite, high performance liquid chromatography (HPLC) column. Fractions from HPLC separations were tested using various enzyme linked immunosorbent assays in order to determine the location of the bispecific antibodies. Positive fractions were pooled and used in microcytostasis assays using the high CEA expressing (SKC01) and low CEA expressing (COLO 320 DM) cell lines. One particular hybridoma (26-61-10) was selected for these *in vitro* assays. The bispecific antibody + DOX test was able to significantly decrease the IC_{50} of DOX with SKC01. The IC_{50} with the COLO 320 DM was not altered, illustrating the specificity of the bispecific antibody for a CEA expressing cell line. The specificity of the results were confirmed when DOX was used in conjunction with a non-specific antibody (Ag8). Bispecific antibody on its own or control antibody on its own, did not have a significant effect on the viability of the cell lines. This supported the theory of the bispecific antibody delivering the drug directly to the CEA expressing cancer cells and that the increased toxicity was not due to direct

antibody killing of cells.

These results provide the first demonstration that the drug doxorubicin can be targeted to a colonic cancer cell line expressing CEA by a bispecific antibody recognising CEA and the drug.

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Many thanks also go out to my wife and family for their moral support and encouragement during this project. I would like to dedicate this thesis to my family in memory of my grandfather, Adolphus Osborne, who passed away due to cancer.

Like all families who have dealt with this disease, we hope that some day there will be a cure.

TABLE OF CONTENTS

| | Page |
|---|-----------|
| ABSTRACT | ii |
| ACKNOWLEDGEMENTS | iii |
| LIST OF TABLES | xii |
| LIST OF FIGURES | xiv |
| LIST OF ABBREVIATIONS | xviii |
| | |
| CHAPTER I: INTRODUCTION | 1 |
| I. 1.0 INTRODUCTION | 1 |
| I. 1.1 Cancer Cell Growth | 1 |
| I. 1.2 Limitations of Cancer Treatment | 3 |
| I. 2.0 ANTIBODY-MEDIATED TARGETING | 5 |
| I. 2.1 Components of Targeting Therapy | 6 |
| I. 2.2 Target Specificity | 6 |
| I. 2.3 Tumour Associated Antigens | 7 |
| I. 2.4 Obstacles in Using Tumour Antigens for Targeting | 8 |
| I. 2.4(a) Antigen Expression and Shedding | 8 |
| I. 2.4(b) Antigen Modulation | 9 |
| I. 2.4(c) Antigen Heterogeneity | 9 |
| I. 2.4(d) Antigen Density | 10 |
| I. 2.4(e) Antibody Immunogenicity | 10 |
| I. 3.0 DRUG CARRIERS | 11 |
| I. 3.1 Monoclonal Antibodies and Cancer Research | 11 |
| I. 3.2 Immunoconjugates | 12 |
| I. 3.2(a) Radiolabelled Antibodies | 13 |
| I. 3.2(b) Immunotoxins | 13 |
| I. 3.3 Drug Immunoconjugates | 14 |
| I. 4.0 BISPECIFIC MONOCLONAL ANTIBODIES | 16 |
| I. 4.1 Bispecific Antibody Production | 17 |
| I. 4.2 Biological Production | 18 |
| I. 4.2(a) Bispecific Antibody Light Chain Expression | 18 |

| | | | |
|------------|------------|---|-----------|
| | | I. 4.2(b) Bispecific Hybridoma Production | 21 |
| | I. 4.3 | Chemical Production | 21 |
| | I. 4.4 | Recombinant DNA Bispecific Production | 22 |
| I. | 5.0 | PURIFICATION OF BISPECIFIC ANTIBODIES | 23 |
| I. | 6.0 | CARCINOEMBRYONIC ANTIGEN | 24 |
| | I. 6.1 | CEA as a Target for Antibody-Mediated Targeting (AMT) | 24 |
| I. | 7.0 | DOXORUBICIN | 26 |
| I. | 8.0 | OBJECTIVES | 27 |
| | | CHAPTER II: MATERIALS AND METHODS | 29 |
| II. | 1.0 | BISPECIFIC HYBRIDOMAS | 29 |
| | II. 1.1 | Colonic Cancer Cell Lines | 29 |
| | II. 1.2 | Tissue Culture Maintenance | 29 |
| | II. 1.3 | Medium for COLO 320 DM and the Bispecific Hybridomas | 30 |
| | II. 1.4 | Medium for SKC01 | 30 |
| | II. 1.5 | Trypsinization of cells (for COLO 320 DM And SKC01) | 30 |
| | | II. 1.5(a) Materials | 30 |
| | | II. 1.5(b) Method | 31 |
| | II. 1.6 | Cell Count | 32 |
| | | II. 1.6(a) Materials | 32 |
| | | II. 1.6(b) Method | 32 |
| | II. 1.7 | Cryopreservation of Cells | 33 |
| | | II. 1.7(a) Materials | 33 |
| | | II. 1.7(b) Method | 33 |
| | II. 1.8 | Thawing of Cells | 33 |
| II. | 2.0 | PRODUCTION OF BSMab ASCITIC FLUID | 34 |
| | II. 2.1 | BSMab Production in Mice | 34 |
| | | II. 2.1(a) Materials | 34 |
| | | II. 2.1(b) Method | 35 |

| | | |
|----------------|---|-----------|
| II. 2.2 | Tapping of Ascitic Fluid | 35 |
| II. 2.2(a) | Materials | 35 |
| II. 2.2(b) | Method | 36 |
| II. 3.0 | PURIFICATION OF BSMab FROM ASCITIC FLUID | 36 |
| II. 3.1 | Protein A-Sepharose Affinity Purification | 36 |
| II. 3.1(a) | Materials | 37 |
| II. 3.1(b) | Protein A Column Preparation | 39 |
| II. 3.1(c) | Ascitic Fluid Filtration | 39 |
| II. 3.1(d) | Purification | 40 |
| II. 3.2 | High Performance Liquid Chromatography (HPLC) | 41 |
| II. 3.2(a) | Materials | 41 |
| II. 3.2(b) | Method I | 43 |
| II. 3.2(c) | Method II | 44 |
| II. 4.0 | DOX-BSA CONJUGATION | 44 |
| II. 4.0(a) | Materials | 45 |
| II. 4.0(b) | Method | 45 |
| II. 4.1 | Gel Filtration | 47 |
| II. 4.1(a) | Materials | 47 |
| II. 4.1(b) | Method | 47 |
| II. 4.2 | CEA-HRP Conjugate Production | 48 |
| II. 4.2(a) | Materials | 48 |
| II. 4.2(b) | Method | 49 |
| II. 5.0 | ELISA | 50 |
| II. 5.1 | Introduction | 50 |
| II. 5.1(a) | Materials | 50 |
| II. 5.2 | Anti-CEA ELISA | 52 |
| II. 5.2(a) | Materials | 52 |
| II. 5.2(b) | Method | 53 |
| II. 5.3 | Anti-DOX ELISA | 54 |
| II. 5.3(a) | Materials | 54 |
| II. 5.3(b) | Method | 54 |
| II. 5.4 | Anti-DOX-BSA ELISA | 54 |
| II. 5.4(a) | Materials | 54 |
| II. 5.4(b) | Method | 55 |
| II. 5.5 | Anti-BSA ELISA | 55 |
| II. 5.5(a) | Materials | 55 |
| II. 5.5(b) | Method | 55 |
| II. 5.6 | Dual Specificity ELISA | 55 |

| | | | |
|-------------|------------|---|-----------|
| II. | 6.0 | MICROCYTOSTASIS ASSAY | 56 |
| | | II. 6.0(a) Materials | 56 |
| | II. 6.1 | Microcytostasis assay Method | 57 |
| | II. 6.2 | Selection of Antibodies for Testing | 59 |
| | II. 6.3 | Antibody Mediated Targeting Method | 59 |
| | | CHAPTER III: RESULTS | 61 |
| III. | 1.0 | PRODUCTION OF DOX-BSA CONJUGATE | 61 |
| III. | 2.0 | PRODUCTION OF CEA-HRP CONJUGATE | 65 |
| III. | 3.0 | ASCITIC FLUID PRODUCTION AND PROTEIN A COLUMN PURIFICATION | 65 |
| III. | 4.0 | HPLC STANDARDIZATION | 69 |
| | III. 4.1 | Standardization using Parental 11-285-14 (anti-CEA monoclonal antibody IgG ₁) | 69 |
| | | III. 4.1(a) HPLC Separation | 69 |
| | | III. 4.1(b) ELISA Results | 71 |
| | III. 4.2 | Standardization using BSMab Separation | 71 |
| | | III. 4.2(a) HPLC Results | 71 |
| | | III. 4.2(b) ELISA Results | 74 |
| | III. 4.3 | HPLC Procedure Modifications | 74 |
| III. | 5.0 | ANALYTICAL HPLC AND ELISA ANALYSIS OF ALL SEVEN HYBRIDOMAS | 77 |
| | III. 5.1 | Bispecific Monoclonal Antibody 26-61-1 | 77 |
| | | III. 5.1(a) HPLC Profile | 77 |
| | | III. 5.1(b) ELISA Results | 79 |
| | III. 5.2 | Bispecific Monoclonal Antibody 26-61-2 | 79 |
| | | III. 5.2(a) HPLC Profile | 79 |
| | | III. 5.2(b) ELISA Results | 79 |
| | III. 5.3 | Bispecific Monoclonal Antibody 26-61-4 | 83 |
| | | III. 5.3(a) HPLC Profile | 83 |
| | | III. 5.3(b) ELISA results | 85 |
| | III. 5.4 | Bispecific Monoclonal Antibody 26-61-10 | 85 |
| | | III. 5.4(a) HPLC Profile | 85 |

| | | |
|-----------------|---|-----|
| | III. 5.4(b) ELISA Results | 88 |
| III. 5.5 | Bispecific Monoclonal Antibody 26-7-35 | 90 |
| | III. 5.5(a) HPLC Profile | 90 |
| | III. 5.5(b) ELISA Results | 90 |
| III. 5.6 | Bispecific Monoclonal Antibody 57-11-25-17 | 93 |
| | III. 5.6(a) HPLC Profile | 93 |
| | III. 5.6(b) ELISA Results | 93 |
| III. 5.7 | Bispecific Monoclonal Antibody 57-9-6-4 | 96 |
| | III. 5.7(a) HPLC Profile | 96 |
| | III. 5.7(b) ELISA Results | 96 |
| III. 6.0 | DUAL ACTIVITY TEST | 99 |
| III. 7.0 | PREPARATIVE HPLC PURIFICATION | 99 |
| III. 7.1 | 26-61-1 5 ml HPLC Separation | 101 |
| | III. 7.1(a) HPLC Profile | 101 |
| | III. 7.1(b) ELISA Results | 105 |
| III. 7.2 | 26-61-4 5 ml HPLC Separation | 105 |
| | III. 7.2(a) HPLC Profile | 105 |
| | III. 7.2(b) ELISA Results | 105 |
| III. 7.3 | 26-61-10 5 ml HPLC Separation | 113 |
| | III. 7.3(a) HPLC Profile | 113 |
| | III. 7.3(b) ELISA Results | 113 |
| III. 7.4 | 57-11-25-17 5 ml HPLC Separation | 118 |
| | III. 7.4(a) HPLC Profile | 118 |
| | III. 7.4(b) ELISA Results | 124 |
| III. 8.0 | <u>IN VITRO</u> MICROCYTOSTASIS ASSAYS | 124 |
| III. 8.1 | Mean IC ₅₀ Values | 124 |
| III. 8.2 | <u>In Vitro</u> Antibody Testing Using the SKC01 Colorectal Cancer Cell Line | 128 |
| | III. 8.2(a) Bispecific Monoclonal Antibody 26-61-10 | 128 |
| | III. 8.2(b) Ag8 Non-Specific Antibody | 131 |
| III. 8.3 | <u>In Vitro</u> Antibody Testing Using COLO 320 DM Colorectal Cancer Cell Line | 139 |
| | III. 8.3(a) Bispecific Monoclonal Antibody 26-61-10 | 139 |
| | III. 8.3(b) Ag8 Non-Specific Antibody | 144 |

| | | | |
|------------|------------|--|-----|
| | | CHAPTER IV: DISCUSSION | 149 |
| IV. | 1.0 | ASCITIC FLUID PRODUCTION AND PROTEIN A COLUMN PURIFICATION | 149 |
| IV. | 2.0 | PRODUCTION OF CONJUGATES | 151 |
| | IV. 2.1 | DOX-BSA Conjugate Formation | 151 |
| | IV. 2.2 | CEA-HRP Conjugate Formation | 151 |
| IV. | 3.0 | STANDARDIZATION OF THE HPLC PROCEDURE | 152 |
| | IV. 3.1 | Purification of 11-285-14 | 152 |
| | IV. 3.2 | Purification of BSMab 26-61-2 | 152 |
| IV. | 4.0 | ANALYTICAL HPLC PURIFICATION OF BISPECIFIC ANTIBODIES (1 ML INJECTIONS) | 153 |
| | IV. 4.1 | Dual Activity Assay | 155 |
| IV. | 5.0 | QUANTITATIVE HPLC PURIFICATION OF BISPECIFIC ANTIBODIES | 156 |
| IV. | 6.0 | IN VITRO CHARACTERIZATION OF 26-61-10 BISPECIFIC ANTIBODY | 158 |
| IV. | 7.0 | CONCLUSION AND SUGGESTED FUTURE WORK | 161 |
| IV. | 8.0 | CHAPTER V: REFERENCES | 163 |

LIST OF TABLES

| | | |
|----|--|-----|
| 1 | Summary of ascitic fluid collection and protein A purification | 68 |
| 2 | Results of dual assays on hybridoma protein A purified ascitic fluid | 100 |
| 3 | ELISA results from 1st 5 ml HPLC separation of the 26-61-1 bispecific hybridoma | 106 |
| 4 | ELISA results from the 2nd 5 ml HPLC separation of the 26-61-1 bispecific hybridoma | 107 |
| 5 | ELISA results from the 3rd 5 ml HPLC separation of the 26-61-1 bispecific hybridoma | 108 |
| 6 | ELISA results from the 1st 5 ml HPLC separation of the 26-61-4 bispecific hybridoma | 111 |
| 7 | ELISA results from the 2nd 5 ml HPLC separation of the 26-61-4 bispecific hybridoma | 112 |
| 8 | ELISA results from the 1st 5 ml HPLC separation of the 26-61-10 bispecific hybridoma | 117 |
| 9 | ELISA results from the 2nd and 3rd 5 ml HPLC separations of the 26-61-10 bispecific hybridoma | 119 |
| 10 | ELISA results from the 1st 5 ml HPLC separation of the 57-11-25-17 bispecific hybridoma | 125 |
| 11 | ELISA results from the 2nd 5 ml HPLC separation of the 57-11-25-17 bispecific hybridoma | 126 |

| | | |
|----|--|-----|
| 12 | ELISA results from the 3rd 5 ml HPLC separation of the 57-11-25-17 bispecific hybridoma | 127 |
| 13 | Summary of IC ₅₀ results | 129 |
| 14 | Calculated IC ₅₀ values of DOX+ 26-61-10 bispecific antibody & DOX + Ag8 non-specific antibody using SKC01 colorectal cancer cell line | 132 |
| 15 | Calculated percent survival of SKC01 when using bispecific antibody (26-61-10) and non-specific antibody (Ag8) controls | 135 |
| 16 | Calculated IC ₅₀ values using DOX+26-61-10 bispecific antibody & DOX +Ag8 non-specific antibody using COLO 320 DM colorectal cancer cell line | 143 |
| 17 | Calculated percent survival of COLO 320 DM when using bispecific antibody (26-61-10) and non-specific antibody (Ag8). | 148 |

LIST OF FIGURES

| | | |
|------|---|----|
| 1 | Expected molecular species in hybrid hybridomas arising by random association of heavy and light chains | 20 |
| 2 | DOX-BSA conjugate purification #1 | 62 |
| 3 | DOX-BSA conjugate purification #2 | 63 |
| 4 | DOX-BSA conjugate purification #3 | 64 |
| 5 | CEA-HRP conjugate purification | 66 |
| 6 | CEA-HRP conjugate specificity and sensitivity ELISA results | 67 |
| 7 | HPLC separation of monoclonal 11-285-14 | 70 |
| 8 | Anti-CEA ELISA results of 11-285-14 HPLC purified peaks | 72 |
| 9(a) | HPLC separation of ascitic fluid containing 26-61-2 bispecific antibody | 73 |
| 9(b) | ELISA results of 26-61-2 separation peaks | 75 |
| 10 | A comparison of 2 HPLC gradient profiles | 76 |
| 11 | HPLC separation of ascitic fluid containing 26-61-1 bispecific antibody | 78 |
| 12 | ELISA results of a 1 ml 26-61-1 HPLC separation | 80 |
| 13 | HPLC absorbance profile of ascitic fluid containing 26-61-2 bispecific antibody | 81 |
| 14 | ELISA results of a 1 ml 26-61-2 HPLC separation | 82 |
| 15 | HPLC absorbance profile of ascitic fluid containing 26-61-4 bispecific antibody | 84 |
| 16 | ELISA results of a 1 ml 26-61-4 HPLC separation | 86 |

| | | |
|----|--|-----|
| 17 | HPLC absorbance profile of ascitic fluid containing 26-61-10 bispecific antibody | 87 |
| 18 | ELISA results of a 1 ml 26-61-10 HPLC separation | 89 |
| 19 | HPLC absorbance profile of ascitic fluid containing 26-7-35 bispecific antibody | 91 |
| 20 | ELISA results of a 1 ml 26-7-35 HPLC separation | 92 |
| 21 | HPLC absorbance profile of ascitic fluid containing 57-11-25-17 bispecific antibody | 94 |
| 22 | ELISA results of a 1 ml 57-11-25-17 HPLC separation | 95 |
| 23 | HPLC absorbance profile of ascitic fluid containing 57-9-6-4 bispecific antibody | 97 |
| 24 | ELISA results of a 1 ml 57-9-6-4 HPLC separation | 98 |
| 25 | 1st quantitative HPLC absorbance profile of ascitic fluid containing 26-61-1 bispecific antibody | 102 |
| 26 | 2nd quantitative HPLC absorbance profile of ascitic fluid containing 26-61-1 bispecific antibody | 103 |
| 27 | 3rd quantitative HPLC absorbance profile of ascitic fluid containing 26-61-1 bispecific antibody | 104 |
| 28 | 1st quantitative HPLC absorbance profile of ascitic fluid containing 26-61-4 bispecific antibody | 109 |
| 29 | 2nd quantitative HPLC absorbance profile of ascitic fluid containing 26-61-4 bispecific antibody | 110 |

| | | |
|----|---|-----|
| 30 | 1st quantitative HPLC absorbance profile of ascitic fluid containing 26-61-10 bispecific antibody | 114 |
| 31 | 2nd quantitative HPLC absorbance profile of ascitic fluid containing 26-61-10 bispecific antibody | 115 |
| 32 | 3rd quantitative HPLC absorbance profile of ascitic fluid containing 26-61-10 bispecific antibody | 116 |
| 33 | Results from dual assays of 26-61-10 bispecific hybridoma | 120 |
| 34 | 1st quantitative HPLC absorbance profile of ascitic fluid containing 57-11-25-17 bispecific antibody | 121 |
| 35 | 2nd quantitative HPLC absorbance profile of ascitic fluid containing 57-11-25-17 bispecific antibody | 122 |
| 36 | 3rd quantitative HPLC absorbance profile of ascitic fluid containing 57-11-25-17 bispecific antibody | 123 |
| 37 | Result from MTT assay using purified BSMab 26-61-10 & DOX with the SKC01 colonic cancer cell line | 130 |
| 38 | Result from MTT assay using purified BSMab 26-61-10 & DOX with the SKC01 colonic cancer cell line | 133 |
| 39 | Result from MTT assay using purified BSMab 26-61-10 & DOX with the SKC01 colonic cancer cell line | 134 |
| 40 | Result from MTT assay using non-specific Mab Ag8 & DOX with the SKC01 colonic cancer cell line | 136 |

| | | |
|----|---|-----|
| 41 | Result from MTT assay using non-specific Mab Ag8 & DOX with the SKC01 colonic cancer cell line | 137 |
| 42 | Result from MTT assay using non-specific Mab Ag8 & DOX with the SKC01 colonic cancer cell line | 138 |
| 43 | Result from MTT assay using purified BSMab 26-61-10 & DOX with the COLO 320 DM colonic cancer cell line | 140 |
| 44 | Result from MTT assay using purified BSMab 26-61-10 & DOX with the COLO 320 DM colonic cancer cell line | 141 |
| 45 | Result from MTT assay using purified BSMab 26-61-10 & DOX with the COLO 320 DM colonic cancer cell line | 142 |
| 46 | Result from MTT assay using non-specific Mab Ag8 & DOX with the COLO 320 DM colonic cancer cell line | 145 |
| 47 | Result from MTT assay using non-specific Mab Ag8 & DOX with the COLO 320 DM colonic cancer cell line | 146 |
| 48 | Result from MTT assay using non-specific Mab Ag8 & DOX with the COLO 320 DM colonic cancer cell line | 147 |

LIST OF ABBREVIATIONS

| | |
|-----------|---|
| ABTS | 2, 2 Azino ¹ - bis (3 - ethyl benzthiazoline - 6 - sulfonic acid) |
| AMT | Antibody mediated targeting |
| BSA | Bovine serum albumin |
| BSMab | Bispecific monoclonal antibody |
| CEA | Carcinoembryonic antigen |
| DOX | Doxorubicin |
| DOX - BSA | Doxorubicin - bovine serum albumin conjugate |
| ECDI | 1 - ethyl - 3 - (dimethylaminopropyl) carbodiimide |
| ELISA | Enzyme linked immunosorbent assay |
| FACS | Fluorescence activated cell sorter |
| FCS | Fetal calf serum |
| HAMA | Human anti-mouse antibody |
| HAT | Hypoxanthine-aminopterin-thymidine |
| HGPRT | Hypoxanthine guanine phosphoribosyl transferase |
| HPLC | High performance liquid chromatography |
| HRP | Horseradish peroxidase |
| IC | Immunoconjugate |

| | |
|------------------|--|
| IC ₅₀ | Concentration of test reagent causing 50% inhibition of cell growth |
| IFN | Interferon |
| Mab | Monoclonal antibody |
| MTT | 3 - [4,5 - dimethylthiazol - 2 - yl] - 2,5 - diphenyltetrazolium bromide |
| NRS | Normal rabbit serum |
| OD | Optical density (absorbance) |
| Pab | Polyclonal antibody |
| PBS | Phosphate buffered saline |
| PRISTANE | 2,6,10,14 tetramethylpentadecane |
| RAID | Radioimmuno detection |
| RAIT | Radioimmuno therapy |
| RAM - HRP | Rabbit anti - mouse immunoglobulin labelled with horseradish peroxidase |
| RT | Room temperature |
| TAA | Tumour associated antigen |
| TSA | Tumour specific antigen |

CHAPTER I

INTRODUCTION

I 1.0 INTRODUCTION

This chapter summarizes the basic characteristics of cancer cell growth and development. It also provides a discussion on antibody mediated targeting (AMT) including the advantages and disadvantages of its use in chemotherapy. This is followed by a discussion on bispecific monoclonal antibodies (BSMab) and methods for their purification.

I 1.1 Cancer Cell Growth

In today's world of advanced technology, cancer remains, for the most part, an unsolved mystery. Great advances in understanding cancer have occurred within the last few decades but not enough to provide a cure for most cancers.

However, research has given us great knowledge about cancer and how these cells differ from normal cells. For instance, normal and cancerous cells are very similar in their biology of cell division and differentiation. But, the failure of cells to regulate proliferation, differentiation and cell-type specific functions results in altered phenotypes and cancer (Fingert et al, 1993).

Normal cells reach a steady state of growth that provides a balance for the whole body. All tissues maintain a steady state by controlling the rate of cell division, the growth

fraction of the cell population and cell death (Baserga, 1981). Physical stimuli can positively or negatively regulate the growth rate of cells. In fact, under certain physiological conditions normal tissue can have a much faster growth rate than that of cancerous tissue. The difference being, that once repair or healing is complete in normal tissue the rate subsides to "normal", whereas the neoplastic tissue continues to grow rapidly (Fingert et al, 1993).

Cancerous cells grow at an exponential rate in the early stages of growth (Skipper et al, 1982). This rate slows in the later stages, following what is called a Gompertzian curve (Tannock, 1978). The smallest tumour that can be detected by current clinical methods contains approximately 10^9 cells, a 1 gram tumour that measures approximately 1 cm in diameter (DeVita, 1989). This should be clonally derived from approximately 30 cell divisions. It takes only 10 extra doublings of this tumour to reach a lethal mass of 1 kg containing 10^{12} cells. By the time that a tumour contains this number of cells there is a very high chance that the tumour has metastasized.

Invasion and tumour metastasis impose limitations to the current treatment of cancer. Approximately 30% of patients with newly diagnosed cancer have clinically detectable metastases. At least 30% - 40% of the remaining patients free of clinically detectable metastases actually harbour occult metastases (Liotta et al, 1993). Therefore, over 60% of patients have either microscopic or clinically evident metastases at the time of diagnosis (Liotta & Stevenson, 1989) and less than a third of newly diagnosed patients can potentially be cured by local therapeutic modalities alone (Liotta et al, 1993).

I 1.2 Limitations of Cancer Treatment

There are three main methods of treating cancer: surgery, radiotherapy and chemotherapy. Surgery is used if the tumour is localized to a specific organ or primary site. This procedure is associated with a high relapse rate due to metastatic seeding before the time of the surgery. Patients are usually followed up with additional radiotherapy and/or chemotherapy (Morton, 1993).

Radiotherapy involves the use of ionizing radiation for the production of short-lived oxygen free radicals inside neoplastic cells. These free radicals then kill cells via oxidation damage (Weichselbaum et al, 1993). Cell death also occurs due to DNA damage. There can be single or double strand breaks in DNA and alteration or loss of nucleotide bases (Weichselbaum et al, 1993). Excess mutations will eventually cause cells to undergo programmed cell death. There are several drawbacks to this procedure. Most large tumours are oxygen deficient (Jain, 1994) and therefore will not contain many free radicals once irradiated. Radioresistant clones may develop after therapy which will not respond to further radiation treatment. Also, there is a problem with excess irradiation of "normal" tissue surrounding the tumour.

