INTERNALIZATION OF A MONOCLONAL ANTIBODY RECOGNIZING CARCINOEMBRYONIC ANTIGEN (CEA) BY HUMAN CANCER CELL LINES

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By

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To my parents
Nelli and Hristos Tsaltas

ABSTRACT

Monoclonal antibody (Mab) internalization by cancer cells has been recently gaining increasing recognition as one of the important factors affecting the action of Mabs or immunoconjugates (ICs) on tumour sites. This project addresses the underexplored subject of internalization, by comparing existing internalization assays in terms of accuracy and consistency, as well as by developing more rapid and concise methods for the detection of internalized antibody. A number of *in vitro* techniques for investigating internalization are evaluated, using a model which consists of a well characterized anti-carcinoembryonic antigen (anti-CEA) Mab and a number of CEA expressing human cancer cell lines. Employing two alternative radiolabelling assays, evidence for internalization of an anti-CEA Mab by a CEA-positive colorectal cancer cell line (LS174T) was obtained throughout the time intervals examined (5 min to 150 min.). A widely employed internalization assay involving the use of a low pH buffer for the dissociation of surface antigen-antibody bonds, has been thoroughly analyzed and shown not to fulfill its alleged role, thereby introducing inaccuracies in the experimental method. Electronmicroscopy employing horseradish-peroxidase labelled anti-CEA Mab, permitted the direct visualization of anti-CEA Mab related staining in intracellular compartments of a high CEA-expressor human colorectal cell line (SKCO1). SDS/PAGE analysis of samples derived from cytosolic and membrane components of solubilized cells from lung and colonic cancer cell lines, provided evidence for non-degraded internalized anti-CEA Mab throughout seven half hour intervals, starting at 5 minutes. Internalized

anti-CEA was detected in all CEA expressing cell lines (LS174T, SKCO1, BENN) but not in the case of a very low CEA expressor line (COLO 320). When the last method was compared to a newly developed internalization assay involving flow cytometry, results were very similar for all the above cell lines. Given that these two methods consistently provided comparable results, use of flow cytometry for the detection of internalized antibody is suggested as a fast and uncomplicated alternative to the internalization assays used at present. Finally, the question of the endocytic route followed by CEA-anti-CEA complexes is addressed through blocking clathrin-mediated endocytosis in the cell lines examined. Preliminary results indicate that such complexes may be internalized only partially by clathrin-coated vesicles, with an alternative uptake-endocytic mechanism also at work.

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ABBREVIATIONS

A	Angstrom
ABTS	2,2-azino'-di-(3-ethylbenzthiazoline sulphonic acid)
Ab	Antibody
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
Ag	Antigen
BFA	Brefeldin A
BGP	Biliary glycoprotein
BSA	Bovine serum albumin
CEA	Carcinoembryonic antigen
cdc2 kinase	cyclin B-$p34^{cdc2}$ kinase complex
cpm	counts per minute
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dox	Doxorubicin
DW	Distilled water
ELISA	Enzyme linked immunosorbent assay
FCS	Fetal calf serum
HAMA	Human anti-mouse antibody response

HMFG	Human milk fat globule
HRP	Horseradish peroxidase
HSA	Human serum albumin
ICs	Immunoconjugates
i.m.	intramuscular
i.p.	intraperitoneal
ITs	Immunotoxins
i.v.	intravenous
LAP	Lysosomal acid phosphatase
LDL	Low density lipoprotein (receptor)
Mab(s)	Monoclonal antibody(ies)
NCA	Nonspecific cross-reacting antigen
NRK cells	Normal rat kidney cells
NRS	Normal rabbit serum
OD	Optical density (absorbance)
Pabs	Polyclonal antibodies
PBS	Phosphate buffered saline
RAID	Radioimmunodetection
RAIT	Radioimmunotherapy
RAM-HRP	Rabbit anti-mouse horseradish peroxidase
RT	Room temperature

RTA	Ricin toxin A chain
s.c.	subcutaneous
SCN-Bz-DTPA	Isothiocyanatobenzyl-diethylenetriamine-pentaacetic acid
TAA	Tumour associated antigen
TILs	Tumour infiltrating lymphocytes
TSA	Tumour specific antigen

GLOSSARY

uptake (of antibody): reflects total amount of antibody per cell (both surface bound and internalized). Equivalent to the term "binding" often used in literature to express this notion.

Room Temperature (RT): 22°C

CHAPTER I

INTRODUCTION

I. 1. CANCER : Definition and History

The disease, or body of diseases, grouped under the term cancer is today the second most frequent cause of death in the developed world. This fear-inspiring disease has probably plagued the human race since the very origins of its existence since there is evidence that cancer may pre-date *Homo sapiens* possibly by millennia. Signs of benign tumours such as of an osteoma and a haemangioma were, for example, found in the remains of a dinosaur that lived during the Cretaceous period, whereas the remains of animals sharing the planet with primitive hominids bear traces of possible benign tumours (Tomatis, 1990).

The term cancer is derived from the Greek word "karkinos" meaning "crab". This term originated in the description of certain malignant tumours which appeared to have a central core and "limbs" (swollen veins looking like crab claws), through which the disease was believed to spread through the rest of the body. In accordance with the somewhat vague term used to describe cancer, a number of misconceptions concerning its "incurable" or "contagious" nature governed general attitudes towards the therapy and medical care of individuals inflicted with the disease. Cancer was considered incurable by Hippocrates and attempts at surgical intervention were generally discouraged, since it was believed that they would render the lesions worse. However cancer of the breast seemed to be a particularly likely candidate for surgery and it is reported that in the second century AD Galen successfully operated on a case of breast

cancer followed by similar attempts by Paul of Egina (seventh century) and Lafranco (fourteenth century) (Tomatis, 1990 and references within).

During the seventeenth century well respected clinicians such as Zacutus Lusitanus and Daniel Sennert reinforced the notion that cancer was contagious (Cassileth, 1983), a myth leading to refusal of admission of cancer patients to many hospitals. It was only in 1740 that the first special hospital for cancer patients was created in Reims, France, due to the efforts of canon Jean Godinot (D' Argent, 1965). The next such initiative was undertaken by surgeon John Howard resulting in the opening of a cancer ward at the Middlesex hospital in London, in 1792 (Hayward, 1965).

Research on cancer was probably first undertaken more than a century later, with the first laboratory being that established in Buffalo, New York, in 1899, under the direction of Dr Roswell Park. Interest in research matured as the accumulation of cases relating cancer development to probable causative agents became increasingly extensive. Increased risk for breast cancer development was noted as early as 1713 when it was reported by Ramazzini that elevated occurrence of breast cancer among nuns might be related to an absence of reproductive life. In the late eighteenth century definite "carcinogenic agents" began to be recognized starting with tobacco snuff (Hill, 1762) and chimney soot which was found to be related to the development of scrotal cancer seen in individuals who had been chimney sweeps (Pott, 1775). A number of other carcinogenic substances were recognized during the nineteenth century, mostly related to the work place, such as aromatic amines which were found to cause bladder cancer

in workers in dye factories (Rehn, 1977). The increasing awareness both of the existence of causative agents for cancer and the possibilities for cure for at least certain types of the disease led to the first "international" cancer congress held in Heidelberg and Frankfurt in 1906.

Surgical intervention as a means for cancer therapy advanced substantially during the last part of the nineteenth and the beginning of the twentieth century. Particularly, progress was made in the surgery of abdominal cancer by the Austrian surgeon Christian Billroth in the 1870's, in the surgery of cancer of the uterus by Wertheim and in the surgery of breast cancer by William Hallsted in 1902.

Roentgen's discovery of X-rays in 1895 was immediately put to use in the radiation treatment of breast cancer in 1896, whereas cancer chemotherapy was only introduced after the Second World War following declassification of war-time experimental findings and particularly those involving nitrogen mustards (Boyland et al., 1949; Cassileth, 1983). However, it was not until oestrogens were first used in the therapy of prostate cancer in the 1940's that chemotherapy was established as the third modality for cancer treatment (Huggins et al., 1941; Hayward, 1965; Huggins, 1967).

Today, surgery, radiotherapy and chemotherapy are often used in combination for the immediate treatment and eradication of the disease. Although surgery, (usually in combination with radiotherapy), remains the initial approach for combatting most major forms of primary cancer, a wide variety of chemotherapeutic protocols are also being used alone. For example, chemotherapy may be the only type of cancer treatment for

many of the tumours that appear early in life, for the eradication of residual disease and in certain types of tumours which respond favourably to particular drugs. Furthermore chemotherapy is expected to play an increasingly important role particularly in the treatment of metastatic disease as more potent cytotoxic agents and more specific ways to deliver them are developed.

As the list of cancer causing agents rapidly increases it also becomes increasingly obvious that one of the focal points in the struggle against cancer should be in the area of prevention. Containment of chemical waste in this era of industrialization, banning of commercially promoted well-known carcinogens such as tobacco and education on protection from both chemical and environmental cancer causing agents should greatly reduce the incidence of cancer especially in the developing countries where it is unlikely that appropriate treatment will be available in the near future.

I. 2. How serious is the Cancer Problem?

The burden of cancer in the world has been estimated both in terms of incidence (new cases of the disease in a unit of time) by Parkin et al (Parkin et al., 1988) and in terms of mortality (number of deaths in a unit of time) by Hakulinen et al (Hakulinen et al., 1986), for the year 1980.

Incidence data indicated that approximately 6.35 million new cases of cancer occurred in 1980, of which 3.25 million were in men and 3.1 million were in women (figures exclude cancer of the skin other than melanoma which are generally not fatal).

The pattern of incidence and types of cancer varied widely among different populations, however the number of cases was rather evenly divided between developed and developing countries (49.3% vs 50.7%). Considering that the population ratio between developed vs developing countries is in the range of 1:3 cancer incidence is clearly much higher in developed countries.

Estimates of number of deaths were based on a statistical model introduced by Preston (Preston, 1976), which gave a summary of mortality from 17 broad groups of cases in over 200 populations in various stages of development. These mortality data showed the world cancer toll to approximate 4.2 million deaths in 1980. At the time, on a world scale cancer ranked third as a cause of death with infectious diseases and diseases of the circulatory system well in the lead (Table 1) (Hakulinen et al., 1986). However these global figures reflect the shorter average life span as well as a much higher proportion of deaths due to infectious diseases for most populations in the developing world.

In the United States alone cancer mortality accounted for 476,927 deaths in 1987 (22.5% of the total number of deaths) and was only second to heart disease which accounted for 35.8% of total deaths according to the report on Vital Statistics of the United States, 1987 (Boring et al., 1991). For 1994 newly diagnosed cancers in the United States are estimated at 1,208,000 and cancer deaths for all sites are estimated at 538,000 (Boring et al., 1994). This steady rise in cancer incidence and mortality corresponds to a population increase, particularly in the aging bracket. Furthermore it

was estimated that 75% to 80% of all cancers in the United States may be due to environmental factors (Doll et al., 1981). The environmental influence on cancer development is supported by data on international variation in cancer incidence (Muir et al., 1987). In Table 2 international variation ranges from 155-fold for melanoma to 5-fold for leukaemia. This variation is not believed to be influenced by factors such as diagnostic and reporting practices among countries, or indeed by genetic factors to any great extent (Fraumeni et al., 1993). Furthermore it is believed that with increased proportions of an aging population and reductions in deaths from coronary artery and cerebral vascular disease, cancer will become the leading cause of death in the United States by the year 2000. Given this dismal prospect, the National Cancer Institute set, in November 1983, the national objective of reducing cancer mortality rates by 50% by the year 2000, through the intensification of preventive, screening and novel treatment programs (Greenwald et al., 1986). In practical terms this goal would translate to a reduction in cancer death rates from 168 per 100,000 in the year 1980 to 84 per 100,000 in the year 2000.

Unfortunately, to date, this objective does not seem likely to be realized. Cancer death rates in the United States amounted to 170.4 per 100,000 in 1987 (a total of 476,927 deaths), whereas the estimated cancer death rate for the year 1994 is 172 deaths per 100,000 (a total of 538,000 deaths) (Boring et al., 1994). Furthermore the cancer incidence for all cancer sites seems to follow a steady increase. For example, a close to 15% increase was noted between 1973 and 1987, according to the Cancer Statistics

Review 1973-1987 in the 1990 edition of the National Cancer Institute's (NCI), annual resource book of cancer data (Newman, 1990), with melanoma of the skin showing an 83% increase, followed by non-Hodgkin's lymphoma with 51% and prostate cancer with 46%. Cancer mortality has also increased by 34.1% for lung and bronchial cancer, 29.8% for melanoma, 23.6% for multiple myeloma and 21.7% for non-Hodgkin's lymphoma. Such dismal statistics obscure success stories such as reductions in mortality of 60% for testicular cancer, 49.5% for Hodgkin's disease and 39.6% for cervical cancer.

A similar trend in incidence and mortality rates seems to be prevailing in Canada. Estimated incidence for all cancers in the year 1992 was 115,000 new cases (Canadian Cancer Statistics, 1992). The average cancer incidence rate for both sexes was 303.5 per 100,000 whereas a total of 58,300 deaths due to cancer were estimated for the same year (an average mortality rate of 144 per 100,000 for both sexes). Estimates for 1993 showed a small increase in both incidence and mortality, with estimated incidence reaching 116,200 new cases (average cancer incidence rates for major cancer sites being 340 for males and 264 for females) (Canadian Cancer Statistics, 1993). Cancer death estimates for 1993 were 59,700, with average mortality rates of 174 per 100,000 for males and 113 per 100,000 for females. Similarly for 1994 estimates of cancer incidence have increased to a total of 121,300 new cases, whereas cancer deaths for this year are estimated to reach 61,000.

Three types of cancer account for 55% of the new cases in each sex: lung,

prostate and colorectal cancers in males, and breast, lung and colorectal cancer in females. A third of the cancer deaths in males are due to lung cancer while lung cancer is now the primary cause of cancer deaths for females with breast cancer a very close second (Canadian Cancer Statistics, 1994). Furthermore, cancer was the leading cause of loss of years of life as measured by potential years of life lost (PYLL) in 1989, with lung cancer, breast cancer and colorectal cancer accounting for about half of the potential life lost. In addition to this enormous drain in potentially productive human life, cancer imposes an immense financial drain in terms of health care considering the long hospitalizations and costly therapy necessary for the cancer patient. For example, the total direct and indirect costs corresponding to all cancers, coronary heart disease, motor vehicle injuries and stroke were estimated in the United States to amount to 23.15, 13.72, 14.44 and 6.46 billions of dollars respectively in 1975 (Thomas, 1991). Recent updates from the National Cancer Institute (NCI) in the United States report that the direct medical costs of cancer are $35 billion a year and rising (American Cancer Society, 1993). Considering the additional morbidity (cost of lost productivity) and mortality costs, the overall costs for cancer were a staggering $104 billion for 1990. Currently screening costs (including mammograms, Pap smears and colorectal exams) add another $3 to $4 billion to overall cancer costs. It is worth mentioning that, despite the enormous therapeutic and financial benefit of early screening, U.S. legislation mandating medicare coverage for cervical and breast cancer screening was only passed by Congress in 1989 and 1990 respectively.

Considerations such as the above along with the apparent fading of the United States' effort to control this devastating disease paint a very bleak picture of its future influence on the developed world. The problem is compounded with an increasingly hostile environment in terms of exposure to an ever expanding variety of chemical, biological and physical carcinogenic factors. It is therefore imperative that efforts be intensified both in terms of cancer prevention and in terms of novel cancer research with an emphasis on the application of this research in the clinical environment.

Table 1

Global and regional patterns of annual deaths by cause, 1980[1]

Region	All causes (# of deaths x1000)	Infection and parasitic diseases (%)	Neoplasms (%)	Diseases of the circulatory system(%)	Other and unknown (%)
World	50911	33.1	8.4	26.2	32.4
Africa	8562	48.7	3.1	12.3	35.9
Latin America	3197	31.0	9.0	24.7	37.3
North America	2081	3.6	21.5	54.5	20.4
East Asia	8842	23.0	10.6	33.7	32.8
South Asia	20315	43.8	4.3	15.6	36.3
Europe[2]	7713	8.6	18.1	53.8	19.6
Oceania	201	17.7	16.0	42.2	24.1
Developed	**10652**	**7.6**	**19.2**	**53.6**	**19.7**
Developing	**40259**	**39.9**	**5.5**	**19.0**	**35.7**

[1] Source: (Hakulinen et al., 1986)
[2] Including the USSR

Table 2
International variation in Cancer Incidence (1978-1982)*

	Ratio (H/L)	High (H) Rate Incidence Area		Low (L) Rate Incidence Area	
Melanoma	155	Queensland (Australia)	30.9	Osaka (Japan)	0.2
Nasopharynx	100	Hong Kong	30.0	South Western (U.K.)	0.3
Prostate	70	Atlanta (U.S., black)	91.2	Tianjin (China)	1.3
Liver	49	Shanghai (China)	34.4	Nova Scotia (Canada)	0.7
Penis	42	Recife (Brazil)	8.3	Israel (Born Eur. & US)	0.2
Oral cavity	34	Bas-Rhin (France)	13.5	Poona (India)	0.4
Cervix uteri	28	Recife	83.2 (Brazil)	Israel (non-Jewish)	3.0
Esophagus	27	Calvados (France)	29.9	Urban Cluj (Romania)	1.1
Stomach	22	Nagasaki (Japan)	82.0	Kuwait	3.7
Multiple Myeloma	22	Alameda (U.S., black)	8.8	Phillipines	0.4
Lung	19	New Orleans (U.S., black)	110.0	Madras	5.8
Colon	19	Connecticut (U.S., white)	34.1	Madras (India)	1.8
Breast	7	Hawaii (Hawaiian)	93.9	Israel (non-Jewish)	14.1
Leukemia	5	Ontario (Canada)	11.6	Nagpur (India)	2.2

* Selected data from (Fraumeni et al., 1993)

I. 3. Biology of Cancer

I. 3.1 Tumour cell Proliferation

Unlimited and uncontrollable proliferation of tumour cells is probably the most outstanding feature of the large panel of diseases grouped under the term cancer. Since stringent controls over cellular proliferation are imposed very early on in embryonic development it has become evident that any cell which does not obey the normal proliferation constraints may give rise to a tumour. Virchow was thus able to realize through microscopic examination of tumours that neoplasms originate from normal cells through uncontrollable growth (Tannock, 1989). Analysis of data on the growth of 780 human tumours in patients not receiving treatment, revealed that tumours follow an exponential growth curve so that a tumour takes a constant time to double its volume (T_D) (typical volume doubling times for common solid tumours are in the range of 2 to 3 months). However, analysis of data from multiple adjuvant studies in breast cancer, revealed that in all instances the cancer growth curves followed Gompertzian growth kinetics (Norton, 1988). In Gompertzian kinetics, the growth fraction of the tumour is not constant but decreases exponentially with time. This type of growth has a serious impact both on predictions of tumour behaviour according to its size, as well as on patterns of regrowth of residual tumour cells following treatment. Since the routine current detection techniques (physical and radiologic examination) can detect tumours of about 1 cm in diameter, the smallest detectable tumour would contain approximately 10^9 cells - which, assuming metastatic potential, will have ample time to seed metastases, and

will have undergone approximately 30 doublings if it is clonally derived from a single transformed cell. A potentially lethal tumour mass of 1 kg would then require only 10 further doubling cycles. Establishing a general model for preclinical tumour growth based on present clinical data, mathematical models and variability of tumour growth potential according to its stage of development, has proven quite difficult (Shackney et al., 1978; Folkman, 1975). Probably the major complicating factor involves the variable stages in tumour development, which generally include an initial need for establishing a blood supply network, evading the host's immune system, the subsequent increasing need for vascularization and increasing cell death due to necrosis for solid tumours.

Tumour cells are remarkably similar to their normal counterparts in terms of basic biochemistries and even in terms of basic cell kinetics. Usually tumour cells do cycle through four temporally distinct phases (Clement et al., 1991) namely M (mitosis), G_1 (the interval between the end of mitosis and the initiation of DNA replication), S (the interval during which DNA synthesis takes place and G_2 (the interval between the end of S phase and the beginning of the next mitosis). Furthermore quiescent (nonproliferating) cells, have for the most part an unduplicated (2n) DNA content and are said to be in G_0. Cells usually enter G_0 when there is a lack of nutrients or growth factors in the environment, or due to negative signals such as in the case of cell contact. Cell survival is favoured by the ability to enter G_0, since cells arrested in this phase generally survive longer than cells arrested in G_1. Cells in both phases have the same DNA content but cells in G_0 usually require twice the amount of time to enter S phase

as compared to cells in G_1. Also transition from G_0 to G_1 to S is sequential with each step requiring different growth factors. The major difference between normal and tumour cells in terms of cell kinetics is that most tumour cells usually lose the ability to enter G_0 (Baserga, 1985; Pardee et al., 1978; Marx, 1986), which can be detrimental both to the cell and the organism. In addition even those tumour cells (such as some tumorigenic fibroblasts) which may retain the ability to enter G_0, when deprived of serum in culture, enter this phase more reluctantly and leave it more readily than normal fibroblasts.

The overall rate of tumour cell proliferation may be approximated by estimating the proportion of cells undergoing DNA synthesis (S phase). Such data have been generated through the use of thymidine labelling techniques and autoradiography or through flow cytometry (McDivitt et al., 1985; Barlogie et al., 1983), for most types of human malignancies and generally indicate that tumours do not have a higher proportion of S phase cells than some normal tissues. Many solid tumours in humans contain at most 10% cells in S phase although this percentage is usually higher in rapidly growing tumours. Furthermore estimates of growth fractions derived by comparing the proportion of S-phase cells to that predicted from the phase distribution of cycling cells are usually in the order of 20% to 30%. Thus a large proportion of tumour cells seem to be out of cycle and this could be due to factors such as differentiation or death due to lack of nutrients as a result of limited vascular accessing especially in larger tumours (Tannock, 1968). The rate of tumour cell loss has been estimated by Steel (Steel, 1967) by comparing potential doubling time (doubling time without cell loss), with measured

volume doubling times, and it was found to be 80% or more of the rate of cell production. Therefore although two characteristics of tumour cells, namely loss of ability to differentiate (anaplasia) and immortality, are considered to act together in order to deter tumour cells from entering an irreversible quiescent state, it seems that tumour growth for most human malignancies is based on a moderate excess in cell production over cell loss.

I. 3.2 Cell Transformation

Ideally, cellular changes due to carcinogenesis should be studied in the intact animal model. However this approach is impractical due to the near impossible task of identifying, isolating and purifying early cancer cells. For this reason cell transformation has been classically studied *in vitro* usually employing normal fibroblasts in culture which are exposed to cancer inducing agents (Hamburger, 1991). The agents that have been used to induce carcinogenesis in this model mostly involve common carcinogens such as chemical agents (for example, benzpyrene on a murine prostate cell line (Chen et al., 1969), or 4-nitro-quinoline-1-oxide on human fibroblasts (Kakunaga, 1984)), irradiation (such as x-rays on rodent and human (Borek, 1980) fibroblasts) and transforming viruses on a number of different cell lines (Burger et al., 1972b; Bissell, 1976).

Changes caused by cell transformation can be grouped into three major categories, namely, changes in growth properties, changes in cell surface and changes in the pattern of oncogene activation (Barbacid, 1987a; Land et al., 1983b; Kahn et al., 1986;

Macdonald et al., 1991). Although such alterations are by no means universal for cancer cells they are the ones most frequently associated with transformation (Hamburger, 1991 and references within).

i. Changes in growth properties. Transformed cells exhibit loss of contact inhibition (Abercrombie, 1970) which leads to a much higher saturation density compared to normal cells; they are able to divide and propagate indefinitely given the necessary nutrients (i.e. they are immortal); they have much lower requirements for nutrients and growth factors (Cuttita et al., 1985); and finally they do not depend on anchorage for growth (MacPherson et al., 1964) (i.e. they do not need to attach to a substratum).

ii. Changes to the cell surface. Although the general structure of the plasma membrane is not altered, transformed cells are agglutinated at a much lower lectin concentration than that required for normal cell agglutination (Burger et al., 1972a); microfilaments and microtubules are frequently found to be more poorly organized in transformed cells; transformed cells seem to have the ability for enhanced transport of nutrients; transformed cells have either drastically reduced levels of fibronectin or completely lack this protein; finally, transformed cells show an increase in secretion of plasminogen activator which causes an increase in concentration of proteases.

iii. Changes in oncogene activation. Transformation is often associated with overexpression of certain oncogenes such as the ras gene (Barbacid, 1987b) as well as the existence of mutations and cooperativity between some oncogenes (Land et al., 1983a).

I. 3.3 Invasion, Metastasis and Angiogenesis

Solid (non-haematologic) tumours are likely to metastasize in approximately 60% of cancer patients. These patients have microscopic and most of them, multiple metastases at the time of primary tumour treatment (Liotta, 1986a; Fidler et al., 1982; Schirrmacher, 1985) and it is this metastatic disease which finally compromises the patient. Furthermore there exists great variability in metastatic potential among tumours, depending on their histologic type, aggressiveness and initial location.

Carcinomatous invasion, refers to the penetration of tumour cells through the basement membrane and into the underlying stroma. Once tumour cells have attached to the matrix, (a process which is possibly mediated by glycoproteins such as laminin and fibronectin, receptors for which exist on tumour cells), there follows disruption of the basement membrane which is believed to be related to specific changes associated with the malignant phenotype (Liotta et al., 1974). One such change is the increased production by cancer cells of lytic enzymes such as plasminogen activator, which cleaves plasminogen in plasma to its active form. Decreased intracellular adhesiveness of cancer cells (probably due to decreased fibronectin production) and increased motility (mostly due to loss of contact inhibition), are also believed to greatly contribute to the invasive ability of malignant cells. Several chemotactic factors, growth factors and motility factors appear to influence the direction of tumour cell locomotion (Garrod, 1990). Autocrine motility factors (Liotta et al., 1986b) are a recently described group of proteins which are believed to be secreted by tumour cells and to act both in an autocrine and paracrine

tumour specific fashion to stimulate both chemotactic (directional) and chemokinetic (random) motility.

Tumour necrosis, which usually arises from a combination of factors such as release of lytic enzymes, release of tumour necrosis factors and vascular insufficiency in rapidly growing tumours, seems to be the process most closely associated with release of tumour cells into the blood stream following invasion. Although it has been estimated that millions of cells may be released daily into the circulation by primary tumours (Liotta, 1986a), it has also been shown experimentally that the majority of cancer cells introduced into the venous circulation of mice are rapidly trapped in the capillary bed of the lungs with less than 1% remaining after 24 hours. Seeding of metastases therefore appears to be a very inefficient process (with less than 0.01% of circulating tumour cells initiating metastatic colonies (Schirrmacher, 1985) mostly because the circulation acts as a very hostile environment for tumour cells (Garrod, 1990). The sites that are most likely candidates for the establishment of metastases are the lungs, liver, lymph nodes and brain. The preferential metastatic potential of these sites may be due to haemodynamic factors, since cancer cells are most likely to be trapped in the capillaries of the organs that they encounter first such as the lungs for cells that enter the circulation before draining into the vena cava or the liver for cells entering the hepatic portal system. Alternatively, or in combination with haemodynamic factors, there may exist the possibility of differential requirements for survival of cancer cells which might be offered by particular local environments (the "seed and soil" hypothesis).

The study of induction of metastatic potential at the genomic level has recently attracted much scientific interest. Research both on oncogenes (Nicolson, 1987; Egan et al., 1987) and "antimetastasis" (Steeg et al., 1988; Leone et al., 1991) genes is beginning to reveal the possibility of controlling metastasis by identifying and possibly inactivating genes which seem to confer metastatic potential when transfected to non-metastasizing lines (Leone et al., 1991; Gunthert et al., 1991; Reber et al., 1990; Hofmann et al., 1991; Arch et al., 1992).

Another possible control point for containing metastatic spread could involve the control of the emergence of a new vascular network designed to provide the necessary nutrients for the growth of newly emerging tumours (Mahadevan et al., 1990).

Angiogenesis is essential for the preservation and growth of solid tumours beyond 2mm in diameter (about 10^6 cells) (Folkman et al., 1987; Folkman, 1985; Folkman et al., 1976). A number of proteins, collectively termed angiogenesis factors (AGFs) have been isolated mostly from tumour cells (tumour angiogenesis factor (TAF) (Folkman, 1974), fibroblast growth factors (FGFs) (Gospodarowicz et al., 1987), transforming growth factor α (TGFα) (Schreiber et al., 1986), but also from macrophages and mast cells (tumour necrosis factor (TNFα) or cachectin (Leibovich et al., 1987) and heparin). Since 1971 there has been an active field of antiangiogenesis research which has focused both on the discovery of antiangiogenic compounds and the development of specific inhibitors of angiogenic factors or related responses.

In short, although the biology of cancer cells does not appear to differ greatly

from that of their normal counterparts in terms of cell cycle kinetics and growth potential, there exist a number of almost universal aberrations in the cancer cell, several of which can be exploited for diagnostic or therapeutic purposes.

I. 4. Cancer Diagnosis

I. 4.1 Conventional Diagnostic Methods

Although screening for certain types of cancer, such as cervical (MacGregor, 1976), breast (Shapiro et al., 1982; Baker, 1982; Tabar et al., 1985), colorectal (Hardcastle et al., 1986; Eddy et al., 1987; Fleisher et al., 1989) and lung (Bomot et al., 1973), has had a very positive impact in terms of early diagnosis, it is very unlikely that screening and preventive programs will play a major role worldwide in arresting a wide variety of cancer types early enough to prevent metastasis, at least in the near future. The key to effective eradication of this disease therefore, seems to lie with improving current diagnostic methods and developing novel techniques. To date, despite technically revolutionary new approaches to cancer diagnosis, it is disheartening to note that diagnostic imaging (which includes the most non-invasive approaches such as ultrasonography, computer tomography, conventional radiography and magnetic resonance imaging) has not shifted the detection threshold for solid tumours to a level lower than 1-2 cm^3 of tumour mass at best. Mammography, which is the most widely used screening technique for the detection of early breast cancer, has improved to the point of detecting cancers of 2 cm or smaller at the noninvasive stage. Trials have

demonstrated that screening reduces breast cancer mortality by approximately 25% (Harris et al., 1993). Unfortunately, as discussed above, a 2 cm tumour will have had ample time for seeding metastases.

In addition the technological advances in terms of diagnostic tools have by no means diminished the effect of the human factor. There now exists a wide choice among diagnostic procedures each of which may be particularly well-suited to tumour detection at certain sites but completely inappropriate for others. Furthermore higher sensitivity in detection techniques can produce quite complex imaging data which can prove difficult to interpret.

I. 4.1.a Ultrasonography (Ainge et al., 1991)

Ultrasonography, which uses high frequency sound waves to generate body images, is a quick and safe procedure which is most effective when used for detecting homogeneous tissue structures such as the liver, pancreas, kidneys, gynaecologic tumours etc. In general, sonography is best suited for detection of lesions greater than 4-5 cm, preferably within a solid organ. For smaller lesions, or where acoustic barriers exist such as gas or bone, other diagnostic methods are preferable.

I. 4.1.b Computed Tomography (Cubberley et al., 1991)

Computed tomographic scanning (CT) which was introduced clinically in 1975, has a spatial resolution comparable to that of ultrasound and better than that of nuclear medicine. CT employs ionizing radiation at levels similar to those used by conventional radiography and provides excellent resolution with the added advantage of the

tomographic transverse perspective. It is the most accurate noninvasive method for detection of primary and secondary hepatic malignancies, cancer of the pancreas, renal carcinoma, adrenal malignancies and lymphomas. However, since CT is relatively expensive, time consuming and uncomfortable for the patient, it is usually considered only where other simpler techniques are deemed insufficient.

I. 4.1.c Nuclear Medicine (Johnston, 1991)

Nuclear medicine involves the intravenous injection of small doses of radioisotopes or radioactively tagged compounds. Choice of the appropriate isotope or compound may lead to its preferential localization or uptake by a particular tissue type, and "diseased" may then be compared with its normal counterpart. Detectors sensing ionizations caused by radioactivity may use gaseous, liquid or solid phases with the most efficient being solid such as sodium iodide crystals. The most popular nuclear medical device translating this radioactivity into images is the scintillation camera. Isotope imaging has proven very useful for the detection of thyroid and parathyroid tumours, brain tumours, questionable lung tumours, primary bone cancer, lesions in Hodgkin's, non-Hodgkin's and Burkitt's lymphoma and in the search for metastatic cancers by liver and bone imaging. However, in no cases were lesions of less than 1 cm detected, with detection sensitivity increasing with lesion sizes between 1 and 5 cm and decreasing after 5 cm.

I. 4.1.d Nuclear Magnetic Resonance Imaging (NMR) (de Lange, 1991)

Nuclear Magnetic Resonance (NMR) imaging (developed in 1973) is the most

recent imaging technique. It is based on protons (mostly hydrogen atoms) emitting a signal of the same frequency as the one they absorb after being bombarded by pulses of electromagnetic energy of the appropriate wavelength. This signal can then be used to construct a computer-generated image of the location of that particular hydrogen. NMR images display excellent tissue contrast, while the technique itself is noninvasive and carries no biological hazards. It is particularly promising in the areas of oncologic examination of the head, neck, spinal chord, brain and soft tissues. However its specificity is still rather low and it is a lengthier and more expensive process than CT scanning which usually still provides higher anatomical detail at most sites outside the CNS. Nevertheless NMR imaging is a diagnostic area of major future potential.

I. 4.1.e Oncological Angiography (Lang, 1991)

Oncological angiography involves the attempt to characterize many neoplasms by their distinctive vascular patterns. Although other, less invasive, diagnostic modalities have diminished its use during the last decade, angiography still exhibits a high sensitivity for the detection of certain tumour types such as parathyroid adenomas and may provide a necessary additional road map prior to resection of lesions. In addition arteriography is recently gaining importance in therapeutic oncology.

Despite the technological leaps involved in diagnostic procedures, they do not remotely approximate the capabilities of endoscopic procedures and analyses of biopsies for early detection and accurate description of tumour characteristics. For example, using current endoscopic procedures such as laparoscopy (a rather inexpensive technique

devised some 80 years ago), lesions of 0.5-2 mm in diameter can be seen from a 10 mm object distance (Berci, 1991). This detection capability cannot of course be compared with either ultrasound or CT, but it has to be borne in mind that endoscopic procedures and biopsies are probably the last diagnostic resort, due to their being highly invasive and with some degree of danger for the patient.

It becomes clear from the above that it would be ideal to perfect the previously mentioned, high-tech, non-invasive techniques so that they can detect tumour lesions of such small sizes that dissemination and metastasis are not likely to have begun. It is likely that the most appropriate method to achieve this goal would come with the ability to "focus in" on the lesion, rather than attempt to scan the suspect neighbourhood. There has been tremendous effort during the last couple of decades in attempting to specifically recognize tumour tissue and differentiate it from its normal surroundings. In the effort to render non-invasive diagnosis more efficient, molecules specifically associated with particular tumours have been searched for, in the hope of "targeting" any particular tumour. Although, to this day, tumour specific markers have only been found for B and T cell leukemias, a number of cancer-associated molecules have been discovered, most of which are actively used both in diagnosis and therapy of some cancer types.

I. 4.2 Cancer Markers

"Cancer markers" are cellular macromolecules many of which are in general produced normally at some early time during development (usually embryonic), but are

present either at very low or undetectable amounts in the adult. The onset of the carcinogenic process may trigger at one point an increase in production of these markers. These "oncodevelopmental" markers, when re-expressed, are usually related to their tissue of origin. Cancer markers include secreted proteins, cell surface molecules, hormones, enzymes and isozymes, cluster designation (CD) markers and karyotypic abnormalities, cytoskeletal markers and more recently oncogenes and suppressor genes.

Specific discussion of each marker is beyond the scope of this work. Suffice it to say that the history of tumour markers might be divided into three eras (Sell, 1991). The first era began in 1846 when it was discovered that urine samples of patients with multiple myeloma (then known as "mollities ossium") contained a heavy precipitate (the Bence-Jones protein (Bence-Jones, 1847)), which 100 years later was recognized as immunoglobulin light chains produced in excess by approximately half of patients with plasmacytomas. Then followed the association of various hormones and enzymes with specific cancers, however significant elevations of such substances are usually not related to any one particular cancer type and may be affected by a variety of biological events, so that they cannot be used as unequivocal diagnostic tools.

The second era of tumour markers is defined by the discovery of two very useful "tumour antigens", namely alphafetoprotein (AFP; G.I. Abelev, 1963 (Abelev, 1971)) and carcinoembryonic antigen (CEA; Gold & Freedman, 1965 (Gold et al., 1965b; Gold et al., 1965a)). Elevated AFP aids in the diagnosis of approximately half the patients with hepatocellular carcinoma, while elevations in CEA are mostly used for the detection

of colorectal malignancies or metastatic cancers and for measuring disease progression. However the most revolutionary discovery of the second era has been in obtaining antibodies of predetermined specificity, after the experiments of Kohler and Milstein in 1975 (Köhler et al., 1975). A variety of murine monoclonal antibodies (Mabs) to human tumours were widely applied against melanoma, colorectal carcinoma, carcinoma of the lung and most other tumours. Although much enthusiasm was initially generated and a relatively large number of diagnostic studies with radiolabelled antibodies, as well as therapeutic studies (usually with monoclonal antibodies alone) were performed, a number of factors unfortunately contributed to the "tapering off" of most researchers' interests, at least on the therapeutic side. One such consideration was the murine origin of those monoclonal antibodies, which generated a human anti-mouse (HAMA) response in most patients, more often expressed as minor side effects such as fever, chills, pruritus, chest tightness, dyspnea, rash, arthralgia, myalgia and hypotension. The major problem created by the HAMA response was that it limited the possibility of prolonged therapy.

However the most serious problem by far was the inability to discover more specific tumour markers so as to ensure exclusive tumour localization. Despite such drawbacks novel approaches to "tumour targeting" with monoclonal antibodiies, such as the use of chimaeric or human antibodies, open up new avenues for their use in the clinical environment.

The third era of tumour markers involves the quickly accumulating information on oncogenes and oncogenic viruses. Expression of several oncogenes has been

associated with cancerous disease states, often of an inheritable nature, thus adding the power of genetic counselling and early diagnosis to the therapist's armamentarium. Furthermore the possibilities for use of molecular biology techniques in recognizing abnormalities (such as gene deletions and mutations) at the level of the genome and possibly rectifying certain of them, open up immense possibilities in the diagnosis and treatment of cancer.

I. 4.3 Immunodiagnosis

Imaging studies with radiolabelled antibodies began as early as 1948 when David Pressman conducted studies demonstrating the localization of radiolabelled antibodies in normal tissues (Pressman et al., 1948) and later in experimental tumours (Pressman et al., 1953; Pressman et al., 1957; Bale et al., 1957). Presently radioimmunodetection (RAID) combined with the use of single photon emission computed tomography (SPECT) has revealed lesions as small as 0.5 to 1 cm (Goldenberg et al., 1992; Goldenberg et al., 1990; Goldenberg et al., 1989; Britton et al., 1989; Bares et al., 1987; Granowska et al., 1986), whereas the smallest detectable lesion using planar imaging was not less than 2 cm. Although RAID is theoretically subject to the usual targeting problems, such as lack of tumour-specific markers, antigen and cell heterogeneity within a tumour and circulating tumour antigen, it seems to circumvent most of these (Goldenberg et al., 1992; Goldenberg, 1988; Nowell, 1990; Goldenberg et al., 1980). Specifically, it seems that RAID does not require entirely cancer-specific antigens, since targeting has been

achieved with only 15% of the tumour cells expressing the target antigen (Doerr et al., 1990) and even very high titers of circulating antigen do not neutralize the injected antibody. Furthermore many of the carrier antibodies presently in use are pan-carcinoma antibodies, making possible the imaging of a large panel of tumours, while there seems to be no need for "tailoring" the antigen-antibody system to individual patients. Antibody dosage, size, affinity and immunogenicity are all points that are under continuous investigation (Goldenberg et al., 1992). Results to date indicate that desirable antibody doses range from 0.25 mg (Goldenberg et al., 1978; Goldenberg et al., 1980) to above 40 mg (Divgi et al., 1991), and that monovalent or bivalent antibody fragments are more desirable for imaging purposes (Wahl et al., 1983; Larson et al., 1983; Chatal et al., 1984; Siccardi et al., 1986). Antibody affinity is a moot point in targeting, but it is becoming increasingly obvious that higher affinity antibodies are not necessarily the most efficient (Sharkey et al., 1990; Weinstein et al., 1987; Fujimori et al., 1989). The issue of HAMA response is as important in RAID as in any other application of targeting and the creation of chimaeric, humanized or human carrier antibodies (LoBuglio et al., 1989; Pinsky et al., 1991) would be the avenue to explore for reducing or eliminating this problem.

Presently, the radiolabels most commonly in use include ^{131}I, ^{123}I, ^{111}In and ^{99m}Tc, with ^{123}I(6 hr half-life) and ^{99m}Tc (13.2 hr half-life) being detected most efficiently. ^{99m}Tc allows for the highest permitted administered dose and results in the best imaging properties among the four RAID isotopes mentioned above (Britton et al., 1989).

However its short half-life precludes its use for detection of targets showing slower uptake (Goldenberg et al., 1990; Goldenberg et al., 1989; Pinsky et al., 1991).

Although the degree of sensitivity in the detection of lesions differs with the antibody-radioisotope system used and the type of lesion examined, an increasing number of clinical trials indicate that use of RAID is very helpful both in confirming cancer sites revealed by current diagnostic techniques and, most importantly, in disclosing occult tumours. A European multicentre clinical study using ^{131}I or ^{111}In-labelled anti-CEA $F(ab')_2$ (Siccardi et al., 1989) revealed that in 20% of the patients RAID disclosed occult lesions and resulted in altering patient management in 13% of the cases. Another multicentre study using ^{99m}Tc-labelled anti-CEA Fab' (Pinsky et al., 1991) found a 42% clinical benefit in colorectal cancer patients. Most RAID studies have focused on colorectal cancer patients partly due to successful detection of nodal metastases in normal size lymph nodes (Nowell, 1990) (which remain undetectable by conventional techniques), as well as higher accuracy in detecting liver metastases (Goldenberg, 1991). Given that at the time of presentation of nodal metastases half the colorectal cancer patients have udetected micrometastases and will die of the disease (Thompson et al., 1987), and about 20% of those patients have liver metastases (Welch et al., 1978), the additional sensitivity provided by RAID is of essence. Most RAID studies on colorectal cancer patients have used anti-CEA antibodies (Goldenberg et al., 1980; Goldenberg et al., 1989; Goldenberg et al., 1983; Larson, 1990; Goldenberg, 1991) linked to any of the four radioisotopes mentioned. In addition, human and chimaeric antibodies have been

employed (Steis et al., 1990; Bischof Delaloye et al., 1991). RAID has also been used in the diagnosis of a large panel of other cancer types with most studies concentrating on ovarian carcinoma and breast cancer (Krag, 1993; Britton et al., 1989; Larson, 1990), and prospective trials have shown RAID to be generally superior to other imaging methods.

I. 5. Cancer Therapy

I. 5.1 Conventional Techniques (Cancer Medicine, 1993)

The three major modalities which have routinely been used either separately or most often in combination, are surgery, radiotherapy and chemotherapy.

I. 5.1.a Surgical Oncology

Surgery will normally only be undertaken if staging indicates that the tumour is localized. Furthermore, surgical excision attempting to achieve cure, is reserved for localized tumours of a histological type which is likely to be confined to the primary site. Unfortunately, there is an extremely high relapse rate for common solid tumours treated surgically, suggesting that at the time of resection of the primary there probably exist occult microscopic metastases. Due to this fact surgery is usually applied in combination with radiotherapy and/or chemotherapy.

I. 5.1.b Radiation Oncology

Radiotherapy is based on the premise that ionizing radiation will result either on a "direct hit" or in the production of free electrons which will react with water to form

highly reactive free radicals. These radicals will act by causing strand breaks in DNA and the radiation sensitivity of a tissue or tumour will depend both on the amount of damage sustained by the cells and the ability of the tissue to repair sublethal damage. The degree of oxygenation of the tumour is also a factor that greatly influences radiation sensitivity. It was discovered that repeated low doses of radiation are safer and more effective and therefore radiation is now delivered in multiple fractions over a period of 1-2 months. Radiation dose is governed by the sensitivity of normal tissues and tumour and these relative sensitivities define the potential for curability.

I. 5.1.c Chemotherapy

Chemotherapy (i.e. systemic therapy using anti-cancer drugs), apart from being frequently used in conjunction with surgery and/or radiotherapy to treat residual disease, may be the only possible treatment for those types of cancer that are not amenable to other forms of treatment (such as leukemias and lymphomas). Most anti-cancer drugs interfere with cell division at some particular stage i.e. they are cell cycle dependent, either for a particular phase (phase-specific drugs), or for any stage of the cell-cycle (cycle-specific drugs). Those drugs that affect cells irrespective of their cycling state are called non-cycle active drugs. Anti-cancer drugs are usually grouped by mode of action (antimetabolites, antitumour antibiotics, alkylating agents), and/or by origin (plant-derived drugs, platinum analogs). In most cases drug action has been extensively characterized along with modes of drug resistance development. There is a host of factors that will affect the onset of chemotherapy, its duration and drug type(s) used. In general

however, some of the most significant questions relating to the application of chemotherapy include tumour burden (which is often a limiting factor), sensitivity of the tumour to the drug used (which usually determines the length of chemotherapy, with most adults receiving a maximum of six to nine treatments), the development of drug resistance and the toxicity of the chemotherapeutic drug which may be tolerated differently by different patients.

I. 5.2 Immunotherapy (Rosenberg, 1993)

The biologic therapy of cancer which has more recently emerged as the fourth modality for cancer treatment, involves the effort to widely mobilize natural host defence mechanisms. Immunotherapy, which is considered a subcategory of biological therapy, gained momentum in the '60's, when data from experimental tumour models indicated that both specific stimulation of the immune system with antigen-bearing tumour cells and non-specific stimulation with bacteria, viruses and other adjuvant-type compounds could enhance the immune response and prevent the recurrence, or delay the growth of experimentally transplanted tumours. Furthermore, this type of treatment seemed to be most effective with small tumour burdens and was considered a possible treatment of residual disease. Immunotherapy involves both "active" (attempting to boost the host's immune responses against his/her own tumour), and "passive" strategies (involving infusion of biologically active agents into the host to mediate an antitumour response). Active immunotherapy is further subdivided into specific and nonspecific approaches.

Active nonspecific immunotherapy employs immune adjuvants such as bacillus Calmette-Guerin (BCG), *Corynebacterium parvum* and levamisole, which can activate monocytes, macrophages and the reticuloendothelial system (RES) to increase tumoricidal activity, or induce the production of cytokines. For the most part this form of immunotherapy has been unsuccessful in the treatment of advanced cancer. However BCG has been found to be effective in the treatment of cutaneous metastases of melanoma patients (Morton et al., 1991) and in the treatment of patients with superficial bladder cancers (Herr, 1991; Morales et al., 1976). More recently, recombinant cytokines such as interferon and interleukin-2 are administered directly or encapsulated in liposomes in order to minimize their toxicity (Rosenberg, 1993).

Active specific immunotherapy involves host immunization with "cancer vaccines" generated either from whole autologous or allogeneic tumour cells using living cells, inactivated cells or cell fragments either alone or in combination with immune adjuvants or viruses. To date neither pre-clinical studies nor clinical trials (Hoover et al., 1991; Hoover et al., 1984; Hoover et al., 1985) have produced any significant results of therapeutic potential.

Passive immunotherapy includes the areas of **adoptive immunotherapy** (the transfer of cells with anti-tumour activity to the tumour-bearing host) and **serotherapy** (the use of antibodies, particularly monoclonal, for a more selective tumoricidal effect).

A number of adoptive immunotherapy studies have been conducted since 1980 when subpopulations of lymphoid cells capable of lysing fresh tumour but not normal

cells were generated from mice and humans (Yron et al., 1980). These **lymphokine activated killer (LAK) cells** are a distinct non-MHC restricted cell population requiring interleukin-2 (IL-2) as the sole signal for their generation. Clinically LAK cells exhibit antitumour responses only in combination with IL-2 (Rosenberg et al., 1985; Rosenberg et al., 1987). Although since the mid 1980's the availability of recombinant IL-2 made such studies feasible, it is required in high doses which result in multiple toxic side effects (Rosenberg et al., 1985; Rosenberg et al., 1987). LAK cell-IL-2 treatment has mostly been applied to patients with melanoma or renal cell cancer and in those studies complete regression of metastatic cancer was observed in approximately 10% of the patients and partial regression in 20%. Some regression of metastatic cancer has also been observed in colorectal, lung, liver, bone, skin, subcutaneous tissue and circulating tumour cells. Another IL-2 stimulated tumour-derived lymphoid cell subpopulation able to destroy some autologous tumour cells has also been identified. **Tumour-infiltrating lymphocytes (TILs)** can be isolated from most types of human tumours and have been found to be 50 to 100 times more potent than LAK cells in mediating the regression of established micrometastases in animals (Rosenberg et al., 1986). To date treatment with TILs has only proven effective in the treatment of melanomas (Rosenberg et al., 1988) and attempts are being made to increase the specificity of treatment using gene therapy techniques (Rosenberg et al., 1990).

Serotherapy or monoclonal antibody mediated tumour cell destruction represents the only cancer therapeutic approach focusing on selectivity for cancer cells at the

cellular level. This approach is of paramount importance since the major common factor in both conventional radiotherapy and chemotherapy (the major therapeutic modalities for combatting metastatic disease), is the lack of specificity of treatment for the cancer tissue versus the normal tissue. Given the fact that both modalities are extremely aggressive and in addition to "curing" the tumour, they may also result in detrimental side effects on normal tissue, it becomes clear that such an alternative treatment, could yield an immense therapeutic benefit.

I. 5.3 Tumour targeting with monoclonal antibodies

The production of monoclonal antibodies in 1975 initiated a renewed wave of interest in the possibility of the use of antibodies for more specific attack on cancer cells via their recognition of tumour antigens. Monoclonal antibodies were used either alone or conjugated to cytotoxic agents such as radioisotopes, toxins and chemotherapeutic drugs, with promising results mostly in transplantation studies. Initial scientific enthusiasm gave rise to a large number of attempts to fulfill Ehrlich's early vision of "the magic bullet". However the inability to discover true tumour specific antigens as well as other difficulties associated with the immunotargeting approach quickly discouraged the academic community. This is an unfortunate fact, since to date no alternative method of specific treatment has been found despite the promise of gene therapy following the revolution in the field of molecular biology. Immunotargeting trials are still in progress both in experimental and clinical settings and a short account of the progress in the use

of immunoconjugates is given in the following sections.

I. 5.3.a Radioimmunotherapy

In contrast to radioimmunoscintigraphy where a gamma ray emitting radionuclide with a relatively long half life is desirable, radioimmunotherapy can take advantage of a wide range of nuclides that would be more suitable for the treatment of tumours of different size, location and radiosensitivity (Ford et al., 1990; Bast et al., 1993; Cobb et al., 1986; Dykes et al., 1987). Currently, beta-emitting radionuclides capable of traversing tissue in the range of millimetres to a few centimetres, may be used for larger tumours and would facilitate the destruction of neighbouring antigen-negative or poorly perfused tumour cells. Beta emitters include ^{131}I, ^{90}Y, ^{188}Re, ^{186}Re, ^{67}Cu, and ^{211}At. Probably the most widely used beta emitter has been ^{131}I, which, when conjugated to either intact antibody or $F(ab')_2$ fragments, has shown very high specificity for tumour xenografts (Vacca et al., 1988; Wahl et al., 1983). Substantial numbers of partial and complete tumour regressions were observed in radioimmunotargeting experiments using athymic rodents (Cheung et al., 1986; Klein et al., 1989; Lee et al., 1988; Sharkey et al., 1987; Chiou et al., 1988; Buchegger et al., 1988). Unfortunately, localization of radiolabelled antibodies in clinical studies was reported to be four orders of magnitude less than those observed in animal models (Buraggi et al., 1985; Carrasquillo, 1989; Colcher et al., 1987b; Epenetos et al., 1986; Farrands et al., 1982; Mach et al., 1981) (in the order of 0.005 % injected dose per gram of tumour). Despite poor tumour uptake

in humans, both partial and complete remissions have been observed in clinical trials with hepatomas and Hodgkin's disease (Leichner et al., 1983; Lenhard et al., 1985; Order et al., 1985; Dillman et al., 1984). Most of these trials have been performed with ^{131}I-labelled polyclonal antiferritin antibodies with no toxic side effects. Clinical trials involving other antibodies conjugated to the same radionuclide have not produced significant results in patients with melanoma (Bjorn et al., 1985), non-Hodgkin's lymphoma and recurrent lymphoma (Bernstein et al., 1990). However partial responses were achieved in 6/6 cutaneous T-cell lymphoma patients treated with ^{131}I-T101 conjugate (Rosen et al., 1987), whereas 4/5 patients with leptomeningeal tumours responded to the intrathecal administration of a variety of ^{131}I-labelled monoclonal antibodies (Lashford et al., 1988). ^{90}Y, a beta emitter of a much higher decay energy than ^{131}I, has been shown to cause specific anti-tumour effects in the animal model, but its use was also associated with bone marrow toxicity (Sharkey et al., 1988). To date, limited clinical trials with this nuclide have not shown much promise (Parker et al., 1990).