Chemotherapy on the other hand often involves the intravenous injection of anti-cancer drugs into the patient. Some of these drugs can also be administered orally. These drugs act either during some stage of a cell cycle (i.e. phase-specific or cycle specific) or irrespective of cell cycle (i.e. non-cycle active drugs) (Tsaltas, 1995). Drugs are also sorted according to their mode of action such as antimetabolites, intercalating agents and

alkylating agents (Epstein, 1990). Since the introduction of chemotherapy in the late 1950's, it has made a significant impact in the treatment of cancer. It has resulted in at least a 30% improvement in survival rates in the past two decades, used either in combination with surgery and radiotherapy or alone (DeVita, 1989). This success has resulted from the treatment of lymphomas, ovarian cancer, leukemias and several other childhood cancers. This success rate involves only about 12% of advanced human tumours (DeVita, 1989). However, the common solid tumours such as lung (15%), colorectal (14%), breast and prostate (27%) account for 56% of total cancer cases and 55% of cancer deaths (Boring et al, 1991).

The biggest limitation of current chemotherapy for cancer is lack of selectivity of the drugs for only the cancer cells (Ford & Casson, 1986). Chemotherapeutic drugs affect normal, "fast - growing" cells of a patient. Affected tissues include hair follicles resulting in alopecia, bone marrow causing anaemia and leukopenia and there is an effect on the lining of the gastrointestinal tract which causes nausea and vomiting (Bushkin, 1993). Drugs such as doxorubicin also cause cardiac complications (cardiomyopathy) (Young et al, 1981). Therefore there is a need for a therapy that is much more selective to the tumour and less toxic to the patient.

Another complication of chemotherapy is drug resistance. Multidrug resistance (MDR) is mediated by the presence of a 170,000 dalton plasma membrane - associated p-glycoprotein (Ford et al, 1991). The amount of cell surface expression of p-glycoproteins correspond to the level of drugs accumulated intracellularly as well as drug resistance (Gerlach et al, 1986). Drugs such as verapamil (a calcium channel blocker) can control

multidrug resistance, in some cases, by competing with the chemotherapeutic drug for binding with p-glycoprotein. Tumour size can also influence drug resistance. The larger the tumour, the greater the interstitial pressure becomes. This internal pressure, due mainly to poor internal drainage and circulation, causes fluid to drain away from the tumour carrying the drugs with it (Jain, 1994). These tumours are usually $> \frac{1}{2}$ cm in diameter and will not be clinically detectable until they reach a diameter of ~ 1 cm. Therefore, there is a growth period for tumours where they become resistant to chemotherapy and remain clinically undetectable. This time period may allow for additional metastases to form.

A more recent form of therapy is Immunotherapy or Biological therapy. This therapy utilizes various biological agents to fight the disease and can be divided into active and passive methods. Active therapy involves boosting the host's immune system against the tumour while passive requires the infusion of biologically active agents into the host in order to mediate an antitumour response. Interest in passive therapy has increased since the development of monoclonal antibodies (Kohler & Milstein, 1975) and with the development of recombinant DNA technology. These technologies have enabled the development of a wider range and more specific forms of biological agents for immunotherapy.

I 2.0 ANTIBODY-MEDIATED TARGETING

Antibodies have been used as carriers for the delivery of toxins, drugs and radioisotopes to cancer cells. The two essential qualities of an efficient antibody-mediated targeting system are: selective delivery of agents to the cancer site and reduced toxin effect

on normal cells (Ford & Casson, 1986, Ford et al, 1990).

This concept of antibody delivery of toxins to diseased cells was developed nearly a century ago by Paul Ehrlich (1854-1915). He was a Nobel Prize laureate who coined the term "antibody" and proposed his theory on how cells of the body have side-chains (receptors) (Dale, 1957) and how anti-toxins (antibodies) are produced in order to eliminate toxins and stop cell damage. He said that this protection could be obtained by immunizing with bacteria that produced these toxins.

Ehrlich also coined the word "chemotherapy" and because of this, he is considered to be the father of modern chemotherapy (Ehrlich, 1908). He realized the need for better drug delivery systems due to the nonspecific toxicity of drugs on normal cells. This concept was the basis of the antibody-mediated targeting therapy.

I 2.1 Components of Targeting Therapy

There are three main components of targeting therapy which include the target, a carrier and a toxic agent. The significance of these components will be discussed in greater detail in the following sections.

I 2.2 Target Specificity

The ideal target cancer cell is one that expresses only one type of antigen. This antigen has to be specific for that tissue type or cancer cell. Even though the search for a tumour specific antigen (TSA) has been ongoing for the past century, the success of such research has been dismal (Schreiber et al, 1988). There has been evidence of tumour

specific antigens in highly inbred mice carrying a tumour induced by a chemical or physical carcinogen (Schreiber et al, 1988), but there is no correlation of these studies to human studies. In fact, the only examples of human TSA to date are the idiotypic markers on certain B and T cell lymphomas (Stevenson et al, 1990).

The major human tumour markers that have been found are termed tumour associated antigens (TAA). The best TAA, of course, is one that is expressed in greater quantities on tumour cells as compared to normal cells. It is also important that this antigen is not expressed in great quantities on "toxin sensitive" normal tissue such as the bone marrow, gut lining and hair follicles.

I 2.3 Tumour Associated Antigens

Three main tumour associated antigens have been characterized for major solid tumours. These are: carcinoembryonic antigen (CEA), alphafetoprotein (AFP) and human chorionic gonadotrophin (HCG). None of these meet the 'ideal tumour marker' classification perfectly. This classification of a marker is as follows: it should be produced only by malignant cells and detectable in body fluids; it should be useful in screening and early detection of cancers; decrease in body fluid levels of the marker should correlate with the efficacy of therapy (Bates & Longo, 1987).

According to this scheme, all three tumour associated antigens are equally important but CEA is probably more applicable to oncology research and AMT. AFP is expressed highly in hepatocellular and testicular carcinomas while HCG is expressed in choriocarcinomas. Testicular cancers and choriocarcinomas have a successful cure rate

of 70% and >90%, respectively (Chabner et al, 1984), therefore the need for a more efficient drug targeting system for these cancers is not as great when compared to colon, lung and breast cancers which have very low cure rates. These solid tumours express high levels of CEA and it is therefore an important marker for screening and AMT for these cancers.

I 2.4 Obstacles in Using Tumour Antigens for Targeting

There are practical problems that have to be taken into consideration when developing and testing monoclonal antibodies in AMT. These problems include antigen expression and shedding, antigen modulation, antigen heterogeneity, antigen density and immunogenicity of the antibody.

I 2.4(a) Antigen Expression and Shedding

Tumour antigens can be membrane-bound, intracellular or secreted from the cells. The level of intracellular antigens expressed needs to be consistent in order for AMT to be successful. Otherwise, there may not be enough antigen available at the surface to allow antibody-antigen recognition. Even though the level is not always consistent, studies have shown that antibody recognition does occur *in vitro* (Dairkee et al, 1988) and *in vivo* (Welt et al, 1987).

Antigen shedding on the other hand is the presence of antigen in the extracellular space. This could theoretically inhibit antibody localization to the tumour cell and thereby negatively affect targeting. Studies have shown, however, that unless the secreted antigen

is in extremely high concentrations, targeting will occur (Begent et al., 1980).

I 2.4(b) Antigenic Modulation

Antigenic modulation involves the redistribution of surface antigens in the presence of bivalent antibodies (Cobbold & Waldmann, 1984). The bivalent binding seems to induce this rapid process and therefore it decreases the internalization of monoclonal antibodies conjugated to toxins or drugs (Shawler et al., 1984). However, this may be overcome by using univalent monoclonal antibodies such as bispecific antibodies.

I 2.4(c) Antigen Heterogeneity

Most tumour associated antigens are expressed on a proportion of the cell population (Greiner, 1986). Some cells may not normally express the TAA or cells may have lost the expression due to mutations (Greensberg, 1994). This range of expression can occur between individual cells of a tumour and will greatly affect the survival time and relapse in patients.

One suggested way to combat this problem is to use a monoclonal antibody "cocktail". This includes antibodies recognising various surface antigens of a tumour (Durrant et al, 1989). Another way to overcome heterogeneity is to use agents which increase antigen expression. Such agents include interferon (Greiner, 1987), growth factors and tumour necrosis factor (Weiner et al, 1988). The use of radionuclides as a toxin is another option. This would extend cell death beyond the target cell, thereby allowing the kill of non-antigen expressors (Order et al, 1990). However, this would also

increase toxicity for normal "bystander" cells.

I 2.4(d) Antigen Density

Another related topic is antigen density. This refers to the density of an antigen on the surface of the tumour cells. Obviously the higher the density the greater the effect immunotherapy will have on the tumour. Low levels of expression may result in subtherapeutic delivery of antibodies to the target site. As with the heterogeneity problem, growth factors have been suggested that may enhance the expression of these antigens. One such growth factor is transforming growth factor β (TGF β) which has been shown to increase the expression of CEA on human colonic carcinomas (Chakrabarty et al, 1988).

I 2.4(e) Antibody Immunogenicity

This does not involve the antigen but rather the mode of targeting i.e. monoclonal antibodies. Antibodies used in targeting research are produced mostly from mice (Ford et al, 1987b; Corvalan & Smith, 1987; Cook & Wood, 1994) which becomes a draw back upon injecting these antibodies into patients. Subsequent injections will cause the patient to develop an antibody response to this foreign antibody or "antigen" in their circulation. This response will inevitably neutralize the effects of the targeting system. This immune response is known as a HAMA (human anti-mouse antibody) response. The HAMA response could be reduced or abolished by developing either a human monoclonal antibody or a "human-mouse" chimeric antibody (Adair, 1992; Ford & Casson, 1986). Chimeric antibodies are developed through molecular engineering by replacing human variable

region heavy and light chain genes with mouse variable heavy and light chain genes (Walls et al, 1993).

I 3.0 DRUG CARRIERS

There are several drug carrier systems developed to date, all of which follow a set of basic requirements. The carrier should deliver the drug or toxin to the target and thereby exert its effect at that site; the linking of toxin to the carrier should not impair the action of the toxin or drug; the complex should remain intact (prolonged half-life) until it is delivered to the target. Also the complex should not be inactivated by the host immune system (Ford & Casson, 1986).

Major carrier systems developed to date include monoclonal antibodies and liposomes. Liposomes are spheres of encapsulating, bilayered phospholipids. Drugs are carried inside the liposome and delivered to the target. Even though specificity of liposomes have been increased by attaching antibodies to their surface (Tanaka et al, 1989; Ahmad et al, 1993), there is still a problem with nonspecific uptake in the reticuloendothelium system. There is also a problem with toxicity and degradation of the liposomes in vitro (Reddy, 1993).

I 3.1 Monoclonal Antibodies and Cancer Research

There are several types of antibody carriers used in cancer research and diagnosis. These include conjugated antibodies, secondary carrier antibodies and bispecific antibodies. Non-conjugated monoclonal antibodies have also played an important role in

the diagnosis and treatment of cancer.

Before the development of monoclonal antibodies in 1975 (Kohler and Milstein, 1975) polyclonal antibodies were used as carriers. These polyclonal antibodies are not good carriers for clinical studies because of antibody heterogeneity and the host's immune response to such large quantities of foreign proteins. On the other hand, monoclonal antibodies have produced much more success due to their paratope specificity for only one particular target antigen. These monoclonal antibodies have been used in both diagnosis and treatment of certain cancers. The treatment of cancer with antibodies alone has included indirect cytotoxicity relying on the antibody dependent cell-mediated cytotoxicity (ADCC) (Reddy, 1993).

Direct cytotoxicity includes the use of antibodies as catalysts to induce changes in cell membranes and proteins of cancer cells (Iverson & Lerner, 1989) or as regulators of cell growth (Queen et al, 1989). Queen et al (1989) illustrated how growth-regulating antibodies, which were specific to growth factor receptors on cancer cells, could be designed. These antibodies controlled the cancer cell growth as a result of binding competition with the growth factors themselves.

I 3.2 Immunoconjugates

Three major chemotherapeutic agents used in immunoconjugates include radioisotopes, toxins and drugs. These agents can be conjugated in a primary or secondary fashion.

I 3.2(a) Radiolabelled Antibodies

Radiolabelled monoclonal antibodies have been used for diagnosis (radioimmunodetection : RAID) and treatment (radioimmunotherapy : RAIT) of carcinomas. Radiolabelling of antibodies is considered to be a simple procedure. The isotopes vary in their half-lives but remain useful in the detection of micrometastases. RAIT has been helpful in the treatment of hepatomas (Order et al, 1985), Hodgkin's disease (Lenhard et al, 1985) and other less common cancers. The underlying problem is with irradiation of and toxicity to non-cancerous cells. Studies have been ongoing to find isotopes with shorter energy path lengths thereby reducing the level of toxicity to normal cells. Such isotopes would include α emitters (Macklis et al, 1988).

I 3.2(b) Immunotoxins

Immunotoxins are produced by conjugating bacterial or plant toxins to monoclonal antibodies. Theoretically, one molecule of endotoxin is enough to kill a cell and because of this it is considered to be more toxic than all chemotherapeutic drugs. The toxin itself consists of three main sections; "o" side chain (species or serotype antigen) and core polysaccharide (genus-specific antigen), which are subsections of the B-chain, and lipid A (toxic moiety) (Peterson, 1991). The B-chain or serologic determinant of the endotoxin is removed before the lipid A is covalently conjugated to an antibody. This serological portion is the cellular moiety therefore removing it reduces the nonspecific binding (Reddy, 1993). The toxin itself acts by inhibiting host cell protein synthesis. Immunotoxins have also been developed using plant toxins such as ricin A chain (Shen et al, 1994) and

diphtheria A chain (Yamaizumi & Mekada, 1978). Such immunotoxins have been used clinically for cancers such as chronic lymphocytic leukemia and B cell leukemia (Hertler & Frankel, 1989) but these immunotoxins have very similar problems to those of immunoconjugates. These problems will be listed in the next section. However, there are now recombinant immunotoxins available which do not involve chemical linkages. The gene for the toxin is spliced into a plasmid next to an antibody gene which allows for direct linkage and cost effective production of immunotoxins (Brinkmann & Pastan, 1994).

I 3.3 Drug Immunoconjugates

Like immunotoxins, drug immunoconjugate (IC) models have been well studied for cancer treatment. Extensive amounts of information are known about these drugs including their effect on normal and neoplastic cells, toxicity levels and their mode of action within cells.

To date, many types of drug immunoconjugates have been developed and some of them have been used in animal studies (Mathe, 1958; Yang & Reisfeld, 1988) and clinical studies (Elias et al, 1990). In order for the IC to be used in a clinical trial, it must first be proven effective preclinically with *in vitro* (human cells) and *in vivo* (animal model) tests.

These IC's have included various combinations of antibodies and drugs such as: doxorubicin linked to an anti-proteoglycan Mab (Yang & Reisfeld, 1988), doxorubicin linked to an anti-leukemic cell Pabs (Hurwitz et al, 1975), vindesine linked to an anti-CEA antibody (Ford et al, 1983) and methotrexate linked to various antibodies (Kulkarni et al, 1981; Deguchi et al, 1986; Garnett et al, 1983).

Those IC's that do reach the clinical stage, usually have not been very successful to date, for reasons such as: not enough drug delivered to the cells, drug resistance and antigen heterogeneity (Kuzel & Rosen, 1994). These problems can be explained by factors such as too few effective drug molecules linked to the antibody, in most cases because a hydrophobic drug is being conjugated to a hydrophilic protein. The conjugation process may inactivate the drug itself or the drug may impair the antigen binding ability of the antibody (Pietersz et al, 1989). Studies have been done in which the conjugation sites of both the drug and the antibody were controlled. This process can still yield a low drug:antibody ratio and poor *in vitro* results (Lau, 1994; Lau et al, 1995 a, b). However, internalization of such antibodies has been proven *in vitro* using techniques such as western blotting (Tsaltas et al, 1992) and FACS (Ford et al, 1996).

Conjugates given *in vivo* may be unsuccessful in reaching the tumour. Like liposome systems, these large antibody-drug complexes may be entrapped in the reticuloendothelial system (Pietersz et al, 1994). This will then cause toxicity to the reticuloendothelial system with minimal effects on the tumour.

If the IC arrives in the vicinity of the tumour, it still may not enter the tumour in large enough quantities to totally destroy it. The tumour vasculature may be unevenly distributed to the point where portions of tumour may not even have any blood vessels (Jain, 1994). These blood vessels may also be convoluted, resulting in slow flow rates in areas (Pietersz et al, 1994). This will result in poor perfusion rates and poor conjugate distribution within the tumour.

Actual internalization of the IC can be slowed or delayed due to antigen

heterogeneity (Ford & Casson, 1986) and increased interstitial pressure (Jain, 1994). The use of Mab conjugates with heterogenic antigens will only delete a small subpopulation of the cells, thus allowing alternate antigen-expressing cells to flourish. This can be controlled by using a "cocktail" of Mab's. Interstitial pressure on the other hand, can be controlled by surgery, if it is possible. As mentioned in section I (1.2), interstitial pressure is a problem with large tumours ($> \frac{1}{2}$ cm in diameter) and not so in smaller metastatic tumours. Therefore, with respect to interstitial pressures, if all detectable masses are removed then IC's will be much more effective in clearing the remaining microfoci of tumour cells.

As mentioned in section I 2.3(e), the HAMA response will of course be a problem. Again, chimeric or humanized antibodies should reduce this problem for targeting purposes.

In spite of these problems, clinical trials have produced some very positive results such as a recent study in colon carcinoma (Takahashi et al, 1993). The study illustrated a much higher survival time of the patients receiving conjugate therapy as opposed to other chemotherapies. Also, survival was increased when larger doses of conjugate therapy were given.

I 4.0 BISPECIFIC MONOCLONAL ANTIBODIES

Even though the production of bivalent antibodies was attempted in 1961 (Paulus, 1985), it did not gain much success until the production of monoclonal antibodies (Kohler & Milstein, 1975).

Early attempts to produce BsMab's by Nisonoff and Rivers (1961) were done with polyclonal antibodies using chemical recombination. The products were therefore highly heterogeneous providing very low yields of the desired bivalent antibody. After discovering that murine B cells could be cultured and manipulated *in vitro*, production of bispecific monoclonal antibodies (Milstein & Cuello, 1983) became much more feasible.

Bispecific antibodies have a number of potential advantages over other antibodies when used for targeting and immunotherapy purposes. Monovalent binding of bispecific monoclonal antibodies is less likely to cause antigenic modulation of target cells, as opposed to their bivalent counter-parts (Nolan & O'Kennedy, 1990). This monovalent binding is very important where target cells express the target antigen in very low densities. Monoclonal antibodies will require a fairly dense distribution of antigen in order to achieve bivalent binding. The monovalent binding of the BSMab will therefore allow more antibodies to bind to a given cell compared to a bivalent Mab. This could correlate with a greater number of drug molecules being delivered to the target cell.

Chemical conjugation of a drug to an antibody to form an antibody-drug conjugate is no longer required. This eliminates the problem of reducing antibody affinity for the target and/or reducing the drug potency.

I 4.1 Bispecific Antibody Production

To date, several techniques have been used to develop bispecific antibodies. Biological, chemical and recombinant DNA production methods will be discussed further in the following sections.

I 4.2 Biological Production

This procedure involves the use of hybridoma technology that was developed by Kohler and Milstein (1975). Like a normal B cell, a hybridoma expresses an immunoglobulin with one type of heavy and one type of light chain through a process called allelic exclusion (Milstein & Cuello, 1983; Songsivilai & Lachmann, 1990). Bispecific hybridoma procedures involve the fusion of two antibody expressing cells resulting in a hybrid hybridoma (Nolan & O'Kennedy, 1990). These cells secrete a mixture of parental monoclonal antibodies, bispecific and seven other possible combinations of heavy and light chains (Suresh et al, 1986). This is due to a codominant expression of both heavy and light chains from both parental cell lines (Milstein & Cuello, 1983).

I 4.2(a) Bispecific Antibody Light Chain Expression

As mentioned, there are 10 possible combinations of heavy and light chains that can occur in the cysternal space of a hybrid hybridoma. Figure 1 illustrates 3 different types of associations. The first involves a random association of two heavy (H1 & H2) and two light (L1 & L2) chains giving the ten listed species. Note that only the cis associations of H and L chains (those from the same parental cell) can form functional paratopes. The second type, random heavy chain association, involves complete restriction on chain assembly. This results in the parental monoclonal antibodies (VI & VII) plus the bispecific monoclonal (I). The third type of association involves random association with partial restriction on the light chain. This will give antibodies with only one functional arm (II, III,

IV, & V). Combinations VIII, IX, & X are completely nonfunctional.

Even though the bispecific combination is only one out of ten possibilities, reports indicate that the percentage of secreted bispecific antibody from these hybridomas can vary from 30% to 50% (Suresh et al, 1986a).

Suresh et al (1986a) demonstrated that ion exchange chromatography purified bispecific antibody ascites into 3 peaks. SDS-PAGE indicated that peaks #1 and #3 were the two parental antibodies, while peak #2 was the bispecific antibody. Other studies have indicated several peaks and light chain compositions from HPLC purified ascites (Xiang et al, 1992; Van Dijk et al, 1989). The differences between these two situations may be due to random combinations of light chains or even the type of purification procedure used. Either way, it illustrates the fact that all hybrids do not express a set number of light chain combinations.

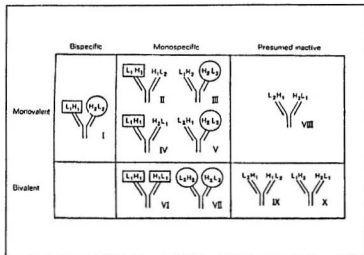


Figure 1. Expected molecular species in hybrid hybridomas arising by random association of heavy and light chains. The parental antibody chain compositions are H1L1 and H2L2 and these functional associations are boxed or circled, while the presumed nonfunctional combinations are not (modified from Suresh et al, 1986a).

I 4.2(b) Bispecific Hybridoma Production

There are two basic biological procedures for producing bispecific antibodies. These involve hybridoma x spleen cell fusion or hybridoma x hybridoma fusion (Songsivilai & Lachmann, 1990). The difference between the two procedures is that the second requires the fusion of two well-established hybridomas each of which is producing a different monoclonal antibody. The success of the hybridoma x spleen cell fusion relies on the immortality of a HAT sensitive hybridoma and the production of hypoxanthine-guanine phosphoribosyl transferase (HGPRT) from an immunized lymphoid cell. The second method requires that both hybridomas be sensitized to two different biosynthetic pathways (Songsivilai & Lachmann, 1990). Therefore, fusion between these two hybridomas will allow one to compensate the other in both synthetic pathways. This will give a successful hybrid-hybridoma.

I 4.3 Chemical Production

This method requires two well established hybridomas producing two different functional antibodies. The hybridomas are not fused as in a hybridoma x hybridoma cell fusion but rather the two different antibodies are purified separately and chemically modified to allow for bispecific antibody formation. This can be done by coupling two whole antibodies and/or derivatives or by dissociation and reassociation of heterologous immunoglobulins (Songsivilai & Lachmann, 1990).

The coupling production of bispecifics immunoglobulins is not very efficient. There

are problems with dissociation of the chemical linker and difficulty with the antibody entering the target cells, due to their relative size (Canevari et al, 1988). The dissociation method requires chemical manipulation to form the Fab' fragment (Knuth et al, 1994) for the formation of derivatives and also in the final linkage step (Brennan et al, 1985; Yagie et al, 1994). This could interfere with the antigen binding site of the Fab fragment and result in short plasma half lives due to their size.

I 4.4 Recombinant DNA Bispecific Production

Bispecific antibodies have been produced using a recombinant DNA procedure. It requires that the V_H and V_L genes of two hybridomas be separately isolated by Polymerase Chain Reaction (PCR) or by ligating overlapping oligonucleotides (Spooner et al, 1994). These are then fused via oligonucleotides which will code directly for a flexible linker between the two gene group products. This final product is usually called a functional single chain bispecific antibody (scFv₂) (Gruber et al, 1994) and is usually expressed in *Escherichia coli*. The placement of the genes coding for scFv₂ in the *E. coli* DNA is usually in association with an amino-terminal secretory signal sequence such as ompA (Gruber et al, 1994). A similar procedure has also included the first constant domains of each hybridoma (Kostelny et al, 1992). Either way, the Fc portions are usually excluded in order to avoid potential HAMA responses in clinical trials.

The process itself is very cost efficient and is not very labour intensive. However, the long-term stability of these molecules is unknown (Spooner et al, 1994) and the exclusion of the Fc portion may reduce its plasma half life.

I 5.0 PURIFICATION OF BISPECIFIC ANTIBODIES

The production of bispecific monoclonal antibodies results in a number of light chain combinations, as illustrated in section I 4.2(a). The purification of bispecific antibodies from mouse ascites will include a number of these combinations as well as other ascitic proteins. The additional antibodies will be responsible for competitive inhibition with the bispecific during *in vitro* as well as *in vivo* assays. Also, the exact concentration of the bispecific antibody will be unknown while the other antibodies remain in the fluid. Immunoaffinity purification, which is the first purification step, is usually done using a Protein A column (Van Dijk et al, 1989; De Lau et al, 1991; Pimm et al, 1990). This will remove non-immunoglobulin proteins in solution by selectively binding to the Fc portion of the antibodies thereby allowing them to be collected in the eluate.

From this point, several procedures of High Performance Liquid Chromatography (HPLC) protocols, including hydroxylapatite (Jantscheff et al, 1993; Xiang et al, 1992) and ion-exchange chromatography (Suresh et al, 1986b; Allard et al, 1992) have been used. The hydroxylapatite column requires the use of phosphate buffers which favours long-term storage of antibodies compared to the organic solvents usually used for ion-exchange chromatography (Stanker et al, 1985). Using organic solvents would require dialysing the antibody in organic solvents before the HPLC procedure and dialysing back to the phosphate buffer afterwards. Using a hydroxylapatite column results in less solvent changing, less dialysis and therefore less antibody loss through precipitation and attachment to dialysis tubing.