In certain cases, such as the treatment of micrometastases, higher and more focused radiation might be considered more appropriate. Radionuclides such as ^{211}At and ^{212}Bi emit alpha particles which have a very high energy and a short tissue range (4-6 cell diameters), thus requiring only 2-25 traversals to kill a cell. Their high cytotoxic potential along with their oxygen-independent mode of action, would make such nuclides very appropriate for the treatment of smaller, easily accessible tumours, such as those of the peritoneal cavity or the circulatory system. *In vitro* and *in vivo* studies with ^{212}Bi

have shown efficacy and specificity in an anti-human IL-2 model (Kozak et al., 1986), as well as very high cytotoxic potential (Kurtzman et al., 1988; Macklis et al., 1988). Clinical studies have yet to corroborate these results.

I. 5.3.b Immunotoxin Therapy

Toxins are large peptide molecules which can catalytically inactivate protein synthesis (Olsnes et al., 1982). The most widely used toxins are ricin and abrin (plant-derived) and diphtheria and pseudomonas exotoxin (bacterial). The intact molecule (holotoxin) consists of a 20-30 kilodalton (kDa) A chain, which makes up the cytotoxin portion of the molecule which is covalently linked to a 30-40 kDa B chain, which binds normal cell surface receptors (Pappenheimer, 1977; Olsnes et al., 1974). For the construction of immunotoxins the holotoxin is modified by removing or blocking the B domain binding site (Blythman et al., 1981; Lambert et al., 1990; Jinno et al., 1988; Murphy et al., 1986) and the resulting molecule is then linked to monoclonal antibodies (usually derivatized to contain a free thiol group), so as to produce a thioether or disulfide bond.

Toxins effect their cytotoxic action in a non-cycle specific fashion, by irreversibly blocking the elongation step of protein synthesis. Their ability to kill resting as well as cycling cells, and their tremendous cytotoxic potential (one toxin molecule effectively internalized, can kill a cell) (Eiklid et al., 1980; Yamaizumi et al., 1978) make it imperative that they be administered as selectively as possible. The choice therefore of

the monoclonal antibody component of an immunotoxin is of paramount importance, since uptake by normal cells should be kept at a minimum, while the immunotoxin should still retain the ability to bind avidly to cells, be rapidly internalized and be routed to appropriate intracellular compartments for effective translocation into the cytosol (Bjorn et al., 1985; Engert et al., 1989; Goldmacher et al., 1989; Press et al., 1988). Due to these requirements only 5% to 25% of specific monoclonal antibodies screened are found adequate for the production of potent immunotoxins (Till et al., 1988).

Because of their high cytotoxic potential, immunotoxins are probably the immunoconjugates that have been most extensively evaluated in clinical trials. The first such trials were performed on patients with refractory solid tumours, including metastatic melanoma (Spitler et al., 1987; Oratz et al., 1990), colorectal carcinoma (Byers et al., 1989), metastatic breast carcinoma (Weiner et al., 1989; Gould et al., 1989) and ovarian carcinoma (Pai et al., 1991). The above were phase I trials, with the exception of two phase II trials on metastatic melanoma (Oratz et al., 1990; Spitler, 1988). Although some partial and mixed responses were achieved, mostly in the case of melanoma patients (where one complete response was also observed), all trials were limited by high unexpected toxicities including vascular leak syndrome (VLS), myalgia, aphasia, neuropathies, encephalopathies and abdominal pain. In the cases of breast and ovarian carcinoma the severe neuropathies were subsequently attributed to possible monoclonal antibody cross-reactivity with epitopes on Schwann cells' myelin and cells in the cerebellum respectively. Furthermore in all these trials 80% to 100% of the patients

treated developed immunological responses either to the ricin A (HARA) component or the antibody (HAMA) component of the immunoconjugate, even after treatment with cyclophosphamide aimed at reducing the immune response of the patients.

Clinical trials involving haematologic malignancies have shown more encouraging results overall, probably due to a wider selection of monoclonal antibodies which do not cross-react with normal cells, higher accessibility of targeted epitopes and rapid replenishment of affected normal cells. Phase I trials involving patients with non-Hodgkin's lymphoma (Grossbard et al., 1992; Vitetta et al., 1991), B-cell chronic leukemia (Hertler et al., 1989), T-cell lymphoma (Le Maistre et al., 1991) and Hodgkin's disease (Le Maistre et al., 1990) have resulted in a higher percentage of complete and partial responses as well as a high number of mixed and transient responses. Furthermore toxic side effects were transient and less severe, while HAMA and HARA responses ranged from 7% to 85%.

However, the most successful clinical applications of immunotoxins have involved the treatment of graft versus host disease (GVHD) (Byers et al., 1990), where a high number of complete and partial responses was induced with tolerable side effects and only 26% of the patients developing HAMA and HARA responses. Presently an anti-CD5-A-Ricin Toxin A (RTA) immunotoxin has been approved for marketing for the treatment of GVHD, while a limited number of phase I and phase II trials involving the use of immunotoxins for the treatment of malignancies are under way.

I. 5.3.c Chemotherapeutic Drug Immunoconjugates

Various murine monoclonal antibodies have been linked to a number of chemotherapeutic agents (Koppel, 1990; Schlom et al., 1990; Ford et al., 1990; Ghose et al., 1987; Pimm, 1988) such as antifolates (Baldwin et al., 1987; Kanellos et al., 1987; Shawler et al., 1988; Smyth et al., 1987a; Affleck et al., 1992), vinca alkaloids (Ford et al., 1987a; Casson et al., 1987; Rowland et al., 1986), anthracyclines (Sheldon et al., 1989; Shouval et al., 1988; Smyth et al., 1991; Trail et al., 1992; Yeh et al., 1992; Iwahashi et al., 1989; Richardson et al., 1989), alkylating agents (Pietersz et al., 1988; Smyth et al., 1987b) and neocarzinostatin (Luders et al., 1985; Kitamura et al., 1992). The most accessible sites for drug attachment onto the antibody carrier are the epsilon amino groups of the lysine residues and the carbohydrate moiety of the C_H2 domain (Koppel, 1990). When $F(ab')_2$ fragments are used the lysine residues would be the most likely attachment sites since those fragments would lack carbohydrate portions.

In addition to the need for conserving the antibody's selectivity for the target antigen as well as the cytotoxic ability of the drug, the drug should be delivered in large enough doses in order to be effective. Since many cell surface antigens have less than 10^5 copies per cell it has been estimated that release of 3×10^6 drug molecules at the cell surface would be marginally sufficient in eliminating the tumour (Bast et al., 1993). Consequently much effort has been concentrated on increasing the number of drug molecules attached to the antibody either by direct linkage using spacer molecules (Yang et al., 1988), or by linking drug carrier molecules such as dextran (Tsukada et al., 1984)

or human serum albumin (HSA, (Ohkawa et al., 1986)) to the monoclonal antibody. Such linkage strategies have yielded immunoconjugates (ICs) which in some cases were found to be more effective than free drug both *in vitro* and *in vivo*. Such ICs include doxorubicin (Dox) linked to a Mab via a cis-aconityl spacer molecule (Yang et al., 1988) or a dextran linker (Yeh et al., 1992; Shih et al., 1991), daunomycin, fluorouridine or methotrexate linked to a monoclonal or polyclonal antibody (Pab) via a dextran bridge (Ford et al., 1990 and references within), (Shih et al., 1990; Shih et al., 1988), and mitomycin C (MCC) linked to antibody via human serum albumin (HSA) (Ohkawa et al., 1986; Affleck et al., 1992).

Another desirable feature of the linker, especially in the case of drugs which act intracellularly, is the ability of the linker to release the drug after entering the cell. Molecules such as the hydrazone linker, which dissociates in low pH environments (such as those of intracellular endosomal or lysosomal compartments) to free the attached drug, have been used with doxorubicin or its derivatives and resulted in immunoconjugates that, under optimal schedules, were found to be more potent than free drug (Braslawsky et al., 1990; Greenfield et al., 1990; Mueller et al., 1990) .

Although there have been discrepancies in terms of *in vivo* and *in vitro* performance of such immunoconjugates (IC's which have been found to be less effective than free drug *in vitro* are more effective than free drug *in vivo* (Rowland et al., 1986; Casson et al., 1987; Dillman et al., 1986)), in many cases the immunoconjugate is found to be less toxic than free drug, thus making possible an increase in tolerated dose (TD).

The concept of enzyme-catalyzed prodrug activation is also being explored, in the hopes that a Mab-linked enzyme which can accumulate in the target tissue may act in a catalytic manner to achieve the conversion of a large molar excess of subsequently administered substrate (drug) (Koppel, 1990; Bagshawe, 1989). Enzyme and prodrug combinations used to date, include carboxipeptidase G2 and a glutamyl derivative of a benzoic acid mustard alkylating agent (Bagshawe et al., 1988), alkaline phosphatase and phosphate derivatives of etoposide or mitomycin C (Senter et al., 1989) and a penicillin-V-amidase and a phenoxyacetamide derivative of doxorubicin (Kerr et al., 1990). In all cases the prodrug was at least 50-fold less toxic than the unconjugated active drug *in vitro* against tumour cells, while combinations of the enzyme conjugate and prodrug administered to tumour-bearing mice were more effective compared to free drug.

Given that the use of chemotherapeutic drugs is probably the most widely used type of cancer therapy and requires much improvement, it is rather peculiar that patience in the scientific world seems to be thinning where drug-Mab immunoconjucates are concerned. To date, clinical trials have been initiated with N-acetylmelphalan and neocarzinostatin (Takahashi et al., 1988) conjugated to antibodies reactive with colorectal cancer and responses have been noted in 2 out of 8 and 3 out of 8 patients respectively.

I. 5.4 Overcoming obstacles to effective tumour targeting

The main reason for the decline in the original enthusiasm generated by the idea of tumour targeting (in addition to the inability to find tumour specific antigens), was that the use of monoclonal antibodies seemed to introduce a variety of practical problems which limited their applicability. Such problems included the existence of shed tumour-associated antigen, antigenic modulation, heterogeneity of antigen expression, limited accessing to tumour sites, immunogenicity of "conventional Mabs" and the variability of Mab potential to be internalized. These problems have been addressed extensively and a number of strategies to circumvent them are still being developed.

I. 5.4.a **Antigen shedding** is a function of tumour size in solid tumours and posed a serious targeting problem since levels of circulating antigen were believed to determine the feasibility of using the targeting approach. However it has been shown that high levels of circulating antigen do not inhibit localization (Goldenberg et al., 1987). The hypothesis suggesting that route of administration of immunoconjugates may facilitate the preferential accessing of tumour-bound antigen has not been supported by pre-clinical or clinical studies for the most part (Ward et al., 1987c; Ward et al., 1987a). Injection of radiolabelled Mab in mice with ovarian cancer xenografts as well as in patients with ovarian cancer has demonstrated that the intravenous (i.v.) route was superior to the intraperitoneal (i.p.) route for solid tumours, although the i.p. route was superior for immunolocalization of ascitic cells. Conversely, direct intratumoral injection of a human

IgM Mab reactive with the GD_2 ganglioside produced partial and complete regression of injected cutaneous melanoma nodules in 4 of 8 patients (Irie et al., 1986). Tumour nodules that failed to respond to repeated injection had relatively lower levels of antigen than did nodules that regressed. Furthermore a mononuclear infiltrate was observed at the sites of antibody injection. Encouraging results have also been obtained with two- or three-step systems. For example, i.p. application of biotinylated Mab in ovarian cancer patients, who subsequently received radiolabelled streptavidin generated mean tumour to normal tissue radioactivity ratios of 9:1 for intra-peritoneal and 45:1 for extra-peritoneal samples respectively on the day of surgery, which is 2 to 3 times the usual values (Paganelli et al., 1992). A three-step approach currently being investigated for immunolocalization (Paganelli et al., 1993; Paganelli et al., 1991), involves injection of biotinylated Mab followed by avidin administration. Avidin serves to precipitate circulating biotinylated Mab and at the same time targets the tumour cells, thus allowing adequate homing in of the subsequently administered biotin. In general, attempts to examine the effect of regional drug administration in conventional chemotherapy were also discouraging and this approach is currently used only for the prophylaxis or management of meningeal metastases .

I. **5.4.b Antigen heterogeneity** (antigen being expressed by only a proportion of cells within the lesion) and **antigenic modulation** (induction of transient disappearance of a cell surface antigen due to binding of antibody), are the two direct ways for tumour

cells to escape treatment with monoclonal antibodies. The existence of antigenic heterogeneity among different tumour lesions has been documented both in experimental models using human carcinoma lines and in biopsies (Horan Hand et al., 1983) and several studies have demonstrated that the antigenic phenotype of a human cell population is the result of dynamic interactions of growth factors, evolution of cell subpopulations, influences of stromal and other cellular elements, as well as the diverse genotypes of the cell population. The result is a cell population that can intrinsically regulate its antigenic phenotype as measured by the binding of Mab. Approaches which attempt to minimize escape of tumor cells from Mab recognition via antigenic heterogeneity include the use of "cocktails" of antibodies (Durrant et al., 1989) which would take advantage of the expression of multiple tumour antigens within a cell population, or the presence of different determinants within the same antigen, and the administration of agents that interfere with cellular differentiation and can induce an enhancement of the antigen's expression (Gasparini et al., 1993). Agents tested for their ability to increase tumour antigen expression include retinoids, growth factors, organic solvents, DNA intercalating agents, vitamin D and tumour necrosis factors, and the most effective in augmenting Mab binding by increasing antigen expression were type I and II recombinant human interferons (rHu-IFNs) (Gasparini et al., 1993; Weinstein et al., 1992b). Another approach to circumventing antigenic heterogeneity could be the use of high-energy radioimmunoconjugates in an effort to extend cell kill to several cell diameters so as to affect antigen-negative tumour cells.

Similar methods may be used to avoid the disappearance of the antigen from the cell surface via antigenic modulation. The ability of most surface antigens to modulate however, often proves beneficial in the immunotargeting context since almost all immunotoxins and most chemotherapeutic drugs have to be internalized by the cell in order to destroy it. Where antigenic modulation is undesirable, the use of antibody Fab or $F(ab')_2$ fragments (discussed in section 1.5.4.c) or bispecific antibodies have been found to diminish it. Bispecific antibodies are functionally univalent molecules each arm of which may recognize different antigens. Apart from their ability to avoid producing antigenic modulation these antibodies circumvent the complications introduced by trying to chemically link effector molecules onto the antibody. In the immunotargeting context bispecific antibodies have been produced, which, after localizing onto the particular tumour by virtue of the antigen they recognize, can recognize and "trap" a subsequently administered effector molecule with the other Fab arm. To date effector molecules recognized by bispecific Mabs include drugs (Corvalan et al., 1987; Reddy et al., 1993), toxins (Webb et al., 1985) as well as epitopes on T lymphocytes in order to directly mobilize the host's own immune system (Bernards, 1987; Lanzavecchia, 1986; Staerz et al., 1985; Kranz et al., 1984; Barr et al., 1987; Staerz et al., 1986b; Staerz et al., 1986a).

I. **5.4.c Tumour accessing** probably poses the most serious barrier to immunotargeting in the case of poorly vascularized tumours or large tumour masses with

extensive necrotic regions. Monoclonal antibodies are large molecules (150,000 Da) which tend to localize on tumour cells adjacent to blood capillaries. Tumour penetration of such large molecules is severely limited by poor vascularization and high interstitial pressure (Jain, 1990; Durrant et al., 1989). For example, biopsy studies from patients who have received labelled antibodies i.v., indicate that only about 0.005% of the injected dose of radioactivity is localized per gram of tumour (Zalutsky et al., 1989). Strategies devised in order to overcome this problem include the use of antibody fragments, external beam irradiation and hyperthermia, use of vasoactive substances and regional administration of immunoconjugates.

Antibody fragments have been generated both by enzymatic and more recently by recombinant techniques (Colcher et al., 1990). In *in vitro* experimental systems employing multicellular spheroids $F(ab')_2$ and Fab antibody fragments were found to be much more effective in penetrating and binding to CEA targets in human colon adenocarcinoma spheroids (Sutherland et al., 1987; Kwok et al., 1988). However more rapid penetration of antibody fragments into tumours is combined with more accelerated clearance of the fragments from the bloodstream as compared to intact antibodies, resulting in a smaller percentage of the injected dose reaching the tumour (Andrew et al., 1986). This shortcoming combined with the fact that antibody fragments lose the ability to mobilize other effectors of the immune system due to the loss of their F_c portion, leaves open the question of whether antibody fragments are indeed the optimal carrier system for immunoconjugates.

The attempt to manipulate tumour haemodynamics using either **external beam irradiation or local hyperthermia** has led to controversial results. Although some results have suggested that pre-irradiation enhanced tumour uptake (Stickney et al., 1987), a more recent study has reported that there was no statistically significant increase in uptake of radiolabelled Mab by human colon carcinoma xenografts (Shrivastav et al., 1989). Analyzing results from this approach is further complicated by the fact that external beam irradiation can decrease tumour mass, which can in itself increase antibody uptake. Local hyperthermia at 42°C was shown to increase the uptake of a radioiodinated $F(ab')_2$ fragment by more than two-fold in a subcutaneous glioma xenograft. Another method used to increase penetration has been the administration of ß-adrenergic blocking agents in order to increase blood vessel permeability and consequently tumour perfusion (Smyth et al., 1987c).

Localized administration of immunoconjugates has already been discussed briefly (section 1.5.4.a). Again, it was hoped that this approach might result in better tumour localization and therefore higher tumour uptake. Iodine-131 labelled immunoconjugates administered i.p. increased uptake in peritoneal colorectal lesions by 2 to 7-fold (Colcher et al., 1987a) and 4 to 7-fold in patients with ovarian carcinoma (Ward et al., 1987b) relative to i.v. administration. Conversely, use of the intracarotid route in patients with malignant glioma did not result in increased intratumoral uptake (Zalutsky et al., 1990). Although randomized therapeutic studies have not been performed to date, non-randomized studies examining the therapeutic advantage of localized immunoconjugate

administration seem to indicate that local administration may be superior for the treatment of non-solid or low volume solid tumours. Intrapleural and intrapericardial administration of ^{131}I-labelled antibodies to human milk fat globule (HMFG1, HMFG2 and AUA1) to patients with malignant pleural and pericardial effusions resulted in a complete response for 10 out of 13 patients (Pectasides et al., 1986). In the treatment of epithelial ovarian carcinoma with ^{131}I labelled antibodies 8 patients with tumours greater than 2 cm in diameter did not respond, whereas 9 out of 16 patients with smaller tumours had an objective response (Epenetos et al., 1987). Intrathecal administration of ^{131}I-labelled antibody to patients with leptomeningeal disease resulted in objective therapeutic responses in 4 out of 5 patients.

Finally, **advances in Mab labelling methodologies** both in metal chelation and radiohalogenation, have resulted in more tumour uptake relative to non-specific accumulation in normal tissue (Bast et al., 1993). Use of an isothiocyanatobenzyl-diethylenetriamene-pentaacetic acid (SCN-Bz-DTPA) chelate for labelling antibodies with ^{88}Y (Roselli et al., 1989), increased tumour uptake in an athymic mouse model more than 4-fold and decreased bone uptake similarly. Also,the use of an N-succinimidyl-3-(tri-n-butylstanyl)benzoate intermediate for radioiodination of antibodies increased the radiation dose to subcutaneous human glioma xenografts by more than a factor of three compared to conventional radioiodination of the same antibody (Zalutsky et al., 1989).

A new approach to the analysis of tumour penetration is the "binding site barrier" notion (Weinstein et al., 1992a; Weinstein et al., 1992b). This theory suggests that

macromolecular ligands could be prevented from penetrating tumours by virtue of their successful binding to the receptor and their subsequent metabolism. According to this hypothesis, the higher the density of target molecules and the higher the antibody affinity, the greater the barrier. The binding site barrier theory is currently under investigation.

I. **5.4.d Immunogenic responses** of the patient to foreign epitopes on monoclonal antibodies produced from rodent (mostly mouse) B-lymphocytes limit the possibility of repeated dosing, because subsequent doses of the foreign antibody or immunoconjugate would be rapidly removed from the circulation by the neutralizing antibody (Dykes et al., 1987; Meeker et al., 1985). Although some advantages are to be gained by the development of anti-idiotypic responses (Traub et al., 1988) and rapid clearance where non-specific irradiation is concerned, the human-anti-mouse response (HAMA) has been a major concern in immunotargeting. Efforts to eliminate this problem include attempts to produce human monoclonals and chimaeric or humanized mouse monoclonal antibodies. The production of human monoclonal antibodies has proven extremely difficult due to their instability, low titer, specificity, isotype and low affinity (James et al., 1987). Despite these problems such antibodies have been produced against breast cancer (Kjeldsen et al., 1988), lung cancer (Saito et al., 1988), lipid A (Ramachandra et al., 1988) and various T-cell dependent antigens (Borrebaeck et al., 1988). An alternative approach has involved molecular engineering of murine and human

immunoglobulin genes to produce chimaeric antibodies with human constant and mouse variable domains or human constant and framework regions with mouse hypervariable regions only (Boulianne et al., 1984; Morrison et al., 1984; Morrison, 1985a; Morrison et al., 1989; Morrison, 1985b; Verhoeyen et al., 1988; Jones et al., 1986; Riechmann et al., 1988). Such Mabs reduce the HAMA response while retaining the ability to result in anti-idiotypic responses. Furthermore, it is possible to construct such recombinant antibodies with constant regions derived from any immunoglobulin subclass to accommodate any desired effector function (Neuberger et al., 1984; Bruggemann et al., 1987). A humanized version of the mouse Mab 17-1A, which reacts with colon carcinomas, did not evoke any HAMA response in 9 out of 10 patients even following multiple administrations (Stickney et al., 1987).

The problem of immunogenicity is particularly serious in the use of immunotoxins since both the antibody and the toxin are immunogenic. In that case humanization of the antibody would not overcome the immunogenicity of the immunotoxin. Consequently, the only alternative to date in both animal and human studies has been the use of immunosuppressive agents (Spitler et al., 1989; Pai et al., 1990).

I. **5.4.e** The question of **immunoconjugate internalization** is particularly relevant in the case of immunotoxins, where the cytotoxic moiety of the immunoconjugate has to enter the tumour cell in order to effectively destroy it. This is not the case for most radioimmunoconjugates and particularly the high energy ones, whose path length can

span many cell diameters, resulting in the destruction of both targeted cells and neighbouring ones. Since the chemotherapeutic drugs currently in clinical or experimental use have very diverse modes of action, internalization of chemoimmunoconjugates may or may not be a prerequisite for their effectiveness. It is therefore important that the internalization potential of each conjugate be determined before its therapeutic potential can be assessed. The topic of internalization will be discussed more analytically in the following section.

I. 6. Internalization

One of the most essential functions for the survival of living organisms is the transport of metabolically important substances into and out of the cell (Bilej et al., 1989). Such substances must cross the dynamic barrier of the cell's semipermeable membrane which serves to separate the integral cellular compartment from the surrounding environment. The currently accepted model describing membrane structure is the "fluid mosaic model" (Singer et al., 1972), in which the cementing framework of the membrane is the lipid bilayer and in which attached or embedded proteins interact with each other and with the lipids, while retaining their capacity to move laterally in the fluid phase. According to this model the predominant types of interactions are hydrophilic and hydrophobic ones.

Transmembrane transport occurs via four main routes, namely free diffusion, passive transport, active transport and endocytosis (reviewed in Bilej et al., 1989).

I. 6.1 Types of transport of materials into cells

Free diffusion takes place along a concentration gradient, with a rate of substance flow directly proportional to their solubility in lipids and no requirement for energy or any particular translocation apparatus. More lipid-insoluble substances (often encountered in bacterial transport), may make use of carrier proteins such as periplasmic permeases, while ions such as Na^+ and K^+ are transported through specific membrane channels.

Passive transport (or facilitated diffusion) is a process in which the substances move across the membrane in a reversible combination with "carrier" membrane proteins, which form an integral part of the membrane structure and are highly specific. This "one way" type of translocation is highly specific and is used by amino acids, mono and disaccharides and some ions which, after penetration, move along the concentration gradient.

Active transport refers to the movement and accumulation of substances against a concentration gradient and consequently it is driven by metabolic energy. Examples of such a type of transport are the sodium pump, glucose transport coupled to Na^+ ions or purine and pyrimidine transport coupled to protons.

Endocytosis is the means by which larger materials move into and out of the cell. Originally the endocytic process, (which is phylogenetically very ancient), served primarily nutritional needs of the cell. During Metazoan evolution however, endocytosis became highly specialized serving mostly as a mechanism for the elimination of undesirable exogenous or endogenous materials and as a defence against pathogenic

microorganisms and viruses. For practically all eukaryotic cells endocytosis is one of the most important and efficient processes for the host's defence.

During non-specific endocytosis, material binds to the cell membrane without the aid of specific receptors and, after crossing the membrane, is rapidly delivered to the lysosomes through fusion with the incoming endosomes. The entire plasma membrane will eventually participate in the process with a turn-over rate of 1-2 hours.