I 6.0 CARCINOEMBRYONIC ANTIGEN

Carcinoembryonic antigen is a tumour associated antigen, as mentioned in section I 2.3. It is found on many of the common and most life threatening solid tumours such as colorectal (> 65%), lung (>50%), pancreas (>60%), ovary and breast (>30%) (Bates & Longo, 1987). CEA is a glycoprotein with a molecular weight of 180,000 Daltons and it is normally expressed in embryonic gut. It belongs to the immunoglobulin (Ig) superfamily and is characterised as being an intercellular adhesion molecule (Abbas et al, 1994). This adhesive ability will increase the survival and metastatic ability of circulating CEA expressing cancer cells. The metastatic ability of colonic cancer cells is further supported by clinical data collected concerning colonic metastases of the liver. In this case, up to 100 % of the metastatic cells expressed CEA (Bates & Longo, 1987). Such metastases have also shown a high expression of CEA mRNA (Benchimol et al, 1989).

I 6.1 CEA as a Target for Antibody-Mediated Targeting (AMT)

As previously mentioned, section I 6.0, CEA is expressed on the surface of many solid tumours. Even though it is expressed to a lesser degree on some normal tissue, it is an antigen that is readily available for targeting purposes. This antigen has been used in the past as a target antigen for anti-CEA immunoconjugates for tumour detection and therapeutic purposes. Immunoconjugates labelled with ¹³¹I have been shown to localise in advanced metastatic adenocarcinomas (Ford et al, 1983). Also, anti-CEA antibodies conjugated to vinca alkaloids have proven successful in reducing tumour growth in

xenograft models (Casson et al, 1987; Corvalan et al, 1987,1988).

This antigen is by far the best available antigen that can be used for AMT purposes on colorectal tumours. Both the *in vitro* binding properties and the *in vivo* therapeutic potential of this antigen has been shown using an anti-CEA monoclonal antibody in our laboratory (Ford et al, 1985, 1986, 1987a, 1987b, 1989, 1990, 1991, 1996; Casson et al, 1987; Reddy & Ford, 1993; Tsaltas et al, 1992, 1993).

The rationale for using the CEA antigen for AMT in the Oncology Research laboratory is best summarized by Reddy (1993);

1. Increased CEA expression on the cell membranes and cytoplasm of the common solid tumours such as colonic, lung and breast cancers, which are presently refractory to therapy.
2. It is the best studied and characterized TAA. The CEA pathway from a gene product to its membrane expression has been studied and elucidated.
3. Many groups including ours have given proof of how important these anti-CEA antibodies are to CEA histopathology of tumours and in its ability to bind and internalize into CEA expressing cells in culture.
4. Our anti-CEA antibody, 11-285-14, has been proven to localise *in vivo* to xenografts, gastrointestinal (GI) malignancies in patients and not to be cross reactive with cross reacting antigens.
5. The efficacy of monoclonal anti-CEA antibody 11-285-14 in association with a vinca alkaloid and DOX has been shown both *in vitro* and *in vivo* using a nude mouse xenograft model.

6. The production of BSMabs specific to CEA and DOX has been complementary to previous immunoconjugate studies done in the laboratory.

I 7.0 DOXORUBICIN

Doxorubicin is one of the most widely used chemotherapeutic drugs, with proven therapeutic effects on a wide variety of tumours (Skladanowski & Konopa, 1993; Myers et al, 1986). This drug has had success against a broad range of human malignant neoplasms including acute leukemia, non-Hodgkins lymphomas, breast cancer, Hodgkin's disease and sarcomas (Keizer et al, 1990). DOX is actually an antineoplastic antibiotic which was isolated from a culture of *Streptomyces peucetius* var. *caesius* and can be produced by chemical synthesis from daunorubicin (Vigevani & Williamson, 1980). There are several reported mechanisms of cytotoxicity that are attributed to DOX. Probably the most reported method of cell kill by DOX is by DNA intercalation whereby the drug prevents the replication of DNA and transcription of RNA (Epstein, 1990). This type of cell kill is said to resemble that of apoptosis (Skladanowski & Konopa, 1993). Another method of cell kill by DOX is through the formation of free radicals. DOX can form an iron complex which induces the formation of hydroxyl and oxygen free radicals. The free radicals induce lipid peroxidation which affects many of the organelles within the cell. This can cause cell membrane damage which leads to cell death (Keizer et al, 1990). It is the actions of these free radicals, compounded with the fact that heart tissue contains large numbers of mitochondria, low levels of superoxide dismutase and catalase and a low rate of glutathione turnover, which accounts for cardiomyopathy when using this drug (Keizer

et al, 1990).

There are side effects and limitations to using DOX for chemotherapy. However, the delivery of the drug to the tumour using an anti-CEA/anti-DOX bispecific antibody may increase its efficacy, especially against metastases.

Anti-CEA/anti-DOX bispecific monoclonal antibodies have been developed by Reddy & Ford (1993). The rationale for developing the bispecific monoclonal antibody against DOX can be summarized as;

1. DOX has widespread applications.
2. AMT will increase the amount of drug reaching the tumour (higher selectivity) and decrease the levels entering the heart tissue (lower toxicity).
3. Targeting more potent analogs of DOX may be possible when using a BSMab, thereby increasing the efficacy of the BSMab.
4. BSMab's overcome the problem of chemically linking the drug to the antibody thereby retaining its potency.

I 8.0 OBJECTIVES

The objectives of this research project were to;

1. Produce ascitic fluid from seven hybrid-hybridomas which had been selected on the basis of their reactivity with CEA and doxorubicin.
2. Affinity purify immunoglobulins from each of the ascitic fluids using Protein A.
3. Analyse Protein A purified immunoglobulins from each of the hybrid-hybridomas using hydroxylapatite HPLC.

4. Screen HPLC fractions of each hybrid-hybridoma for anti-CEA, anti-DOX, anti-DOX-BSA, anti-BSA and dual activity by enzyme linked immunosorbent assays.
5. Select the most promising bispecific antibodies for further investigation (based on 1 - 4 above) and scale up the HPLC for preparative purification of these antibodies.
6. Test the ability of one of these antibodies to target doxorubicin to CEA expressing or CEA non-expressing human colonic cancer cell lines using a colorimetric microcytostasis assay.

CHAPTER II

MATERIALS AND METHODS

II 1.0 BISPECIFIC HYBRIDOMAS

The bispecific antibody producing hybrid-hybridomas used in this thesis were developed in the Oncology Research Laboratory by Reddy and Ford (Reddy, 1993; Reddy & Ford, 1993). These hybrids were selected according to their anti-CEA, anti-DOX and dual activity.

II 1.1 Colonic Cancer Cell Lines

The colonic cell lines used in this project as test cell lines included the SKC01 which is a high CEA expressing cell line as well as the COLO 320 DM which is a low or negative CEA expressor (Ford et al, 1987). These lines were used for *in vitro* assays (section II 6.0) and all were obtained from the American Type Culture Collection (ATCC) Rockville, Maryland, U.S.A.

II 1.2 Tissue Culture Maintenance

The hybrid hybridoma suspension lines and the colonic cancer monolayer lines were grown in 75 cm² tissue culture flasks (Falcon, Becton-Dickinson). These cells were maintained at 37°C in a 5% carbon dioxide atmosphere in a humidified incubator. Cell lines were maintained in the required medium as listed in sections 1.5 and 1.6. The

medium was replenished daily in order to maintain optimal growth of cells.

II 1.3 Medium for COLO 320 DM and the Bispecific Hybridomas

The colonic cancer cell line COLO 320 DM and the seven bispecific hybridomas required RPMI medium.

- (1) RPMI-1640, 500 ml (# 21870-050, GIBCO Laboratories, Life Technologies, Inc., Grand Island, N.Y.)
- (2) Fetal calf serum (FCS), 50 ml (# 3000, P.A. Biologicals Co. PTY LTD., Sydney, Australia)
- (3) Penicillin/streptomycin, 12 ml (# 15070-014, GIBCO Laboratories)
- (4) L-glutamine, 6 ml (# 25-005-L1, CELLGRO, Mediatech, Washington, D.C.)

II 1.4 Medium for SKCO1

- (1) Minimal essential medium (MEM), 500 ml (# 11090-057, GIBCO Laboratories)
- (2) FCS, 50 ml (#3000, P.A. Biologicals Co. PTY LTD.)
- (3) Penicillin/Streptomycin, 12 ml (# 15070-014, GIBCO Laboratories)
- (4) L-glutamine, 6 ml (# 25-005-L1, CELLGRO, Mediatech)
- (5) Non-essential amino Acids, 6 ml (# 25-025-L1, CELLGRO, Mediatech)

II 1.5 Trypsinization of cells (for COLO 320 DM and SKC01)

II 1.5(a) Materials

- (1) Trypsin-EDTA (x 10) (Gibco Laboratories, Life Technologies Inc., Grand

Island, New York).

- (2) Phosphate buffered saline (PBS) pH 7.2, 1 tablet per 100 ml distilled water, (Oxoid, Unipath Ltd., Basingstoke, Hampshire, England).
- (3) Benchtop centrifuge (IEC HN SII model)
- (4) Conical centrifuge tubes, 15 ml (Falcon, Becton-Dickinson Labware, Becton-Dickinson & Company, Lincoln Park, New Jersey. 07035).

II 1.5(b) Method

- (1) Dilution (1:10, 5 ml) of trypsin and PBS was prepared aseptically.
- (2) Cultures which were confluent but still in log phase of growth were then washed in 5-10 ml of sterile PBS and the washing discarded.
- (3) The trypsin-PBS solution was added to the flask and incubated for 10 minutes at 37°C.
- (4) The cell suspension was then added to a 15 ml conical centrifuge tube and the flask washed in 5 ml PBS.
- (5) Once the flask was washed, the suspension was centrifuged at 1000 rpm (220 x g) for 5 minutes in a benchtop centrifuge.
- (6) The supernatant was decanted and the pellet was resuspended in 10 ml MEM. A viability count was carried out as described below.

II 1.6 Cell Count

Viability counts of cells were done using a fluorescence microscope and a Neubauer hemocytometer. Cells were mixed with an acridine orange (AO) and ethidium bromide (EB) solution. The Acridine orange stains viable cells a fluorescent green while the ethidium bromide will stain the dead cells a brownish-red colour when viewed under ultraviolet light.

II 1.6(a) Materials

- (1) Neubauer hemocytometer (with cover slip).
- (2) Acridine orange (AO) (#A-6014, Sigma Chemical Co., St. Louis, Missouri).
- (3) Ethidium bromide (EB) (#E-8751, Sigma Chemical Co.). 1 ml aliquots of AO and EB (0.001%) were made up in 100 ml of PBS and stored at -20°C.
- (4) Fluorescent microscope with 50 Watt mercury lamp (Ortholux II, Leitz).

II 1.6(b) Method

Cells from the culture flasks were transferred to a centrifuge tube and centrifuged for five minutes at 1000 rpm (175 x g). The supernatant was discarded and the pellet was resuspended in 10 ml of medium. One drop of this suspension was mixed with one drop of AO/EB solution. A portion of this mixture was placed under the cover slip of a hemocytometer and placed under a fluorescent microscope. Counts were then done and the percent viability was calculated as given:

$$\% \text{ viability} = (\text{total AO count} / \text{total cell count}) \times 100\%$$

II 1.7 Cryopreservation of Cells

II 1.7(a) Materials

Freezing media contains 10% (1:10) dimethyl sulfoxide (DMSO) (# D-8779, Sigma Chemical Co.) in fetal calf serum (FCS). This solution was filtered through a Millipore 0.22 μ m syringe filter (Millipore Products, Bedford, MA 01730) and stored at -20°C.

II 1.7(b) Method

- (1) Cells were counted (sections II 1.5 & 1.6).
- (2) The cells were then centrifuged for 5 minutes at 1000 rpm (175 x g).
- (3) The supernatant was discarded and the pellet resuspended in cold freezing medium (on ice) at a concentration of 5×10^6 cells per 1 ml.
- (4) 1 ml aliquots were made, labelled and placed at -70°C overnight.
- (5) The 1 ml aliquots at -70°C were then transferred to liquid nitrogen the following day.

II 1.8 Thawing of Cells

- (1) Medium (9 ml) was aseptically transferred to a 15 ml centrifuge tube.
- (2) The appropriate vial of cells was removed from the liquid nitrogen canister and thawed in a 37°C waterbath.
- (3) Before complete thawing the vial was quickly transferred to a flow cabinet and pipetted into the 9 ml of medium, on ice.

- (4) This mixture was then centrifuged for 5 minutes at 1000 rpm (175 x g).
- (5) The supernatant was discarded and cells were resuspended in 1 to 2 ml of medium.
- (6) The cells were then transferred to a 25 cm² culture flask and incubated at 37°C in a humidified, 5% carbon dioxide atmosphere incubator.

II 2.0 PRODUCTION OF BSMab ASCITIC FLUID

The selected hybrid-hybridomas from section II 1.0 were thawed as in section II 1.8 and maintained as described earlier (sections II 1.2 & 1.3). Once the cells had achieved sufficient numbers (section II 1.6), the appropriate volumes of cells were removed and injected into pristane primed mice (section II 2.1). Remaining cells were then frozen down to approximately 5×10^6 cells/ml. This procedure is detailed in section II 1.7.

II 2.1 BSMab Production in Mice

II 2.1(a) Materials

- (1) Pristane (# T-7640, Sigma Chemical Co., St. Louis, Missouri.).
- (2) Ethyl ether (# 9249-06, J.T.Baker Inc., Phillipsburg, NJ.).
- (3) Refrigerated centrifuge (Model TJ-6, Beckman Instruments Inc., Palo Alto, CA.).
- (4) 15 ml centrifuge tubes (# 000-2097-STR, Elkay products (UK) LTD., Basingstoke, Hampshire, UK).
- (5) 5 ml syringes (# 9604, Becton Dickinson and Co., Rutherford, NJ.).

- (6) 50 ml centrifuge tubes (Fisher Scientific, Ontario).
- (7) 20 gauge needles (# 305175, Becton Dickinson and Co.).
- (8) Bispecific hybrid hybridomas
- (9) Balb/c mice (Jackson Laboratories, U.S.A.)

II 2.1(b) Method

- (1) Balb/c mice were primed with an intraperitoneal injection of 0.5 ml of pristane two weeks prior to the hybrid injection.
- (2) Cells were maintained in culture, as in section II 1.2 & 1.3.
- (3) Cells were centrifuged (5 minutes, 750 x g), resuspended in 10 ml of medium and counted (as in section II 1.6).
- (4) Suspensions were recentrifuged and resuspended in sterile PBS at 1.0×10^6 viable cells per 0.5 ml.
- (5) Each hybrid was then transferred separately to 5 ml syringes.
- (6) Hybrids were injected intraperitoneally into ether anaesthetised, pristane primed Balb/c mice at 0.5 ml per mouse.
- (7) The mice were regularly monitored for abdominal swelling and ascitic fluid build-up. Withdrawal of ascitic fluid started around 10 days post inoculation.

II 2.2 Tapping of Ascitic Fluid

II 2.2(a) Materials

See section II 2.1(a).

II 2.2(b) Method

- (1) Hybrid-inoculated mice were tapped by first anaesthetising the animal with ether.
- (2) The intraperitoneal fluid was removed using 20 gauge needles and 5 ml syringes.
- (3) The ascitic fluid from different mice inoculated with the same hybrid-hybridoma was drawn into a syringe and pooled in a labelled 50 ml centrifuge tube (on ice).
- (4) Each mouse was then placed into a recovery cage before being transferred back to its appropriate cage. Mice were not tapped more than three times at which time they were terminated. Any animal not looking well was also terminated.
- (5) Once withdrawn, the ascitic fluid was centrifuged (10 minutes, 4000 rpm, 750 x g).
- (6) The supernatant was then removed and pooled in appropriately labelled bottles and stored at -20°C.

II 3.0 PURIFICATION OF BSMab FROM ASCITIC FLUID

II 3.1 Protein A-Sepharose Affinity Column Purification

Protein A is a bacterial cell wall protein of *Staphylococcus aureus*. It is a single polypeptide chain of 42 kDa with glycoproteins (Hermanson et al, 1992). It is classified as a type I bacterial Fc receptor according to the Myhre and Kronvall classification of bacterial immunoglobulin-binding proteins (Boyle, 1990). Mouse IgG₁ does not bind to this protein as well as IgG_{2a}, IgG_{2b} & IgG₃ (Hermanson et al, 1992), whereas Protein G is noted as being a better binding protein for the mouse IgG₁ subclass. However, according to Western blotting results obtained in the Oncology Research Laboratory, use of Protein G did not result in any better purification of IgG₁ than did Protein A

(unpublished observations). Also, Protein G is not recommended for the purification of mouse IgG, as its removal from Protein G can be difficult (Andrew & Titus, 1994). Therefore, Protein A was used to purify the seven BSMabs from ascitic fluid in this investigation.

II 3.1(a) Materials

(1) Purification buffers;

- A. 0.5 M sodium phosphate buffer pH 8.0. Sodium di-hydrogen phosphate (monobasic), NaH_2PO_4 , H_2O (# ACS 795, BDH Inc., Toronto) 69.0 g/L, di-sodium hydrogen phosphate, anhydrous, Na_2HPO_4 (# B30 158, BDH Inc.) 71.0 g/L with 0.01% sodium azide, NaN_3 (# B30 111, BDH Inc.). 69 g of phosphate monohydrate and 0.1 g of sodium azide were dissolved in 1 litre of distilled water. This was added to an anhydrous phosphate solution (71.0 g in ~ 800 ml of distilled water with 0.1 g of sodium azide). This mixture was adjusted to pH 8.0 and made up to 2 litres in distilled water. This stock was stored at -20°C . The required 0.1 M buffer was obtained by diluting the 0.5 M solution 1:5 in distilled water.
- B. 0.1 M citrate-phosphate buffer pH 3.0-7.0. Citric acid, $\text{C}_6\text{H}_8\text{O}_7$, H_2O (# B27 780, BDH Inc.) 21.01 g/L, di-sodium hydrogen phosphate, Na_2HPO_4 (BDH Inc.) 0.1 M, 14.2 g/L. A 0.1 M citric

acid solution was obtained by dissolving 21.01 g of citrate crystals in 1 litre of distilled water. The pH 6.0 buffer was made by titrating the 0.1 M phosphate solution with the citric acid until the desired pH was obtained. The pH 3.5 solution was obtained by titrating the citric acid solution with the phosphate buffer until a pH of 3.5 was obtained.

C. 1.0 M Tris-hydrochloric acid buffer pH 9.0. Tris(hydroxymethyl) aminomethane 1 M (# T-1503, Sigma Chemical Co.) 121 g/L, Hydrochloric acid 1 M. Dissolve 121 g of Tris in 1 litre of distilled water. Mix 25 ml of 1 M Tris with 2.5 ml of 1 M HCl while Titrating to a pH of 9.0.

(2) PBS

(3) Whatman # 2 filters (Whatman Industries Ltd., Maidstone, England).

(4) Sterile 0.22 μ m filter (Millipore Corporation, Bedford, Mass.).

(5) Amicon ultrafiltration unit (Amicon Corporation, Lexington, Mass.).

(6) Spectrophotometer (Beckman Instruments INC.).

(7) PHM82 standard pH meter (Bach-Simpson Limited, London, Ontario).

(8) Gel filtration column (Pharmacia column length 30 cm, diameter 2 cm).

(9) Peristaltic pump P-3 (Pharmacia Fine Chemicals, Uppsala, Sweden)

(10) Protein A-Sepharose 4B (Pharmacia Fine Chemicals)

(11) Dialysis tubing (Spectra/Por Membrane MWCO 12-14,000. Spectrum

Medical Industries, Inc., Houston, Texas).

(12) Pressurized filtering apparatus (Millipore Corp.).

(13) Filtering membranes; 1.2 μm , 0.45 μm , 0.22 μm filters, membrane spacers and prefilter membranes (Millipore Corp.).

II 3.1(b) Protein A Column Preparation

(1) Freeze-dried Protein A powder was swollen in 0.1 M sodium phosphate buffer (pH 6.0).

(2) The swollen beads were washed on a 15 ml sintered glass filter funnel.

(3) The beads were then resuspended in phosphate buffer (pH 6.0) and poured into a 30 ml Pharmacia column.

II 3.1(c) Ascitic Fluid Filtration

(1) Each of the pooled ascitic fluid samples was thawed and passed through a Whatman # 2 filter.

(2) The volume of the filtered sample was measured and then diluted in 3 volumes of 0.1 M sodium phosphate buffer, pH 8.0 (at 4°C).

(3) The ascitic fluid was filtered using a Millipore pressurized filtering system. The series of Millipore filters were set up in the following manner: first, a pre-filter followed by a spacer then a 1.2 μm filter, a spacer, a 0.45 μm filter, a spacer and finally a 0.22 μm filter.

(4) The filtered ascitic fluid was then removed from the apparatus aseptically and stored at -20°C in approximately 50 ml aliquots.

II 3.1(d) Purification

- (1) The protein A column (section # II 3.1(b)) was equilibrated by washing with phosphate buffer, pH 8.0 at 4°C until an O.D. reading of < 0.02 at 280 nm was obtained.
- (2) Whatman filtered ascitic fluid was then added (25 ml) to the column allowing all the material to run into the gel. The unabsorbed material was collected, labelled NONBOUND MATERIAL and stored at -20°C.
- (3) Once the ascitic fluid had entered the gel an equal volume of phosphate buffer pH 8.0 was added. The collected eluate was labelled WASH #1 and stored at -20°C. Both the NONBOUND and WASH #1 could be pooled and repurified later.
- (4) The column was then washed overnight with phosphate buffer pH 8.0. This was continued until the O.D. of the eluting fluid was < 0.02.
- (5) An equal volume of 0.1 M citrate/phosphate buffer pH 6.0 was then added to the column. Eluted antibody was collected using a flask containing 1 ml of 1.0 M Tris buffer pH 9.0 to immediately neutralize the pH. This fraction was labelled pH 6 ELUTED and stored at -20°C.
- (6) The remaining bound antibodies were eluted using an equal volume of 0.1 M citrate buffer pH 3.5 and collected in a separate flask.
- (7) The collected pH 6 ELUTED samples, for each batch of ascitic fluid, were pooled and concentrated to approximately 2 to 3 mg/ml using Amicon ultrafiltration.
- (8) At the same time the column was washed with phosphate buffer pH 8.0 until the O.D. was < 0.02.

(9) The concentrated antibodies were then dialyzed against 3 changes of cold PBS.

Each session was about 8 hours.

(10) The dialyzed solution was then transferred to a sterile tube. It was then filtered using a 0.22 μm Millipore filter and a O.D. reading taken. The sample was then labelled and stored at 4°C.

(11) The NONBOUND and WASH#1 were pooled and repurified by Protein A column chromatography to check for any bispecific antibodies that may not have been bound to the column during the first application.

II 3.2 High Performance Liquid Chromatography (HPLC)

HPLC was used as a final purification procedure for the bispecific monoclonal antibodies. Each purified ascitic fluid sample was analyzed using HPLC and further analyzed according to its HPLC separation absorbance (280 nm) profile. One minute fractions were taken of each HPLC separation. Fractions corresponding to particular HPLC peaks were further analyzed using ELISA.

II 3.2(a) Materials

(1) Hydroxylapatite column and guard (BIO-RAD Laboratories (Canada) Ltd., Mississauga).

(2) Phosphate Buffers:

A. 60 mM, pH 6.8. NaH_2PO_4 , H_2O (# ACS 795, BDH Inc.) 0.704 g/l,
sodium phosphate (dibasic) Na_2HPO_4 , 7 H_2O (# S373-500, Fisher

Scientific Co., Ontario) 1.314 g dissolved in 1 litre of distilled water.

The pH was adjusted to 6.8 using 2.0 M NaOH.

B. 180 mM, pH 6.8. NaH_2PO_4 (BDH Inc.) 4.22 g/l, Na_2HPO_4 (Fisher Scientific) 7.88 g dissolved in 1 litre of distilled water. The pH was adjusted to 6.8 using 2.0 M NaOH.

C. 360 mM, pH 6.8. NaH_2PO_4 (BDH Inc.) 12.66 g, Na_2HPO_4 (Fisher Scientific) 23.64 g dissolved in 1 litre of distilled water. The pH was adjusted using 2.0 M NaOH.

All of the above buffer solutions contained 0.05% NaN_3 (# B30111, BDH Inc.) 0.5 g/l and 0.01 mM calcium chloride CaCl_2 (# 1-1308, J.T.Baker Chemical Co.) 0.00111 g/l. Solutions were vacuum suction filtered through 0.44 μm and 0.22 μm filters and stored at 4°C.

- (3) HPLC analog interface module 406, Programmable Detector Module 166, Autosampler 507, 110B Solvent Delivery Module (System Gold Model 460AT, Beckman Instruments (Canada) Inc.).
- (4) Nylon filter membranes 0.45 μm (CAT # NO4SP04700) and 0.22 μm (CAT # NO2SP04700). Micron Separations Inc., Westboro, MA. C/O Fisher Scientific.
- (5) Suction filtration funnel
- (6) Suction flask (Pyrex, U.S.A).
- (7) Helium gas
- (8) Nitrogen gas

- (9) Fraction collector with glass test tubes (Pharmacia fraction collector FRAC-100, Pharmacia Fine Chemicals)

II 3.2(b) Method I

HPLC and hydroxylapatite columns have been used previously for purifying bispecific antibodies by Xiang et al, 1992 and Dietsch et al, 1993. The following methods involve slight modifications of these procedures.

- (1) Both the 60 mM and the 180 mM phosphate buffers were degassed for ten minutes each using helium gas.
- (2) Each buffer sample line was then bled in order to clear the system of air bubbles. The pump pressure was immediately set to approximately 500 psi.
- (3) Vial containing the protein A purified bispecific ascitic fluid sample was placed in the assigned position on the autosampler carousel. Then, the nitrogen gas was turned on, which was needed to activate the sampling apparatus in the autosampler.
- (4) The appropriate file was then activated on the System Gold software program. This step started the HPLC procedure. The computer controlled the rest of the procedure, analyzed the results and stored the results in the file that was activated.
- (5) The autosampler removed the preset volume of ascitic fluid from the appropriate vial and loaded it onto the column.
- (6) At the same time the loading buffer (60 mM) was being pumped by pump #1 (100 %) at a preset rate of 1.5 ml/min. This continued for 20 minutes.
- (7) Pump #2 was activated, removing 180 mM eluting buffer from a second flask.

Buffer was pumped from 0 % to 100 % over a 80 minute period. Pump #1 changed from 100 % to 0 % over the same time period. This resulted in the 60-180 mM phosphate buffer gradient.

(8) At the onset of the HPLC analysis 1 ml/min fractions were collected every minute.

These fractions were then used for ELISA.

(9) Once the analysis was completed the gradient was reversed and the column was equilibrated to a baseline O.D. of 0.

II 3.2© Method II

This procedure was the same as II 3.6 (b) Method I with the exception of using a 360 mM eluting phosphate buffer instead of a 180 mM buffer.

II 4.0 DOX - BSA CONJUGATION

Doxorubicin was conjugated to bovine serum albumin (BSA) using ECDI [1-ethyl-3-(dimethylaminopropyl) carbodiimide] as a heterobifunctional crosslinker. BSA was the carrier macromolecule used during the immunizations with DOX-BSA, which had been used for the development of the hybrids being used in this thesis (Reddy & Ford, 1993). The following conjugation method is a modification of a procedure previously developed in the Oncology Research Lab (Reddy, 1993; Reddy & Ford, 1993).

II 4.0(a) Materials

- (1) Doxorubicin hydrochloride (Adriamycin HCl; Adria laboratories Inc, Columbus, Ohio).
- (2) Phosphate buffered saline (PBS).
- (3) ECDI [1 - ethyl - 3 - (dimethylaminopropyl) carbodiimide], (# E-6383, Sigma Chemical Co.).
- (4) BSA (A - 7888, Sigma Chemical Co.).
- (5) Spectrophotometer (Beckman Instruments(Canada) Inc.).
- (6) Glass cuvettes (Beckman Instruments(Canada) Inc.).

II 4.0(b) Method

- (1) 70 mg of BSA was dissolved in 2 ml of PBS (made from tablets; Oxoid). This was left at RT for 1½ hours to dissolve.
- (2) 2 ml of DOX (2 mg/ml) was added to the BSA solution dropwise, with occasional light stirring.
- (3) ECDI (15 mg in 1 ml of PBS; pH 7.2) was added dropwise to the DOX - BSA solution, with occasional stirring. This mixture was then left at RT for 4 hours (covered in aluminum foil) and gently stirred every 30 minutes.
- (4) The final pH of the solution was 6.78. The solution was stored at 4°C overnight and then at RT for 2 hours in order to redissolve the precipitate that formed overnight.
- (5) The solution was then slowly added to a Sephadex G - 25 gel filtration column (see

sections II 4.1(a) & (b)), using a Pasteur pipette.

(6) Fractions were collected every five minutes with a total of 50 fractions collected.

The conjugate was eluted using PBS.

(7) The absorbance of each fraction was then analyzed at 280 and 495 nm using a spectrophotometer. The molar ratios were calculated as follows;

where, A_{280} = absorbance reading at 280 nm

A_{280}' = calculated absorbance reading for DOX at

280 nm

A_{280}'' = true absorbance reading for BSA at 280 nm

A_{495} = absorbance reading at 495 nm

c = molar concentration of DOX

c' = molar concentration of BSA

13,000 = extinction coefficient of DOX at 495 nm (Dement & Sehested, 1993)

8,000 = extinction coefficient of DOX at 280 nm (Lau, 1994)

44,200 = extinction coefficient of BSA at 280 nm (Dement & Sehested, 1993)

The concentration of DOX at 495 nm:

$$A_{495} = 13,000 (c)$$

solve for c .

The concentration of DOX at 280 nm:

$$A_{280}' = 8,000 (c), \text{ solve for } A_{280}'$$

The true absorbance of BSA at 280 nm;

$$A_{280} - A_{280}' = A_{280}''$$

The concentration of BSA:

$$A_{280}'' = 44,200 (c'), \text{ solve for } c'.$$

DOX : BSA ratio:

$$C(\text{DOX}) : C(\text{BSA})$$

II 4.1 Gel Filtration

II 4.1(a) Materials

- (1) Sephadex G25 (# SC202722, Pharmacia Fine Chemicals, Uppsala, Sweden).
- (2) Gel filtration column (Pharmacia column, Length 30 cm, diameter 2 cm).
- (3) Peristaltic pump P-3 (Pharmacia Fine Chemicals)
- (4) Fraction collector with glass test tubes (Pharmacia programmable fraction collector FRAC - 100, Pharmacia Fine Chemicals).
- (5) Pasteur pipettes

II 4.1(b) Method

- (1) Sephadex G - 25 superfine beads were used at a predetermined concentration of 1 g to 5 ml of PBS. The amount used in the 30 cm column was 12 g in 60 ml of PBS .
- (2) The beads were expanded in PBS at 4°C overnight.
- (3) The column was poured in the cold room (at 4°C). A peristaltic pump was attached to allow for an even flow rate for fraction collecting (1 ml/minute).

II 4.2 CEA-HRP Conjugate Production

This conjugate was produced in order to test the dual activity of the bispecific monoclonal antibody.

II 4.2(a) Materials

- (1) Carcinoembryonic antigen (CEA) (supplied by Oncology Research Laboratory and purified from liver metastases as published by Ford et al., 1987b).
- (2) 0.01 M sodium carbonate bicarbonate buffer, pH 9.5:
 - (a) sodium carbonate 5.3 g (# AC-8290, Anachemica Ltd., Montreal) in 500 ml distilled water (1 M solution)
 - (b) sodium bicarbonate 4.2 g (# 7412, Mallinckrodt Inc., Kentucky) 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) 1% Dinitrofluorobenzene (# D-6879, Sigma Chemical Co.). 100 μ l in 10 ml of absolute ethanol.
- (4) 0.08 M Sodium periodate NaIO_4 (# 1867070, BDH Chemicals, England) 17.12 mg dissolved in 1 ml of distilled water.
- (5) Ethylene glycol monoethylether (# E-180, Fisher Scientific Co., New Jersey). 16 μ l Ethylene glycol in 10 ml of water.
- (6) Sodium borohydride NaBH_4 (2 mg/ml) (# S-9125, Sigma Chemical Co.)

- (7) Horseradish peroxidase. Hydrogen-peroxide oxido-reductase, Type VI.
(Sigma Chemical Co.)

II 4.2(b) Method

This procedure was previously used by the Oncology Research group to label 11-285-14 monoclonal antibodies with HRP (Woodhouse, 1982a). The procedure was slightly modified by Reddy, 1993, in order to label the CEA with HRP.

- (1) To 6.2 mg of HRP (type VI, RZ = 3) in 1 ml carbonate buffer (0.01 M), 100 μ l 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 changes of carbonate buffer at 4°C.
- (5) CEA 1 mg/ml in carbonate buffer was added to the above solution and mixed gently for 3 hrs 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 (section II 4.1 (a) & (b) using Sephadex G-75 instead of G-25).

II 5.0 ELISA

II 5.1 Introduction

Enzyme linked immunosorbent assays are assays used to test the specificity of antibody-antigen reactions. ELISAs have an advantage over radioimmunoassays in that no radioactive substances are required. Also, the reagents for ELISA are less expensive and can be stored for long periods of time.

An anti-CEA ELISA was developed by Woodhouse et al (1982b) using micro cuvettes. Reddy (1993) modified this procedure and developed procedures for anti-DOX, anti-DOX-BSA and anti-BSA detection. He also developed a procedure for a dual assay using a CEA-HRP conjugate. These procedures are listed below.

II 5.1(a) Materials

- (1) 8 channel digital micro pipette (Model 7000, Nichiryo, Japan).
- (2) 2,2 - Azino - bis (3 - Ethylbenzthiazoline - 6 -Sulfonic Acid) (ABTS) (# A - 1888, Sigma Chemical Co.).

ABTS was prepared as stock at 27.8 mg/ml. ABTS solution was made up in 100 ml of distilled water, aliquoted out in 100 μ L samples and stored at -20°C. Freshly prepared 0.2224 g/L of ABTS as substrate for each ELISA contained 100 μ l of ABTS stock, 12.5 ml of citrate phosphate buffer, and 1 μ l of hydrogen peroxide.

- (3) Hydrogen peroxide, 30% (# ACS 399 - 74, BDH Chemicals Co., Toronto, Canada.).

(4) Rabbit anti-mouse immunoglobulins labelled with HRP (RAM-HRP)(P161, Dako, Denmark).

(5) ELISA 96 well Microtitration Plates (ICN Biomedicals Inc.).

(6) ELISA buffers:

A. Citrate phosphate buffer, pH 4.0. Citric acid crystals (# 013402, J.T.

Baker Chemical Co., Philipsburg, New Jersey, USA) 4.53 g, sodium hydrogen phosphate (# 114288, BDH Inc., Toronto, Canada)

Na_2HPO_4 5.083 g. This was dissolved in double distilled water, pH adjusted to 4.0 with 12 M HCl and made up to a final volume of 500 ml.

B. Carbonate - Bicarbonate buffer, pH 9.2, sodium carbonate (AC -

8290, BDH Inc.) Na_2CO_3 0.795 g, sodium bicarbonate (S - 8875,

Sigma Chemical Co.) NaHCO_3 1.465 g dissolved in distilled water. A pH over or under 9.2 was adjusted by using 12 M HCl or 2 M NaOH, respectively. The final volume was made up to 500 ml.

C. 1 % NRS in PBS - Tween-20. NRS (Normal rabbit serum, Animal

Care, MUN) 1.0 ml and 100 μl of Tween detergent (RO6435 - 74, BDH Chemical Co.) dissolved in and made up to 100 ml in PBS (pH 7.2).

D. 1 % NRS in carbonate - bicarbonate buffer (pH 9.2). NRS 1.0 ml

was mixed in 99 ml of carbonate - bicarbonate buffer.

E. 0.15 M NaCL with 0.1 % Tween-20. Sodium chloride (ACS 783, BDH Inc.) 35.4 g and 4 ml of tween detergent were dissolved in 4 litres of distilled water.

(8) Plate reader (Bio-Tek Instruments Inc., Burlington, Vermont).

II 5.2 Anti - CEA ELISA

II 5.2(a) Materials

- (1) CEA (supplied by Oncology Research Laboratory), purified as detailed in Ford et al, 1978b and dissolved in carbonate bicarbonate buffer.
- (2) 11-285-14 anti-CEA monoclonal antibody (IgG₁ positive control).
- (3) Ag8 (P3X63Ag8; IgG₁ negative control. American Type Culture Collection, Rockville, Maryland).
- (4) RAM-HRP (Rabbit anti-mouse immunoglobulin horseradish peroxidase, P161, Dako, Denmark).
- (5) ABTS (refer to section II 5.1(a)).
- (6) 1% NRS (Normal Rabbit Serum) in carbonate buffer, pH 9.2.
- (7) ELISA buffers (section II 5.1(a)).
- (8) HPLC fractionated antibodies

II 5.2(b) Method

- (1) 96 well ELISA plates were coated with CEA (100 μ l per well at 2.5 μ g/ml). These plates were incubated at 37°C for 3 hours. Then, the plates were stored at 4°C overnight.
- (2) The coating solution was removed and the plates were washed 6 times each with 0.15 M NaCl/Tween-20 solution using a wash bottle.
- (3) 200 μ l of 1% NRS in carbonate buffer was added to each well as a blocking solution and the plates were then incubated at 37°C for 1 hour.
- (4) Plates were washed as before (step #2).
- (5) The HPLC fractions were then added to the test wells at 100 μ l per well.

The anti-CEA ELISA controls were: background (1% NRS in PBS/Tween-20) positive control (11-285-14 Mab) at 10, 1, 0.1, & 0.01 μ g/ml in 1% NRS in PBS/Tween-20; negative control (Ag8) 10 μ g/ml in 1% NRS in PBS/Tween. The controls were added in triplicate.
- (6) Plates were then incubated at 37°C for 3 hours.
- (7) Plates were then washed as before (step #2).
- (8) RAM-HRP was added at a 1:1000 dilution in 1% NRS PBS/Tween-20 at 100 μ l per well.
- (9) Plates were then incubated at 37°C for 3 hours.
- (10) Plates were washed as before (step #2).
- (11) ABTS solution was then added at 100 μ l per well and incubated at RT (in the dark) for 1 hour.

(12) Plates were read using a plate reader at a wavelength of 405 nm.

II 5.3 Anti - DOX ELISA

II 5.3(a) Materials

- (1) ELISA buffers (section II 5.1(a)).
- (2) Doxorubicin (DOX) at 2 $\mu\text{g/ml}$.
- (3) HPLC fractions of ascitic fluid.
- (4) RAM-HRP (section II 5.1(a))
- (5) ABTS (refer to section II 5.1(a))

II 5.3(b) Method

The procedure was similar to the anti-CEA ELISA with the following exceptions. The coating antigen used was DOX (2 $\mu\text{g/ml}$) and there was no positive antibody control. The controls were: background (1% NRS in PBS/Tween-20); negative control (Ag8 immunoglobulins in PBS/Tween-20).

II 5.4 Anti - DOX - BSA ELISA

II 5.4(a) Materials

- (1) DOX-BSA conjugate at 2 $\mu\text{g/ml}$ of DOX (section II 4.0)
- (2) 1% NRS in PBS/Tween-20 (pH 9.2).
- (3) Negative control (Ag8 antibody)
- (4) Materials in section II 5.1(a).

II 5.4(b) Method

The procedure was similar to the anti-DOX ELISA with the exception of using DOX-BSA as the coating antigen.

II 5.5 Anti - BSA ELISA

II 5.5(a) Materials

- (1) Coating with BSA at the same concentration used in the DOX-BSA conjugate (at 2 $\mu\text{g/ml}$ of DOX).
- (2) Negative control (Ag8 antibody)
- (3) 1% NRS in PBS/Tween-20 (pH 9.2).
- (4) Materials (section II 5.1(a)).

II 5.5(b) Method

The procedure was very similar to the anti-DOX-BSA ELISA with the exception that the coating antigen used was bovine serum albumin (BSA).

II 5.6 Dual Specificity ELISA

This procedure was similar to the other ELISA assays. The only differences were: the wells were coated with DOX-BSA at 2 $\mu\text{g/ml}$ DOX (100 μl per well) and CEA-HRP was used (1/100 dilution in 1% NRS/PBS/tween) instead of RAM-HRP.

An ELISA was done first to test the selectivity of the CEA-HRP conjugate for the anti-CEA 11-285-14. Wells were coated with 5 $\mu\text{g/ml}$ of 11-285-14 antibody or

non-specific antibody Ag8 and blocked with 1 % NRS/PBS/Tween-20. The conjugate was added in quadruplicate in doubling dilutions (1/5, 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640, 1/1280, and 1/2,560).

II 6.0 MICROCYTOSTASIS ASSAY

The procedure used was that published by Ford et al (1989) with the modifications indicated. This assay was used to determine the sensitivity of the cell lines to the drug plus antibody and drug alone. The MTT or tetrazolium salt (yellow solution) reacted with succinate dehydrogenase to give a formazan precipitate (purple solid). The precipitate was dissolved in DMSO to give a purple solution before absorbance readings were taken. Succinate dehydrogenase is a functional mitochondrial enzyme found only in the live or viable cells. The amount of formazan precipitate formed in each well correlated directly to the level of functional enzyme or the number of live cells in that well. Therefore, the test sample absorbance readings were an indication of cell survival, when compared to control absorbance levels.

II 6.0(a) Materials

- (1) SKC01 and COLO 320 DM colonic cancer cell lines.
- (2) Multichannel digital micro pipetter (Nichiryo Co., LTD. 1, Kanda-Matsunaga-cho Chiyoda-Ku, Tokyo, Japan)
- (3) Sterile pipette tips (200 µl) (ICN FLOWS, ICN Biomedicals, Inc., Costa Mesa, CA.)

- (4) Linbro 96-well (1 x 0.7 cm, 0.35 ml well capacity) flat bottomed tissue culture plates (# 76-003-05 Flow Labs).
- (5) RPMI and MEM medium containing 10% fetal calf serum and 6 ml of glutamine.
- (6) Sterile PBS
- (7) MTT (3 [4,5 Dimethylthiazol - 2 - yl] - 2,5 - diphenyltetrazolium bromide) (#M-2128, Sigma Chemical Co.)
- (8) Clinical rotator (Fisher Scientific Co., New Jersey)
- (9) Doxorubicin (DOX) (Adria Laboratories)
- (10) Bispecific antibodies or Ag8 antibody

II 6.1 Microcytostasis Assay Method

The following is an outline of the microcytostasis assay (MTT) procedure used in the Oncology Research Laboratory. The 96 well plates used in the assay had 12 columns of wells labelled 1-12 and 8 rows of wells labelled A-H.

- (1) Cultured cells were trypsinized (section II 1.5) and counted (section II 1.6). 10^4 cells was required per well in a 96 well plate at 100 μ l per well.
- (2) 10 ml of cells at 10^4 cells / well (10^6 total) gives 10^5 cells per ml. These cells were plated and incubated at 37°C (5% CO₂) for 24 hours. Column 12, rows A-H of the assay plate did not receive any cells.
- (3) Medium was then carefully suctioned from the wells using a multichannel pipetter, making sure not to disturb the cells.

- (4) Doxorubicin was diluted in sterile PBS and then added to the plate in quadruplicate (rows A to D) in log dilutions (starting with column 1 with 0.000001 ng/ml to column 11 having 10000 ng/ml, respectively). These dilutions were added at 100 μ l per well.
- (5) Rows E - H (columns 1 to 11) were controls and received 100 μ l of medium. The plate was then incubated at 37°C in a humidified incubator (5% CO₂) for 24 hours.
- (6) Both drug solutions and medium were then suctioned from the wells. This was followed by three washes with sterile PBS (200 μ l PBS per well).
- (7) Medium was then added to each well (100 μ l per well) followed by a 24 hour incubation in a 37°C humidified incubator (5% CO₂).
- (8) Medium was suctioned from the wells. 100 μ l of MTT solution (5 mg/ml stock diluted 1:10 in medium) was added to each well. The plate was then incubated in a humidified 37°C incubator for 4 hours.
- (9) The MTT solution was then suctioned from each well. DMSO was added (100 μ l per well) and the plate was placed on a shaker for 10 minutes. Finally, the absorbance of each well was determined with a plate reader using dual wavelengths of 630 and 570 nm.
- (10) Data points were entered in a statistical computer program and the points were fitted to a sigmoid curve. The IC₅₀ value was determined by inputting Y=50, corresponding to 50% survival and then receiving the corresponding concentration or X value.

II 6.2 Selection of Antibodies for Testing

Each of the bispecific antibodies were tested for anti-CEA, anti-DOX, anti-DOX-BSA, anti-BSA and dual (anti-CEA, anti-DOX) activity, at first using HPLC fractions from 100 μ l injections. Those samples with the highest anti-CEA and anti-DOX activity were selected for further analysis. Out of those selected, the samples with the largest volume and concentration of antibodies were finally chosen for preparative separation, using a 5 ml manual sample injection loop, and with 1 ml HPLC injections. These samples were tested again using ELISA and then all of the remaining samples were separated by using HPLC with repeated 5 ml injections.

II 6.3 Antibody-Mediated Targeting Method

This procedure was very similar to the DOX IC₅₀ assay with the exception of the following steps. This assay was carried out using the BSMabs and the nonspecific Ag8 antibody in various concentrations with SKC01 and COLO 320 DM cell lines.

The changes to the microcytostasis assay procedure in section II 6.1 occur in step #4. After the medium is removed, the antibody (BSMab or Ag8) was diluted in medium at 10, 1, 0.1, and 0.01 μ g/ml. The 10 μ g/ml dilution was added to columns 1-5 (rows A-D), 1 μ g/ml to columns 6-10 (rows A-D), 0.1 μ g/ml to columns 1-5 (rows E-H), and 0.01 μ g/ml to columns 6-10 (rows E-H). Columns #5 and #10 served as antibody control wells for each antibody dilution. The remaining 4 columns of 4 wells for each dilution were used as test wells. Column 11 was the PBS control while column 12 was a blank. The antibodies were incubated for 15 minutes at 4°C.

The supernatant was then removed and the drug dilutions were added. Dilutions were added from the lowest to the highest concentrations in columns 1 to 4 and columns 6 to 9, respectively. The 8 wells of each column received 100 μ l of the assigned drug dilution. The dilutions used for the SKC01 cell line were 1000, 100, 10 and 1 ng/ml while the COLO 320 DM dilutions were 10,000, 1000, 100 and 10 ng/ml. These dilutions were made according to the IC_{50} values for each cell line. The plates were then incubated at 37°C for 24 hours and the procedure continued as in section II 6.1 (step #8).

At the same time, a second plate was set up using DOX alone at the same dilutions. This plate served as a DOX control plate.

CHAPTER III

RESULTS

III 1.0 PRODUCTION OF DOX-BSA CONJUGATE

DOX-BSA conjugate was required as one of the coating reagents for the anti-DOX-BSA ELISA, as well as for the dual assay. Three conjugates were prepared and purified using Sephadex G-25 chromatography. Two-ml fractions were collected at 5 minute intervals and spectrophotometric readings taken for each fraction. The first sample (Figure 2) had a DOX:BSA ratio of 1.66. The second sample readings showed a very similar level of conjugation with a DOX:BSA ratio of 1.83 (Figure 3). Approximately 4 ml was retrieved from the first experiment and 4 ml from the second. There was some precipitation of the DOX during these experiments but this was kept to a minimum by adding the drug slowly and maintaining very slow stirring.

A third conjugation was performed and the DOX:BSA ratio was calculated as 3.15 (Figure 4). This was performed without the overnight incubation and resulted in a clear product with no precipitation. There was more consistent slow stirring using a very small magnet which may have accounted for the better product. Fraction #9 also had a high reading for DOX with a DOX:BSA ratio of 20.8.

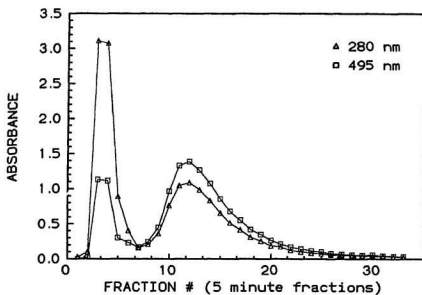


Figure 2. DOX-BSA conjugate purification #1. Conjugate separation was performed using a Sephadex G-25 gel filtration column. 5 minute fractions were collected and analysed spectrophotometrically at 450 and 280 nm.

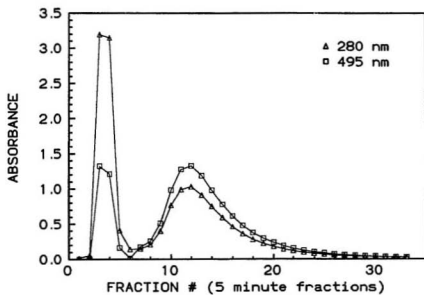


Figure 3. DOX-BSA conjugate purification #2. Procedures were carried out as in Figure 2.

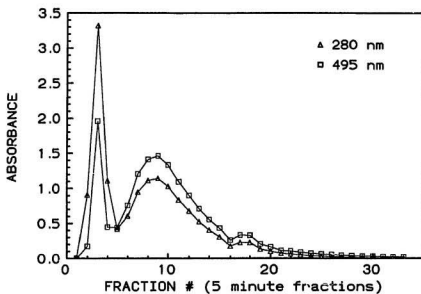


Figure 4. DOX-BSA conjugate purification #3. Fractions were collected from a Sephadex gel column at 5 minute intervals. The legend is the same as for Figure 2.

III 2.0 PRODUCTION OF CEA-HRP CONJUGATE

CEA-HRP was the reagent required in the dual assay, replacing the RAM-HRP used in the other ELISAs. This procedure was developed by Reddy, (1993); Reddy & Ford, (1993) for this particular purpose.

All the HRP in solution appeared to have conjugated to CEA (Figure 5) as HRP was only detected at the same peak as the CEA peak at 280 nm. In order to demonstrate that this conjugate was functional, a selectivity ELISA was done using the anti-CEA 11-285-14 Mab (Materials and Methods section II 5.6). Once the ABTS substrate was added the conjugate showed high levels of HRP activity (Figure 6). There was high activity up to 1/80 dilution of the pooled fractions when tested against the anti-CEA 11-285-14 antibody. The control (nonspecific) antibody resulted in very low HRP activity for all dilutions of the conjugate. This result demonstrated the activity and specificity of the conjugate for anti-CEA antibody.

III 3.0 ASCITIC FLUID PRODUCTION AND PROTEIN A COLUMN PURIFICATION

Ascitic fluid was obtained by injecting the growing BSMab producing hybridomas intraperitoneally into Balb/c mice (Materials and Methods section 2.0). Varying amounts of ascitic fluid were collected for each hybrid (Table 1). This depended on how much ascitic fluid was produced by each mouse and how long the mouse lived to tap the fluid from it.

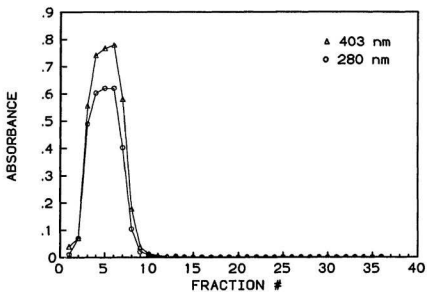


Figure 5. CEA-HRP conjugate purification. The separation was performed using a Sephadex G-75 gel filtration column. Fractions were collected at 2 minute intervals at a rate of 0.75 ml/minute. Fractions were spectrophotometrically analysed at 403 and 280 nm.

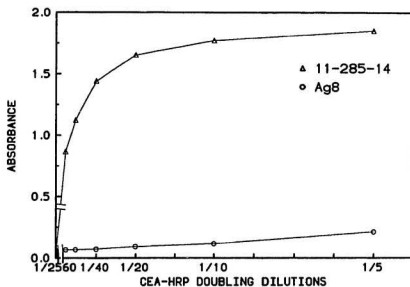


Figure 6. CEA-HRP conjugate specificity and sensitivity ELISA results. ELISA plates were coated with 5 $\mu\text{g/ml}$ of test antibody (11-285-14, anti-CEA) or control antibody (Ag8, non-specific). Conjugate was added in doubling dilutions from 1/5 to 1/2560.