Receptor-mediated endocytosis refers to internalization of materials following attachment to specific cell receptors mostly found on the cell surface. This most efficient mechanism for the uptake of macromolecules from the extracellular fluid, was first demonstrated by the uptake of yolk proteins into mosquito oocytes via specialized regions of the membrane described by Roth and Porter as a "bristle coat" (Roth et al., 1964). This lattice-like coating exists on less than 2% of the cytoplasmic face of the membrane where there exist discontinuities in the microfilament network closely associated with the membrane. This coating was later identified as composed of a single 180-kDa protein called **clathrin** (Pearse, 1975; Pearse et al., 1990) and associated adaptors (Pearse et al., 1990).

I. 6.2 Membrane components necessary for receptor-mediated endocytosis

Clathrin makes up a "honeycomb"-like coat lattice while an inner shell of the coat is made up by adaptors which in turn interact with the cytoplasmic domain of cell surface receptors. Immunofluorescence studies show that clathrin and adaptors are co-localized both in the Golgi region and on the plasma membrane with a dotted distribution indicating thousands of coated pits and vesicles per cell (Robinson, 1987). The basic structural unit of clathrin is a complex of three molecules of clathrin heavy chain (180kDa) and three molecules of clathrin light chain (LC, 30-40 kDa) in a three legged structure termed **triskelion** (Kirchhausen et al., 1981). The clathrin heavy chain is highly conserved among different species, with 99% homology between human, rat and bovine brain clathrins (Lemmon et al., 1991; Keen, 1990). There are two structurally distinct types of clathrin light chains (Lc_α and Lc_β) which are encoded by different genes (Kirchhausen et al., 1987). Light chains are not required for *in vitro* assembly of the lattice structure (Winkler et al., 1983) and their absence does not seem to affect lattice stability (Schmid et al., 1984), but they have been found to bind calcium in a 1:1 molar ratio (Mooibroek et al., 1987) and to stimulate phosphorylation of the AP-2 adaptor polypeptide in coated vesicles. Although their functional significance has not yet been determined, their strong conservation among different species and numerous tissue-specific differences in light chain messages (Kirchhausen et al., 1987; Jackson et al., 1988) are strong indications that each light chain plays a critical and specialized role in

cellular coated membrane functions, which may be related to determining the precise shape of individual triskelia.

Triskelia are symmetric structures with three 45-50 nm legs joined at a common hub, with each leg having a characteristic bend about 16-19 nm from the central hub (Keen, 1990). Deep-etching electron microscopy of clathrin adsorbed to mica surfaces has shown that all the legs in a given triskelion display a uniform clockwise bend when viewed from the cytoplasmic perspective. The legs have a uniform thickness of approximately 2-4 nm but a completely folded globular domain (terminal domain) approximately 5-8 nm in diameter exists at the distal end of each leg. This domain contains about 25 % of the total mass of the heavy chain leg and is thought to be attached by a flexible linker to the triskelion leg since it can be readily released into solution by various proteases (Kirchhausen et al., 1984). The triskelion is thought to have a "resting" non-planar conformation with the legs hanging beneath a raised vertex.

Clathrin triskelia are induced to assemble into complete lattices by interaction with another group of proteins, termed **adaptors** (Pearse et al., 1981) (the terms assembly proteins, associated proteins and the abbreviation AP have also been used (Keen, 1990)). Two distinct adaptors HA-1 and HA-2 (or AP-1, AP-2) were identified and so named due to their ability to be separated from each other by hydroxyl-apatite chromatography (Pearse et al., 1984; Keen, 1987). A third protein AP180 is specific to the brain. HA-1 adaptors are found in coated vesicles derived from the Golgi complex (Robinson, 1987), whereas HA-2 adaptors are associated with coated pits on the plasma membrane. Both

molecules are heterotetramers and appear by freeze-etch electron microscopy to consist of a central head flanked by two smaller appendages or ears (Heuser et al., 1988). The HA-1 adaptor is composed of a γ adaptin, a β' adaptin (subunits of 100-110 kDa), a 47 kDa protein and a 20 kDa polypeptide. The HA-2 adaptor is a 270 kDa tetramer consisting of an α and β adaptin (subunits of 100-110 kDa) and two polypeptides of 50 and 17 kDa. There is little homology between the α, β and γ polypeptides of the two adaptors but the β and β' adaptin subunits are closely related. Limited sequence similarities exist between the α-adaptin of the HA-2 adaptor and the γ adaptin of the HA-1 adaptor but only in the N-terminal region, which suggests that it is information within the C-terminal region that targets the α adaptin to coated pits and the γ adaptin to the Golgi apparatus. Adaptor molecules are only found associated with their specific target membranes but it is believed that a sequence binding to cytoplasmic tails of receptors may be present in the C-region of these molecules. The role of the adaptor molecules in linking clathrin to the membrane was demonstrated with elastase-treated isolated coated vesicles. When these vesicles were stripped of clathrin and subsequently treated with elastase, to which the 100-kDa proteins are very sensitive, clathrin would not rebind to the vesicles in contrast to non-elastase-treated vesicles (Unanue et al., 1981; Virshup et al., 1988). The suggested role of adaptors linking the cytoplasmic tail of receptors to clathrin was investigated by *in vitro* reconstitution studies with the mannose-6-phosphate receptor (Pearse, 1985) and purified clathrin, as well as the Low Density Lipoprotein (LDL) receptor that was shown to bind HA-2 adaptors in a competitive fashion (Pearse,

1988). Most adaptins have now been cloned and a transfection system for the expression of cloned adaptins in intact cells has been developed (Robinson, 1990), which is hoped will aid in the precise identification of adaptor targeting sequences.

I. 6.3 Signals for ligand receptor recognition

The order in which clathrin, adaptor molecules and ligand receptors combine prior to internalization has not yet been elucidated. A likely hypothesis is that receptor tails bind to adaptors, followed by attachment to the triskelion. The resulting coated pits have been shown to exclude resident membrane proteins such as Thy-1 (Bretscher et al., 1980), which lacks a cytoplasmic tail, whereas receptors such as the ones transferring nutrients into the cell (eg. LDL and transferrin receptors) are found in plasma membrane coated pits in high concentrations (Pearse et al., 1990). Furthermore, deletion of the cytoplasmic tail of receptors reduced the efficiency with which these receptors internalize to about 10% or less of the wild-type receptor (Goldstein et al., 1985; Rothenberger et al., 1987; Mostov et al., 1986). Cell surface receptors which are constitutively recycling (such as the LDL, transferrin, asialoglycoprotein, polymeric Ig and mannose-6-phosphate receptors) and which typically have a large ligand binding extracellular domain, a single helix traversing the membrane and a small cytoplasmic portion, have been found to contain internalization signals in their cytoplasmic tails.

Initial information on such internalization signals was obtained through the LDL receptor, when mutations resulting in truncations of its cytoplasmic tail inhibited

internalization (Lehrmann et al., 1985). Furthermore, genomic DNA analysis of the terminal exons of an LDL receptor derived from skin fibroblasts of a patient suffering from familial hypercholesterolemia, showed that it differed from its wild-type counterpart by a single base substitution at residue 807 (18 residues from the membrane), which converted a tyrosine codon to a cysteine codon. Although this mutant was identical to the wild-type receptor in both size and charge, introduction of the mutation into a cDNA clone of the wild-type receptor resulted in a reduction of internalization to 25% of the normal receptor. Later studies indicated that the LDL internalization signal consists of a four amino acid motif which structurally forms a tight turn (Chen et al., 1990; Bansal et al., 1991). This tight turn was also found to fit the secondary structure formed by the four codon internalization signal (YXRF) of the transferrin receptor (Collawn et al., 1990), as well as the hexapeptide PGYRHV which promotes internalization of lysosomal acid phosphatase (LAP) (Lehmann et al., 1992). Again, the critical element of this receptor's signal seems to be the presence and position of the tyrosine residue (Eberle et al., 1991).

The importance of the tyrosine residue for internalization was further demonstrated when the replacement by tyrosine of a specific cysteine at position 543 of the influenza virus haemagglutinin, which is not normally internalized, resulted in its efficient internalization (Lasarovits et al., 1988). Furthermore, these experiments clearly showed that it was not only the presence of tyrosine but also the context in which it was presented that promoted internalization.

A critical tyrosine residue also appears in the cytoplasmic portion of the poly Ig receptor (Breitfield et al., 1989). In this case a second tyrosine found 16 residues from the membrane spanning region in a position analogous to the crucial tyrosine of the LDL receptor, does not seem to be important for its internalization. This is not the case for the mannose-6-phosphate receptor which seems to contain two crucial tyrosines in its 163 residue-long cytoplasmic tail. The two tyrosines in this receptor seem to be part of two distinct sorting signals, in agreement with the dual function of this receptor. One, contained in the region proximal to the membrane, is necessary and sufficient for efficient endocytosis of secreted lysosomal enzymes and insulin-like growth factor II (Kornfeld, 1987), while the other, found in the distal region of the tail, makes possible the sorting of lysosomal enzymes from the trans-Golgi network to a pre-lysosomal compartment (Oka et al., 1986). Furthermore, part of the hexapeptide YKYSKV in positions 24-29 required for rapid internalization, (specifically its most essential peptides YSKV), could be moved to a more proximal region of the cytoplasmic tail with only a modest loss of activity (Jadot et al., 1992). The same work also showed that the signal could be replaced by the four residue signals of seven other receptors and membrane proteins known to undergo rapid endocytosis, without serious compromise in internalization capacity. These results suggested that at least such 4-residue signals are interchangeable even among Type I and Type II membrane proteins.

In agreement with information on commonalities of signals for such receptors, a generic tyrosine internalization signal of 8-10 aminoacids which is both necessary and

sufficient for internalization has been proposed (Ktistakis et al., 1990). This hypothesis proposes that a tyrosine residue should be located in the segment of the sequence furthest from the membrane. In addition, within the sequence, there exist a number of polar or positively charged residues at conserved positions.

Recent studies on other plasma membrane receptors such as the group of G-protein-coupled receptors (GPCRs), have provided less conclusive data in terms of consensus signals, although their internalization signals seem to also be located in their cytoplasmic tail (carboxyl terminus) (Bouvier et al., 1988; Strader et al., 1987; Hertel et al., 1990; Rodriguez et al., 1992). One GPCR, the β_2-adrenergic receptor has been shown to be internalized and recycled via the same pathways used for the transferrin receptor (Von Zastrow et al., 1992) and interestingly, it was also shown that truncation of this receptor at position 365 caused the mutant to internalize to a greater extent than wild type. Similarly, truncation of 21 and 43 aminoacids from the cytoplasmic tail of the luteinizing hormone/ chorionic gonadotropin receptor resulted in increased internalization versus wild type, whereas deletion of 58 aminoacids from this region resulted in a receptor that was not expressed on the plasma membrane. Recently, using site-directed mutagenesis, the thyrotropin-releasing hormone receptor (TRHR) complex was found to contain two dissimilar domains in its cytoplasmic tail (between residues 335-368) which are necessary for efficient internalization (Nussenzveig et al., 1993). Furthermore the presence of a proximal cysteine residue was found to be critical. In the case of muscarinic cholinergic receptors an intracellular domain rich in serine and threonine

residues seems to regulate internalization (Moro et al., 1993). Such small domains, containing multiple S/T residues and necessary for receptor internalization, have also been found in several other GPCR receptors, such as catechol and peptide ones. Internalization in this group of receptors is believed to serve in down-regulating the relevant hormones.

Internalization seems to serve a similar function in the case of growth factor regulation, the most well-studied of which is the epidermal growth factor (EGF). Internalization of the EGF receptor complex is preceded by a complex array of events leading to signal transduction. Despite detailed knowledge of these events however, the requirements for the onset of internalization are not well understood. Interestingly, a region of this receptor's complex cytoplasmic tail, containing a negatively charged 18 aminoacid helical segment (Chen et al., 1989), shows about 30% identity to the region containing the tyrosines in the mannose-6-phosphate receptor.

Most endocytosed receptors exhibit protein kinase activity and contain potential phosphorylation sites, which led many investigators to believe that this may be the trigger to initiate the process of internalization. However, this hypothesis has created a lot of controversy. Mutation of phosphorylation sites in the LDL (Davis et al., 1987) and transferrin receptor (Rothenberger et al., 1987) did not seem to impair their internalization or recycling properties. In the insulin receptor model there are studies that both support (Graco et al., 1992), and dispute (Margolis et al., 1989), the requirement for an active tyrosine kinase for internalization. In the case of the asialoglycoprotein

receptor which contains the Y_5-E_6-N_7-F_8 internalization signal in its cytoplasmic tail (Ozaki et al., 1993), a protein kinase inhibitor abrogated its endocytosis (Fallon et al., 1992).

Interestingly, a reverse type of internalization signal seems to operate in the case of the CD4 glycoprotein. This glycoprotein is constitutively internalized and recycled in nonlymphoid cells, but is excluded from the endocytic pathway in lymphocytes. Inhibition of CD4 endocytosis is dependent on CD4 expressing an intact cytoplasmic chain and is only observed in cells where CD4 can interact with the protein tyrosine kinase $p56^{lck}$, a member of the *src* gene family (Pelchen-Matthews et al., 1992; Sleckman et al., 1992).

Internalization signals for lymphocytes and antigen-presenting cells (APCs) still remain elusive. It was recently shown that endocytosis of the antigen receptor surface Ig (sIg) on B lymphocytes is a process independent of B lymphocyte activation, since calcium (Ca^{2+}) and protein kinase C (PKC) were found not to be involved in the endocytic process of cross-linked sIg (Shuler et al., 1993). Internalization by APCs is an extremely active process mostly undertaken by macrophages, B lymphocytes and, as shown recently, by dendritic cells to an equal degree (Levine et al., 1992). A recent study on internalization and antigen presentation via type III receptors for IgG (FcγRII) demonstrated that a tyrosine-containing motif that transduces cell activation signals also induces internalization. Macrophage FcγRII receptors consist of α (ligand binding) and γ (signal-transducing) subunits. When murine FcγRIII was transfected into FcγR-

negative antigen-presenting B-lymphoma cells it mediated rapid ligand internalization and strongly increased the efficiency of antigen presentation when antigen was complexed to Ig. This process furthermore, was dependent on a structural motif containing two tyrosine residues present in the cytoplasmic domain of associated γ chains (Amigorena et al., 1992). The hypothesis proposed by these researchers is that, since high affinity IgE receptors (FcεRI), as well as T- and B-cell antigen receptors contain subunits structurally related to those on FcγRIII, there may be a general role for these associated chains in the internalization of multimeric receptors. The relation, if any, between initiation of internalization and signal transduction in this system however remains to be found.

Recognition of receptor signals is the first stage in the endocytic process, which begins with transmembrane transport and is followed by intracellular transport and possible recycling of components of the receptor-ligand complex. Both endocytic steps in eukaryotic cells also require the cooperation of the cytoskeletal network, which is interconnected with the plasma membrane.

I. 6.4 Association of the cytoskeleton with the endocytic process

The cytoskeletal network, which consists of microtubules, microfilaments, intermediate filaments and associated proteins, forms a three-dimensional array in the cytoplasm of all eukaryotic cells. The membrane-bound cytoskeleton is believed to be involved in the undertaking of processes such as cellular contact, endocytosis and the organization of the membrane structure (reviewed in Bilej et al., 1989). Some

cytoskeletal components are integral membrane proteins and may affect the functional properties of the plasma membrane such as motional freedom of membrane domains, electrical properties, activity of ion channels and membrane permeability. Furthermore, cytoskeletal structures have also been found associated with various intracellular organelles such as endosomes, lysosomes, Golgi elements and elements of the endoplasmic reticulum (ER).

Microtubules are hollow cylinders 18 to 30 nm in diameter, usually consisting of 13 protofilaments running parallel to the tubule axis, and consist of protein subunits, named α- and β- tubulin, which polymerize into microtubules. Tubulin is believed to be an integral part of certain kinds of membranes and as such it is distinct from soluble tubulin (2). Although the function of membrane tubulin is not yet well understood, it has been found that the microtubule system can directly influence membrane microvicosity and fluidity (Berlin et al., 1977). Furthermore, microtubule depolymerization results in increased motional freedom of membrane proteins and alters the membrane potential (Aszalos et al., 1986). Intracellularly, microtubule depolymerization has induced the fragmentation of the Golgi apparatus and the dispersion of its elements throughout the cell, which has led to the conclusion that microtubules may serve as tracks directing the vectorial transport of Golgi elements and secretory vesicles to the plasma membrane. Saltatory lysosome movement is also associated with the integrity of microtubules.

Microfilaments are composed of actin subunits (a 42 kDa globular protein), helically arranged in a double-stranded filament with a diameter of 4 to 7 nm. These are

the major structures responsible for cellular mobility. Membrane-microfilament interactions have also been reported in a wide variety of cells ((Bilej et al., 1989) and references within).

Intermediate filaments (IF), include five classes of proteins which share a common ultrastructure. They are insoluble cytoplasmic fibers of about 10 nm in diameter and they exist in all differentiated cells. Their function is still unclear, since there is no known specific inhibitor of their polymerization. However, this system is believed to be involved in a tissue- and cell-cycle specific interaction between the genetic apparatus and the outer environment of the cell. Since the IF network breaks down during mitosis, the expression of IF proteins is tissue-specific and IF-membrane associations have been found.

The role of the cytoskeletal network in the intramembrane and intracellular stages of endocytosis will be discussed in the following section (1.6.5).

I. 6.5 The endocytic process

The mechanism of endocytosis may be divided into four interrelated stages. Cross-linking of cell surface receptors via attachment of ligand (for non-constitutively endocytosed receptors), results in the formation of clathrin lattices on the plasma membrane. Growth of such lattices in turn results in the invagination of the "pit". Secondly, deeply invaginated coated pits are released into the cytoplasm following membrane scission. Thirdly, the coated vesicle is rapidly uncoated and is believed in

most cases to fuse with endosomes for initial sorting of receptors and ligands. Finally, released ligands may be delivered to lysosomes for degradation, other endocytic compartments for processing and presentation at the cell surface (antigen presenting cells), or they may be transported to storage granules for cell consumption. Similarly, released receptors may be targeted to lysosomes for degradation or may be re-routed to the cell surface to repeat the process.

I. 6.5.a Coated-pit formation and invagination

Following binding of the adaptor molecules to the receptor tails and clathrin triskelia (in an order that is as yet undetermined), the resulting complex diffuses in the membrane until it encounters other such complexes in a nucleation site for the formation of a new pit. Such assembly sites have already been found in sonicated fibroblasts (Moore et al., 1987). Triskelia then polymerize to form a planar array of hexagons. Pits are thought to invaginate by the conversion of hexagons to pentagons, with a possible heptagon intermediate (Heuser et al., 1980). The appearance of new coated pits and their scission to form coated vesicles (but not invagination), were shown to be dependent on ATP and cytosol addition using perforated A431 cells (Smythe et al., 1989). The use of stage-specific assays in the same system, tracing the fate of transferrin ligands, also showed that a combination of purified adaptors, cytosolic clathrin and unidentified cytosolic factors stimulate coated pit assembly and ligand sequestration into deeply invaginated coated pits, whereas their addition does not affect later endocytic events. In

a different system using isolated human fibroblast membranes attached to a solid substratum by their extracellular surface, addition of coat proteins extracted from purified bovine-coated vesicles resulted in the formation of new coated pits which were identical to the native ones (Mahaffey et al., 1989). This system also required the presence of cytoplasm as a source of clathrin (and possibly other cytoplasmic components), but in contrast to the previously described experiment, the addition of ATP was not required. Formation of coated pits in this system was also inhibited by low pH conditions. Recently, the requirement for GTP-binding proteins for the processes of coated pit assembly, invagination and coat vesicle budding was studied using stage-specific assays. In this study, which used perforated A431 cells, coated pit invagination and coated vesicle budding were both stimulated by the addition of GTP and inhibited by GDPβS (Carter et al., 1993). Furthermore, antagonists of G protein function such as GTPγS, AlF_4- and mastoparan inhibited late events involved in coated vesicle budding.

Involvement of GTP-binding proteins has also been addressed in a second class of transport vesicles, referred to as "nonclathrin-coated vesicles" or "COP-coated vesicles" (COP-CV) (Carter et al., 1993), which have been shown to mediate vesicular traffic along the exocytic pathway (Orci et al., 1986). The coat constituents of COP-CV include polypeptides of 160 (α-cop), 110 (β-cop), 98 (γ-cop) and 68 (δ-cop) kDa subunits, smaller subunits of 36 and 35 kDa (Maholtra et al., 1989) as well as ADP-ribosylation factor (ARF), a 20 kDa GTP-binding protein (Serafini et al., 1991). Both classes of coated pits assemble from a cytosolic pool of coat proteins. In contrast

however to clathrin and adaptors which exist as distinct soluble pools that appear to assemble sequentially to form clathrin-coated pits (Mahaffey et al., 1990; Smythe et al., 1992), COPs are present in the cytosol as a large multimeric precursor, termed a "coatomer" which presumably self-assembles onto membranes to form COP-coated pits. These COP-coated pits appear to be nonselective, mediating bulk-flow transport events, and although they were identified very recently, their mechanism of transport is probably better understood than the clathrin-mediated one (Schmid, 1992).

The process of invagination has proven more difficult to study due to the lack of specific inhibitors and the constitutive nature of endocytosis. However, it has been shown that invagination does not require ATP or cytosolic factors. Recently, the observation that endocytosis in mammalian cells is inhibited during mitosis (Fawcett, 1965), was used to demonstrate that coated pits are arrested during mitosis at all stages of invagination, suggesting the iterative action of one enzyme or a set of enzymes (Pypaert et al., 1987). Researchers now hope to identify these enzymes via a reconstruction of this inhibition on perforated cells using either mitotic cytosol or cyclin B-p34^{cdc2} kinase complex (cdc2 kinase)(Pypaert et al., 1991).

The release of a coated vesicle is mediated by an energy-dependent membrane scission event. The 100 kDa protein dynamin (van der Bliek et al., 1991), with GTPase activity and an *in vitro* microtubule sliding action, has been implicated in this event, using the temperature-sensitive *shibire* mutant of *Drosophila melanogaster* (Kosaka et al., 1983). After the pulling effect imposed by dynamin, it is possible that other cytosolic

factors may act as a 'collar' bringing the membrane bilayers together so that they can interact and pinch off (Smythe et al., 1991).

I. 6.5.b Intracellular endocytic events

Following membrane scission, the resulting coated vesicle is rapidly uncoated due to the action of an "uncoating ATPase", an enzyme which has the ability to release clathrin from isolated vesicles in an ATP-dependent manner (Schlossman et al., 1984), although it is not yet clear how it differentiates between coated vesicles and coated pits that are still membrane bound. Uncoating occurs in less than one minute after vesicle formation and requires the presence of clathrin light chains for the initial attachment of the enzyme. Uncoating ATPase exists in a 3:1 excess over cytosolic clathrin and has a higher affinity for lattice-bound clathrin, so that the enzyme rapidly dissociates from clathrin once the latter is released from the lattice (Schmid et al., 1985). Adaptor molecules are not removed during this initial phase of uncoating, but since they are known to recycle back to the plasma membrane, they are thought to become uncoated in a later, as yet unidentified stage.

Only a decade ago, vesicle fomation and the intracellular transport of entrapped materials were the focus of much speculation. The lack of markers to identify intracytoplasmic vesicles and their composition led to speculation on both the fate of clathrin after invagination of the coated pit and the formation of further vesicles. Contrary to popular scientific belief at the time, favouring the pinching off of coated pits

to form coated vesicles, Pastan and Willingham (Pastan et al., 1983) raised doubts as to the recycling of clathrin based on the inability of current work to show any appreciable amounts of soluble clathrin and the inability of anti-clathrin antibodies to trap and precipitate clathrin in the cytosol. Furthermore they suggested that coated pits are stable structures always associated with the cell surface and that "receptosomes" (a term which has been used alternatively to early endosomes), form directly from coated pits by pinching off from the region adjacent to coated pits, into which the receptor ligand complexes somehow slide. However, the almost parallel demonstration of the existence of endocytic coated vesicles as independent organelles (Petersen et al., 1983), defied this hypothesis.

During the same year Helenius et al. (Helenius et al., 1983) put forth two alternative possibilities for the transport of endocytosed material through different endosomal and lysosomal compartments. The **maturation model,** suggested that the endosomes are transient structures which undergo maturation as they continually carry the dissociated ligand, until they deliver it by fusion with a lysosome. Conversely, the **vesicle shuttle model**, suggested that endosomes are stable structures through which the internalized material passes by fusion of incoming vesicle and budding of leaving vesicles. Evidence favouring the vesicle shuttle model was produced by labelling endosomal proteins of highly purified early and late endosomes and showing that there were no significant levels of proteins common to both plasma membrane and late endosomes (Schmid et al., 1988), which should have been the case if endosomes were

derived from the plasma membrane. Conversely, there was a subset of proteins common to both early and late endosomes, which suggests that late endosomes may arise from early ones (Gruenberg et al., 1989). Presently, the endocytic network for the transport of material internalized through RME has been shown to consist of the following set of vesicles.

Early endosomes are tubulo-vesicular structures with vesicular structures of 0.4 - 1 μm and tubular projections of several micrometres. Kinetically they are defined as the first compartment to be reached by endocytic tracers. Similar to all other endosomal-lysosomal compartments, they retain an acidic environment generated by means of an ATP-driven proton pump (Galloway et al., 1983). In the case of early endosomes this acidification is modulated by a Na^+, K^+-ATPase which reduces the degree of acidification relative to late endosomes and lysosomes. This early endosomal pH between 5-6, seems to be optimal in promoting dissociation of a large number of ligand/receptor complexes, following which receptors are recycled back to the cell surface.

The process by which segregation of receptors from ligands is accomplished is not clear. Earlier immunolocalization studies on the separation of asialoglycoprotein from its receptor, demonstrated that receptors were clustered into tubular elements away from the vacuolar portions where the ligand remains (Geuze et al., 1983). This gave rise to the speculation that segregation may involve either interaction of receptor tails with cytoskeletal elements or aggregation of receptors.