Table 1. Summary of Ascitic Fluid Collection and Protein A Purification.

| Bispecific Hybridoma number | # Animals Inoculated | Pooled Unpurified Ascitic Fluid (ml) | Protein A Purified Ascitic Fluid (ml) | Protein Concentration (mg/ml) |
|------------------------------------|-----------------------------|---|--|--------------------------------------|
| 26-61-1 | 20 | 85 | 16.5 | 2.52 |
| 26-61-4 | 11 | 58 | 9 | 1.78 |
| 26-61-10 | 17 | 50 | 20.5 | 2.04 |
| 57-11-25-17 | 14 | 120 | 17.5 | 2.36 |
| 26-61-2 | 11 | 62 | 5 | 2.16 |
| 26-7-35 | 20 | 39 | 6.5 | 0.55 |
| 57-9-6-4 | 11 | 45 | 6.0 | 2.07 |

Once protein A purified, the ascitic fluid samples were concentrated (Table 1). Some samples had higher protein concentration than others and each differed in the final volume available for further analysis.

III 4.0 HPLC STANDARDIZATION

In order to begin the HPLC separation of the seven bispecific monoclonal antibodies, the procedure had to be standardized first. Fifty microlitres of each protein A purified antibody was separated using a 60 mM to 180 mM phosphate buffer gradient (Xiang et al, 1992). This was undertaken in order to determine how each antibody would separate under these conditions.

III 4.1 Standardization Using Parental 11-285-1 (anti-CEA monoclonal antibody IgG₁)

III 4.1(a) HPLC Separation

Fifty microlitres (2.50 mg/ml) of the protein A purified parental monoclonal antibody was loaded onto the HPLC column. The elution took place over a 60 minute period from time 20 to 80 minutes as seen in Figure 7, using a 60 mM to 180 mM buffer gradient. The first peak, seen at approximately 4 minutes, is a small loading peak which is an artifact of all HPLC separation procedures. There is a major peak which starts at approximately 58 minutes and ends at 65 minutes. Both peaks were collected manually and analyzed by ELISA for anti-CEA activity.

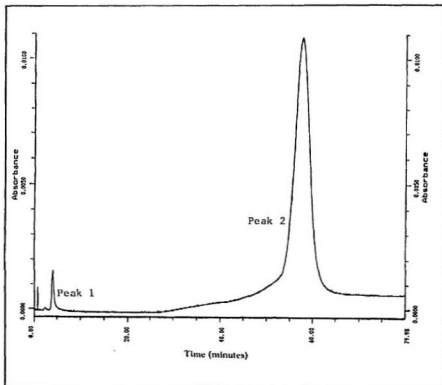


Figure 7. HPLC separation of monoclonal 11-285-14. 50 μ l of a 2.5 mg/ml sample was loaded onto a hydroxylapatite column and eluted using a 60 mM to 180 mM phosphate buffer gradient. The absorbance of the eluate was analysed at 280 nm and fractions collected at 1 minute intervals.

III 4.1(b) ELISA Results

The anti-CEA ELISA results revealed that peak 1 contained very little anti-CEA activity (Figure 8). The resulting absorbance reading was very similar to that of the control wells. The control mean absorbance plus 2 standard deviations was 0.321. Peak 2 gave a very high anti-CEA activity. The absorbance reading was double that found for the 10 and 1 $\mu\text{g/ml}$ dilutions of the positive test control. The positive test control in this case was the unfractionated 11-285-14 parental monoclonal antibody.

III 4.2 Standardization of BSMab Separations

Only one of the bispecific antibodies was used for this initial standardization. The 26-61-2 antibody was chosen for this procedure.

III 4.2(a) HPLC Results

The 50 μl sample revealed 6 distinct peaks (Figure 9(a)) with a small shoulder between peaks 5 and 6. Peak 6 showed an elution time very similar to that of peak 2 of Figure 7. There were no more protein peaks following the set time period shown in Figure 9(a) where the absorbance reading returned to baseline (results not shown). Each of the peaks was collected manually into 10 ml tubes. The volumes collected in the tubes varied from peak to peak as each peak varied in overall size. This manual collecting did result in peak overlap in each tube as the tubes were changed only when a "new" peak started to elute.

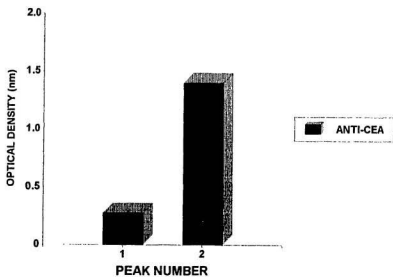


Figure 8. Anti-CEA ELISA results of 11-285-14 HPLC purified peaks. Peak fractions were tested using CEA coated ELISA plates and a 1/1000 dilution of RAM-HRP.

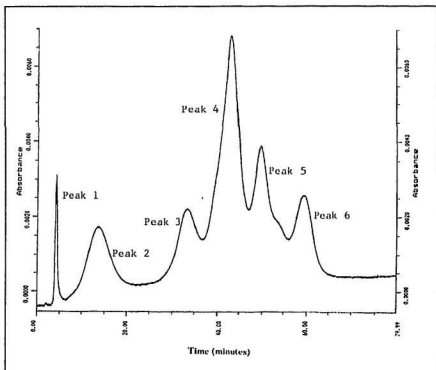


Figure 9(a). HPLC separation of ascitic fluid containing 26-61-2 bispecific antibody. 50 μ l of a 2.0 mg/ml sample was loaded onto a hydroxylapatite column and eluted using a 60 mM to 180 mM phosphate buffer gradient. Eluate was analysed at 280 nm and collected at 1 minute intervals with a flow rate of 1 ml/minute.

III 4.2(b) ELISA Results

There were four ELISA's performed on the initial HPLC separation. These tests included anti-CEA, anti-DOX, anti-DOX-BSA and anti-BSA. The anti-CEA results (Figure 9(b)) revealed no activity in peak 1, as in the anti-CEA result of peak 1 with the parental 11-285-14 (Figure 8). Peaks 2 through to 6 did show increasing anti-CEA activity. Peak 6 showed the highest anti-CEA activity similar to that seen with peak 2 of the parental 11-285-14 (Figure 8).

The highest anti-DOX activity was found in peak 2 with an absorbance reading of 1.462. The level of activity decreased from peak 2 through to peak 6 with peak 6 having the lowest level.

The anti-DOX-BSA ELISA results (Figure 9(b)) were very similar to those of the anti-DOX ELISA. The highest absorbance reading was in peak 2. The level of absorbance decreased from peak 2 to peak 6. The intensity of the readings were not as high as for the anti-DOX ELISA.

The anti-BSA ELISA (Figure 9(b)) did not give the same pattern of absorbance readings as did the anti-DOX and the anti-DOX-BSA ELISAs. Peaks 1 and 6 gave very low readings while peaks 4 and 5 gave the highest levels.

III 4.3 HPLC Procedure Modifications

A 60 mM to 360 mM gradient was tried with the same conditions (Xiang et al, 1992) in an attempt to try and improve the separation. Upon viewing these differences (Figure 10) it was decided to proceed with the 60 mM - 360 mM gradient due to its

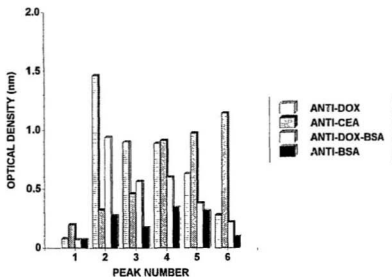


Figure 9(b). ELISA results of 26-61-2 HPLC separation peaks. Anti-CEA, anti-DOX, anti-DOX-BSA and anti-BSA ELISA's were performed on each peak fraction using 100 μ l of sample and a 1:1000 dilution of RAM-HRP.

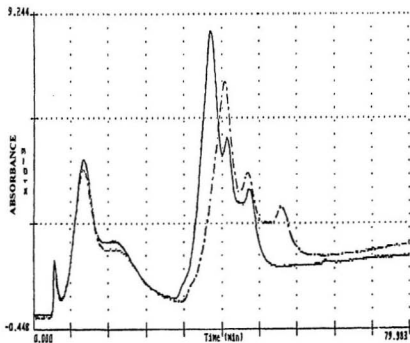


Figure 10. A comparison of two HPLC gradient profiles. The solid line (___) illustrates a 60-180 mM phosphate buffer (PB) gradient elution profile of a 50 μ l injection of 26-61-4 ascitic fluid. The dashed line (• - •) illustrates the same volume of sample eluted with a 60-360 mM PB gradient.

better discrimination of peak areas.

Due to the inconsistencies of collecting the fractions manually, a FRAC-100 fraction collector was programmed to collect these fractions at 1 minute intervals. The tube numbers then corresponded to each minute of the time scale on the x axis of the HPLC profile. This permitted a more precise analysis of each peak.

III 5.0 ANALYTICAL HPLC AND ELISA ANALYSIS OF ALL SEVEN HYBRIDOMAS

For this procedure 1 ml of each bispecific monoclonal antibody was loaded onto the column and fractions collected. This was performed using the 60 to 360 mM phosphate buffer gradient protocol. Each fraction was analysed for anti-CEA, anti-DOX, anti-DOX-BSA and anti-BSA activity.

III 5.1 Bispecific Monoclonal Antibody 26-61-1

III 5.1(a) HPLC Profile

The 1 ml HPLC profile of 26-61-1 illustrates two major peaks (Figure 11). The first peak starts at approximately 12 minutes as a sharp peak and tails out between 15 and 28 minutes. The second peak region starts at approximately 50 minutes and ends at approximately 65 minutes. This region contains two distinct peaks (54 and 57 minutes, respectively) with a shoulder at 53 minutes, just before the first peak in that region.

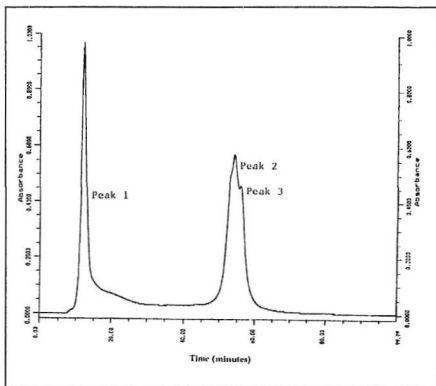


Figure 11. HPLC separation of ascitic fluid containing 26-61-1 bispecific antibody. A 1 ml sample was loaded on to a hydroxylapatite column and eluted using a 60 mM to 360 mM phosphate buffer gradient. The eluate was analysed at 280 nm and fractions were collected at 1 minute intervals.

III 5.1(b) ELISA Results

ELISA results of the 26-61-1 HPLC separated fractions showed high anti-DOX and anti-DOX-BSA activity clustered between 10 and 20 minutes and between 48 and 65 minutes (Figure 12). The anti-DOX-BSA was highest in the 50 to 65 minute region. Anti-CEA results started to increase at approximately 10 minutes and reached a maximum at 50 minutes, stabilised up to 65 minutes and decreased down to near baseline at 70 minutes. The anti-BSA activity of this hybridoma was low in all the fractions. There was one area of activity between 50 and 58 minutes but the absorbance was not higher than 0.25 nm.

III 5.2 Bispecific Monoclonal Antibody 26-61-2

III 5.2(a) HPLC Profile

The HPLC profile of the 26-61-2 ascitic fluid (Figure 13) indicates two major peak regions. The first eluted at approximately 8 minutes with a flat plateau region between 17 and 37 minutes. The second peak region contained three discrete peaks each eluting at 41, 45 and 52 minutes, respectively. The absorbance level did not return to 0 following this last peak but rather it remained at a very low level for the last 40 minutes.

III 5.2(b) ELISA Results

The ELISA results (Figure 14) indicate high anti-DOX-BSA activity in the first peak region with decreasing levels in the between the two major peak regions. There is

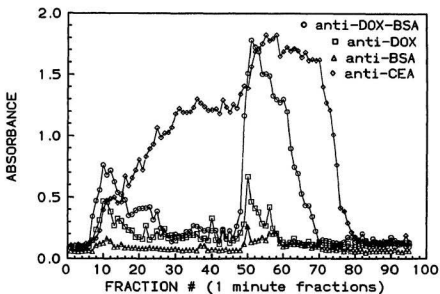


Figure 12. ELISA results of a 1 ml 26-61-I HPLC separation. 1 minute fractions were tested for anti-CEA, anti-DOX, anti-DOX-BSA and anti-BSA activity. The absorbance was read at 405 nm.

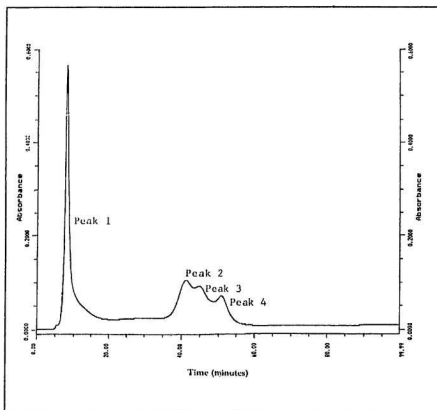


Figure 13. HPLC absorbance profile of ascitic fluid containing 26-61-2 bispecific antibody. A 1 ml sample was loaded and eluted from a hydroxylapatite column using a 60 mM to 360 mM phosphate buffer gradient. Absorbance was read at 280 nm and 1 minute fractions were collected.

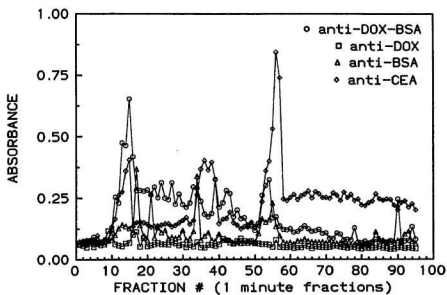


Figure 14. ELISA results of a 1 ml 26-61-2 HPLC separation. Fractions were tested for anti-CEA, anti-DOX, anti-DOX-BSA and anti-BSA activity. The absorbance was read at 405 nm.

also an increase in activity at approximately 60 minutes and then a decrease in optical density to baseline level.

The anti-CEA activity increased at approximately 14 minutes, returned to near baseline levels and increased again at 34 minutes. The anti-CEA activity was much greater at 56 minutes, reflected by an absorbance of 0.80. The optical density decreased very quickly after this point but did not go below 0.2 for the remainder of the fractions.

The anti-DOX results for the 26-61-2 purified ascitic fluid did not show any major peaks throughout the entire gradient. There was an increase in anti-BSA activity at approximately 18 minutes into the HPLC separation. Levels then returned to baseline until fraction #59. Here the optical density increased to nearly 0.2 and then returned to baseline for the remainder of the fractions.

III 5.3 Bispecific Monoclonal Antibody 26-61-4

III 5.3(a) HPLC Profile

This 1 ml sample separated into a sharp peak at approximately 9 minutes and a broader peak area at approximately 63 minutes (Figure 15). The first peak started eluting at 7 minutes and ended at 11 minutes. After this point, a broad shoulder and a low plateau region appeared from 12 minutes up to approximately 41 minutes. The second peak area started to elute very slowly at approximately 48 to 59 minutes. Then the amount of eluting protein increased very rapidly at approximately 60 minutes to give two peaks at 64 and 67 minutes. The absorbance level then decreased rapidly to give another low shoulder region from approximately 69 to 80 minutes.

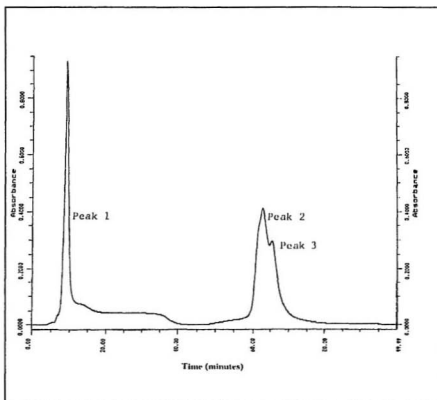


Figure 15. HPLC absorbance profile of ascitic fluid containing 26-61-4 bispecific antibody. A 1 ml sample was loaded and eluted from a hydroxylapatite column using a 60 mM to 360 mM phosphate buffer gradient. Fractions were collected at 1 minute intervals and absorbance read at 280 nm.

III 5.3(b) ELISA Results

The anti-CEA activity of these fractions (Figure 16) followed a different pattern to that of the other hybridoma fractions. The activity increased rapidly at approximately 8 minutes and stabilised until 45 minutes. Then the optical density levelled for 9 minutes before it increased very rapidly again. This time it stabilised from 59 minutes until 86 minutes and then dropped back to background level. The anti-BSA and anti-DOX-BSA activity peaked between 8 and 12 minutes at approximately the same level. Both activities then stabilised from 12 to 42 minutes before returning to background activity levels. Both activities again increased at 58 minutes and slowly decreased from 66 to 80 minutes. The anti-DOX activity was not as great as that of the anti-BSA and anti-DOX-BSA test results. The pattern of activity was similar to that of the previously mentioned results but with much lower activity between 8 and 12 minutes. From 13 to 60 minutes there was only background activity and no plateau as observed previously for the anti-DOX and anti-DOX-BSA tests. The level of anti-DOX activity then increased at 60 minutes and followed a similar pattern to that of the anti-BSA results.

III 5.4 Bispecific Monoclonal Antibody 26-61-10

III 5.4(a) HPLC Profile

There were two distinct peak areas within the HPLC profile of the bispecific monoclonal antibody 26-61-10 (Figure 17). The first peak appeared at 8 minutes into the elution procedure and ended at 20 minutes with a slight tail effect. There was then a plateau region from 20 minutes to 45 minutes. A shoulder (peak 2) appeared between

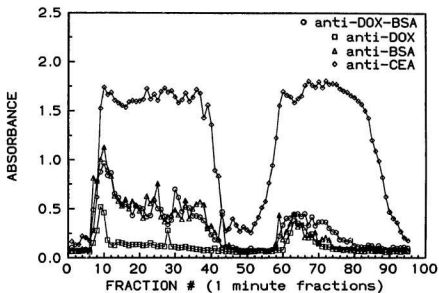


Figure 16. ELISA results of a 1 ml 26-61-4 HPLC separation. Fractions were tested for anti-CEA, anti-DOX, anti-DOX-BSA and anti-BSA activity. The absorbance was read at 405 nm.

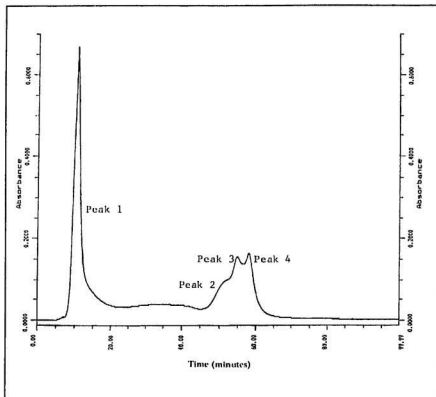


Figure 17. HPLC absorbance profile of ascitic fluid containing 26-61-10 bispecific antibody. A 1 ml sample was loaded and eluted from a hydroxylapatite column using a 60 mM to 360 mM phosphate buffer gradient. Fractions were collected at 1 minute intervals and absorbance read at 280 nm.

51 and 53 minutes and the third peak at 54 minutes. The fourth peak of the profile appeared at 58 minutes and finished at approximately 62 minutes.

III 5.4(b) ELISA Results

The 26-61-10 anti-CEA results, as with most of the BSMab's, showed a gradual increase in optical density with time (Figure 18). This activity reached a maximum level at approximately the 50 minutes fraction where it stabilized for a 20 minute period and then decreased very quickly. At the 76 minute period, the activity had dropped back to background level.

The anti-BSA activity was very high within the first peak region between 9 to 14 minutes (Figure 18). The activity decreased after this point but did not drop below an absorbance of 0.75. The activity fluctuated within the 14 and 46 minute fractions and increased again at the 47 minute fraction. The level of activity again fluctuated until the 62 minute fraction at which point the anti-BSA activity quickly dropped down to background level. The activity did not change after this point.

The anti-DOX-BSA activity of this HPLC separation first showed an increase in activity at approximately the 8th fraction (Figure 18) which was within the first peak region of the HPLC profile (Figure 17). The activity level then slowly decreased until the 40 minute period. The absorbance increased over a 10 minute time span but did not increase much beyond 0.5. After this point the absorbance dropped to approximately 0.25 and slowly returned to the background level thereafter.

The anti-DOX activity was the greatest within the first peak region of the

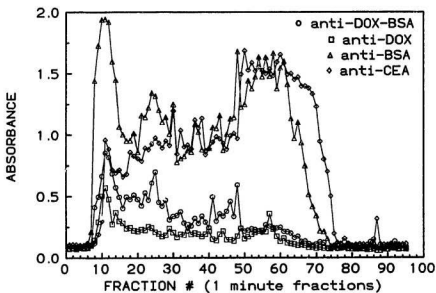


Figure 18. ELISA results of a 1 ml 26-61-10 HPLC separation. Fractions were tested for anti-CEA, anti-DOX, anti-DOX-BSA and anti-BSA activity. The absorbance was read at 405 nm.

HPLC separation with a peak level just beyond 0.5. The activity level then decreased after this point and did not increase any more, slowly decreasing to a background level throughout the remainder of the fractions.

III 5.5 Bispecific Monoclonal Antibody 26-7-35

III 5.5(a) HPLC Profile

The HPLC profile of this hybridoma, like the other hybridomas, gave two major peak regions (Figure 19). The first eluted between 5 and 15 minutes while the second eluted between 35 and 65 minutes. The second peak region contained two distinct peaks which reached maximum absorbance levels at 44 and 54 minutes, respectively. Following the second peak region the absorbance remained flat but did not return to the reference level (0).

III 5.5(b) ELISA Results

The anti-CEA activity of the 26-7-35 hybridoma was very similar to that of the other hybrids where it increased slowly over the first half of the HPLC separation (Figure 20). The level of activity reached a maximum over the second peak region, in this case between the 35 and 72 minute fractions. However, the major portion of the second peak region was eluted by the 60 minute fraction with a tail portion that extended to approximately the 75 minute fraction.

The anti-BSA activity was high within the two peak regions of the HPLC profile (Figure 20) with peak values occurring in the 7 and 49 minute fractions,

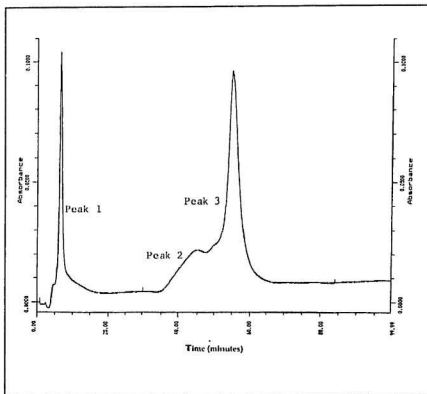


Figure 19. HPLC absorbance profile of ascitic fluid containing 26-7-35 bispecific antibody. A 1 ml sample was loaded and eluted from a hydroxylapatite column using a 60 mM to 360 mM phosphate buffer gradient. Fractions were collected at 1 minute intervals and absorbance read at 280 nm.

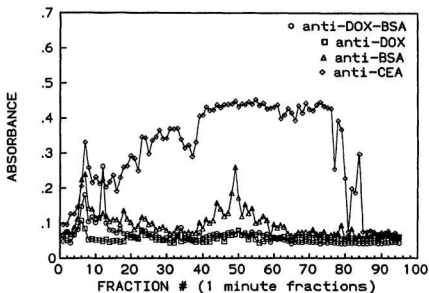


Figure 20. ELISA results of a 1 ml 26-7-35 HPLC separation. Fractions were tested for anti-CEA, anti-DOX, anti-DOX-BSA and anti-BSA activity. The absorbance was read at 405 nm.

respectively.

Anti-DOX-BSA assay (Figure 20) gave two points of high activity in the first peak region of the HPLC separation profile (Figure 19). This activity occurred in the 7 and 12 minute fractions, respectively. This activity then decreased and remained at a background level throughout the remainder of the fractions.

The anti-DOX activity in this hybridoma was not very strong for most of the fractions (Figure 20). The only real indication of activity occurred in the first peak region in fraction number 6.

III 5.6 Bispecific Monoclonal Antibody 57-11-25-17

III 5.6(a) HPLC Profile

The 57-11-25-17 protein A purified ascitic fluid also separated into two major peak areas (Figure 21). The first peak was very sharp and occurred between 8 and 13 minutes. The second peak region contained two peaks. The first was at approximately 54 minutes and the second at approximately 56 minutes. The peak region spanned the 50 to 60 minute time period with a low tail region that reached baseline absorbance at approximately 80 minutes. The absorbance between the two peak regions did not return to baseline, but rather it showed a low plateau pattern.

III 5.6(b) ELISA Results

This BSMab showed very high anti-BSA (Figure 22) activity in both peak regions of the HPLC profile (Figure 21). The anti-DOX and the anti-DOX-BSA

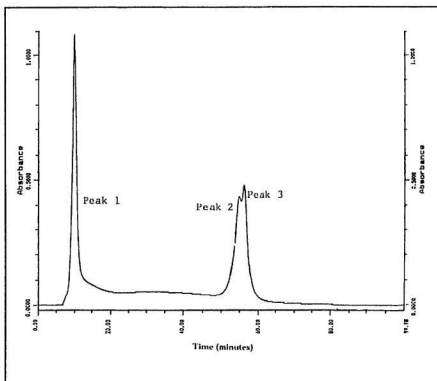


Figure 21. HPLC absorbance profile of ascitic fluid containing 57-11-25-17 bispecific antibody. A 1 ml sample was loaded and eluted from a hydroxylapatite column using a 60 mM to 360 mM phosphate buffer gradient. Fractions were collected at 1 minute intervals and absorbance read at 280 nm.