Fusion between endosomal compartments has been monitored by measuring the

ability of ligands to fuse with another population of vesicles after being internalized for various intervals. It was found that only those populations internalized for short time periods retained the ability to fuse (Diaz et al., 1989). It was not clear however, whether the loss of fusogenic ability was due to maturation of an early endosomal compartment or to transport to later endosomal vesicles which don't have this ability. Although some evidence to support the second possibility was produced (Gruenberg et al., 1989), it was recently shown that delivery of ligands from early (sorting) to late endosomes occurs by maturation of sorting endosomes (Dunn et al., 1992). In this study, the properties of individual endosomes were examined by digital image analysis, to distinguish between the two mechanisms for entry of ligands into late endosomes. Using LDL as a marker it was shown that a pulse of fluorescent LDL is retained by individual sorting endosomes and that, with time, sorting endosomes lose their ability to fuse with primary endocytic vesicles. These data were consistent with a maturation mechanism where the sorting endosome retains and accumulates lysosomally directed ligands until it loses its ability to fuse with newly formed endocytic vesicles and matures into a late endosome. A specific early endosomal marker, the *rab5* protein which is a member of the *rab* family of GTP binding proteins, has recently been identified (Chavrier et al., 1990). Another member of this family, the *rab7* protein, has also been identified as a marker for late endosomes.

Late endosomes are mostly located in the perinuclear region of the cell. They are large spherical structures that are frequently found to have deep membrane invaginations

giving rise to multivesicular bodies. As with early endosomes, late endosome-lysosome interactions have been reconstituted in cell-free systems, such as the one using rat liver late endosomes interacting with lysosomes when incubated at 37°C in the presence of cytosol and an ATP generating system (Mullock et al., 1989). Their major function seems to involve the transport of ligand to the lysosomal compartment (Schmid et al., 1988), presumably using the microtubular network, since nocodazole, a microtubule-disrupting agent, inhibited the transport of a marker molecule from an endosomal compartment to pre-lysosomes (Gruenberg et al., 1989).

Pre-lysosomes differ from late endosomes in that they are rich in acid hydrolases. This compartment was distinguished from the endosomal compartment by virtue of the fact that it was not labelled by endocytic tracers in cells held below 20°C, a temperature at which the transfer of materials from endosomes to lysosomes is inhibited (Helenius et al., 1983). Morphologically, pre-lysosomes have been shown to have deep membrane invaginations in normal rat kidney (NRK) cells (Griffiths et al., 1988) and it was postulated that this structure may represent the site of overlap of the endocytic and biosynthetic pathways where newly synthesized lysosomal enzymes are delivered. The receptor for mannose-6-phosphate (which is involved in the transport of newly synthesized lysosomal enzymes) is present in pre-lysosomes and it provides a way of distinguishing this compartment from lysosomes which do not carry this receptor (Kornfeld et al., 1989).

Lysosomes are dense organelles found in the perinuclear area. They have a pH

of about 4.5 and are rich in acid hydrolases. They are the last compartment reached by internalized material in the endocytic pathway and play a digestive role. Endosomes are known to move along microtubular "tracks", and lysosomal saltatory movements are similarly correlated with the integrity of the microtubular system, as shown in indirect immunofluorescence experiments with cultured fibroblasts (Collot et al., 1984).

I. 6.5.c Inhibitors of endocytosis

In view of the extensive and varied network involved in the endocytic process, it is not surprising that inhibitory agents can block this process at practically every stage. Inhibitory agents can act in a specific way to block transport through particular compartments, or they can act in a general fashion, for example by abolishing energy requirements, necessary at most internalization stages. Indeed, endocytic inhibition has been shown to occur at the level of the membrane, elements of the cytoskeletal network, endosomal and lysosomal compartments, as well as the Golgi network. Although the particulars on the different types of inhibition are beyond the scope of this thesis, a brief account will be given on the most important types of inhibitors and their level of action.

Several processes can be specifically inhibited at the membrane level. Primary amines and transglutaminases have been shown to block clustering of ligands (Pastan et al., 1981; Davies et al., 1980). Inhibition of clathrin-lattice formation or budding of clathrin-coated vesicles has been achieved through a variety of methods affecting cellular potassium levels and intracellular pH. Hypotonic shock and potassium depletion were

shown to reduce viral endocytosis in Hep2 cells (Madshus et al., 1987), whereas cytosol acidification by several methods, such as pre-pulsing with ammonium chloride, addition of small amounts of acetic acid and addition of ionophores such as nigericin and valinomycin, was shown to inhibit endocytosis via coated pits (Sandvig et al., 1988), as well as transport from the trans-Golgi network to the cell surface (Cosson et al., 1989). Cytoplasmic acidification affects clathrin lattice morphology by promoting the formation of unusually spherical lattices with empty microcages around their edges. Hypotonic media and retention of a neutral pH with nigericin or cell sticking on culture dish surfaces with polylysine produces a preponderance of unusually flat lattices, whereas potassium depletion and hypertonicity seem to cause surface pits to disappear with cytoplasmic clathrin precipitates into unusually small microcages (Heuser, 1989; Heuser et al., 1989). Using immunofluorescence microscopy it was recently demonstrated that cytosol acidification does not affect clathrin or adaptor molecules but interferes with budding of clathrin-coated vesicles from the plasma membrane as well as from the trans-Golgi network. Conversely, potassium depletion and exposure to hypertonic media remove membrane-associated clathrin lattices by preventing clathrin-adaptor interactions (Hansen et al., 1993).

As previously mentioned, the cytoskeletal network affects various components of the endocytic mechanism, and several agents which disrupt cytoskeletal functions have an inhibitory effect on endocytosis, mostly by disrupting intracellular trafficking. Colchicine, a drug isolated from *Colchicum autumnale*, inhibits endocytosis by binding

to the α-tubulin subunit thus inhibiting microtubule polymerization. Other drugs with similar antimicrotubule properties include nocodazole, podophyllotoxin, rotenon, steganacin and the vinca alkaloids vincristine, vinblastine and vindesine (strangely, another alkaloid, taxol has a microtubule stabilizing effect) (Bilej et al., 1989). In contrast to the variety of antimicrotubule agents, the only agents known to disrupt microfilaments belong to a class of fungal metabolites, the cytochalasins. Cytochalasin B reduces the rate of actin polymerization by inhibiting monomer addition to the barbed end of actin filaments (Bilej et al., 1989).

On the intracellular level, transfer of internalized ligands from endosomes to lysosomes is reportedly delayed by carboxylic ionophores such as monensin and nigericin (Manske et al., 1989; Tartakoff, 1983). Ligand degradation can be inhibited by neutralizing intralysosomal pH using both carboxylic ionophores (Carriere et al., 1985) and lysosomotropic amines such as chloroquine, ammonium chloride and amantadine (Ziegler et al., 1982; Krogstad et al., 1987), or by inhibiting lysosomal enzymes such as leupeptin (Chang et al., 1984; Press et al., 1990).

In systems such as the asialoglycoprotein receptor one, where coated pit receptor-mediated endocytosis is believed to be modulated by the activity of protein kinases and protein phosphorylation, protein kinase inhibitors such as staurosporine were found to be potent and rapid inhibitors of receptor trafficking by a yet unknown mechanism, which was found to be independent of the inhibition of protein kinase C (Fallon et al., 1992). Inhibitors of endocytosis have recently been used to dissect the endocytic process on the

cell surface of polarized epithelial cells (Gottlieb et al., 1993). Using cytochalasin D the specific inhibitor of actin microfilaments, it was possible to show that these microfilaments play a critical endocytic role in the apical but not the basolateral surface of these cells. Similarly, receptor-mediated phospholipase C activation in cultured rat proximal tubule cells, was shown to require cytoskeleton-dependent endocytosis for apical but not basolateral surfaces.

Finally, some evidence pointing to possible similarities between the endocytic and biosynthetic pathways, was produced by a comparison of the action of the fungal antibiotic brefeldin A (BFA) on the Golgi network and on endosomes (Hunziker et al., 1992; Low et al., 1992). BFA inhibits protein secretion and causes the breakdown of the Golgi network presumably by dissociating a non-clathrin cytoplasmic coat from Golgi membranes through the redistribution of at least three components of the coat material, namely β-COP, ADP ribosylation factor and γ-adaptin from the Golgi membrane to the cytosol. Both β-COP and γ-adaptin share significant homology with the β-subunit of the clathrin adaptor complex of endocytic coated vesicles and BFA has been found to significantly increase the tubular appearance of the early endosomal compartment involved in the recycling of internalized transferrin receptors. However BFA does not markedly affect internalization, recycling or delivery of internalized ligands from early endosomes to degradative endocytic compartments. Currently the role of BFA in the exocytic and endocytic pathway is an area of intense interest.

I. 7 Antibody Internalization

Initial interest in the ability of antibodies to be internalized was generated following the observation that antibodies can induce the disappearance of membrane antigens from the cell surface using immunofluorescence techniques (Chatenoud et al., 1984). This phenomenon of **antigenic modulation** was first described in 1963 (Boyse et al., 1963), when it was found that thymic leukaemia cells expressing the TL antigen lost their sensitivity to the complement-mediated cytotoxic action of anti-TL antibodies when they had been previously exposed to this antibody.

A decade ago the study of antibody-induced antigenic modulation had progressed to the point of awareness of processes taking place on the cell membrane following exposure to antibodies against surface immunoglobulins and a limited number of other surface antigens such as T-cell differentiation antigens (mostly those already identified as leukaemic markers) (Chatenoud et al., 1984 and references within). The stages identified following exposure to anti-Ig antibodies included:

a) **patching**, or the clustering of surface Ig into "patches" on the membrane within 1-2 minutes after exposure, a process which was energy independent, inhibited by low temperatures and influenced by valency of the ligand;

b) **capping**, or the concentration of patches at a cell pole (the uropod) over the Golgi region within 3-4 minutes, a process which is energy dependent and thus inhibited by inhibitors of the respiratory chain (eg. sodium azide and dinitrophenol) or of glycolysis (eg. fluoride, iodoacetamide) and,

c) **endocytosis** (and subsequent degradation) of surface Ig which is again energy dependent. Cytoskeletal elements were found to be involved in the lateral mobility of cellular determinants as well as capping although there have been contradictory results with inhibitors of both microtubules and microfilaments, some of which seem to inhibit while others seem to promote capping.

The two first stages (patching and capping), were identified by immunofluorescence, whereas endocytosis was identified by loss of anti-Ig induced cytotoxicity and could not be effectively differentiated from loss of surface markers by shedding. Furthermore, the same process applied to the modulation of the other surface antigens studied, except for the stage of polarized capping, since other surface antigens did not exhibit a preferential membrane capping region. Also, other antigens exhibited variable degrees of endocytosis. It was also observed that antibody fragments do not generally induce antigenic modulation whereas antibody crosslinking (eg via the use of a second antibody), enhances the process. The type of cell and antigen involved in this type of endocytosis was found to have a profound effect on the process, since it was noted that histocompatibility antigens internalize following this mechanism only in non-lymphoid cells (eg fibroblasts), and then only after extensive cross-linking (Pernis, 1985). During the same period the effects of antigenic modulation were confirmed in the therapeutic setting, when antigenic modulation observed with indirect immunofluorescence, was induced by the murine T101 antibody (recognizing the T65 antigen on normal and neoplastic human T cells), on cells of patients with chronic

lymphocytic leukemia and T cell lymphoma (Shawler et al., 1984).

With more extensive studies on the use of monoclonal antibodies as mediators of more specific anti-cancer action (either as carriers of cytotoxic agents or as mobilizers of components of the host's immune system), it became increasingly apparent that both their ability to induce antigenic modulation and to internalize had profound effects on their use in targeting (see discussion). Aspects of antigenic modulation have been widely studied (as discussed in the context of immunotargeting, section 1.5.4.b), however intense interest in the internalizing ability of the antibody, irrespective of the induction of antigenic modulation has only developed since the mid 1980's. Presently, the internalizing potential of the antibody(ies) used in various immunotargeting models is an area of increasing interest. The potential for, and characteristics of, antibody internalization, is of particular importance in cases where the antigen being targeted is not well characterized in terms of endocytic potential, as is the case with CEA, which serves as the target antigen throughout this project.

I. 8 CEA structure and anticipated function

An extensive amount of literature is devoted to CEA, but despite the fact that this antigen has been well characterized, there has been no evidence for the triggering of a ligand-induced internalization mechanism as is the case with a number of other receptors discussed previously.

As previously mentioned (section I.4.2), CEA is the primary tumour marker

associated with colorectal cancer, the second most lethal neoplastic disease in North America. Its primary clinical use is in monitoring response to therapy and tumour reccurence, and CEA serum levels are followed by most surgeons prospectively after a potentially curative resection (Woolfson, 1991; La Rocca et al., 1992; Tabuchi et al., 1992). This tumour marker is also extensively used for early detection of other tumours of epithelial origin including lung, breast and ovarian carcinoma (Kim et al., 1992), and more recently as a prognostic marker in HIV-related pneumonias (Bédos et al., 1992), in addition to its role as a target antigen for immunotargeting.

The CEA gene family belongs to the immunoglobulin supergene family due to the fact that the main backbone of all CEA-related molecules is made up of a variable number of immunoglobulin-like domains. This gene family can be divided into two main subgroups based on sequence comparisons, namely the CEA subgroup, containing the genes encoding the membrane-anchored molecules CEA, the CEA cross-reacting antigens (such as NCA) and the biliary glycoprotein 1 (BGP1), and the PSG subgroup containing the genes encoding the pregnancy specific glycoproteins which are secreted (Thompson et al., 1991 and references within).

In humans the CEA gene family is clustered on the long arm of chromosome 19 and consists of approximately 20 genes. The general structure of the human CEA subgroup (Thompson et al., 1991; Hefta et al., 1992), consists of a 34 aminoacid signal peptide (leader), which is used to direct nascent proteins to the plasma membrane and is cleaved in the mature protein. The leader peptide is followed by one N-terminal

domain (108-110 aminoacids long) whose secondary structure reveals homology to immunoglobulin-like variable domains. This domain is followed by immunoglobulin-like constant domains of 92-96 amino acids (A domains) or 86 amino acids (B domains), the number of which is highly variable among CEA-like molecules. Finally, nascent CEA-like peptides contain a hydrophobic membrane domain which is replaced by a glycosyl phosphatidylinositol moiety post-translationally, serving as a membrane anchor.

Although the relevance of GPI anchorage (Cross, 1990) is still unknown, GPI-anchored proteins reveal a more rapid lateral mobility on membranes than integral membrane proteins (Cross, 1990), with probable functional consequences, and they also seem to mediate a selective sorting mechanism, since they are more often found to be located toward the apical than the basal region in polarized cells (Lisanti et al., 1990). Conversely BGP-like peptides contain a hydrophobic transmembrane domain which is followed in most cases by a cytoplasmic domain.

CEA family members are heavily glycosylated (60% glycosylation in mature CEA). All of the 28 potential glycosylation sites in CEA are thought to be glycosylated with average carbohydrate chains of about 20 monosaccharide residues, considering the molecular weight of CEA (180 kDa). However, there is a large degree of variability in oligosaccharide structures of CEA molecules depending on their source, resulting from post-translational modifications. Through sequence comparisons to the immunoglobulin superfamily it was anticipated that CEA-like molecules would serve as cell adhesion molecules (Thompson et al., 1991). Indeed, it has been extensively demonstrated that

CEA mediates homotypic Ca^{2+}-independent cell aggregation (Benchimol et al., 1989). This function of CEA as an intercellular adhesion molecule, is dependent on its N-domain since N-domain deletion mutants lose their adhesion properties (Eidelman et al., 1993). Recently the third internal repeat domain of CEA was shown to be essential for CEA mediated cell adhesion (Zhou et al., 1993). The model derived from this study proposed interactions between immunoglobulin supergene family members in which especially strong binding seems to be effected by double reciprocal interactions between the V-like domains and C-like domains of antiparallel CEA molecules on apposing cell surfaces. Furthermore, expression of functional CEA on the cell surface blocks terminal differentiation and maintains cell proliferative potential (Eidelman et al., 1993). Generally the high expression of this cell adhesion molecule in embryonic tissue is essential for intercellular interactions involved in organogenesis (Thompson et al., 1991), whereas its elevated expression in malignancy is believed to play an important role in the creation of metastases, especially to the liver, by increasing hepatic retention of tumour cells via binding onto specific Kuppfer cell receptors (Thomas et al., 1990; Jessup et al., 1993). The role of CEA in the maintainance of proliferative potential of malignant cells, as well as its relation to the reversion of adult epithelial architecture to an embryonic (multilayered) one, is currently being investigated (Eidelman et al., 1993).

I. 9 Aims of this project

A number of studies clearly showed that different antigen-antibody systems exhibit considerable quantitative and qualitative differences in internalization processes. Studies examining unconjugated antibodies targeting different antigens expressed on malignant cells, revealed that those antibodies which internalize, do so at considerably variable rates (Press et al., 1989; Wang et al., 1987; Sutherland et al., 1987; Aboud-Pirak et al., 1988; Matzku et al., 1987; Matzku et al., 1988b; Matzku et al., 1988a; Matzku et al., 1990; Matzku et al., 1986; Della Torre et al., 1987). However, those of the studies performed in an immunological context, were mostly focused on the functional outcome rather than the mechanics of endocytosis and did not emphasize dissection of the process and understanding the differences which would account for such variabilities. In general, very similar techniques were used in studies of internalization and often a number of indirect techniques inadvertantly incorporated artifacts which were not accounted for, or alternatively disregarded the biochemistry of ligand-receptor interactions. Thus these techniques started from a basis of inaccurate assumptions (see section III.2 and Tsaltas et al., 1993).

In view of the foregoing the aims of this project were to:

1) **examine whether the anti-CEA monoclonal antibody used in a CEA-targeting model system, is internalized by CEA-expressing human cancer cell lines.** This basic information on the model system is necessary in order to optimize the effect of anti-CEA immunoconjugates being produced in this laboratory.

2) **determine an accurate and efficient way to study antibody internalization**, and in so doing **compare** the accuracy and efficiency of some of the **most established methods** (past and current) used in similar studies.

3) **use inhibitors of endocytosis, to study the CEA endocytic mechanism at the cell surface level.**

The model used throughout this project, consists of a CEA anti-CEA targeting system, along with CEA-expressing human cancer cell lines. The monoclonal anti-CEA antibody studied (11-285-14) has been well characterized previously (Ford et al, 1987a) and has been extensively evaluated for immunotargeting *in vitro* (Allum et al., 1986b; Gatter et al., 1982), *in vivo* (Pimm et al., 1985; Macdonald et al., 1986; Rowland et al., 1984; Casson et al., 1987; Richardson et al., 1989), and clinically (Hockey et al., 1984; Allum et al., 1986a). This antibody is produced and purified in the Oncology Research laboratory and it has already been used to produce vindesine immunoconjugates which have been extensively evaluated (Ford et al., 1987a; Casson et al., 1987; Ford et al., 1990). Doxorubicin-11-285-14 immunoconjugates have also been produced and characterized in this laboratory (Richardson et al., 1989), while further such conjugates are currently being produced here, using alternative conjugation methods.

CHAPTER II

MATERIALS AND METHODS

II. 1.0 Cell lines

Cancer cell lines used in this study were of human origin and were propagated in culture in the Oncology Research Laboratory. The human lung cancer line BENN was obtained from Dr. M. Ellison, Ludwig Institute for Cancer Research (Sutton, U.K.) and the other lines were obtained from the American Type Culture Collection (Rockville, Maryland, U.S.A.).

Cell lines utilised and their source	
Cell line	**Source**
LS174T	Human Colon Adenocarcinoma
SKCO1	Human Colon Adenocarcinoma
COLO320DM	Human Colon Adenocarcinoma
BENN	Human Lung Carcinoma
11-285-14	Murine hybridoma
P3-X63-Ag8	Murine Myeloma

The above cell lines have been extensively characterized in terms of CEA expression in the same laboratory,using three monoclonal antibodies which recognize different epitopes of the CEA molecule (Ford et al., 1987a). Cell lines were aliquoted in 1.8 ml NUNC cryotubes (Inter Med, Rocksilde, Denmark) at 4-6 x 10^6 cells per sample and were kept frozen in liquid nitrogen cannisters (Minnesota Valley Engineering Inc., New Prague, MIN.). Cell growth was supported by the media and supplements listed in table 3.

Table 3: Cell Culture Media *		
Cell line	**Medium**	**Supplements**
LS174T	Minimum essential medium (500 ml)	50ml fetal calf serum (8.8%) 6 ml glutamine 6 ml non essential aminoacids 12 ml penicillin-streptomycin
SKCO1	Minimum essential medium (500 ml)	50ml fetal calf serum (8.8%) 6 ml glutamine 6 ml non essential aminoacids 12 ml penicillin-streptomycin
COLO320DM	RPMI-1640 (500 ml)	50ml fetal calf serum (8.8%) 6 ml glutamine 12 ml penicillin-streptomycin
BENN	M199 (250 ml) Dulbecco's (250 ml)	50ml fetal calf serum (8.8%) 6 ml glutamine 12 ml penicillin-streptomycin
11-285-14	RPMI-1640 (500 ml)	50ml fetal calf serum (8.8%) 6 ml glutamine 12 ml penicillin-streptomycin
P3-X63-Ag8	Dulbecco's modification of Eagle's medium	50ml fetal calf serum (8.8%) 6 ml glutamine 12 ml penicillin-sreptomycin 4.5 g/lit glucose

* All media were supplied by either Flow laboratories (McLean, Virginia, U.S.A.), or Gibco laboratories (Life Technologies Inc., Grand Island, N.Y., U.S.A.).

II. 1.1 Cell Culture Maintenance

The cell lines were grown as monolayers in 75 cm^2 perspex tissue culture flasks (Falcon, Becton-Dickinson), and maintained in a 5 % carbon dioxide atmosphere at 37°C, in a humidified incubator. Cell growth was monitored by viewing the monolayer under an inverted viewing phase microscope (Diavert, Leitz). Fresh medium was supplied as needed to support optimal cell growth. On average, 25-30 ml of medium were added twice per week depending on cell growth. Cells were always handled under sterile conditions, using a laminar air flow enclosure (Contamination Control Inc.), sterile equipment and standard techniques such as flaming the containers and equipment and avoiding their contact with work surfaces.

II. 1.2 Trypsinization

When cells in the tissue culture flasks became confluent the cell monolayer was detached from the bottom of the flask by trypsinization and a cell suspension was prepared. A small cell sample was then removed in order to perform a count of the number of viable cells in the suspension.

II. 1.2 (a) Materials

(1) Phosphate buffered saline (PBS) pH 7.2, 0.15 M.

PBS tablets (Oxoid Ltd.,Hampshire, England), were dissolved in distilled water as per manufacturer's instructions and sterilized by autoclaving.

(2) Trypsin-EDTA (Gibco); 1:10 dilution in PBS.

II. 1.2 (b) Method

(1) Medium in culture flasks was poured off under sterile conditions and discarded.

(2) The cell monolayer was washed twice with approximately 20 ml PBS and the PBS was then discarded.

(3) 5.0 ml trypsin-EDTA/PBS solution were added to the flask, which was then incubated for 10 min at 37°C.

(4) The trypsin solution containing the cells was then poured into a 15 ml sterile centrifuge tube (Falcon) to which approximately 5-6 ml PBS were added in order to wash off any remaining cells from the flask.

(5) The cells were then centrifuged for 5 min at 200 x g (IECHN-SII centrifuge, Damon/IEC).

(6) The supernatant was discarded and the cell pellet was resuspended by tapping the tip of the tube.

(7) 10 ml of the appropriate medium was then added to the cells and a cell count was performed.

II. 1.3 Cell Count

The cell concentration in the suspension was calculated using a Neubauer haemocytometer. Cell viability was determined by viewing the cells under a fluorescence

microscope after staining them using the dyes acridine orange (which stains viable cells brilliant green) and ethidium bromide (which stains dead cells brown).

II. 1.3 (a) Materials

(1) Haemocytometer (Neubauer) with cover slip.

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

(3) Ethidium Bromide, EB (#E-8751, Sigma Chemical Co.).

AO and EB used as a 0.001% solution and mixed together, were stored in 1 ml aliquots at -20°C.

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

II. 1.3 (b) Method

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

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

II. 1.4 Cryopreservation of cells

II. 1.4 (a) Materials

Medium for freezing cells:

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

II. 1.4 (b) Method

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

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

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

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

(5) The vials were transferred in a bucket of ice to the freezer and stored at -70°C.

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

II. 1.5 Thawing cells

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

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

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

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

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

(6) The supernatant was discarded.

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

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

II. 2.0 Antibodies

The test antibody used throughout this project was a protein-A purified murine anti-CEA monoclonal IgG1 (Corvalan et al., 1984). This antibody which is identified by the code number 11-285-14 has been extensively evaluated for immunotargeting *in vitro* (Allum et al., 1986b; Gatter et al., 1982), *in vivo* (Pimm et al., 1985; Macdonald et al., 1986; Rowland et al., 1984; Casson et al., 1987; Richardson et al., 1989), and

clinically (Hockey et al., 1984; Allum et al., 1986a). Purified ascitic fluid from the P3-X63-Ag8 mouse myeloma (American Type Culture Collection), was used as a control. P3-X63-Ag8 myeloma secretes an IgG1 (γ,κ) of unknown binding specificity (the code identifying this antibody is Ag8).

The control ascites was a commercial product purchased from Bethesda Research Laboratories (Life Technologies Inc., Gaithersburg, Maryland). The ascites fluid was produced by injecting MOP2-21 plasmocytoma cells into BALB/c mice. This fluid contained IgG immunoglobulin, as well as all the other substances normally induced during the inflammatory response to the growth of plasmacytoma cells. Control ascites fluid shows low reactivity in an ELISA, making it a suitable control for immunological tests in which other ascites fluids are being used, notably, hybridoma testing. Control ascites is supplied in lyophilized form in three vials, each containing 0.5 ml of lyophilized ascites.

Certain assays required that antibodies be iodinated (^{125}I) or conjugated to an enzyme (horseradish peroxidase or HRP) or a fluorochrome (fluorescein). The secondary antibody used in most indirect assays was rabbit-anti-mouse immunoglobulin conjugated to horseradish peroxidase (RAM-HRP, Dako Corporation, Santa Barbara, CA, U.S.A), whereas some radiolabelling assays required the iodination of rabbit-anti-mouse immunoglobulins (Dako Corporation).

II. 2.1 Purification of antibodies

II. 2.1 (a) Materials

(1) **0.5 M Phosphate buffer pH 8.0.**

Sodium di-hydrogen phosphate/hydrate ($NaH_2PO_4H_2O$, BDH Chemicals Ltd, Toronto) 69.0 g/l.

Di-sodium hydrogen phosphate, anhydrous (Na_2HPO_4, Mallinckrodt Inc., Paris, Kentucky, U.S.A) 71.0 g/l.

Prepare stock solutions of each salt in water and add 0.01% sodium azide (BDH) as a preservative. Store at room temperature.

Mix the two solutions to obtain the required pH using a pH meter and then adjust to the desired molarity.

(2) **0.1 M Citrate Acid buffer pH 3.5 and pH 6.0**

Citric acid ($C_6H_8P_7.1H_2O$, BDH) 0.1 M (21.01 g/l)

Di-sodium hydrogen phosphate (Na_2HPO_4, Mallinckrodt Inc.) 0.1 M (14.2 g/l).

For pH 6.0 titrate phosphate with citric acid; for pH 3.5 titrate citric acid with phosphate.

(3) Protein A-Sepharose C1-4B Affinity Gel (Pharmacia, Uppsala, Sweden).

The freeze-dried powder was swollen in the 0.1 M sodium phosphate pH 8.0 buffer and washed on a 15 ml sintered glass filter funnel. This gel was poured into a Pharmacia C16/20 column with one adaptor at the base and a conical RC16 packing reservoir. Gel volume was approximately 50 ml and the reservoir held

approximately 100 ml.

(4) Spectrophotometer.

(5) Magnetic stirrer.

(6) pH meter.

(7) 4°C cooling chamber.

(8) Pharmacia programmable fraction collector FRAC-300 with a peristaltic pump. The pump was set at 5.0 and the tubing internal diameter was 2.1 mm. This delivers a flow rate of 1 drop / 5 sec.

(9) 1.0 M Tris-hydrochloric acid buffer pH 9.0

Tris (hydroxymethyl) aminomethane (Sigma) 1 M (1.21 g/l)

Hydrochloric acid 1 M, pH 9.0.

25 ml of 1 M tris was mixed with 2.5 ml 1 M HCl.

(10) Amicon concentrator with PM10 filter membrane (Amicon Corporation, Lexington, Mass.).

(11) Dialysis tubing (Spectrum Medical Industries Inc., Los Angeles, CA., U.S.A.).

(12) Phosphate buffered saline pH 7.2.

(13) Millipore filters 0.8, 0.45 and 0.22 μm (Millipore Corp., Bedford, Mass.).

II. 2.1 (b) Method

(1) Clots were removed from the ascitic fluid, which was clarified by centrifugation followed by filtration through millipore filters, starting with 0.8 then through 0.45

and finally 0.22 μm.

(2) The clarified fluid was transferred to a graduated cylinder and 3 volumes of 0.1 M phosphate buffer pH 8.0 were added with magnetic stirring; the pH was adjusted to 8.1 with 1.0 M tris at room temperature and the fluid was stored at -20°C in approximately 100 ml aliquots. Aliquots were eventually thawed to 4°C to run through the column.

(3) The Protein A column was equilibrated with phosphate buffer pH 8.0 at 4°C. One aliquot (100 ml) of diluted fluid was added to the conical reservoir - the fraction collector was run for 250 min. All material was allowed to run into the gel.

(4) The unadsorbed fraction was collected, labelled and stored at -20°C to be put down the column again.

(5) 100 ml of 0.1 M phosphate buffer pH 8.0 was added with the fraction collector set for 250 min. This material was collected, labelled wash #1 and stored at -20°C for recycling.

(6) The column was flushed with pH 8.0 phosphate buffer by filling the conical reservoir and a 1 litre plastic bottle with buffer and making sure the system was air tight. The fraction collector was set to 999 min. The O.D. was checked at 280 nm for about 10 ml of eluate after about 600 ml had washed through the column. When O.D. was less than 0.04 the Ig was eluted.

(7) Bound IgG_1 was eluted with 0.1 M citrate/phosphate buffer pH 6.0

(approximately 100 ml). The fraction collector was set to 250 min.

About 0.5 ml Tris pH 9.0 was added to the collecting pot to adjust the pH to 7.0.

This material was labelled pH6 ELUTED and stored at -20°C.

(8) Remaining Ig was eluted from the gel with 100 ml of 0.1 M citrate buffer pH 3.5.

(9) The column was re-equilibrated with phosphate buffer pH 8.0.

(10) All eluted pH 6.0 material was pooled and concentrated using the Amicon.

(11) The concentrate was dialysed against 3 changes of PBS to remove the azide.

(12) It was then filtered through a 0.22 μm millipore filter and the optical density was read at 280 nm.

The protein concentration was calculated using the formula:

$$\frac{\text{O.D.}}{\text{E.C.}} \times \text{dil} = \frac{\text{O.D.}}{14.3} \times \text{dil}$$

$E.C._{1cm}^{1\%}$ (extinction coefficient) = 14.3 for IgG

II. 2.2 Conjugation of antibodies to HRP

The conjugation of antibodies to HRP was performed according to the method of Wilson and Nakane (Wilson et al., 1978). The application of enzymes as markers for antibodies was introduced in 1966 by Nakane and Pierce (Nakane et al., 1966). Since then the coupling of HRP to antibodies has been achieved using a variety of one-step or two-step methods. The one-step method involves mixing the antibody, HRP and the

coupling agent, following which the reaction either stops spontaneously or is terminated by an agent. The two-step method, which is more controlled and provides a more homogeneous conjugate, involves activating the enzyme or the antibody with a coupling agent. The excess coupling agent is then removed, the other reactant is added to the activated protein and the reaction is allowed to proceed. The coupling agents usually have two active groups, one coupling to the protein, the other coupling to the second reactant.

Both the 11-285-14 and Ag8 antibodies were conjugated to HRP using sodium periodate as a coupling agent. This agent oxidizes the sugar moieties on the polysaccharide shell of HRP so that aldehyde groups are introduced. The HRP-aldehyde then reacts with the amino groups of the antibody thus forming the HRP conjugate.

II. 2.2 (a) Materials

(1) Horseradish peroxidase (HRP), RZ=3 (Sigma).

(2) Sodium periodate 0.1 M ($NaIO_4$, Analar, BDH Ltd, Poole, England).
2.14 g $NaIO_4$ in 100 ml distilled water.

(3) Sodium acetate buffer 0.001 M, pH 4.4 (Fisher, New Jersey, U.S.A)
0.136 g sodium acetate in 1 litre distilled water.

(4) Sodium carbonate (Na_2CO_3, Fisher).

(5) Sodium bicarbonate ($NaHCO_3$, Fisher).

(6) Sodium carbonate-bicarbonate buffer 0.2 M, pH 9.5.
2.12 g Na_2CO_3 dissolved in 100 ml distilled water.

1.68 g $NaHCO_3$ dissolved in 100 ml distilled water.

Na_2CO_3 solution was added to $NaHCO_3$ solution at a ratio of approximately 1:3 until the pH was raised to 9.5.

(7) Sodium carbonate-bicarbonate buffer 0.01 M, pH 9.5.

Prepared by further diluting (6).

(8) 11-285-14 or Ag8 antibodies.

(9) Freshly prepared sodium borohydride ($NaBH_4$, Baker, New Jersey, U.S.A.); 4 mg/ml distilled water.

(10) Sephadex G200 superfine (Pharmacia).

To prepare a 100 cm x 2 cm column (approximately 40 ml of material), 2 g Sephadex were allowed to swell in distilled water for 48 hours at 4°C. The column was then packed using a peristaltic pump at 25 ml/hour.

II. 2.2 (b) Method

(1) 4 mg HRP were dissolved in 1 ml distilled water.

(2) 200 μl of 0.1 M $NaIO_4$ were added and mixed gently for 20 minutes.

(3) Reactants were dialysed in 0.001 M sodium acetate pH 4.4 at 4°C for 20 hours to remove any excess sodium periodate.

(4) The pH was then raised to 9.5 through the addition of 20 μl of 0.2 M sodium carbonate-bicarbonate buffer pH 9.5.

(5) 8 mg antibody dissolved in 1 ml of 0.01 M sodium carbonate-bicarbonate buffer

pH 9.5 were immediately added, the solution was mixed gently and the coupling reaction was allowed to proceed for 2 hours at room temperature.

(6) The reaction was terminated by the addition of 0.1 ml fresh sodium borohydride. The solution was again left to stand for 2 hours at 4°C.

(7) The conjugate was chromatographed using a Sephadex G200 superfine gel bed at an approximate flow rate of 4 ml/cm^2/ hr. Forty 2 ml fractions were collected and read on a spectrophotometer at wavelengths of 280 nm (antibody detection) and 403 nm (enzyme detection) and the conjugate was pooled and stored in 1 ml aliquots at 4°C.

II. 2.3 Iodination of antibodies

Both 11-285-14 and RAM antibodies were radiolabelled with ^{125}Iodine for use in two different internalization assays. The Chloramine T method (Hudson et al., 1980) was selected since it introduces the radioactive tracer without significantly altering the immunological properties of the labelled molecules and previous work had shown that it could be used with 11-285-14 and Ag8 antibodies (Ford et al., 1987a).

II. 2.3 (a) Materials

(1) 400 μg antibody diluted in phosphate buffered saline (PBS) at 1:2 dilution factor; total volume 200 μl.

(2) Sodium phosphate buffer ($NaPO_4$, Baker) 0.05 M, pH 7.5.

(3) Chloramine T (Amersham Corp., 4 mg/ml in 0.05 M $NaPO_4$ buffer pH 7.5).

(4) Potassium Iodide (KI, Sigma) 4 mg/ml in PBS.

(5) Sodium bisulfite ($Na_2S_2O_4$, Sigma) 10 mg/ml in PBS.

(6) 1% bovine serum albumin (BSA, Sigma) in PBS.

(7) Sodium ^{125}Iodine (Amersham Corp.).

(8) Sephadex G-25 medium (Pharmacia), 6 g in 20-25 ml PBS.

II. 2.3 (b) Method

(1) A 25 ml glass pipette was mounted on a stand and plugged with a very small amount of glass wool.

(2) The Sephadex column was poured and equilibrated with 1% BSA in PBS.

(3) The antibody was placed into a 1.5 ml Eppendorf tube and 1 mCi of $Na^{125}I$ was added.

(4) 200 μl Chloramine T were added and the tube was shaken for 1 minute.

(5) 200 μl freshly made sodium bisulfite were added.

(6) 400 μl potassium iodide were then added and the reaction was applied onto the pre-equilibrated column.

(7) 26 x 2 ml fractions were collected and a radioactivity count was taken.

(8) The samples included in the first peak were pooled and the percent bound radioactivity was determined using the trichloroacetic acid (TCA) precipitation method described below (section II.2.3 (b) supplement).

II. 2.3 (b)supplement: TCA precipitation

(1) A 1:200 dilution of a small sample of the pooled fractions in 1% BSA / PBS (5 μl sample in 995 μl diluent), was prepared.

(2) A 250 μl aliquot of (1) was then removed and labelled sample A (representing total [bound + nonbound] radioactivity.

(3) Another 250 μl aliquot was removed from solution in (1). 250 μl TCA (20 % weight/volume) were added to it.

(4) Sample was vortexed and let to stand 15 - 20 min, following which it was centrifuged at 350 x g (Beckman TJ-6 centrifuge), at 4°C for 5 minutes.

(5) 250 μl were then removed from the supernatant and labelled sample B (representing non protein bound radioactivity).

(6) Radioactivity counts were obtained for samples A and B using a gamma counter, and the percent protein bound radioactivity was obtained using the following formula: **% bound activity = 100 - 2 x (B/A) x 100.**

II. 2.4 Labelling of Antibodies With Fluorescein

11-285-14 antibody as well as control ascites were labelled with a fluorochrome (fluorescein isothiocyanate - FITC) in order to perform internalization assays employing flow cytometric techniques. During those assays commercially available anti-FITC was used to quench surface fluorescence in order to make possible the differentiation between surface and internalized activity.

II. 2.4 (a) Materials

(1) Sodium carbonate buffer, $NaHCO_3$ (0.1 M), pH 9.0, 16.8 g (Mallinckrodt Inc. Paris, Kentucky) prepared in 2 litres of distilled water.

(2) Fluorescein-5-isothiocyanate, FITC, (1.3 mg) (# F-143, Lot # 1121-4, Molecular Probes Inc. Pitchford Ave. Eugene, OR.)

(3) Dimethylsulphoxide, DMSO, (1.2 ml) (AnalaR, BDH Chemicals, Toronto).

(4) Sephadex G-25 (1.0 g) added to 10 ml of PBS (Pharmacia Fine Chemicals AB, Uppsala, Sweden).

(5) Phosphate Buffered Saline (PBS) pH 7.2 (Oxoid. Unipath Ltd. Basingstoke, Hampshire, England).

(6) NaOH (2 N) (Fisher Scientific Company).

(7) Spectrapor Membrane dialysis Tubing (10mm x 50ft) (Spectrumedical Industries Inc. 60916 Terminal Annex, Los Angeles 90054).

(8) 11-285-14 antibody at 1.68 mg ml^{-1}

II. 2.4 (b) Method

(1) A 2 litre 0.01 M $NaHCO_3$ solution was made up and equilibrated.

(2) 11-285-14 was added to dialysis tubing at a concentration of 1.68 mg ml^{-1} and was dialysed in the $NaHCO_3$ buffer for 24 hours at 4°C with continuous stirring.

(3) A 1.2 mg ml^{-1} solution of FITC-DMSO was prepared.

(4) 300 μl of this was added to the 11-285-14 sample in 10 μl aliquots, wrapped in

foil and stored at room temperature for 5 hours.

(5) The purified conjugate was obtained by layering the conjugate mixture in 1 ml aliquots over swollen G-25 Sephadex beads. These beads were packed in a 5 ml syringe and were then centrifuged for 1 minute at 560 x g at 0^0C.

(6) The eluent containing the FITC-conjugated monoclonal antibody was then collected.

(7) The fluorescein/protein (antibody), F/P, ratio of the collected conjugate was determined by reading optical densities at 280 nm and 495 nm. These readings were then incorporated into the following equations:

Molar Concentration of protein P= OD_{280} - 0.35 x OD_{495} / 1.43

1.43 = Molar Absorption Coefficient of IgG at 280 nm

Molar Concentration of Fluorescein F = OD_{495} / 0.7 x 10^5

(Haaijman, 1983).

(8) The product was then aliquoted out (0.743 mg ml^{-1}) into Eppendorf tubes and stored at -20^0C.

(9) An identical procedure as the one described above was used to label the control ascites.

10 mg ml^{-1} of ascites was used in the dialysis. The dialysis product was stored at 1.0 mg ml^{-1} at -20^0C.

II. 3.0 Immunoadsorbent CEA Purification

Carcinoembryonic antigen was extracted from liver metastases and purified according to the method of Ford et al. (Ford et al., 1987b), which utilizes a monoclonal anti-CEA affinity column.

II. 3.1 Preparation of immunoadsorbent column

The anti-CEA monoclonal antibody 11-285-14 was used for the preparation of an immunoadsorbent column to be employed in the last stages of carcinoembryonic antigen purification.

II. 3.1 (a) Materials

(1) Semi-purified 11-285-14 (see section II.2.1).

(2) Cyanogen bromide-activated Sepharose 4B column (C26/40, Pharmacia, Canada).

(3) HCl solution, 1 mM.

(4) Ethanolamine solution 1 M, pH 8.0.

(5) Borate buffer (Fisher) 0.1 M in 0.5 M NaCl pH 8.0.

(6) Acetate buffer (Fisher) 0.1 M in 0.5 M NaCl pH 8.0.

(7) Acetate buffer (Fisher) 0.1 M in 0.5 M NaCl pH 4.0.

II. 3.1 (b) Method

(1) 11-285-14 was semi-purified from ascitic fluid by precipitating with ammonium sulphate and then desalting and buffer exchanging on Sephadex G25, as described in section II.2.1.a.

(2) 15 g cyanogen-bromide-activated Sepharose 4B were swollen and washed with 1 mM HC1.

(3) The semi-purified monoclonal antibody was mixed at a concentration of 1 mg/ml with the activated gel and rotated overnight at 4°C.

(4) Remaining activated groups were blocked with 1 M ethanolamine, pH 8.0, and excess uncoupled protein was washed off by alternating buffers; first 0.1 M borate buffer pH 8.0, then 0.1 M acetate buffer pH 8.0 and finally 0.1 M acetate buffer pH 4.0, for a total of 3 times each.

(5) The gel was then equilibrated with the borate buffer and a C26/40 column was prepared with a gel bed of 2.6 x 7.5 cm.

II. 3.2 CEA purification

200g liver metastasis from a primary colonic adenocarcinoma were minced under sterile conditions and homogenised in 800 ml ice-cold distilled water in an Polytron homogeniser (Brinkmann Instruments, Wexdale, Ontario). Carcinoembryonic antigen was then extracted from the tumour homogenate as follows:

II. 3.2 (a) Materials

(1) Phosphate buffered saline (PBS; 0.1 M phosphate, 0.2 M NaCl pH 5.5).

(2) Glass fibre filter (GFA, Whatman).

(3) Ultrafiltration PM10 Diaflo membrane (Amicon Corp., Lexington, Mass.).

(4) Ultrafiltration cell (Amicon Corp.).

(5) Borate buffer (Fisher) 0.1 M, pH 8.0 in 1 M NaCl.

(6) Sephadex G25 column (Pharmacia).

(7) 0.45 μm filter (Millipore Corp., Bedford, Mass.).

(8) Acetic acid 1 M.

(9) Sodium Carbonate anhydrous (Fisher), 3 M.

(10) Fraction collector.

(11) Spectrophotometer.

(12) Spectrapor molecularporous dialysis tubing (Spectrum Corp.).

(13) Ultracentrifuge.

II. 3.2 (b) Method

(1) Liver homogenate was extracted on ice for 30 min with an equal volume of phosphate buffered saline and centrifuged at 13,000 x g for 30 min at 4°C.

(2) The supernatant was filtered through a glass fibre filter (GFA, Whatman) until clear and then concentrated by ultrafiltration to 100 ml (1 ml/g original tumour) with a PM10 Diaflo membrane.

(3) Successive aliquots were buffer-exchanged on Sephadex G25 equilibrated with 0.1 M borate buffer in 1 M NaC1 pH 8.0 and pooled fractions re-concentrated to 1 ml/g original tumour.

(4) After filtration (0.45 μm, Millipore) successive aliquots were run into the immunoadsorbent column until protein was just detectable at the outflow. The column was stopped each time for 20 min to allow any unbound material to be washed through with borate buffer. When all the material had been added to the column it was washed with borate buffer until the absorbance of the eluate at 280 nm was zero.

(5) CEA was eluted with 1 M acetic acid and, after rapid neutralization with sodium carbonate, the pooled fractions were dialysed against distilled water, lyophilised and weighed after dessication.

II. 4.0 Immunoperoxidase assays

Immunohistochemical techniques were introduced as early as 1934 (Marrack, 1934) when it was recognized that antibodies could be suitable histochemical reagents provided that they could be linked to appropriate markers in a manner that would not impair their capacity to react with specific antigens. Initially only fluorescent dyes proved to be strong enough markers but in 1966 Wilson and Pierce (Nakane et al., 1966) successfully applied enzymes as antibody markers, while in the same year the enzyme horseradish peroxidase was introduced as one of the most powerful markers. Since that

time the term "immunoperoxidase methods" has been reserved for the demonstration of antibodies using HRP as a marker, a method used routinely in immunocytochemistry (Ford et al, 1981).

II. 4.1 (a) Materials

(1) Air-dried, ethanol-fixed smears of human cancer line cells.

(2) Xylene.

(3) Ethanol : Absolute, 75%, 60%, 30%.

(4) Hydrogen peroxide (H_2O_2, Mallinckrodt Inc., Paris, Kentucky, U.S.A) 7.5% in distilled water.

(5) Periodic acid (BDH), 2.28% in water.

(6) Potassium borohydride (BDH), 0.02% prepared fresh in water.

(7) Phosphate buffered saline (PBS).

(8) Bovine serum albumin (Sigma), 1% (w/v) in PBS (1% BSA).

(9) Brij 35 (BDH), Non-ionic detergent.

(10) Rabbit-anti-mouse immunoglobulins conjugated to horseradish peroxidase (Dako Corp.).

(11) Normal rabbit serum pool.

(12) 3, 3'-Diaminobenzidine (DAB, Sigma), 0.5 mg ml^{-1} in PBS + 0.03% H_2O_2.

(13) Mayers haemalum stain (BDH).

(14) Lithium carbonate (BDH) saturated aqueous solution.

II. 4.1 (b) Method

(1) The slides were bleached for 5 minutes with 7.5% H_2O_2 and washed with tap water.

(2) Endogenous peroxidase was blocked using 2.28% periodic acid for 5 minutes followed by washing in tap water.

(3) Aldehyde groups were blocked with freshly made 0.02% potassium borohydride for 2 minutes. Slides were washed with tap water followed by PBS.

(4) 100 μl normal rabbit serum diluted 1/25 were incubated on the sections for 10 minutes in a moist chamber to block non-specific binding of conjugate. The slides were washed with PBS.

(5) The slides were transferred to a wash bath containing PBS and 0.001% Brij and were agitated for 5 minutes.

(6) 100 μl of the first test anti-CEA antibody (11-285-14) were added to the slide with appropriate positive (for example liver metastases) and negative (for example control ascites in 1% BSA diluent) controls.

(7) Following a 30 minute incubation in a moist chamber the first antibody was washed off using PBS.

(8) The slides were transferred to a wash bath containing PBS-Brij and were agitated for 15 minutes.

(9) 100 μl of appropriate enzyme (HRP) labelled antibody directed against the first antibody and appropriately diluted in PBS (usually 1/50 or 1/100), were incubated

on the sections for 30 minutes in a moist chamber. This secondary labelled antibody was washed off using PBS.

(10) The slides were transferred to a wash bath containing PBS-Brij and were agitated for 15 minutes.

(11) Freshly prepared diaminobenzidine in PBS was incubated on the slides for 5 minutes and was then washed off with PBS.

(12) Mayer's haemalum was used to counterstain the sections followed by blueing in saturated lithium carbonate.

(12) Sections were dehydrated through the alcohols, cleared in xylene and mounted.

II. 5.0 Enzyme linked immunosorbent assays (ELISAs)

Use of this solid phase immunoassay makes possible the immobilization of proteinaceous substances such as antigens or antibodies by adsorption onto a solid surface such as a polystyrene bed or a plastic microtitre plate. Antigen, (purified or cell-bound), is usually adsorbed to the solid phase first, followed by the addition of the antibody being tested. This antibody is then detected by a second labelled antibody directed against the first one. Both radioisotopes and enzymes have been used as labels on the detector (secondary) antibody, however enzymes are both safer in terms of handling and can be stored for very long periods of time so that they are now almost exclusively used in routine ELISAs. Outlines of some representative ELISAs are presented at the end of this section (figure 1).

II. 5.1 Materials

(1) 0.5%, 1% or 2% bovine serum albumin (BSA, Sigma) in PBS/Tween (Sigma) (0.5, 1 or 2 g BSA in 100 ml PBS + 100 μl Tween).

(2) Carbonate - bicarbonate buffer, pH 9.2 (1.59 g sodium carbonate (Na_2CO_3, Fisher) + 2.93 g sodium bicarbonate (Na_2HCO_3, Fisher) dissolved in 1 litre of distilled water).

(3) 1% BSA in carbonate - bicarbonate buffer (1 g BSA in 100 ml carbonate-bicarbonate buffer).

(4) Citrate-phosphate buffer (BDH), pH 4.0 (9.06 g citric acid ($C_6H_8O_7.H_2O$) + 8.16 g disodium hydrogen phosphate (Na_2HPO_4, BDH) dissolved in 1 litre distilled water).

(5) Sodium chloride (Fisher)/ 1% Tween (Sigma) wash solution 0.15 M (8.76 g sodium chloride + 1 μl Tween dissolved in 1 litre distilled water).

(6) 2,2-azino-di-3-ethylbenzthiazoline sulphonic acid (ABTS, Sigma; 100 μl of a 27.8 mg ml^{-1} stock diluted in 12.5 ml citrate-phosphate buffer + 1μl hydrogen peroxide (BDH)).

(7) Linbro 96 well sterile enzyme immunoassay plates (Flow Laboratories).

(8) Soft round bottom polyvinyl chloride (PVC) 96 well plates (Dynatech).

(9) Glycine-HCl buffer 0.05 M, pH 2.8 in 0.1 M NaCl (5.844 g NaCl in 1 litre distilled water + 3.753 g glycine (ammonia-free Glycine, Sigma, St. Louis, Mo., U.S.A. or 98% pure glycine, Aldrich, Milwaukee, Wis., U.S.A.).

(10) Polylysine (PLL, 1 mg ml^{-1} in PBS).

(11) 11-285-14 and Ag8 antibodies.

(12) Rabbit anti-mouse antibody conjugated to horseradish peroxidase (RAM-HRP, section II.2.2).

(13) Live or fixed CEA expressing cells derived from the human cancer cell lines LS174T, SKCO1 or BENN (section II.1.0).

(14) CEA derived from human liver metastases and purified in the Oncology Research Laboratory, Memorial University, NF (section II.3.0).

(15) Bio-Tek EL310 ELISA plate reader.

II. 5.2 Method: ELISA on live cells (Morris et al., 1982)

(1) Cells from all three lines were grown overnight on soft PVC plates at a concentration of 5 x 10^5 cells ml^{-1} (100 μl/well or 5 x 10^4 cells/well).

(2) Cells were washed x 3 in 0.5% BSA/PBS by centrifugation at 200 x g for 5 minutes.

(3) 100 μl 11-285-14 (test) or Ag8 (control) antibody in 2% BSA/PBS at a concentration of 5 μg ml^{-1} were added to the appropriate wells and incubated for 2 hours at 37°C.

(4) Wells were washed x 4 in 0.5% BSA/PBS and 100 μl glycine-HCl buffer (test) or 0.1 M NaCl (control) were added to the appropriate wells and incubated either at room temperature or 37°C for 20 minutes.