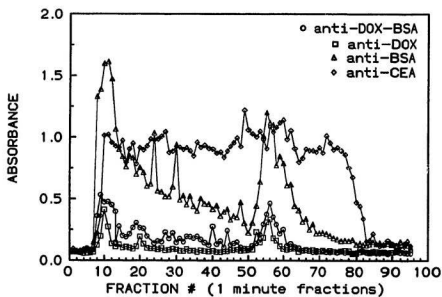


Figure 22. ELISA results of a 1 ml 57-11-25-17 HPLC separation. Fractions were tested for anti-CEA, anti-DOX, anti-DOX-BSA and anti-BSA activity. The absorbance was read at 405 nm.

readings were much lower with the highest levels occurring in the two peak regions. The anti-CEA results, on the other hand, were high from the 10 minute fraction up to the 80 minute fraction.

III 5.7 Bispecific Monoclonal Antibody 57-9-6-4

III 5.7(a) HPLC Profile

The HPLC profile of the 57-9-6-4 1 ml sample separation was different from that of the other hybridomas (Figure 23). As with the other samples, there was a major peak region at approximately 9 minutes but the second peak region gave very low absorbance readings. There was an indication of a second peak between 37 and 56 minutes of the separation.

III 5.7(b) ELISA Results

There were positive ELISA results from the 57-9-6-4 HPLC separation and they were no lower than the 26-7-35 ELISA results (Figure 24). The anti-CEA ELISA results gave a very similar pattern of activity to that of the other hybridomas whereby the activity began to increase within the first peak region and continued to plateau over the second peak region. The activity remained high beyond the second peak region and quickly decreased to background levels after the 74th fraction.

The anti-BSA activity was high within the two peak regions of the HPLC separation. The first high activity peak occurred at 9 minutes and the second between 39 and 48 minutes (Figure 24).

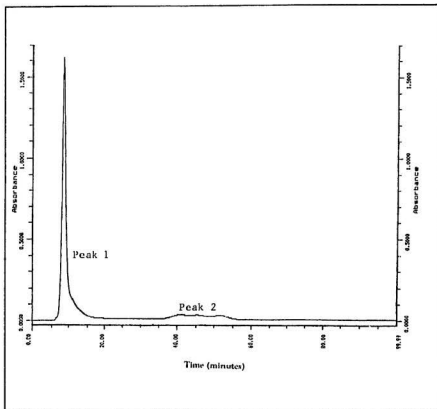


Figure 23. HPLC absorbance profile of ascitic fluid containing 57-9-6-4 bispecific antibody. A 1 ml sample was loaded and eluted from a hydroxylapatite column using a 60 mM to 360 mM phosphate buffer gradient. Fractions were collected at 1 minute intervals and absorbance read at 280 nm.

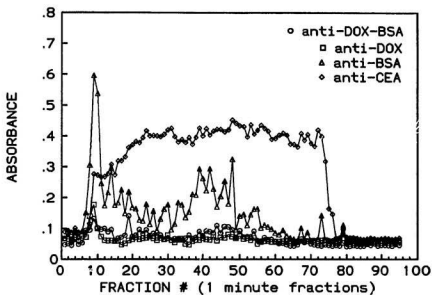


Figure 24. ELISA results of a 1 ml 57-9-6-4 HPLC separation. Fractions were tested for anti-CEA, anti-DOX, anti-DOX-BSA and anti-BSA activity. The absorbance was read at 405 nm.

The anti-DOX-BSA and anti-DOX activity was very low for this particular hybridoma (Figure 24). Both tests showed high activity in the 9 minute fraction but remained very low throughout the other fractions.

III 6.0 DUAL ACTIVITY TEST

The presence of dual activity was tested in dilutions of the original protein A ascitic fluid samples. The results in Table 2 indicate that not all the samples responded strongly in the test. The strongest reaction came from the protein A concentrated sample of 26-61-10. Next to this were the 26-7-35 and the 57-11-25-17 results. 57-9-6-4 gave the lowest response. The values obtained for this particular hybridoma were not greater than the control mean plus two standard deviations (0.0647), therefore, they were not considered to be significant. The results with 26-61-1, 26-61-2 and 26-61-4 were significant but still gave low readings.

The protein A washes, which were collected for each of the hybrids, were also checked for dual activity. This was checked for possible loss of valuable protein in the initial washing of the protein A column. Results indicated very low activity. All but the 26-61-1 wash had values that were not significant (results not shown).

III 7.0 PREPARATIVE HPLC PURIFICATION

To be able to scale up the purification of each of the bispecific antibodies a 5 ml loading loop was used. This was required in order to load larger amounts of protein A purified ascitic fluid onto the HPLC column. The process required less time and

Table 2. Results of dual assays on hybridoma protein A purified ascitic fluids.

| BSMab HYBRID # | ABSORBANCE READINGS * | | | | |
|-------------------|-----------------------|-------|-------|-------|-------|
| | SAMPLE CONCENTRATION | | | | |
| | STOCK | 1/5 | 1/10 | 1/20 | 1/40 |
| 26-61-1 | 0.081 | 0.075 | 0.071 | 0.067 | 0.067 |
| 26-61-2 | 0.071 | 0.056 | 0.063 | 0.057 | 0.056 |
| 26-61-4 | 0.068 | 0.069 | 0.058 | 0.054 | 0.054 |
| 26-61-10 | 0.169 | 0.120 | 0.089 | 0.053 | 0.053 |
| 26-7-35 | 0.103 | 0.094 | 0.071 | 0.061 | 0.062 |
| 57-11-25-17 | 0.107 | 0.098 | 0.089 | 0.080 | 0.071 |
| 57-9-6-4 | 0.061 | 0.064 | 0.067 | 0.054 | 0.054 |

* Tests performed on doubling dilutions

resulted in larger concentrations of antibody per fraction.

Four of the original seven hybridoma samples were selected for the final analysis. This selection was based on the following criteria. First, each required a significant result from the dual assay ELISA ($> \text{control mean} \pm 2 \text{ S.D.}$). Second, there was a requirement for a significant volume of protein A purified sample. A large initial volume meant that there was more antibody available to work with in case problems did arise further into the project. Thirdly, there was a requirement for a reasonable concentration of protein in each protein A purified ascitic fluid sample. Fourthly, the samples had to have positive ELISA results for anti-CEA, anti-DOX, anti-DOX-BSA. It was preferred that if there was any anti-BSA activity that it would be equal to background absorbance or no greater than that of the anti-DOX-BSA results.

In order to conserve reagents, and based on the analytical results, ELISA testing was concentrated on fractions from the second major peak area from the HPLC separation. Also, because only 5 ml could be separated at a time and samples from each of the secreted hybridomas consisted of 9 or more ml, several injections had to be done. Therefore, each hybridoma had several HPLC profiles and as many sets of fractions to be analysed.

III 7.1 26-61-1 5 ml HPLC Separation

III 7.1(a) HPLC Profiles

There were three 5 ml injections of this hybridoma. Figures 25, 26 & 27 illustrate how the protein peaks eluted at approximately the same time and gave very

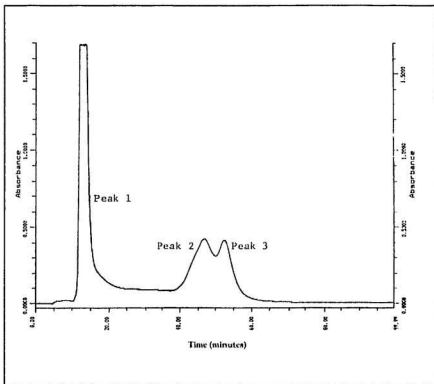


Figure 25. First quantitative HPLC absorbance profile of ascitic fluid containing 26-61-1 bispecific antibody. A 5 ml sample was loaded and eluted from a hydroxylapatite column using a 60 mM to 360 mM phosphate buffer gradient. Fractions were collected at 1 minute intervals and absorbance read at 280 nm.

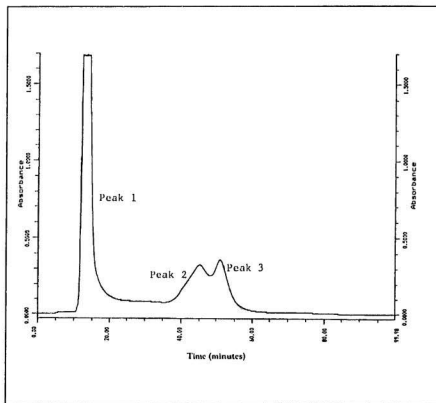


Figure 26. Second quantitative HPLC absorbance profile of ascitic fluid containing 26-61-1 bispecific antibody. Rest of details as for legend to Figure 25.

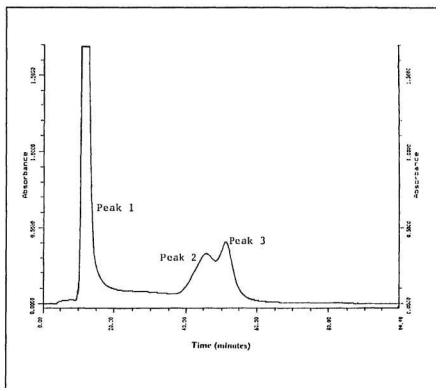


Figure 27. Third quantitative HPLC absorbance profile of ascitic fluid containing 26-61-1 bispecific antibody. Rest of details as for legend to Figure 25.

similar absorbance values.

III 7.1(b) ELISA Results

The ELISA results (Tables 3, 4 & 5) of the first, second and third separations, respectively, all illustrate high levels of anti-CEA activity. The anti-DOX and anti-DOX-BSA activity levels start off lower than the anti-CEA activity with a peak region in the middle fractions. However, the dual assay results (Tables 3,4 & 5) reveal that the dual response of this hybridoma was low. Fraction # 43 was the only fraction of the first separation that had an absorbance reading greater than 0.076 (control mean \pm 2 standard deviations).

III 7.2 26-61-4 5 ml HPLC Separation

III 7.2(a) HPLC Profiles

The HPLC profiles of the two 5 ml separations (Figures 28, 29) illustrate the same elution times for the two peak regions. However, there was a slight difference in the appearance of the second peak region which was the area of interest for ELISA screening.

III 7.2(b) ELISA Results

The assay results for the collected fractions (Tables 6 & 7) revealed that the anti-CEA results were very similar however the anti-DOX and anti-DOX-BSA results were a little different. The two assays gave peaks in approximately the same fractions

Table 3. ELISA results from the first 5 ml HPLC separation of the 26-61-1 bispecific hybridoma.

| FRACTION # | 26-61-1 1st 5 ml Separation | | | |
|------------|--------------------------------|------------|-----------------|----------------|
| | ANTI-CEA* | ANTI-DOX** | ANTI-DOX-BSA*** | DUAL ASSAY**** |
| 40 | 1.276 | 0.198 | 0.379 | 0.060 |
| 41 | 1.296 | 0.364 | 0.291 | 0.066 |
| 42 | 1.049 | 0.232 | 0.447 | 0.071 |
| 43 | 1.224 | 0.349 | 0.504 | 0.084 |
| 44 | 1.294 | 0.457 | 0.764 | 0.062 |
| 45 | 1.296 | 0.643 | 0.790 | 0.071 |
| 46 | 1.299 | 0.767 | 0.977 | 0.056 |
| 47 | 1.288 | 0.773 | 0.845 | 0.057 |
| 48 | 1.301 | 0.943 | 0.800 | - |
| 49 | 1.248 | 0.736 | 0.845 | 0.059 |
| 50 | 1.209 | 0.626 | 1.013 | 0.063 |
| 51 | 1.274 | 0.650 | 1.082 | 0.061 |
| 52 | 1.224 | 0.564 | 0.751 | 0.060 |
| 53 | 1.151 | 0.668 | 0.622 | - |
| 54 | 1.133 | 0.566 | 0.759 | - |
| 55 | 1.221 | 0.419 | 0.585 | 0.050 |
| 56 | 1.215 | 0.326 | 0.481 | 0.057 |
| 57 | 1.182 | 0.334 | 0.330 | - |

Control Mean + 2 S.D.: * 0.1440 ** 0.0780 *** 0.1538 **** 0.0760

Table 4. ELISA results from the second 5 ml HPLC separation of the 26-61-1 bispecific hybridoma.

| FRACTION # | 26-61-1 2nd 5 ml Separation | | | |
|------------|--------------------------------|------------|-----------------|----------------|
| | ANTI-CEA* | ANTI-DOX** | ANTI-DOX-BSA*** | DUAL ASSAY**** |
| 40 | 1.088 | 0.214 | 0.407 | 0.066 |
| 41 | 0.957 | 0.260 | 0.447 | 0.061 |
| 42 | 0.973 | 0.383 | 0.519 | 0.063 |
| 43 | 0.929 | 0.491 | 0.636 | 0.051 |
| 44 | 0.912 | 0.488 | 0.654 | 0.055 |
| 45 | 0.869 | 0.656 | 0.866 | 0.060 |
| 46 | 0.997 | 0.643 | 0.775 | 0.060 |
| 47 | 0.969 | 0.658 | 0.850 | - |
| 48 | 0.984 | 0.593 | 1.057 | - |
| 49 | 0.897 | 0.609 | 0.891 | 0.054 |
| 50 | 0.703 | 0.718 | 0.669 | 0.058 |
| 51 | 0.639 | 0.574 | 0.566 | 0.065 |
| 52 | 0.922 | 0.622 | 0.742 | 0.059 |
| 53 | 1.053 | 0.631 | 0.497 | - |
| 54 | 1.077 | 0.594 | 0.736 | 0.051 |
| 55 | 1.035 | 0.406 | 0.425 | 0.053 |
| 56 | 0.967 | 0.327 | 0.454 | 0.048 |
| 57 | 0.710 | 0.289 | 0.757 | 0.057 |

Control Mean + 2 S.D.: * 0.1440 ** 0.0780 *** 0.1340 **** 0.0760

Table 5. ELISA results from the third 5 ml HPLC separation of the 26-61-1 bispecific hybridoma.

| FRACTION # | 26-61-1 3rd 5 ml Separation | | | |
|------------|--------------------------------|------------|-----------------|----------------|
| | ANTI-CEA* | ANTI-DOX** | ANTI-DOX-BSA*** | DUAL ASSAY**** |
| 40 | 0.970 | 0.170 | 0.361 | 0.047 |
| 41 | 1.042 | 0.204 | 0.480 | 0.050 |
| 42 | 1.021 | 0.341 | 0.842 | 0.050 |
| 43 | 1.091 | 0.396 | 0.557 | 0.047 |
| 44 | 1.036 | 0.471 | 0.693 | 0.049 |
| 45 | 1.070 | 0.535 | 0.660 | 0.056 |
| 46 | 1.065 | 0.620 | 0.671 | 0.052 |
| 47 | 0.894 | 0.628 | 0.765 | 0.064 |
| 48 | 0.999 | 0.610 | 0.706 | 0.052 |
| 49 | 1.059 | 0.689 | 0.726 | 0.063 |
| 50 | 1.095 | 0.819 | 0.912 | 0.054 |
| 51 | 1.102 | 0.603 | 0.823 | 0.069 |
| 52 | 1.138 | 0.647 | 0.652 | 0.055 |
| 53 | 1.090 | 0.597 | 0.714 | 0.058 |
| 54 | 1.041 | 0.517 | 0.475 | 0.051 |
| 55 | 1.027 | 0.410 | 0.564 | 0.050 |
| 56 | 1.035 | 0.400 | 0.419 | 0.055 |
| 57 | 1.039 | 0.391 | 0.791 | 0.056 |

Control Mean + 2 S.D.: * 0.1440 ** 0.0780 *** 0.1340 **** 0.0760

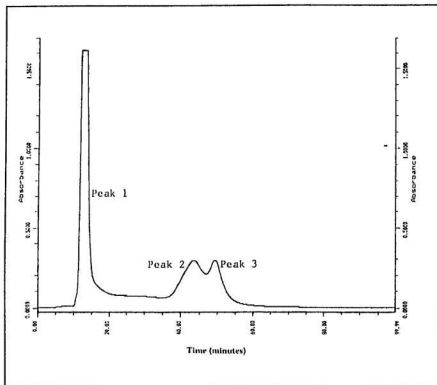


Figure 28. First quantitative HPLC absorbance profile of ascitic fluid containing 26-61-4 bispecific antibody. A 5 ml sample was loaded and eluted from a hydroxylapatite column using a 60 mM to 360 mM phosphate buffer gradient. Fractions were collected at 1 minute intervals and absorbance read at 280 nm.

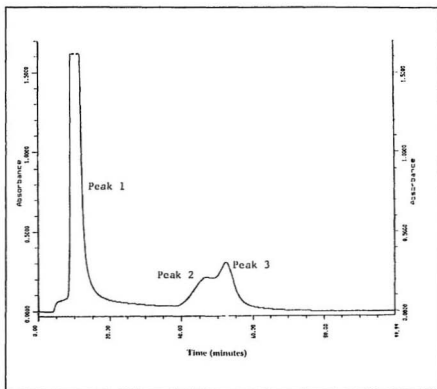


Figure 29. Second quantitative HPLC absorbance profile of ascitic fluid containing 26-61-4 bispecific antibody. Rest of details as for legend to Figure 28.

Table 6. ELISA results from the first 5 ml HPLC separation of the 26-6-4 bispecific hybridoma.

| FRACTION # | 26-61-4 1st 5 ml Separation | | | |
|------------|--------------------------------|------------|-----------------|----------------|
| | ANTI-CEA* | ANTI-DOX** | ANTI-DOX-BSA*** | DUAL ASSAY**** |
| 36 | 0.966 | 0.203 | 0.205 | 0.048 |
| 37 | 1.009 | 0.174 | 0.204 | 0.047 |
| 38 | 1.072 | 0.235 | 0.201 | 0.049 |
| 39 | 1.090 | 0.418 | 0.218 | 0.058 |
| 40 | 0.971 | 0.800 | 0.356 | 0.048 |
| 41 | 1.035 | 1.099 | 0.375 | 0.047 |
| 42 | 0.984 | 1.389 | 0.666 | 0.063 |
| 43 | 0.936 | 1.586 | 0.761 | 0.061 |
| 44 | 0.991 | 1.609 | 0.603 | 0.049 |
| 45 | 1.032 | 1.550 | 0.659 | 0.050 |
| 46 | 1.010 | 1.395 | 0.755 | 0.052 |
| 47 | 0.989 | 1.350 | 0.685 | 0.045 |
| 48 | 0.959 | 1.060 | 0.661 | 0.058 |
| 49 | 0.686 | 1.187 | 0.623 | 0.052 |
| 50 | 1.005 | 1.139 | 0.877 | 0.062 |
| 51 | 1.026 | 1.210 | 0.790 | 0.064 |
| 52 | 1.014 | 1.127 | 0.526 | 0.053 |
| 53 | 1.090 | 1.098 | 0.757 | 0.074 |
| 54 | 1.120 | 1.005 | 0.440 | 0.059 |

Control Mean + 2 S.D.: * 0.1582 ** 0.0578 *** 0.1538 **** 0.0760

Table 7. ELISA results from the second 5 ml HPLC separation of the 26-61-4 bispecific hybridoma.

| FRACTION # | 26-61-4 2nd 5 ml Separation | | | |
|------------|--------------------------------|------------|-----------------|----------------|
| | ANTI-CEA* | ANTI-DOX** | ANTI-DOX-BSA*** | DUAL ASSAY**** |
| 40 | 1.092 | 0.181 | 0.575 | 0.067 |
| 41 | 1.087 | 0.156 | 0.410 | 0.057 |
| 42 | 1.135 | 0.191 | 0.728 | 0.060 |
| 43 | 0.987 | 0.319 | 0.827 | 0.049 |
| 44 | 1.076 | 0.518 | 0.991 | 0.052 |
| 45 | 1.138 | 0.641 | 0.886 | 0.052 |
| 46 | 1.194 | 0.926 | 1.178 | - |
| 47 | 1.091 | 0.957 | 1.204 | 0.050 |
| 48 | 1.085 | 1.113 | 1.090 | 0.047 |
| 49 | 1.190 | 1.277 | 0.996 | 0.061 |
| 50 | 1.110 | 1.180 | 0.972 | 0.063 |
| 51 | 1.185 | 1.295 | 1.129 | 0.065 |
| 52 | 1.152 | 0.810 | 1.062 | 0.062 |
| 53 | 1.212 | 1.196 | 1.207 | 0.059 |
| 54 | 1.189 | 1.132 | 1.201 | 0.054 |
| 55 | 1.013 | 1.263 | 1.127 | 0.059 |
| 56 | 1.021 | 0.843 | 1.042 | 0.048 |

Control Mean + 2 S.D.: * 0.1582 ** 0.0978 *** 0.1538 **** 0.0760

for both samples but the intensity for each sample was not the same.

The dual assay results (Tables 6 & 7) for these samples were also minimal where none of the wells gave absorbance readings greater than the control mean \pm 2 standard deviations.

III 7.3 26-61-10 5 ml HPLC Separation

III 7.3(a) HPLC Profiles

There were three consecutive 5 ml HPLC separations for this hybridoma. The profiles (Figures 30, 31 & 32) were not exactly the same. The first separation (Figure 30) gave a second peak region which eluted between 53 and 64 minutes whereas the second (Figure 31) and third (Figure 32) separations gave a second peak region between 35 and 55 minutes.

III 7.3(b) ELISA Results

Although the separation profile of sample 1 (Figure 30) was different from the other samples, the assay results revealed the same general pattern of high activity in the middle fractions (Table 8). The dual activity assay gave a peak activity region which corresponded with peak regions of the anti-CEA, anti-DOX and anti-DOX-BSA assay results. The mean control absorbance value for the dual assay in this case was 0.0830. The mean + 2 standard deviations was 0.0948. Fractions 55 through to 63 remained above this value.

Only the anti-CEA assay was completed for the second separation sample. This

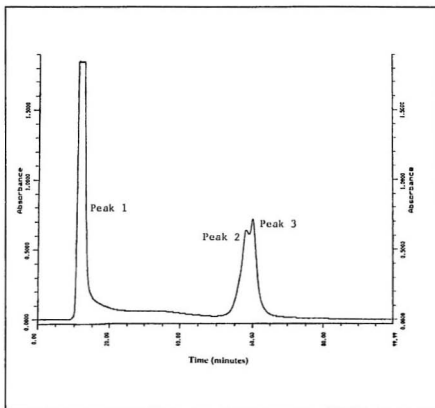


Figure 30. First quantitative HPLC absorbance profile of ascitic fluid containing 26-61-10 bispecific antibody. A 5 ml sample was loaded and eluted from a hydroxylapatite column using a 60 mM to 360 mM phosphate buffer gradient. Fractions were collected at 1 minute intervals and absorbance read at 280 nm.

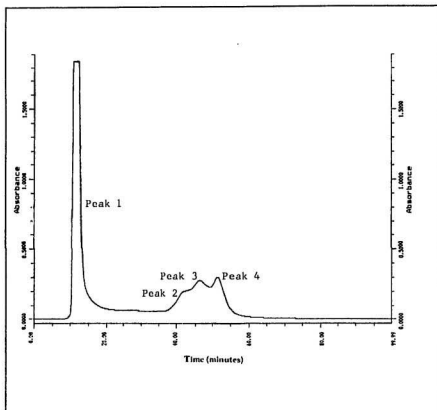


Figure 31. Second quantitative HPLC absorbance profile of ascitic fluid containing 26-61-10 bispecific antibody. Details as for legend to Figure 30.

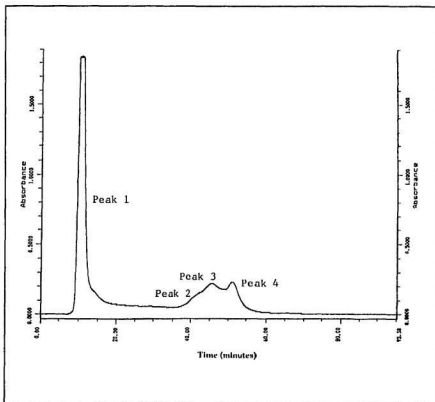


Figure 32. Third quantitative HPLC absorbance profile of ascitic fluid containing 26-61-10 bispecific antibody. Details as for legend to Figure 30.

Table 8. ELISA results from the first 5 ml HPLC separation of the 26-61-10 bispecific hybridoma.

| FRACTION # | 26-61-10 1st SAMPLE | | | |
|------------|------------------------|------------|---------------------|-------------------|
| | ANTI-CEA* | ANTI-DOX** | ANTI-DOX- BSA*** | DUAL ASSAY**** |
| 53 | 1.185 | 0.221 | 0.187 | 0.098 |
| 54 | 1.087 | 0.138 | 0.207 | 0.095 |
| 55 | 1.105 | 0.223 | 0.271 | 0.101 |
| 56 | 1.109 | 0.306 | 0.406 | 0.111 |
| 57 | 1.067 | 0.383 | 0.680 | 0.115 |
| 58 | 1.168 | 1.031 | 0.837 | 0.110 |
| 59 | 1.336 | 0.588 | 0.912 | 0.147 |
| 60 | 1.315 | 0.968 | 0.853 | 0.101 |
| 61 | 1.266 | 1.495 | 0.942 | 0.101 |
| 62 | 1.051 | 0.306 | 0.538 | 0.092 |
| 63 | 1.113 | 0.307 | 0.468 | 0.098 |
| 64 | 1.296 | 0.187 | 0.434 | 0.090 |

Control Mean + 2 S.D.: * 0.1910 ** 0.1155 *** 0.0916 **** 0.0948

was done in an attempt to compare the second sample fractions to the third. Once a similarity was shown, the remaining tests would only need to be completed on one of the samples as opposed to both. This was a safeguard that would allow more of the fraction volumes to be retained and would result in a larger volume of the final pooled product. The other tests were therefore undertaken with the third sample. The anti-CEA results were very similar for samples 2 and 3 (Table 9). The anti-DOX and anti-DOX-BSA assay results indicated gradual peak areas in the middle fractions. This was very similar to the dual assay results (Table 9) which gave increasing absorbance values at fraction 43 and peaked at fraction number 49 (Figure 33). The mean absorbance value for the controls in this dual activity test was 0.0648. The control mean \pm 2 standard deviations was 0.0734. Fractions 40 to 56 were higher than this value.

Dual activity testing was also completed for the first sample. Fractions 53 to 64 were tested (Figure 33) where fraction number 59 gave the highest dual activity. Several fractions were significantly higher than the control mean \pm 2 standard deviations (0.0948).

III 7.4 57-11-25-17 5 ml HPLC Separation

III 7.4(a) HPLC Profiles

There were slight variations in the three HPLC profiles of the 57-11-25-17 (Figures 34, 35 & 36). All three had a second peak region which contained two distinct peaks. The second peak region of the first separation (Figure 34) started eluting at approximately 37 minutes into the procedure and finished near 58 minutes. The second

Table 9. ELISA results from the second and third 5 ml HPLC separations of the 26-61-10 bispecific hybridoma

| FRACTION # | 26-61-10 2nd SAMPLE | 26-61-10 3rd SAMPLE | | | |
|------------|---------------------|---------------------|------------|-----------------|-------------|
| | ANTI-CEA* | ANTI-CEA* | ANTI-DOX** | ANTI-DOX-BSA*** | DUAL ASSAY@ |
| 35 | 0.921 | 1.017 | 0.119 | 0.279 | 0.074 |
| 36 | 0.934 | 1.021 | 0.141 | 0.285 | 0.074 |
| 37 | 0.975 | 1.005 | 0.150 | 0.428 | 0.073 |
| 38 | 0.986 | 0.932 | 0.157 | 0.490 | 0.071 |
| 39 | 1.004 | 0.986 | 0.215 | 0.319 | 0.072 |
| 40 | 0.984 | 1.015 | 0.160 | 0.230 | 0.080 |
| 41 | 1.033 | 1.003 | 0.176 | 0.588 | 0.077 |
| 42 | 0.886 | 0.964 | 0.211 | 0.403 | 0.083 |
| 43 | 0.970 | 0.853 | 0.248 | 0.379 | 0.111 |
| 44 | 0.985 | 0.868 | 0.289 | 0.398 | 0.125 |
| 45 | 1.070 | 0.831 | 0.328 | 0.698 | 0.123 |
| 46 | 1.213 | 0.852 | 0.437 | 0.469 | 0.136 |
| 47 | 1.116 | 0.864 | 0.440 | 0.556 | 0.154 |
| 48 | 1.064 | 1.045 | 0.328 | 0.641 | 0.183 |
| 49 | 0.935 | 1.064 | 0.323 | 0.438 | 0.396 |
| 50 | 1.070 | 1.044 | 0.310 | 0.433 | 0.163 |
| 51 | 1.091 | 0.876 | 0.273 | 0.370 | 0.179 |
| 52 | 1.196 | 1.055 | 0.259 | 0.389 | 0.176 |
| 53 | 1.059 | 0.887 | 0.255 | 0.467 | 0.157 |
| 54 | 1.151 | 1.010 | 0.223 | 0.350 | 0.163 |
| 55 | 1.043 | 0.939 | 0.176 | 0.344 | 0.174 |
| 56 | 1.046 | 1.021 | 0.144 | 0.362 | 0.123 |

Control Mean + 2 S.D.: *0.1910 ** 0.1155 *** 0.0916 @ 0.0734

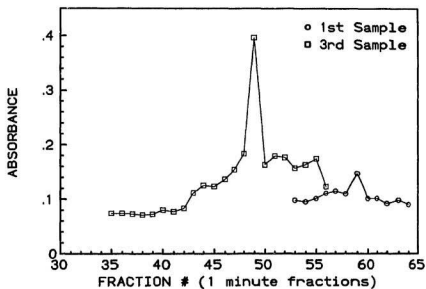


Figure 33. Results from dual assays of 26-61-10 bispecific hybridoma. Testing was completed on all 95 fraction of samples 1 and 3. Only the second peak region gave positive results. The absorbance was read at 405 nm.

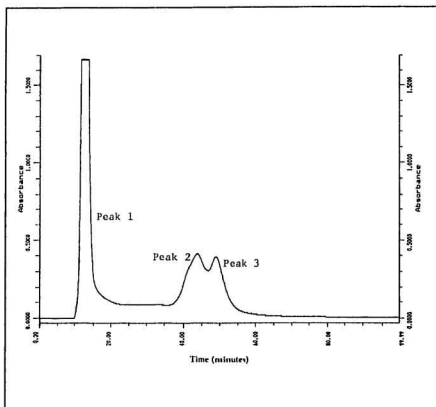


Figure 34. First quantitative HPLC absorbance profile of ascitic fluid containing 57-11-25-17 bispecific antibody. A 5 ml sample was loaded and eluted from a hydroxylapatite column using a 60 mM to 360 mM phosphate buffer gradient. Fractions were collected at 1 minute intervals and absorbance read at 280 nm.

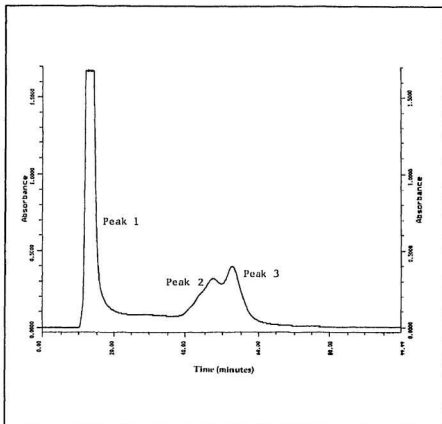


Figure 35. Second quantitative HPLC absorbance profile of ascitic fluid containing 57-11-25-17 bispecific antibody. A 5 ml sample was loaded and eluted from a hydroxylapatite column using a 60 mM to 360 mM phosphate buffer gradient. Fractions were collected at 1 minute intervals and absorbance read at 280 nm.

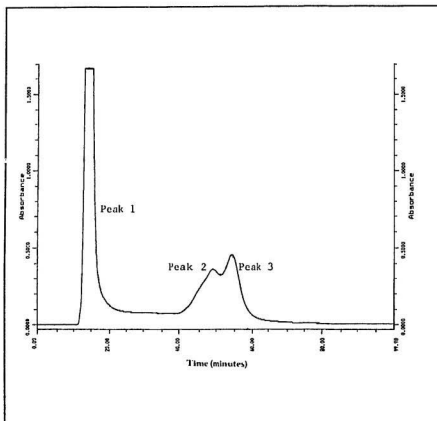


Figure 36. Third quantitative HPLC absorbance profile of ascitic fluid containing 57-11-25-17 bispecific antibody. A 5 ml sample was loaded and eluted from a hydroxylapatite column using a 60 mM to 360 mM phosphate buffer gradient. Fractions were collected at 1 minute intervals and absorbance read at 280 nm.

and third separations (Figures 35 & 36) showed the second peak region eluting between 40 and 62 minutes. Both of these separations were very similar in appearance. However, the first separation was slightly different in appearance where the first peak of the second peak region was slightly larger.

III 7.4(b) ELISA Results

The anti-CEA results for all three separations gave a very similar pattern to the 1 ml separation results for that particular peak region. The anti-DOX and anti-DOX-BSA results for each of the separations (Tables 10, 11 & 12) were very similar in intensity and revealed the general pattern of peaking within the middle fractions. The dual assay results revealed that there were some wells in each of the separations that had absorbance readings significantly above the control. The control mean \pm 2 standard deviations was 0.0661. However, there were not many results above this value. Those that were greater were not as significant as the results with the 26-61-10 hybridoma (Tables 8 & 9). Fraction #38 (Table 10) gave a very high volume. However, since this was the only fraction whose volume was considered to be insufficient to permit concentration and then subsequent testing in the microcytostasis assay.

III 8.0 IN VITRO MICROCYTOSTASIS ASSAYS

III 8.1 Mean IC_{50} values

A high CEA expressing colonic cancer cell line (SKCO1) and a low CEA expressing colonic cancer cell line (COLO 320 DM) were tested for their response to

Table 10. ELISA results from the first 5 ml HPLC separation of the 57-11-25-17 bispecific hybridoma.

| FRACTION # | 57-11-25-17 1st 5 ml Separation | | | |
|------------|------------------------------------|------------|-----------------|----------------|
| | ANTI-CEA* | ANTI-DOX** | ANTI-DOX-BSA*** | DUAL ASSAY**** |
| 37 | 0.976 | 0.126 | 0.449 | 0.060 |
| 38 | 0.877 | 0.179 | 0.454 | 0.069 |
| 39 | 1.039 | 0.176 | 0.590 | 0.062 |
| 40 | 0.959 | 0.272 | 0.574 | 0.060 |
| 41 | 0.870 | 0.286 | 0.615 | 0.062 |
| 42 | 0.845 | 0.513 | 0.933 | 0.059 |
| 43 | 0.898 | 0.681 | 0.933 | 0.066 |
| 44 | 0.733 | 0.567 | 0.876 | 0.059 |
| 45 | 1.009 | 0.517 | 0.885 | 0.067 |
| 46 | 0.985 | 0.429 | 0.791 | 0.055 |
| 47 | 1.111 | 0.426 | 0.777 | 0.054 |
| 48 | 1.170 | 0.423 | 0.889 | 0.060 |
| 49 | 1.075 | 0.479 | 0.902 | 0.057 |
| 50 | 1.019 | 0.982 | 0.702 | 0.057 |
| 51 | 1.092 | 0.380 | 0.956 | 0.055 |
| 52 | 1.062 | 0.396 | 1.150 | 0.057 |
| 53 | 0.982 | 0.330 | 0.863 | 0.047 |
| 54 | 1.107 | 0.347 | 0.990 | 0.052 |

Control Mean + 2S.D.: * 0.1454 ** 0.0635 *** 0.0728 **** 0.0661

Table 11. ELISA results from the second 5 ml HPLC separation of the 57-11-25-17 bispecific hybridoma.

| FRACTION # | 57-11-25-17 2nd 5 ml Separation | | | |
|------------|------------------------------------|------------|-----------------|----------------|
| | ANTI-CEA* | ANTI-DOX** | ANTI-DOX-BSA*** | DUAL ASSAY**** |
| 40 | 1.016 | 0.153 | 0.444 | 0.067 |
| 41 | 0.921 | 0.195 | 0.508 | 0.075 |
| 42 | 0.823 | 0.216 | 0.779 | 0.063 |
| 43 | 0.916 | 0.330 | 1.030 | 0.089 |
| 44 | 0.908 | 0.387 | 0.970 | 0.064 |
| 45 | 0.906 | 0.386 | 0.979 | 0.077 |
| 46 | 0.894 | 0.485 | 1.216 | - |
| 47 | 0.984 | 0.567 | 1.044 | - |
| 48 | 1.005 | 0.615 | 1.056 | 0.060 |
| 49 | 0.897 | 0.483 | 1.192 | 0.062 |
| 50 | 1.002 | 0.672 | 1.158 | 0.064 |
| 51 | 1.058 | 0.544 | 0.984 | 0.058 |
| 52 | 0.933 | 0.536 | 1.146 | 0.056 |
| 53 | 0.901 | 0.701 | 0.844 | 0.058 |
| 54 | 0.878 | 0.722 | 0.943 | 0.060 |
| 55 | 1.105 | 0.659 | 1.018 | 0.065 |
| 56 | 1.140 | 0.797 | 1.154 | 0.056 |
| 57 | 0.941 | 0.594 | 1.148 | 0.056 |
| 58 | 0.936 | 0.379 | 0.842 | 0.072 |
| 59 | 0.947 | 0.368 | 0.764 | 0.065 |

Control Mean + 2 S.D.: * 0.1319 ** 0.1155 *** 0.0916 **** 0.0661

Table 12. ELISA results from the third 5 ml HPLC separation of the 57-11-25-17 bispecific hybridoma.

| FRACTION # | 57-11-25-17 3rd 5 ML Separation | | | |
|------------|------------------------------------|------------|-----------------|----------------|
| | ANTI-CEA* | ANTI-DOX** | ANTI-DOX-BSA*** | DUAL ASSAY**** |
| 40 | 1.084 | 0.262 | 0.333 | 0.056 |
| 41 | 1.115 | 0.130 | 0.378 | 0.053 |
| 42 | 1.073 | 0.143 | 0.525 | 0.054 |
| 43 | 0.790 | 0.366 | 0.722 | 0.064 |
| 44 | 1.117 | 0.158 | 0.714 | 0.056 |
| 45 | 1.092 | 0.198 | 0.872 | 0.064 |
| 46 | 1.044 | 0.276 | 0.901 | 0.057 |
| 47 | 1.155 | 0.250 | 1.065 | 0.067 |
| 48 | 0.917 | 0.409 | 0.969 | 0.078 |
| 49 | 0.813 | 0.562 | 1.006 | 0.060 |
| 50 | 1.082 | 0.686 | 1.192 | 0.063 |
| 51 | 1.151 | 0.539 | 1.131 | 0.057 |
| 52 | 1.120 | 0.544 | 0.978 | 0.060 |
| 53 | 1.119 | 0.465 | 0.940 | 0.058 |
| 54 | 0.566 | 0.511 | 0.914 | 0.064 |
| 55 | 0.918 | 0.623 | 0.930 | 0.058 |
| 56 | 1.190 | 0.591 | 1.008 | - |
| 57 | 1.141 | 0.668 | 0.951 | 0.057 |
| 58 | 1.024 | 0.464 | 0.861 | 0.057 |
| 59 | 0.669 | 0.588 | 0.545 | 0.055 |

Control Mean + 2 S.D.: * 0.1454 ** 0.0847 *** 0.0728 **** 0.0661

doxorubicin. The drug alone was added to the cells in log dilutions. The toxicity was determined by comparing the absorbance in test wells to that of the control wells. This permitted calculation of a % cell survival value for each dilution of drug. These values were then placed on a sigmoid curve to determine which dilution gave 50 % inhibition of cell growth, or the IC_{50} value. The cytotoxicity assay with DOX alone was performed at the same time as the testing with BSMab and DOX.

An IC_{50} value of DOX for the SKC01 cell line had been determined in this laboratory previously (Ford et al, 1989). The mean IC_{50} value was reported as being 27 ng/ml. The value calculated in my experiments was 28.5 ng/ml with a standard deviation of 3.14 (Table 13). However, the value for the COLO 320 DM cell line had not been determined prior to this project. It was determined as being 1163 ng/ml with a standard deviation of 167.9 (Table 13).

III 8.2 In Vitro Antibody Testing Using the SKC01 Colorectal Cancer Cell Line

III 8.2(a) Bispecific Monoclonal Antibody 26-61-10

These tests were completed in triplicate in order to verify the effect of the test antibody. Figure 37 illustrates a decrease in survival of the cells with increasing concentration of drug. It also shows a distinct difference in percent survival between the drug alone and the test values using the log dilutions of antibody and drug. The test values were clustered together and did not show any differences in percent survival when comparing test results for each antibody dilution. However, the IC_{50} values

Table 13. Summary of IC₅₀ Results

| Cell line | Mean IC ₅₀ DOX (ng/ml)* | Mean IC ₅₀ 26-61-10+DOX (ng/ml)** | | | | Mean IC ₅₀ Ag8+DOX (ng/ml)** | | | |
|--------------------|------------------------------------|--|-----------|------------|-----------|---|--------------|--------------|----------|
| | | Antibody Concentration (µg/ml) | | | | Antibody Concentration (µg/ml) | | | |
| | | 10 | 1 | 0.1 | 0.01 | 10 | 1 | 0.1 | 0.01 |
| COLO 320 DM | 1163 +/- | 1533 +/- | 880 +/- | 1147 +/- | 984.7 +/- | 1721 | 1054.7 | 1283.7 | 1204.6 |
| | 167.9 | 254 | 410.5 | 639.4 | 3.14 | +/- 618 | +/- 239.9 | +/- 712.5 | +/- 63.9 |
| SKC01 | 28.5 +/- | 14.0 +/- | 14.0 +/- | 12.0 +/- | 12.7 +/- | 31.3 | 24.7 | 39.0 | 31 +/- |
| | 3.14 | 9.6 | 5.3 | 1.81 | 3.17 | +/- | +/- | +/- | 7.21 |
| | | (p<0.05)* | (p<0.01)* | (p<0.001)* | (p<0.01)* | 9.5 | 4.9 | 19.7 | |

* based on 6 tests

** based on 3 tests

+ Significantly different to IC₅₀ for DOX by student T test

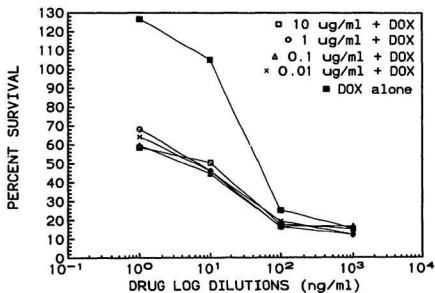


Figure 37. Result from MTT assay using purified BSMab 26-61-10 and DOX with the SKC01 colorectal cell line. The BSMab was added in log dilutions from 10 to 0.01 $\mu\text{g/ml}$. DOX was added with the bispecific and alone (control) in log dilutions from 1000 to 1 ng/ml .

(Table 13 & 14) indicate that all of the test results were smaller than the mean IC_{50} value for DOX alone with this cell line.

Figure 38 also illustrates the greater decrease in survival of the SKC01 cells when using the bispecific 26-61-10 and drug dilutions together as opposed to using doxorubicin alone. The IC_{50} values (Table 14) for the antibody dilutions were significantly lower than those for doxorubicin alone.

The third graph for SKC01 using 26-61-10 and doxorubicin (Figure 39) also indicates a percent survival difference between the DOX alone result and antibody + drug tests. The calculated IC_{50} values for these results are shown in Table 14. The mean IC_{50} values for these three assays are summarized in Table 13 and from this it can be seen that BSMab + DOX gave a substantially reduced IC_{50} compared to DOX alone for all BSMab concentrations that were tested.

The % survival values for the antibody alone control wells for each of the figures (Table 15) illustrate that there was little or no effect of the antibody alone on the cells. Most values fluctuated near 100% with no great drop in % survival as was seen with the antibody + drug test values.

III 8.2(b) Ag8 Non-Specific Antibody

All three tests using the non-specific Ag8 antibody and doxorubicin dilutions with the SKC01 cell line (Figures 40, 41 & 42) were very similar. All three figures illustrated the close proximity of the test results to those of the DOX alone control values. In Figure 40, there was one IC_{50} result (0.1 $\mu\text{g/ml}$ Ag8 + DOX) which

Table 14. Calculated IC_{50} values of DOX + 26-61-10 bispecific antibody and DOX + Ag8 non-specific antibody using the SKC01 colorectal cancer cell line.

| BISPECIFIC 26-61-10 ANTIBODY TESTS | CALCULATED IC_{50} | | |
|---|--|------------------|------------------|
| | Figure 37 | Figure 38 | Figure 39 |
| 10 μg/ml | 10.33456 | 7.205612 | 24.64955 |
| 1 μg/ml | 8.113348 | 17.59263 | 15.75564 |
| 0.1 μg/ml | 8.971718 | 15.00386 | 11.6288 |
| 0.01 μg/ml | 7.610129 | 12.86101 | 17.95198 |
| DOX alone | 41.03033 | 23.45008 | 32.42578 |
| NON-SPECIFIC Ag8 ANTIBODY TESTS | Figure 40 | Figure 41 | Figure 42 |
| 10 μg/ml | 41.14354 | 21.68152 | 30.84934 |
| 1 μg/ml | 27.15968 | 18.54718 | 27.55846 |
| 0.1 μg/ml | 60.52837 | 22.94955 | 33.42546 |
| 0.01 μg/ml | 29.26531 | 39.03962 | 25.38895 |
| DOX alone | 38.31267 | 18.72450 | 18.44690 |

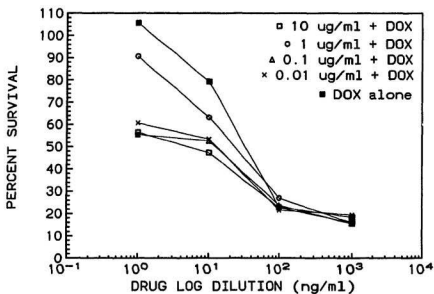


Figure 38. Results from MTT assay using purified BSMab 26-61-10 and DOX with the SKC01 colorectal cancer cell line. The BSMab was added in log dilutions from 10 to 0.01 $\mu\text{g/ml}$. DOX was added with the bispecific and alone (control) in log dilutions from 1000 to 1 ng/ml .

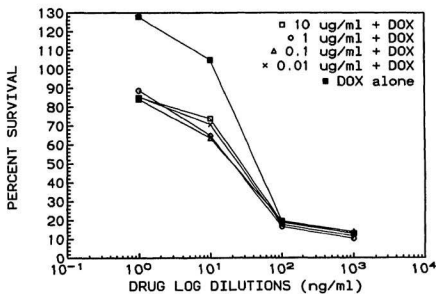


Figure 39. Results from MTT assay using purified BSMab 26-61-10 and DOX with the SKC01 colorectal cancer cell line. The BSMab was added in log dilutions from 10 to 0.01 µg/ml. DOX was added with the bispecific and alone (control) in log dilutions from 1000 to 1 ng/ml.

Table 15. Calculated percent survival of SKC01 when using bispecific antibody (26-61-10) and non-specific antibody (Ag8) controls.

| BISPECIFIC 26-61-10 ANTIBODY CONTROL | % SURVIVAL | | |
|---|-------------------|------------------|------------------|
| | Figure 37 | Figure 38 | Figure 39 |
| 10 µg/ml | 87.1 | 96.7 | 88.9 |
| 1 µg/ml | 89.4 | 117.8 | 102.3 |
| 0.1 µg/ml | 76.4 | 108.6 | 91.9 |
| 0.01 µg/ml | 72.4 | 103.5 | 108.3 |
| NON-SPECIFIC Ag8 ANTIBODY CONTROL | Figure 40 | Figure 41 | Figure 42 |
| 10 µg/ml | 96.6 | 90.4 | 105.4 |
| 1 µg/ml | 93.7 | 88.1 | 91.0 |
| 0.1 µg/ml | 104.1 | 97.5 | 105.9 |
| 0.01 µg/ml | 111.7 | 89.4 | 81.4 |

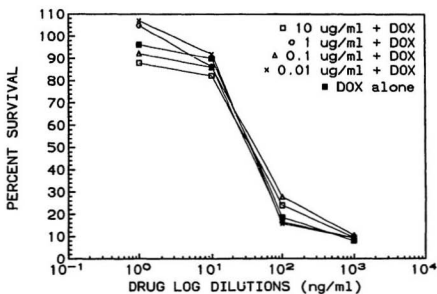


Figure 40. Results from MTT assay using non-specific Mab Ag8 and DOX with the SKC01 colorectal cancer cell line. The non-specific antibody was added in log dilutions from 10 to 0.01 $\mu\text{g/ml}$. DOX was added with Ag8 and alone (control) in log dilutions from 1000 to 1 ng/ml .

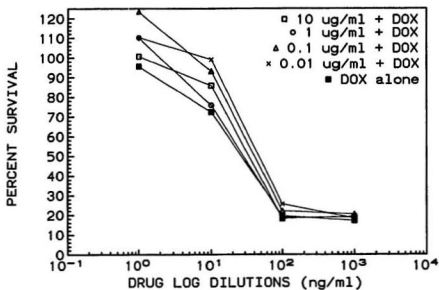


Figure 41. Results from MTT assay using non-specific Ag8 Mab and DOX with the SKC01 colorectal cancer cell line. The non-specific antibody was added in log dilutions from 10 to 0.01 $\mu\text{g/ml}$. DOX was added with Ag8 and alone (control) in log dilutions from 1000 to 1 ng/ml .

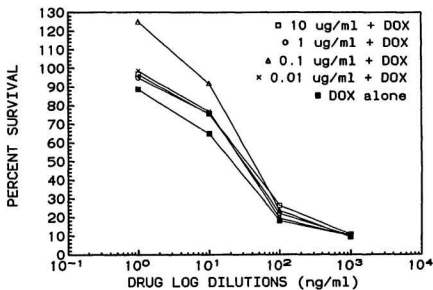


Figure 42. Results from MTT assay using non-specific Mab Ag8 and DOX with the SKC01 colorectal cancer cell line. The non-specific antibody was added in log dilutions from 10 to 0.01 $\mu\text{g/ml}$. DOX was added with Ag8 and alone (control) in log dilutions from 1000 to 1 ng/ml .

substantially exceeded the 100% value for the control wells with medium only. This is shown in Table 14.

Figure 42 shows a greater variation than the results shown in Figures 40 and 41. However, all test IC_{50} values (Tables 13 & 14) were within range of the mean \pm 2 standard deviations of the calculated doxorubicin IC_{50} for that cell line.

The % survival for the Ag8 antibody controls in all three of these tests (Table 15) were close to the 100% value. As with the antibody alone controls for the 26-61-10 bispecific Mab, these values did not show any great decrease in % survival in contrast to those seen with the test BSMab + DOX samples.

III 8.3 *In Vitro* Antibody Testing Using COLO 320 DM Colorectal Cancer Cell Line

III 8.3(a) Bispecific Monoclonal Antibody 26-61-10

The *in vitro* results when using the bispecific Mab 26-61-10 + DOX with the low CEA expressing cell line, COLO 320 DM, were consistent. All of the test values fluctuated around the DOX control result (Figures 43, 44 & 45) showing no significant drop in the IC_{50} values. Most of the calculated IC_{50} values for this series of tests (Table 16) were within the mean \pm 2 standard deviations range of the calculated IC_{50} values for COLO 320 DM with DOX alone (table 13). The only values lower than this were the 1, 0.1 and 0.01 μ g/ml 26-61-10 bispecific antibody + DOX samples in Figure 45.

The percent survival of the antibody controls using the bispecific antibody alone (Table 16) revealed variations in survival which were very similar to those seen with

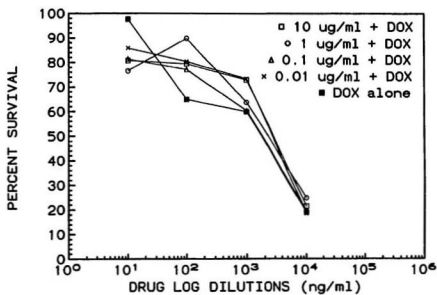


Figure 43. Results from MTT assay using purified BSMab 26-61-10 and DOX with the COLO 320 DM colorectal cancer cell line. The BSMab was added in log dilutions from 10 to 0.01 $\mu\text{g/ml}$. DOX was added with the BSMab and alone (control) in log dilutions from 10000 to 10 ng/ml .