(5) Wells were washed again x 4 and 100 μl RAM-HRP diluted 1:1000 in 2% BSA/PBS were added to the wells and incubated for 2 hours at 37°C.

(6) Wells were washed x 4 and the soft plates were snap-fitted onto rigid LINBRO EIA plates which had been previously coated with 100 μl polylysine for 30 minutes at room temperature.

(7) Holes were punched in the center of the wells with an 8 gauge needle and the plates were centrifuged causing the cell suspension to be transferred onto the rigid plates.

(8) The ABTS substrate solution was added and incubated for 1 hour at room temperature.

(9) Absorbance at a wavelength of 405 nm was then measured on the platereader.

II. 5.3 Method: ELISA on fixed cells

(1) LS174T, SKCO1 and BENN cells ($2.5x10^5$ cells ml^{-1}) were plated out in LINBRO tissue culture plates at 100 μl / well and incubated overnight at 37°C.

(2) Cells were fixed by incubation in methanol for 5 minutes at room temperature.

(3) Uncoated plastic was then blocked with 1% BSA in carbonate-bicarbonate buffer at 200 μl/well, for 1 hour at 37°C.

(4) Wells were washed x 6 with 0.15 M NaCl and 100 μl/well test (11-285-14) or control (Ag8) antibodies at a concentration of 20 μg ml^{-1} were added and incubated for 90 minutes at 37°C.

(5) Wells were washed x 6 again and 100 μl glycine-HCl buffer were added in the appropriate wells and incubated for 20 minutes at room temperature or at 37°C.

(6) Cells were washed again x 6 and, in some assays, test antibody was re-applied in certain wells for another 90 minutes followed by six further washes.

(7) RAM-HRP (diluted 1:1000 in 1% BSA/PBS) was then applied to the wells at 100 μl/well and was incubated for 3 hours at 37°C.

(8) Cells were again washed x 6 and 100 μl ABTS substrate solution were added to each well and incubated for 1 hour at room temperature.

(9) Absorbance at 405 nm was then measured as in section II.5.2 above.

II. 5.4 Method: ELISA using antibody-HRP conjugates

(1) Linbro plates were coated with 100 μl/well 11-285-14-HRP (test) or Ag8-HRP (control) antibodies (section II.2.2) in carbonate-bicarbonate buffer at a concentration of 1 μg ml^{-1}.

(2) The plates were incubated at 37°C for 3 hours and left at 4°C overnight.

(3) The same procedure as for fixed cells was then followed (section II.5.3 (3)-(9)).

II. 5.5 Method: ELISA using purified CEA coated plates (Reddy et al, 1993).

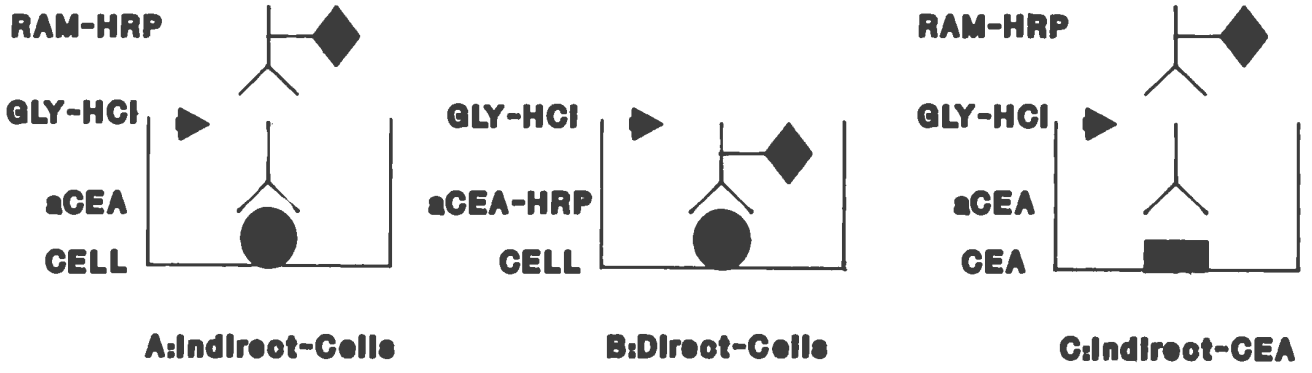
(1) Linbro plates were coated with 100 μl purified CEA (2.5 μg ml^{-1} in carbonate-bicarbonate buffer, pH 9.2).

(2) The same procedure as for antibody-HRP conjugates (section II.5.4 (2) onward), was used.

(3) After application of the glycine-HCl buffer and thorough washing (x6) to remove dissociated antibody, test and control antibodies were applied for a second time in some groups of wells in order to examine the degree of antibody reassociation to antigen.

Schematic drawings of some representative ELISA assays performed, are provided on the following page (Figure 1).

ELISAs testing the dissociating effect of Glycine-HCl on antigen-MAb bonds



aCEA = anti-CEA

Figure 1

II. 6.0 Radioimmunoassays

To estimate amount of antibody internalized by CEA-expressing cancer cell lines, two types of radioimmunoassays were employed. The first, referred to as the "glycine-HCl internalization assay" makes use of a low pH glycine buffer to dissociate surface antigen-antibody bonds. The second, referred to as the "double labelling assay" makes use of a secondary antibody which detects surface anti-CEA antibody at any time interval. Both assays make use of iodine-125 labelled antibodies and they both provide semi-quantitative estimates of internalized antibody.

II. 6.1 Glycine-HCl Internalization Assay

Uptake and internalization of iodinated 11-285-14 antibody were measured by a modified internalization assay (Matzku et al., 1986). Cells were incubated with radiolabelled antibody for various time intervals after which glycine-HCl buffer was applied to test groups in order to dissociate surface antigen-antibody bonds. In these groups remaining radioactivity after buffer treatment was presumed to reflect internalized (ie. non buffer accessible) antigen-antibody complexes.

A graphic representation of this assay is provided in Figure 2 (following page).

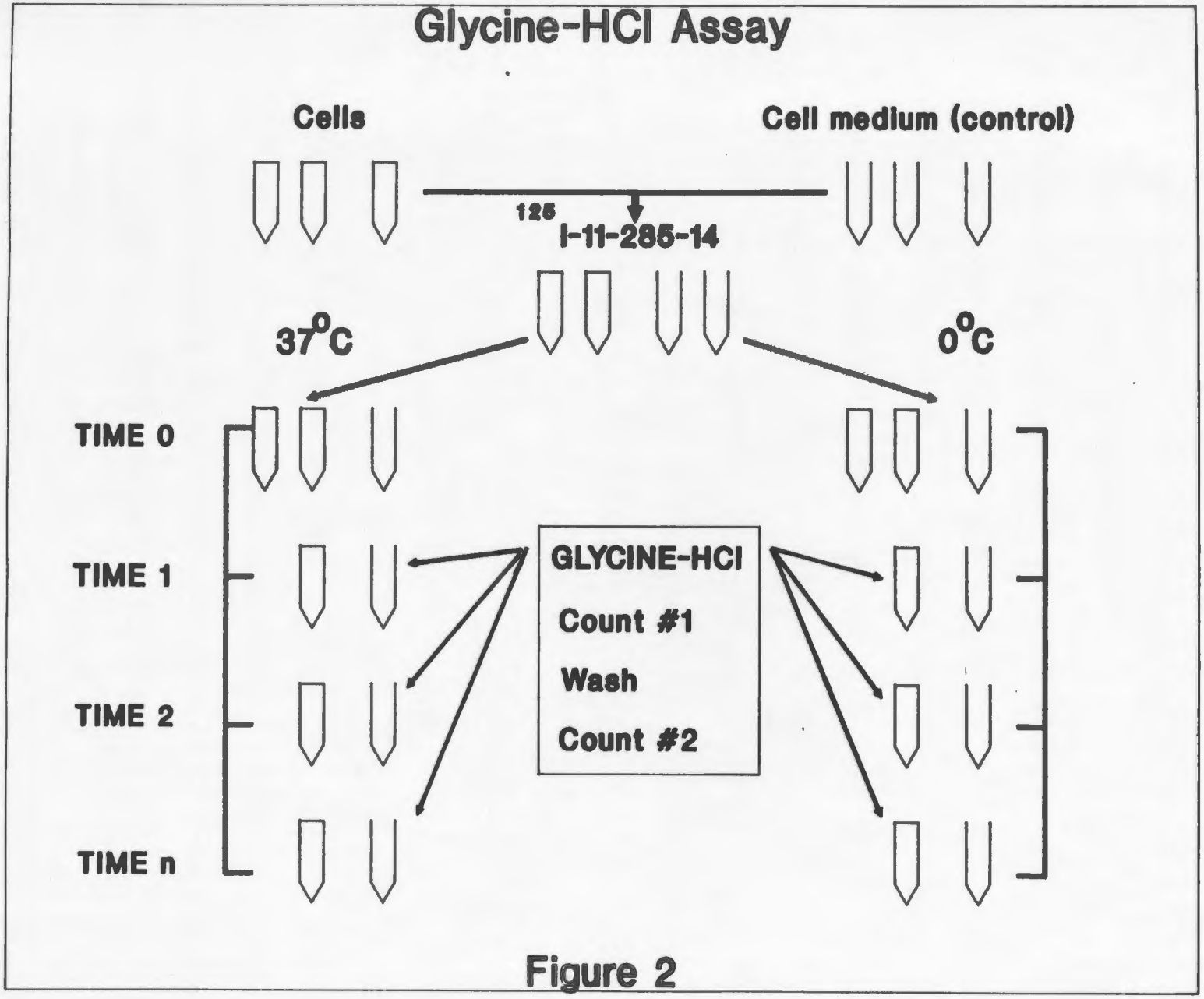

Glycine-HCl Assay
Cells
Cell medium (control)
125 I-11-285-14
37°C
0°C
TIME 0
TIME 1
TIME 2
TIME n
GLYCINE-HCl
Count #1
Wash
Count #2

Figure 2

II. 6.1 (a) Materials

(1) LS174T human colorectal cancer cells.

(2) ^{125}I labelled 11-285-14 anti-CEA MAb (section II.2.3.).

(3) 1% fetal calf serum (FCS, Gibco) in PBS.

(4) Glycine-HCl buffer 0.05 M, pH 2.8 (section II.5.1 (9))

(5) Beckman G 7000 gamma counter.

II. 6.1 (b) Method

(1) LS174T cells were dispensed into Eppendorf tubes (2.5×10^5 cells per tube in 100 μl medium at RT). Controls consisted of tubes containing medium only.

(2) Iodinated antibody in saturating doses determined in previous assays (10 μl of 200 μg/ml antibody) was immediately dispensed into each tube.

(3) Duplicate tubes were placed either at 37°C or 0°C and were subsequently removed at half hour intervals and processed as follows:

(4) Cells were washed three times with 1 ml of 1% FCS in PBS and supernatants were discarded at the end of each wash.

(5) Cell pellets were resuspended in 1 ml glycine buffer (0.05 M glycine-HCL pH 2.8 in 0.1 M NaCl) for a period of 20 minutes at RT, after which time a radioactivity count was taken for each sample.

(6) The samples were then washed again three times with 1 ml of 1% FCS in PBS in order to wash off antibody released due to treatment with the glycine buffer.

(7) A second radioactivity count of the cell pellet was then taken to be used as a

measure of the amount of remaining cell bound antibody. In some assays 100 μl medium along with 10 μl ^{125}I labelled Mab were added to the samples which were then incubated at 0°C for 30 min.

(8) Following incubation cells were washed 3 times with 1% FCS in PBS and radioactivity counts of cell pellets were taken. These samples were used to evaluate the effect of glycine buffer on antigenic sites (or the degree of CEA re-expression).

II. 6.2 Double Labelling Assay for Internalization (Rosenthal et al., 1980)

This assay indirectly estimates the amount of internalized anti-CEA antibody through the use of a radiolabelled secondary antibody which depicts the amount of surface anti-CEA at the end of each incubation interval. Anti-CEA (test) or Ag8 (control) antibody treated cell samples were separated into two groups. All samples of the first group were treated with secondary radiolabelled antibody so that their radioactivity levels at the end of each incubation period would be indicative of both surface-bound and internalized antibody. Samples of the second group are treated with secondary antibody at the end of each incubation time interval so that radioactivity levels in that case would be indicative of surface bound antibody only. Subtracting second group radioactivity counts from first group counts could then be used as a measure of internalized (residual) antibody for each time interval. An outline of the double labelling assay is provided on the following page (Figure 3).

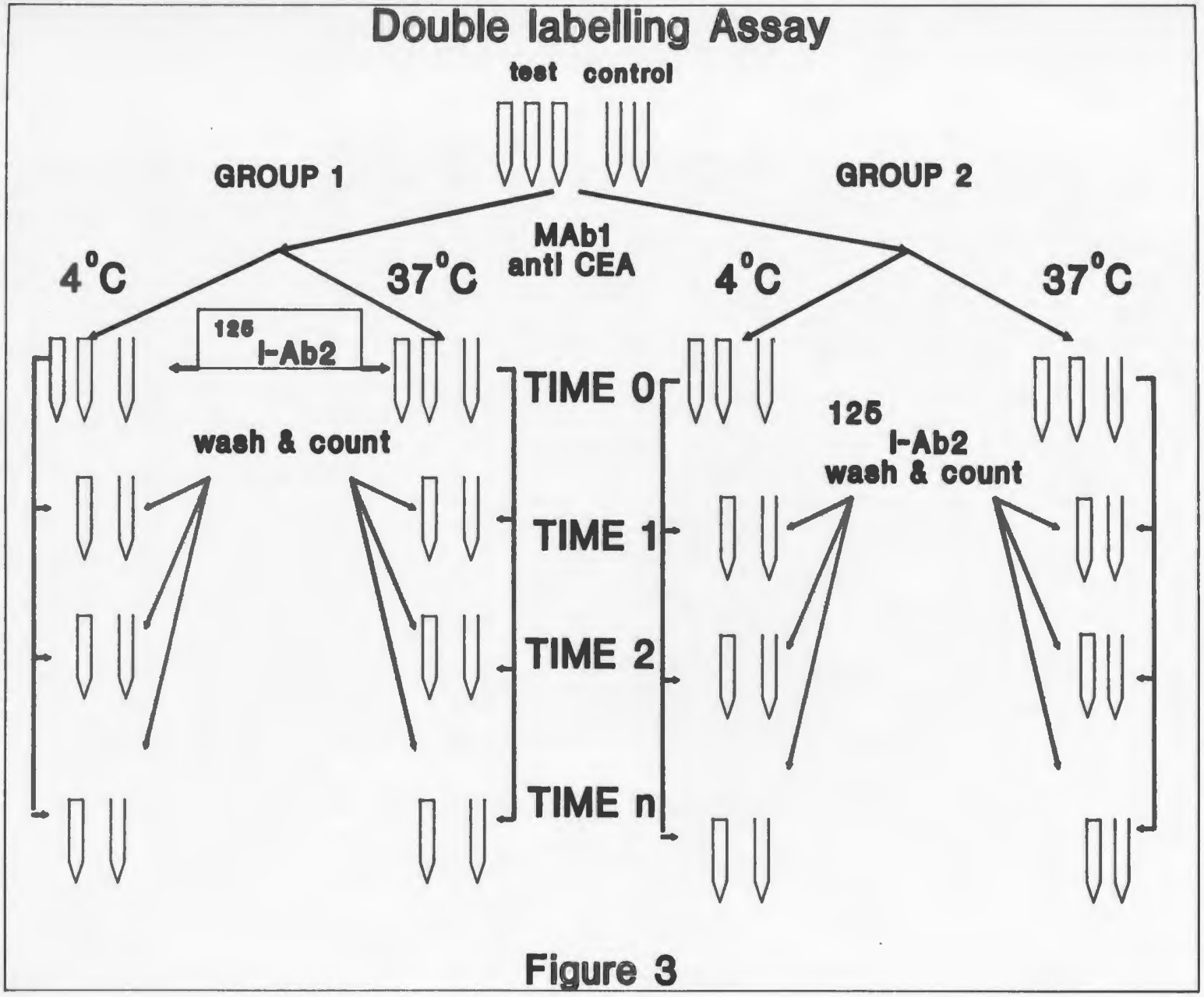

Double labelling Assay
test
control
GROUP 1
GROUP 2
MAb1
anti CEA
4°C
37°C
4°C
37°C
125
I-Ab2
TIME 0
wash & count
125
I-Ab2
wash & count
TIME 1
TIME 2
TIME n

Figure 3

II. 6.2 (a) Materials

(1) LS174T colorectal cancer cells (section II.1.0)

(2) 11-285-14 and Ag8 antibodies (section II.2.0)

(3) ^{125}Iodine labelled rabbit-anti-mouse antibody (section II.2.3)

(4) 2% fetal calf serum (FCS, Gibco) in PBS.

(5) 1.5 ml plastic Eppendorf tubes (polypropylene microcentrifuge tubes,Bioplas Inc).

(6) Gamma counter (Beckman LS-7500).

II. 6.2 (b) Method

(1) 100 μl of LS174T cells at a concentration of 2.5 x 10^6 cells ml^{-1} were dispensed into Eppendorf tubes (2.5 x 10^5 cells/tube) at RT.

(2) Test antibody (11-285-14) or control antibody (Ag8) were added to respective tubes at 2 μg/tube. In certain tubes to be used as controls for nonspecific binding of secondary antibody, no primary antibody was added.

(3) To establish initial antibody binding, all tubes were incubated at 37°C for 30 minutes.

(4) Following this incubation period, samples were washed x 3 in 1 ml 2% FCS/PBS, divided into two groups and treated as follows:

(5a) Secondary ^{125}I-labelled RAM antibody was added to all tubes of one group (1 μg RAM or 20.8 μl per tube based on previous saturation assays) and incubated at room temperature for 30 min to allow binding of the secondary antibody. This

group of tubes (Group 1) would eventually be used for assessment of total antibody binding.

(5b) After the 30 min incubation period samples were washed 3 times in 1 ml of 2% FCS in PBS and were then divided into subgroups - to be incubated either at 4°C or at 37°C.

(5c) Samples were subsequently retrieved in duplicate at pre-specified time intervals, were washed again x 3 and radioactivity counts of the cell pellets were taken.

(6) The second group of tubes (Group 2) was treated as follows:

(6a) Samples were left at 37°C for pre-specified incubation intervals (same as for Group 1).

(6b) At the end of each interval duplicate samples were retrieved and ^{125}I labeled RAM was added to each (1 μg/tube).

(6c) Samples were incubated for 30 min at RT, and were then washed 3 times in 1 ml of 2% FCS-PBS.

(6d) Radioactivity counts of the cell pellets were then taken. This group of samples would be used for the assessment of surface Ab binding during each time interval.

(7) Radioactivity counts obtained for group 2 tubes (surface antibody) were then subtracted from counts obtained for group I tubes (total antibody) for each time interval.

II. 7.0 Electron microscopy assays.

Direct visualisation of internalized antibody was made possible by the use of electron microscopy. Localization of intracellular proteins (such as antibodies), normally requires that the antibody be labelled with an electron-opaque (eg. ferritin) (Singer, 1959; Sternberger, 1967), or heavy metal marker (eg. uranium or colloidal gold) (Sternberger, 1967; Sternberger et al., 1965). The introduction of methods for conjugating enzymes to antibodies (Nakane et al., 1966) has opened up the possibility of using enzymic labels for electron microscopy. Horseradish peroxidase has a number of properties which make it particularly suitable for such applications such as ease of detection, commercial availability, low endogenous peroxidase levels in biological tissue and small size relative to most other labels currently available. Consequently the label of choice for the electron microscopic assays was horseradish peroxidase conjugated to the anti-CEA and control antibodies.

II. 7.1 (a) Materials

(1) SKCO1 colorectal cell line.

(2) 11-285-14 and Ag8 antibodies conjugated to horseradish peroxidase (section II.2.2).

(3) Millicell-HA membranes (0.45 μm culture plate insert, 12 mm diameter, Millipore, Bedford, MA).

(4) Sterile PBS.

(5) Half-strength Karnovsky's solution (Merivac Ltd. Halifax, Nova Scotia).

(6) Cacodylate buffer 0.1 M (Merivac Ltd.).

(7) Diaminobenzidine (DAB, Sigma) 0.5 mg ml^{-1}.

(8) Tris-HCl buffer 0.2M, pH 7.4 + 0.0009% hydrogen peroxide.

(9) 1% osmium tetraoxide (Merivac Ltd.).

II. 7.1 (b) Method

(1) SKCO1 cells at a concentration of 4 x 10^6 cells ml^{-1} in medium were applied onto membranes at 100 μl/membrane, and incubated overnight at 37°C.

(2) Following incubation the cells were washed x 3 in PBS and 200 μl test (11-285-14-HRP), or control (Ag8-HRP) antibodies diluted in sterile PBS at concentrations of 25, 50 or 100 $\mu g\ ml^{-1}$, were dispensed on each membrane.

(3) A two-hour incubation period at 37°C followed, after which the membranes were washed x 3 in PBS and fixed in half-strength Karnovsky's solution for 15 min.

(4) Membranes were then washed x 2 by rocking in 0.1 M cacodylate buffer, and suspended in 225 μl diaminobenzidine in 0.2 M Tris-HCl buffer (pH 7.4) with 0.0009% hydrogen peroxide (H_2O_2).

(5) After another 15 min incubation at 4°C the membranes were washed, postfixed in 1% osmium tetraoxide and processed for EM analysis.

II. 8.0 SDS/PAGE on membrane and cytosolic portions of solubilized cells

An alternative method of visualizing surface-bound and internalized antibody involved the separation of cellular components (plasma membrane from cytosol) followed by the electrophoresis and blotting of these components for the identification of pre-incubated antibody.

Following incubation of cells with either CEA-specific or non CEA-specific antibodies, cells were broken open and separated into solubilized membrane and cytosol. Such samples were then electrophoretically separated into their protein components and Western blotted. Relevant antibodies were then identified by immunostaining.

II. 8.1 Separation of plasma membrane from cytosol

The separation process took place following incubations of cells with the appropriate antibodies for pre-specified time intervals, therefore the description of incubations will be included in the present section.

II. 8.1 (a) Materials

(1) LS174T, SKCO1, BENN and COLO320DM cell lines.

(2) 11-285-14 and Ag8 antibodies.

(3) Sonicator (Virsonic Cell Disrupter 16-850, Virtis Co, Gardiner, N.Y.).

(4) Ultracentrifuge (Beckman L5-65).

(5) TNEN buffer, pH 8.0 (0.242 g Tris base + 0.585 g NaCl + 0.037 g EDTA + 50 μl Nonidet P40 in 100 ml distilled water).

II. 8.1 (b) Method

(1) LS174T, SKCO1, BENN and COLO320 cells were grown as described above and divided into samples of 1 x 10^8 cells each.

(2) Samples were incubated with 11-285-14 or Ag8 (200 μg/sample) for incremental half hour time intervals, at 37°C.

(3) Samples were then washed 3 times in 1 ml PBS and frozen at -70°C.

(4) Cells were subsequently solubilized and membrane and cytosolic portions were extracted as follows:

(5) Cells were sonicated twice for 15 sec on ice and then centrifuged at 100,000 x g for 1 hr at 4°C.

(6) The supernatants (cytosolic portions) were then stored at -70°C whereas 1 ml TNEN was added to the pellets which were again sonicated twice for 15 sec on ice.

(7) The sonicated pellets were then centrifuged again for 1 hr at 100,000 x g at 4°C, and the supernatants (membrane portions) were stored at -70°C.

(8) Protein concentrations for all samples were then calculated in a Bio Rad protein assay kit.

II. 8.1 (b) supplement: Bio Rad protein assay

(1) Seven dilutions (140 μg-20 μg) of protein standard (lyophilised bovine gamma globulin), were prepared.

(2) 100 μl of standards and appropriately diluted samples were placed in clean, dry test tubes.

(3) 100 μl sample buffer was placed in "blank" test tube.

(4) 5 ml of diluted dye reagent (1 volume of Dye Reagent Concentrate diluted with 4 volumes of high quality distilled or deionized water and filtered through a Whatman No. 1 paper) were added to the samples which were immediately vortexed.

(5) Following a 1 hour incubation optical density at a wavelength of 595 nm was measured versus the reagent blank.

(6) OD_{595} versus concentration of standards was then plotted and the unknowns were read from the standard curve.

II. 8.2 SDS-PAGE

II. 8.2 (a) Materials

(1) Acrylamide (Bio Rad).

(2) N,N,-Methylene-bis-Acrylamide (Sigma).

(3) THAM (Tris Hydroxymethyl) Aminomethane (Fisher).

(4) SDS (sodium dodecyl sulfate) (BDH) or lauryl sulfate (Sigma).

(5) Ammonium persulfate ($(NH_4)_2S_2O_8$, Analar).

(6) TEMED (N,N,N,N-Tetramethylethylenediamine,(Sigma)).

(7) **STOCK SOLUTIONS**

A. **Acrylamide : BIS (30%T, 2.67%C)**

29.2 g acrylamide (Bio Rad).

0.8 g N, N, - Methylene-bis-acrylamide (Sigma or Bio Rad).

Brought to a final volume of a 100 ml with distilled water. Filtered and stored at 4°C in the dark for a maximum of 30 days.

B. **1.5 M Tris-HCl, pH 8.8**

18.15 g Tris base (Sigma).

50 ml distilled water.

Adjusted to pH 8.8 with 1 M HCl and brought to a final volume of a 100 ml with distilled water.

C. **0.5 M Tris-HCl pH 6.8**

3.0 g Tris base.

20 ml distilled water.

Adjusted to pH 6.8 with 1 M HCl and brought to a final volume of 50 ml with distilled water.