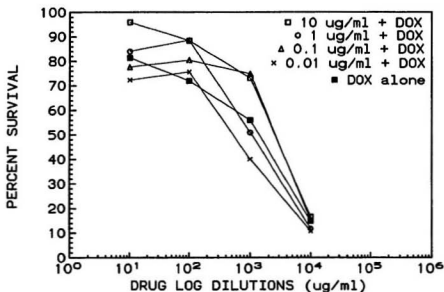


Figure 44. Results from MTT assay using purified BSMab 26-61-10 and DOX with the COLO 320 DM colorectal cancer cell line. The BSMab was added in log dilutions from 10 to 0.01 $\mu\text{g/ml}$. DOX was added with the BSMab and alone (control) in log dilutions from 10000 to 10 ng/ml .

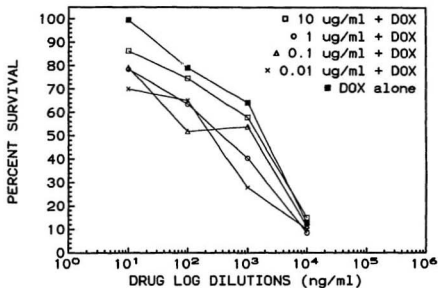


Figure 45. Results from MTT assay using purified BSMab 26-61-10 and DOX with the COLO 320 DM colorectal cancer cell line. The BSMab was added in log dilutions from 10 to 0.01 $\mu\text{g/ml}$. DOX was added with the BSMab and alone (control) in log dilutions from 10000 to 10 ng/ml .

Table 16. Calculated IC_{50} values of DOX + 26-61-10 bispecific antibody and DOX + Ag8 non-specific antibody using COLO 320 DM colorectal cancer cell line.

| BISPECIFIC 26-61-10 ANTIBODY TESTS | CALCULATED IC_{50} | | |
|---|--|------------------|------------------|
| | Figure 43 | Figure 44 | Figure 45 |
| 10 $\mu\text{g/ml}$ | 1605.644 | 1743.164 | 1249.983 |
| 1 $\mu\text{g/ml}$ | 1212.356 | 1006.606 | 421.4929 |
| 0.1 $\mu\text{g/ml}$ | 1395.70 | 1624.738 | 420.8688 |
| 0.01 $\mu\text{g/ml}$ | 1701.714 | 912.1766 | 340.2694 |
| DOX alone | 872.8328 | 1202.872 | 1364.761 |
| NON-SPECIFIC Ag8 ANTIBODY TESTS | Figure 46 | Figure 47 | Figure 48 |
| 10 $\mu\text{g/ml}$ | 2422.376 | 1782.880 | 1256.173 |
| 1 $\mu\text{g/ml}$ | 1122.603 | 1484.328 | 788.4096 |
| 0.1 $\mu\text{g/ml}$ | 2065.267 | 1253.187 | 1116.004 |
| 0.01 $\mu\text{g/ml}$ | 1137.350 | 689.7904 | 1213.305 |
| DOX alone | 1103.412 | 1263.868 | 1175.075 |

the BSMab + DOX values.

III 8.3(b) Ag8 Non-Specific Antibody

As with the bispecific Mab 26-61-10, the results for the Ag8 non-specific antibody + DOX dilutions were very similar to that of the DOX control for each set of tests (Figures 46, 47 & 48). Many of the Ag8 + DOX tests had IC_{50} values that were considerably higher than the mean IC_{50} values for DOX with the COLO 320 DM cell line (Table 13). When the mean IC_{50} for Ag8 + DOX for all 3 assays were compared to the mean IC_{50} for DOX for this cell line, none of the differences reach statistical significance. The % survival of the Ag8 antibody alone controls for these tests fluctuated between 68 to 100% (Table 17).

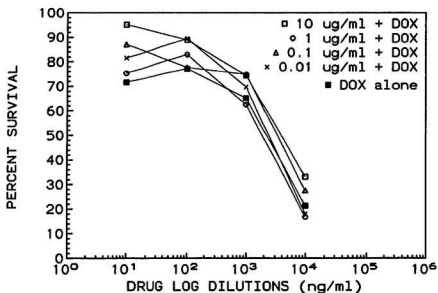


Figure 46. Results from MTT assay using non-specific MabAg8 and DOX with the COLO 320 DM colorectal cancer cell line. The non-specific antibody was added in log dilutions from 10 to 0.01 $\mu\text{g/ml}$. DOX was added with Ag8 and alone (control) in log dilutions from 10000 to 10 ng/ml .

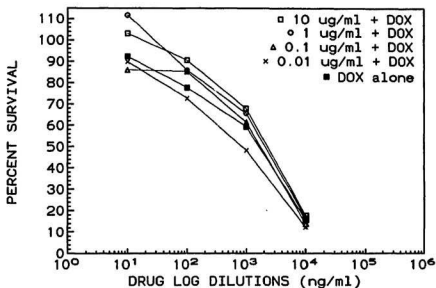


Figure 47. Results from MTT assay using non-specific Mab Ag8 and DOX with the COLO 320 DM colorectal cancer cell line. The non-specific antibody was added in log dilutions from 10 to 0.01 $\mu\text{g/ml}$. DOX was added with Ag8 and alone (control) in log dilutions from 10000 to 10 ng/ml .

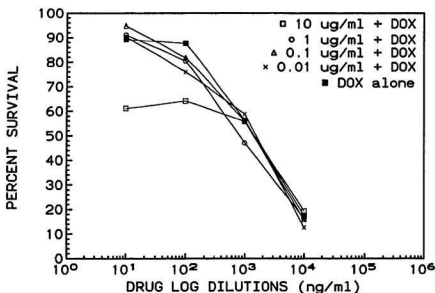


Figure 48. Results from MTT assay using non-specific Mab Ag8 and DOX with the COLO 320 DM colorectal cancer cell line. The non-specific antibody was added in log dilutions from 10 to 0.01 µg/ml. DOX was added with Ag8 and alone (control) in log dilutions from 10000 to 10 ng/ml.

Table 17. Calculated percent survival of COLO 320 DM when using bispecific antibody (26-61-10) and non-specific antibody (Ag8).

| BISPECIFIC 26-61-10 ANTIBODY CONTROL | % SURVIVAL | | |
|---|-------------------|------------------|------------------|
| | Figure 43 | Figure 44 | Figure 45 |
| 10 µg/ml | 91.2 | 88.1 | 79.5 |
| 1 µg/ml | 105.1 | 80.6 | 64.0 |
| 0.1 µg/ml | 102.0 | 83.0 | 87.8 |
| 0.01 µg/ml | 90.2 | 90.9 | 64.4 |
| NON-SPECIFIC Ag8 ANTIBODY CONTROL | Figure 46 | Figure 47 | Figure 48 |
| 10 µg/ml | 90.0 | 98.2 | 97.6 |
| 1 µg/ml | 81.8 | 100.1 | 100.5 |
| 0.1 µg/ml | 72.5 | 68.3 | 97.8 |
| 0.01 µg/ml | 76.9 | 77.5 | 88.1 |

CHAPTER IV

DISCUSSION

IV 1.0 ASCITIC FLUID PRODUCTION AND PROTEIN A COLUMN PURIFICATION

The initial requirement for this study was to produce more of the secreted product of the seven bispecific hybridomas than could be obtained from tissue culture alone. To do this, Balb/C mice were inoculated intraperitoneally with the hybridomas. In the experiments described in this thesis, 104 mice were inoculated and for each hybridoma approximately 50 - 100 ml of pooled ascitic fluid was obtained. However, after initial purification by protein A affinity chromatography the volume of recovered semi-purified ascitic fluid was greatly reduced (range 5 - 20.5 ml). For six of the hybridomas the protein concentration, as assessed by absorbance at 280 nm, was reasonably consistent (range 1.78 - 2.52 mg/ml). However, the yield from hybrid 26-7-35 was very low at 0.545 mg/ml. There is no explanation for this apparent discrepancy other than that this particular hybrid has a low rate of secretion of antibody or that a particular antibody subclass was not binding to protein A. However, if there was a subclass not binding, then it did not include the BSMab as all the unbound material and column washings were tested for dual activity and were found to be negative. Much of the protein A-purified material for the seven BSMabs was used for the initial standardisation procedures, resulting in a shortage of purified product for HPLC

preparative purification and *in vitro* microcytostasis assays.

Protein A purification is noted as being a much better affinity purifying procedure for murine IgG_{2a}, IgG_{2b} and IgG₃ but to be not so good for IgG₁ purification (Hermanson et al, 1992). The parental 11-285-14 anti-CEA used in the Oncology Research lab is an IgG₁ and has usually been purified in this manner with efficient recovery of product. Many groups have used protein A purification as a primary bispecific antibody purification procedure (Morelli et al, 1994; Dietsch et al, 1993; Duke-Cohan et al, 1993). However, all cases involved the use of at least one IgG_{2a} parental monoclonal antibody. Protein G is suggested for use with antibodies that do not bind well to protein A (Hermanson et al, 1992) and has been used in such cases (Xiang et al, 1992). The decision to use protein A rather than protein G in the results presented in this thesis was based on the fact that although protein G has been used by a number of investigators, it is not recommended for the purification of mouse IgG as its removal from protein G can be difficult (Andrew et al, 1994). Also, the parental 11-285-14 monoclonal antibody is an IgG₁ and preliminary attempts to purify it with protein G has resulted in a very low recovery of anti-CEA antibody, presumably due to the problem of not being able to completely disrupt its binding to the protein G (Ford, unpublished observations). Hence it seemed more appropriate to use protein A for the initial purification of the BSMabs.

The only other option that could improve the initial purification step is having a purification column which was labelled with CEA. This would directly purify any antibody with anti-CEA activity, including the BSMabs. Antibodies without the anti-

CEA activity would be washed out of the column leaving fewer subclasses to analyze with the HPLC method. However, in the absence of such a column, the protein A column would be the more logical choice.

IV 2.0 PRODUCTION OF CONJUGATES

IV 2.1 DOX-BSA Conjugate Formation

Three separate DOX-BSA conjugations were done. The first two (Figures 2 & 3) were very similar in their ratio of protein to drug. The conjugated product, being of larger mass, eluted out from the Sephadex column at the first peak. The second peak consisted of non-conjugated drug. In the third conjugation (Figure 4) there was more drug per protein molecule, as can be seen from the high reading at 495 nm. The only difference in the procedure between the first two conjugations and the last was the first two procedures were done over two days. It involved an overnight storage period at 4°C while the last procedure was completed within one day. This may be a possible explanation for the difference seen between the first two conjugations and the third.

IV 2.2 CEA-HRP Conjugate Formation

With the CEA-HRP conjugation (Figure 5) the entire 403 nm peak was seen within the 280 nm peak indicating that all the HRP was associated with CEA. The eluted material was proven to be CEA-HRP by testing it against the parental anti-CEA antibody (Figure 6). The CEA-HRP conjugate only bound to the coating parental antibody but not to the plate coated with control Ag8 antibody. The 1/40 (5%) dilution

gave a high absorbance reading and yet was sufficiently dilute to conserve the reagent, and this was chosen as the dilution to be used in the dual activity assays.

IV 3.0 STANDARDIZATION OF THE HPLC PROCEDURE

IV 3.1 Purification of 11-285-14

In order to obtain experience with HPLC analysis, the parental anti-CEA monoclonal antibody was the first antibody purified on the HPLC column. This was done using a 60 mM to 180 mM phosphate buffer gradient and was found to give one anti-CEA peak, as expected. The profile (Figure 7) illustrated two peaks. However, anti-CEA ELISA testing (Figure 8) demonstrated that this first peak was an injection peak. This was an artifact of all HPLC purifications and did not contain any protein. This experiment also indicated, approximately, where one could expect the parental antibody to elute when the BSMabs were being purified.

IV 3.2 Purification of BSMab 26-61-2

The formation of a hybridoma x spleen cell or hybrid x hybridoma bispecific antibody results in the formation of multiple antibodies (Figure 1). Not only does one get the parental antibodies but also hybrid molecules with the binding characteristics of each of the fusion partners (Suresh et al, 1986). Because of this one would expect to find various numbers of immunoglobulin chain combinations per hybridoma. This should then correspond to various elution peaks within a HPLC purification profile.

26-61-2 gave six peaks (Figure 9(a)) but again the first was shown to be

insignificant (Figure 9(b)). The elution time of the sixth peak (Figure 9(a)) corresponded to that of the elution time of the parental anti-CEA monoclonal antibody (Figure 7). The ELISA results in Figure 9(b) also indicated that the two peaks were the same antibody. Likewise, the second peak gave high anti-DOX and anti-DOX-BSA activity. This indicated that this peak probably contained the anti-DOX parental antibody. Peaks four and five gave high anti-CEA activity and relatively high anti-DOX and anti-DOX-BSA activity (Figure 9(b)) indicating potential bispecific activity in these peaks. On the basis of these ELISA results, this peak region was chosen for the study of the other BSMabs.

IV 4.0 ANALYTICAL HPLC PURIFICATIONS OF BISPECIFIC ANTIBODIES (1 ml INJECTIONS)

The products of seven hybridomas were initially analysed using 1 ml injections. As expected from the possible combinations of heavy and light chain immunoglobulin genes (Suresh et al, 1986) all seven hybridomas secreted products with different elution profiles. Various shaped absorbance peaks and different peak locations indicated that each sample had slightly different combinations and different amounts of various combinations of gene products. Hydroxylapatite HPLC separates macromolecules by differential surface binding to phosphate or calcium sites on the matrix. The differential surface binding is ionic, but also has specific affinity properties, and it is these properties that have made it a common method for the separation of proteins, especially enzymes and antibodies. This is why it was selected for use in this thesis.

The ELISA results also varied with each hybridoma. In all cases, the anti-CEA activity started within the first ten fractions. It then gradually increased and peaked around the region of the last absorbance peak for that hybridoma. The activity then decreased rapidly. This indicates that the anti-CEA parental combination was located in the last peak and that earlier peaks probably contained antibody with only one anti-CEA combining site. The increasing ELISA activity indicated that the amount of this monovalent combination was increasing in concentration in subsequent peaks. In fact twice as many monovalent antibodies could bind per plate than the parental bivalent antibody. Therefore, if monovalent concentrations are high enough then these peaks could give as strong or stronger anti-CEA response than the parental antibody itself. This could explain the strong anti-CEA activity in the regions outside of the parental anti-CEA monoclonal antibody combination.

Apart from the anti-CEA activity, there was anti-DOX, anti-DOX-BSA and anti-BSA activity in the various fractions. The anti-DOX was expected in the first peak region as well as in the second peak regions of each HPLC profile. The first, as we expected, was the parental anti-DOX combination. The second peak would have anti-DOX because of the bispecific antibody in that region. All hybridomas had high anti-DOX activity in the first peak region but not all had high activity in the second peak region. In some cases the anti-DOX-BSA or the anti-BSA activity was much greater (Figure 18). It was suspected that if the anti-DOX activity was low and the anti-BSA was high in the second peak region, then the efficacy of the bispecific with DOX would be poor. 26-61-10 (Figure 18) is an example of such a case. Anti-DOX activity was

very low and the anti-DOX-BSA activity was higher. Even more discouraging was the anti-BSA activity for this hybridoma. The anti-BSA activity was just as high as the anti-CEA, but yet the dual activity assay (Table 2) indicated that this hybridoma had the best bispecific activity of all the hybridomas. The high anti-BSA activity of several of these hybridomas cannot be fully explained, particularly as the hybrids were originally selected on the basis of their high anti-DOX-BSA activity and low anti-BSA activity in 'subtraction' ELISAs before they were cloned (Reddy, 1993; Reddy & Ford, 1993). However, it appears that this is not an indicator of a poor anti-CEA/anti-DOX bispecific antibody. In fact, the results from these assays may indicate that anti-BSA activity is not a critical assay. The most important test for a bispecific antibody activity is the dual specificity assay.

Most studies of bispecific antibodies using HPLC only include the ELISA results of the main elution peak fractions (Xiang et al, 1992; Dietsch et al, 1993). However, in this study all fractions were tested and the ELISA results were graphed. This was done in order to get an idea of how each hybridoma would respond to the various ELISAs and to help verify which fractions should be collected for the *in vitro* microcytostasis assays.

IV 4.1 Dual Activity Assay

The dual activity assay did not produce as high absorbance readings as the other ELISAs. This can be explained by the nature of the test itself. The dual specificity assay relies on the affinity of both antibody combining sites ie. the anti-DOX arm of the

BSMab to bind to the DOX or DOX-BSA coated plate and the anti-CEA arm of the BSMab to bind to CEA-HRP. Both of these steps will be affected by affinity to a greater degree than the other ELISAs, as explained below. The anti-CEA ELISA for BSMab relies on only one of the antibody combining sites, the anti-CEA, the rest of the assay relies on the binding of an anti-mouse Ig to bind to the heavy and light chains of the BSMab and does not rely on the affinity of the other antibody combining site of the BSMab. The same applies to the anti-DOX ELISAs. From a theoretical point of view much lower absorbance readings would be expected in the dual specificity assay, and this is exactly what was found. As a consequence of this, a reading greater than the mean plus 2 standard deviations of the control value was taken as being significant.

IV 5.0 QUANTITATIVE HPLC PURIFICATION OF BISPECIFIC ANTIBODIES

Four of the original seven hybridomas (26-61-1, 26-61-4, 26-61-10 & 57-11-25-17) were selected for further preparative analysis based on ELISA results, protein concentration and final volume of protein A purified ascitic fluid. As mentioned earlier, the anti-BSA ELISA results were used originally as a major determining factor for this selection process. If the anti-BSA activity for a given hybridoma was higher than the anti-DOX response then it was originally thought that this hybridoma would not be a good choice for further testing. However, the *in vitro* response of 26-61-10 (Figures 37, 38, 39, 43, 44 & 45) proved that this was not so. The ELISA test with the most reliance was of course the dual activity assay. The best response, as determined by the

dual assay, came from 26-61-10, 57-11-25-17 and 26-7-35 (Table 2). 26-7-35 was not selected for quantitative analysis because both its total volume and the protein concentration were too low (Table 1). 26-61-2 and 57-9-6-4 were also rejected on the basis of volumes available, as well as, in the case of 57-9-6-4, its low response in the dual assay (Table 2).

The HPLC profiles for the 5 ml injections were not significantly different from those of the 1 ml profile for each hybridoma with the exception of the first 5 ml injection for 26-61-10 (Figure 30). This was ascribed to poor equilibration of the column before the elution process started.

The ELISA assays were completed on the second peak region of all four hybridomas because previous ELISAs indicated that the bispecific activity was in this region. The ELISAs were consistent with the results obtained in the previous tests done on the 1 ml HPLC separations. However, the dual assay results were different. The 26-61-10 dual assay results (Figure 33) showed much higher values than those obtained with the protein A purified stock sample (Table 2). However, this was not the case with 57-11-25-17 (Tables 10, 11 & 12) where the values were much lower than those obtained with the purified protein A stock (Table 2). Based on this test alone, hybridoma 26-61-10 was selected for further testing. The ELISA results indicated that the HPLC procedure was an effective method of further purifying such antibodies from protein A purified ascitic fluid. It not only gave fractions that were not too dilute for ELISA testing, but also the precise monitoring procedure allowed for easy selection of fractions with bispecific activity.

IV 6.0 **In Vitro CHARACTERIZATION OF 26-61-10 BISPECIFIC ANTIBODY**

The in vitro tests on the high CEA expressing cell line, SKC01, using 26-61-10 and DOX demonstrated a dramatic decrease in the doxorubicin IC_{50} value for that cell line (Table 13). Figures 37, 38 and 39 illustrate that with DOX alone there is a definite decrease in survival with increasing drug concentrations. However, the decrease in survival is more evident with antibody + drug. When using the non-specific Ag8 antibody, it was observed that the IC_{50} did not change (Table 13) thus proving that 26-61-10 was indeed reacting in an anti-DOX specific manner by increasing the killing of the CEA expressing colonic carcinoma cells and acting as a 'drug receptor', focusing DOX on the cells. The BSMab controls clearly indicate that antibody alone was not causing this effect. To confirm the specificity of the anti-CEA arm of the BSMab the low CEA expressing COLO 320 DM colonic cancer cell line was used. The results (Table 15) show that there was no increase in cytotoxicity as compared to DOX alone. This indicates that 26-61-10 did indeed react in an anti-CEA specific manner, focusing doxorubicin on the CEA expressing SKC01 cells and thereby increasing cytotoxicity to these cells (Tables 13, 15 & 17). The fact that Ag8, a non-specific antibody, did not have any effect, confirmed the specificity of the results obtained. The specific targeting ability of 26-61-10 is clearly seen in the summary in Table 13.

The results clearly demonstrate that BSMab 26-61-10 reduces the IC_{50} for DOX but they also show that over the close range tested (0.01 $\mu\text{g/ml}$ - 10 $\mu\text{g/ml}$) the amount of BSMab does not seem to make much of a difference (see figures 37,38 &

39). A plausible explanation for this is that at the lowest concentration of BSMab used (0.01 $\mu\text{g/ml}$) all the CEA molecules expressed by the target cells are saturated with antibody. Adding 100 or 1000 times more BSMab does not result in any more targeting of doxorubicin to the target cells and, therefore, the IC_{50} remains fairly constant. To confirm that this explanation is indeed correct, one would need to titrate the BSMab from 0.01 $\mu\text{g/ml}$ i.e. 0.001, 0.0001, 0.00001 etc. and see what effect that this would have on the IC_{50} . The anticipated result would be that as the BSMab concentration decreased, the IC_{50} would gradually approach that of DOX alone.

The actual killing of these cells by BSMab which has focused doxorubicin to the cell surface is suspected to be via antibody internalization. Of course, the efficacy of such an antibody would depend on the nature of the target antigen (Duke-Cohan et al, 1993). Parental 11-285-14 anti-CEA antibody has been shown to be internalized by CEA expressing cancer cells, including SKC01 (Tsaltas et al, 1992; Tsaltas, 1995; Ford et al, 1996). The anti-CEA activity of the bispecific antibody was illustrated in the anti-CEA ELISAs and was often as intense as the parental antibody itself. The CEA-binding ability and the internalizing properties of the parental anti-CEA should, therefore, have been retained in the bispecific. Retaining the ability to be internalized would be very important for such an antibody. Other studies have shown that not only do such antibodies selectively deliver the drug to the tumour, but that they can also exert an antidotal or antitumour activity (Morelli et al, 1994). These authors also found that their antibody was able to significantly decrease the DOX concentration in the intestines of test mice. Also, they found that there was significant antidotal effects in

these mice when exposed to otherwise lethal doses of DOX. It is very likely that the 26-61-10 was effective due to internalisation and may respond *in vivo* in a similar way.

Bispecifics created for the treatment of B-cell lymphomas have also been used successfully *in vitro* and *in vivo* (Demanet et al, 1994). Such an antibody was developed to specifically bind to different antigens than the 26-61-10 but still it contained the same dual specificity and antibody mediating targeting properties shown by many bispecifics. The use of an indium labelled bispecific antibodies in a clinical study proved that the tumour to blood and tumour to liver uptake ratios of colorectal cancer patients was improved (Le Doussal et al, 1993). However, 7 out of 11 patients did develop a HAMA response. Therefore, more research is needed to improve such antibodies for the treatment of human tumours *in vivo*.

A similar case occurred with an ovarian cancer study where the bispecific activity was inhibited by the HAMA response (Lamers et al, 1995). This is obviously the most limiting factor of using such antibodies in clinical studies and will definitely have to be addressed with 26-61-10, if it is to be used in clinical studies. An attempt is currently being made to develop a chimeric humanized Mab derived from the parental 11-285-14. The Fc portion will, in fact, become a human Fc thereby reducing the chances of a HAMA response. It will be reduced because most of the HAMA response is directed towards the foreign mouse Fc. Once the process is completed a chimeric version of 26-61-10 could be produced. This would reduce the chances of this antibody eliciting a HAMA response, complexing with anti-mouse antibodies and thereby being prevented from reaching the desired tumour target.

IV 7.0 CONCLUSIONS AND SUGGESTED FUTURE RESEARCH

Seven hybridomas, selected previously for anti-CEA and anti-DOX activity (Reddy, 1993; Reddy & Ford, 1993) have been purified using protein A affinity chromatography and analytical HPLC profiles have been obtained for all of them. Analysis of the HPLC fractions by ELISA for anti-CEA, anti-DOX, anti-DOX-BSA, anti-BSA and dual activity to CEA and DOX indicated which hybridomas to choose for further research. Four were selected for preparative HPLC analysis and of these, one, 26-61-10, was shown to have the highest dual specificity activity. Fractions from preparative HPLC runs with this antibody were pooled, concentrated and used to evaluate the ability of 26-61-10 to target doxorubicin to a CEA expressing colonic cancer cell line. This study clearly demonstrated that 26-61-10 causes a statistically significant reduction in the IC_{50} value for doxorubicin with SKC01 cells. This effect is specific as: an 'irrelevant' antibody, Ag8, did not cause this effect; the IC_{50} value for doxorubicin with a low CEA expressing cell line was not affected by the BSMab and drug together; 26-61-10 alone, without the presence of doxorubicin, had only a minimal effect on the viability of SKC01 cells.

It would have been of interest to analyse all seven BSMabs in the in vitro microcytostasis assay. However it was not the priority of this study. The main objective was to determine if HPLC could be used to purify these BSMabs and then to select the antibodies of most interest for evaluation in the future. It was only possible to evaluate one of the purified antibodies in the microcytostasis assay in this study due to limitations in the amount of antibodies available. In the future, to do the other

hybridomas justice, there would have to be much more ascitic fluid produced for each one. This should be a future goal for 26-7-35 in particular as it showed good dual specificity activity but could not be evaluated in the *in vitro* microcytostasis assay because there was insufficient purified product. It was demonstrated in this study that four of the seven protein A purified hybridomas had very low activity in the dual specificity assay. It is possible that the immunoglobulin genes of these hybridomas have mutated or have been lost with resultant loss of bispecific activity. This is speculation and it would have to be verified by chromosomal analysis.

Finally, the next step for 26-61-10 is that it should be further evaluated for its targeting potential in a preclinical *in vivo* model with SKC01 and COLO 320 DM growing as xenografts in nude mice. If results from this study were promising, then Phase I clinical evaluation would be warranted in patients. The results obtained in this thesis have provided the necessary background for such evaluations in the future.

CHAPTER V

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