(8) **Separating Gel Preparation** -10.7 Gel, 0.4 M Tris, pH 8.8

Distilled water	27.0 ml
1.5 M Tris-HCl pH 8.8 (B)	20.0 ml

10% (w/v) SDS (sodium dodecyl sulfate, BDH)	0.8 ml
Acrylamide : BIS (30%T, 2.67%C) (A)	26.7 ml
10% ammonium persulfate (fresh)	0.2 ml
TEMED	0.04 ml

(9) **Stacking Gel Preparation** 4.8% Gel, 0.154 M Tris, pH 6.8, 1.5 mm thick stacking gels

Distilled water	8.2 ml
0.5 M Tris-HCl, pH 6.8 (C)	5.0 ml
10% (w/v) SDS (BDH)	0.2 ml
Acrylamide : BIS (30%T, 2.7%C) (A)	2.6 ml
10% ammonium persulfate (fresh)	0.2 ml
TEMED	0.02 ml

(10) **Sample Buffer**

Distilled water	4.0 ml
0.5 M Tris-HCl, pH 6.8	1.0 ml
Glycerol (Sigma)	0.8 ml
10% (w/v) SDS	1.6 ml
2-Mercaptoethanol	0.4 ml
0.05% (w/v) Bromophenol Blue	0.2 ml

(11) **Electrode Buffer, pH 8.3**

Tris base	12 g
Glycine	57.6 g
SDS	4 g

pH to 8.3 with 1 M HCl, bring to a final volume of 2 litres with distilled water and dilute 1 : 1 with distilled water to run gel.

(12) Protean Cell apparatus (Bio Rad).

II. 8.2 (b) Method

(1) To prepare the monomer solutions (separating gel and stacking gel), all reagents except the ammonium persulfate and TEMED were combined and de-aerated under vacuum for 5-15 minutes.

(2) To initiate polymerization, the ammonium persulfate and TEMED were added and swirled gently to mix.

(3) The Protean Cell instructions were followed for casting the gel.

(4) The separating gel was allowed to polymerize overnight.

(5) The next day the stacking gel was allowed to polymerize for 30-45 minutes.

(6) Samples were diluted with sample buffer so as to achieve a final protein concentration of 0.1 mg/ml for all samples and were added at 50 μl / well.

(7) The gel was run at 100 mA per 1.5 mm slab gel (usually overnight). For two gels of the same thickness, the run was performed at 190 mA overnight.

(8) Once the run was completed the power was turned off and the glass plates were removed from the chamber.

(9) The sandwich clamps were removed and one plate was prodded out using a third spacer as a wedge. When one plate was removed distilled water from a squirt bottle was used to pry the gel from the second plate.

(10) The gel was then placed in transfer buffer for Western blotting.

II. 8.3 Transfer Procedure (Western Blotting)

II. 8.3 (a) Materials

(1)

Transfer Buffer	**1000 ml**
Tris	3.0 g
Glycine	14.4 g
SDS	1.0 g
*Methanol	200 ml

* Pure Grade Fisher A-4128

(2) Filter paper (Whatman #3), 24.0 cm.

(3) Nitrocellulose membrane (Schleicher & Schuell) pore size 0.45 μM.

(4) Bio Rad Transfer system with cooling element.

(5) Bio Rad Model 250/2.5 power supply.

II. 8.3 (b) Method

(1) After completing the electrophoresis run, the gel was placed in transfer buffer to pre-equilibrate (pre-equilibration facilitates the removal of contaminating electrophoresis buffer salts and neutralization salts i.e. salts resulting from the denaturation of nucleic acids prior to transfer). 1.5 mm gel thickness requires about 60 minutes in transfer buffer at room temperature.

(2) Transblot chambers were filled with buffer.

(3) Scotch-brite pads were soaked with transfer buffer. Whatman #3 filter paper and nitrocellulose membranes were also soaked before using (gloves were used for the handling of nitrocellulose membranes).

(4) The nitrocellulose membrane was placed over the gel (gel and membrane were kept wet with transfer buffer) and all air bubbles were removed.

(5) Water was run through the chilling unit and a stirring bar was placed inside the transfer cell to maintain uniform conductivity and temperature during electrophoretic transfer.

(6) The transfer was performed at a constant current of 750 mA for 5-6 h.

II. 8.4 Immunoblotting

Blotting was performed according to the method of Towbin et al., 1979, with some modifications.

II. 8.4 (a) Materials

(1) 3% BSA in PBS + 0.1% Tween.

(2) 0.1% BSA in PBS + 0.1% Tween.

(3) Horseradish peroxidase-conjugated rabbit-anti-mouse immunoglobulins (Dako Corp., 1:1000 dilution in (2)).

(4) Methanol.

(5) Tris-HCl 50 mM, pH 7.4; Trizma base (Tris hydrohymethyl aminomethane, Sigma), pH to 7.4 with HCl.

(6) Hydrogen peroxide (Mallinckrodt).

(7) 4-Chloronaphthol (Sigma).

II. 8.4 (b) Method

(1) After protein transfer from the gel to nitrocellulose membranes, blots were blocked by incubation in 3% BSA, 0.1% Tween 20 in PBS for 1 hr.

(2) Blots were then incubated overnight in RAM immunoglobulins linked to HRP (100 ml of 1:1000 solution).

(3) After washing the blots 3 times in PBS the enzyme reaction was developed by the addition of substrate (30 mg 4-chloronaphthol in 1.5 ml methanol + 50 μl 50 mM Tris-HCl pH 7.4 + 25 μl hydrogen peroxide), for approximately 30 minutes

II. 9.0 Flow Cytometry

Flow cytometry is a sophisticated analytical tool with current clinical applications in haematology and in cellular immunology (Melamed et al., 1990). It is also an analytical method of choice for classifying leukaemias and lymphomas. It involves the use of fluorescent cell markers to identify specific cells and cell components. The major advantages of flow cytometry are rapid cell processing, cell-by-cell analysis and the simultaneous evaluation of multiple markers (Stewart, 1992). The marker is detected as right-angle light scattering of an argon laser beam as it passes perpendicularly through a water jet containing labelled cells (Steen, 1990).

II. 9.1 Internalization assays using flow cytometry

The fluorochrome of choice for our internalization assays was fluorescein isothiocyanate which emits a green fluorescence. The advantage of using fluorescein in such assays had been established previously in the Oncology Research laboratory (Hopper et al., 1990; Osborne, 1992).

Cell samples treated for flow cytometric processing were run in parallel with samples prepared for solubilization and analysis using SDS gels and Western blots for comparative purposes. In order to keep conditions identical for the two sets of samples, slight modifications were introduced in the incubation protocol for the samples to be used for Western blotting. Otherwise these samples were processed as described in section II.8. The concentrations of all antibodies used for flow cytometry (fluoresceinated 11-

285-14 and control ascites as well as anti-FITC antibody) had been determined previously (Osborne, 1992). The concentrations of antibodies used on Western blot samples were as previously determined (section II.8).

Flow cytometry was performed with a Becton -Dickinson FACS^{tar} analyzer with a 100 mW air-cooled argon laser at 488 nm. Power was at 40 mW for each test. Filters used were 560 DM reading green fluorescence at 530 +/- 30 nm. Acquisition was in list mode on 3.5" floppy disks. Fluorescence settings were adjusted to obtain suitable fluorescence histograms.

II. 9.1 (a) Materials

(1) LS174T, SKCO1 and COLO320 cells cultured as described in section II.1.

(2) Fluorescein labelled 11-285-14 antibody (see section II.2.4).

(3) Control ascites labelled with FITC (Bethesda Research Laboratories, Life Technologies. Gaithersburg, Maryland) (see section II.2.4).

(4) Anti-fluorescein antibody (H+L) (Anti - FITC) (# A-889, Lot # 68131, Molecular Probes Inc. 4849, Pitchford Ave. Eugene, OR)

(5) Minimal essential medium (see section II.1.0, Table 2)

(6) Propidium iodide, (PI),(5 μl) (Sigma)

(7) Phosphate buffered saline (PBS) pH 7.2 (Oxoid, Unipath Ltd, Basingstoke, Hampshire, England).

(8) Pasteur pipettes.

(9) Cotton wool.

(10) Centrifuge (Beckman TJ-6 with TJ-R refrigeration unit).

(11) FACS^{tar} analyzer.

II. 9.1 (b) Method

In all cases, cell samples which were to be used for either Western blotting or FACS analysis were incubated with antibodies in parallel, under identical conditions.

(1) LS174T, SKCO1 and COLO320 cells were grown as described above and divided into samples of 1 x 10^8 cells (for Western blots) or 1 x 10^6 cells (for FACS analysis) each.

(2) Samples were incubated with either FITC-conjugated (100 μl/sample at 1 μg/ml for the FACS analysis) or unconjugated (200 μl at 1 mg/ml for Western Blots), 11-285-14 antibody or control ascites for 30 minutes on ice in order to allow antibody uptake while inhibiting internalization. The antibody concentrations used were previously determined in appropriate titration assays.

(3) Samples were then washed in 1 ml cold PBS x 2 by centrifugation at 560 x g , followed by the addition of 1 ml cell medium and incubation for 0, 30 and 90 minutes at 37°C. Incubations were staggered to ensure simultaneous performance of the final step of the assay.

(4) Following antibody incubations, cells were washed with PBS x 1. Samples which were to be used for Western blots were frozen at -70°C for later processing, while

samples destined for FACS analysis were processed immediately.

(5) **FACS Analysis**

Following incubation with the anti-CEA Mab, test samples were incubated with 100 μl of anti-FITC antibody (anti-fluorescein IgG, Molecular Probes) at a 1:20 dilution in PBS, based on previous titration assays, whereas positive control samples were incubated with 100 μl PBS for 30 minutes on ice. An additional cell sample which had not been previously exposed to antibody treatment was resuspended in 100 μl control ascites (1 μg/ml) and incubated similarly.

(6) After the incubation, cells were centrifuged at 560 x g for 5 minutes and resuspended in 1 ml PBS.

(7) 5 μl propidium iodide (Sigma) were added to all samples

(8) Samples were then filtered in pasteur pipette/ nylon wool filters and were analyzed immediately using a Becton-Dickinson FACS^{tm} analyzer.

II. 9.2 Use of Flow cytometry to detect possible inhibition of clathrin-mediated anti-CEA Mab internalization

A variety of biochemical approaches have been used to dissect the process of internalization (see section I.6.5.c), however the ones that seem to affect clathrin assembly and invagination (i.e. the very first stages of the endocytic process), basically include three approaches: that of the use of potassium depletion coupled to cell bathing in hypertonic media, that of cytosol acidification, and finally simple exposure to

hypotonic media (Heuser, 1989; Heuser et al., 1989; Hansen et al., 1993). Since the fate of CEA-anti-CEA complexes has only been classified within the large group of reactions involved with antigen-antibody complex internalization, the studying of such surface phenomena seemed rather appropriate. In addition, the flow cytometric assay we currently employ is exclusively suited to studying surface phenomena alone. With the above in mind, we decided to make use of the method employing hypertonic medium to inhibit clathrin-mediated internalization, both due to its relative simplicity and minimal cell manipulation, as well as because in that case endocytic arrest should occur exclusively on or under the cell surface (further reasoning of this choice will be provided in section 4- Discussion).

11-285-14 Mab was again FITC labelled (see section II.2.4), and optimal concentrations were again determined in flow cytometric titration assays (and occasionally through the use of fluorescence microscopy). Optimal concentration was also re-established similarly for anti-FITC. SKCO1 was the cell line of choice since it had previously shown the highest anti-CEA Mab uptake and was easily "manageable" experimentally.

II. 9.2 (a) Materials

(1) SKCO1 cells cultured as described in section II.1.

(2) Fluorescein labelled 11-285-14 antibody (see section II.2.4).

(3) Anti-Fluorescein Antibody Rabbit IgG (H+L) fraction

(Anti - FITC) (# A-889, Lot # 5541-1, Molecular Probes Inc. 4849, Pitchford

Ave. Eugene, OR)

(4) Minimal essential medium (see section II.1.0, Table 2).

(5) Sucrose, grade I crystalline, anhydrous, M.W. 342.3 (Sigma F-9378, Lot 13F-0021).

(6) Minimal essential medium containing 0.45 M sucrose.

(7) Propidium iodide, (PI),(5 μl) (Sigma)

(8) Pasteur pipettes.

(9) Nylon wool.

(10) Centrifuge (Beckman TJ-6 with TJ-R refrigeration unit).

(11) FACS^{tar} analyzer.

II. 9.2 (b) Method

(1) SKCO1 cells were grown as described before and left to incubate in medium for 30 min. at 37°C prior to aliquoting.

(2) Cells were then separated in 1 x 10^6 cell aliquots and were washed once in 1 ml either minimal essential (regular) or regular medium containing 0.45 M sucrose (hypertonic), by centifuging at 560 x g for 5 min at 4°C.

(3) Cells were then incubated in respective media (regular or hypertonic), for 30 min. at 37°C (treatment samples specified in the results section 3.7).

(4) Cells were centrifuged as before and 100 μl of 1 μg ml^{-1} 11-285-14-FITC in either regular or hypertonic medium was then added to the appropriate tubes,

while the remaining tubes received the same amount of regular or hypertonic medium.

(5) All samples were washed as before twice in respective media.

(6) 200 μl respective medium was then added in the tubes and the appropriate samples were incubated either at 37°C (to allow internalization), or on ice (to arrest internalization), for the pre-defined time intervals (0 min., 30 min., and 90 min.).

(7) All samples were again washed once in respective media.

(8) 100 μl anti-FITC at a dilution of 1:20 was added to the appropriate samples, with the remaining samples receiving an identical quantity of either regular or hypertonic medium as necessary. All samples were incubated on ice for 30 min.

(9) All samples were centrifuged, the supernatant was discarded, and 1 ml of regular medium was added to all tubes followed by 5 μl PI.

(10) All samples were filtered in pasteur pipette / nylon wool filters and were analyzed immediately using a Becton-Dickinson FACS^{tar} analyzer as before.

II. 9.2 supplement: Alternative method

(a) In order to examine the effect on cell Mab uptake, of the pre-treatment of the cells with hypertonic medium, steps (1) to (3) were omitted. The remainder of the assay proceeded as before.

CHAPTER III

RESULTS

III. 1. Initial Radioimmunoassay For The Estimation Of Internalized anti-CEA Antibody (employing a low pH glycine-HCl buffer)

Until recently this assay had been the one most frequently employed for the determination of internalized antibody (see section IV.3 and Tsaltas et al., 1993). It relies on the assumption that a low pH environment is disruptive to previously formed, surface antigen-antibody complexes. Since internalized antibody is inaccessible to the low pH buffer, the residual activity lines in Figures 1.1, 1.2 and 1.3 (a,b) are assumed to represent internalized antibody. In our initial experiment (Figures 1.1.a, 1.1.b) incubation of cells at 0°C (a temperature at which internalization is inhibited) (Figure 1.1.a), resulted in moderate antibody uptake throughout the incubation intervals whereas residual (internalized) activity was below control levels, suggesting that antibody was not internalized. Conversely, when cells were incubated at 37°C, total antibody uptake increased significantly (more than doubled) and modest residual activity was observed (Figure 1.1.b). Furthermore the peak of "internalization" at 37°C, was reached after an incubation interval of 90 minutes at which point residual radioactivity represented approximately 30 percent of total antibody uptake. The same incubation period of 90 minutes represented the highest point of antibody internalization in the case of samples incubated at 0°C (Figure 1.1.a). The decrease in radioactivity values (indicating decreased antibody uptake beyond the 90 [0°C], or 120 [37°C], minute interval), which

was observed during this experiment, was not seen in a subsequent experiment (Figures 1.2, a and b). Here, antibody uptake continued to increase throughout all the incubation intervals at both temperatures. Furthermore, although the uptake profile was similar to that of the previous experiment for the first 2 hours of incubation (uptake at 0°C approximately half that of 37°C), there was a large increase in uptake at 0°C during the last interval. Residual activity (representing internalized antibody) was observed at both temperatures and in general seemed to increase with higher antibody uptake. Residual activity in this case seemed to account for approximately 30-40% of total antibody throughout the incubation intervals at 37°C. Antibody uptake at 37°C was considerably lower for a subsequent assay (Figures 1.3 b), while residual activity was at control levels at both temperatures. It is not clear whether this was due to decay of the radiolabel (since this assay was performed approximately two weeks following radiolabelling), or an uncharacteristically low antigen expression on the particular passage of LS174T cells. In this assay radiolabelled antibody was re-applied onto the 90 minute samples after glycine treatment in order to examine the possibility of its re-association with antigenic sites (Table 1.4). The 90 minute samples were chosen since a high degree of residual activity relative to antibody uptake was generally observed at this time interval. Radioactivity counts of the treated samples increased to pre-glycine treatment levels following a second antibody treatment, suggesting that binding of Mab to CEA was not affected by treatment with the low pH buffer. The actual radioactivity readings obtained for the three assays discussed above, are incorporated in Tables 1.1, 1.2 and 1.3 (a and b), which are

presented opposite the respective figures (following). Table 1.4 contains reassociation values for the anti-CEA Mab following exposure of Mab-treated cells to the glycine-HCl buffer and a second incubation with the same Mab, as described in the legend of this table.

Figures 1.1, 1.2 and 1.3 (a and b)
Radioimmunoassays investigating the kinetics of total uptake and residual (post glycine-HCl buffer treatment) binding of 11-285-14 Mab.

2.5×10^5 LS174T cells/sample were incubated with 2 μg of I^{125} labelled 11-285-14 Mab for the time intervals shown. Control samples (cell medium only) establish the background for non specific Mab adherence to plastic.
(a) Incubations at 0°C.
(b) Incubations at 37°C.
The corresponding tables opposite each figure include the actual radioactivity readings in counts per minute (cpm) for each sample (test sample values are the average of duplicate tube readings)

Table 1.1.a
RIA employing Glycine-HCl to Estimate Mab Internalization(0°C); Experiment 1
Means of duplicate tubes

TIME(min)	SAMPLE	COUNT 1	COUNT 2
5	1	28792	11250
	1c	12360	9727
30	2	27568	9843
60	3	33992	10852
90	4	64186	26202
	4c	31063	25247
120	5	51721	13288
150	6	29846	8150
	6c	9275	6910

Control sample tube : c
COUNT 1 : radioactivity counts taken prior to treatment with the glycine-HCl buffer.
COUNT 2 : counts taken after buffer treatment.

LS174T Uptake/Release of 125-I-Mab After Glycine-HCl Treatment (0°C)

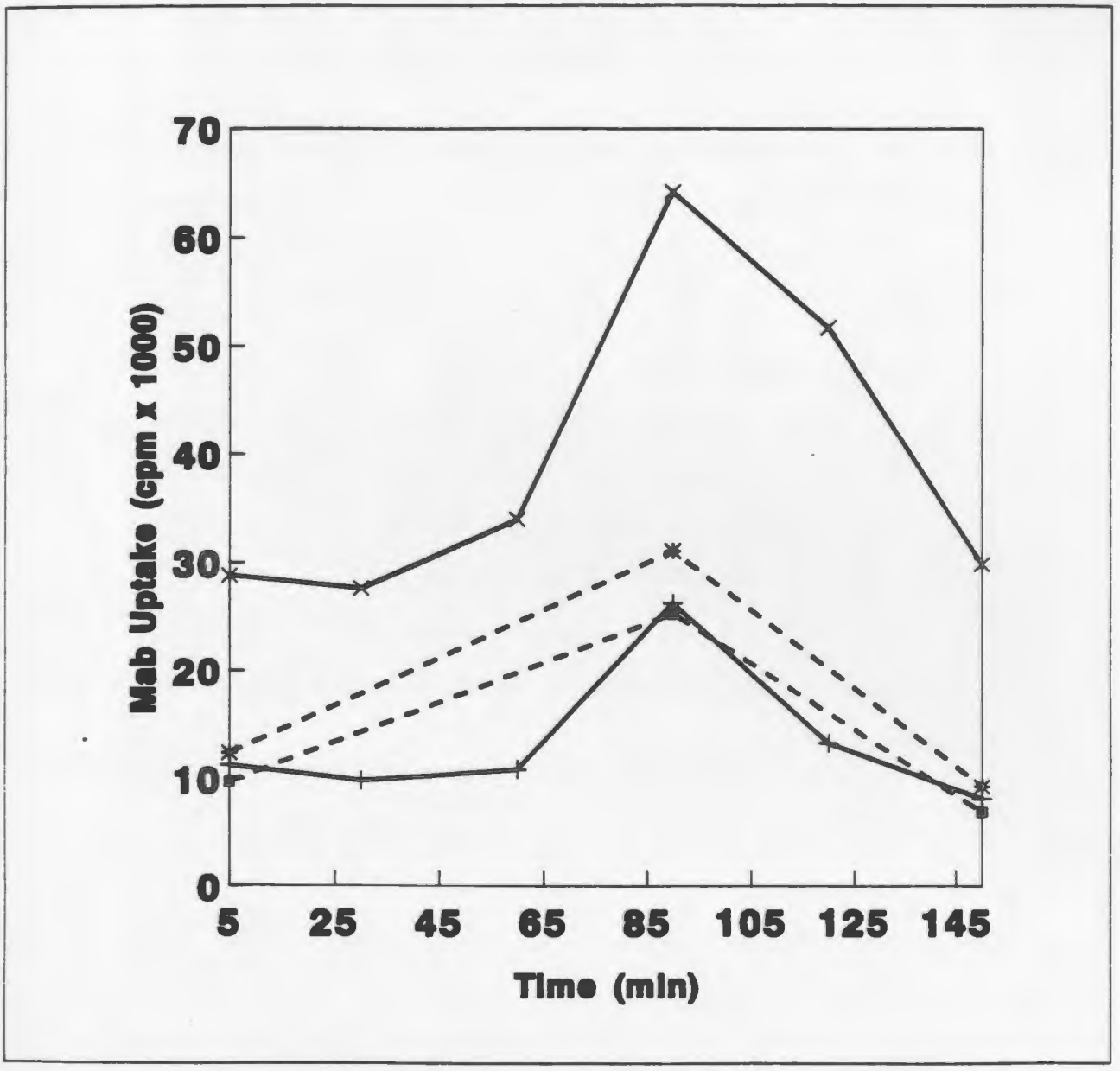


Figure 1.1.a

Table 1.1.b
RIA employing Glycine-HCl to Estimate
Mab Internalization(37°C)
Experiment 1
Means of duplicate tubes

TIME(min)	SAMPLE	COUNT 1	COUNT 2
5	7	29264	13500
	7c	8987	11264
30	8	47811	13766
60	9	90788	24566
90	10	94687	27846
	10c	9459	9081
120	11	121184	22278
150	12	98586	22602
	12c	9128	8113

Control sample tube : c
COUNT 1 : radioactivity counts taken prior to treatment with the glycine-HCl buffer.
COUNT 2 : counts taken after buffer treatment.

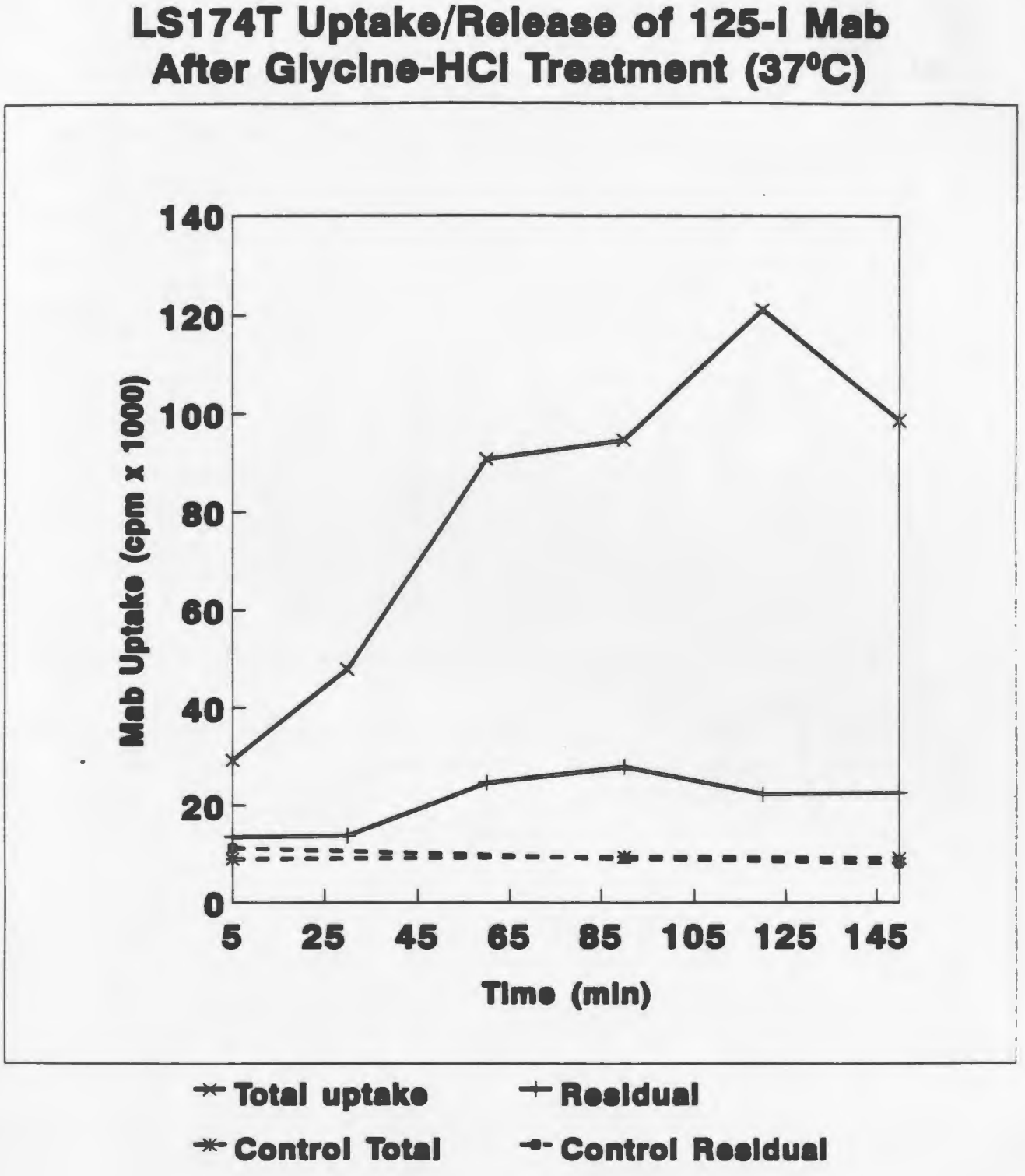

LS174T Uptake/Release of 125-I Mab
After Glycine-HCl Treatment (37°C)
Mab Uptake (cpm x 1000)
140
120
100
80
60
40
20
0
5
25
45
65
85
105
125
145
Time (min)
Total uptake
Residual
Control Total
Control Residual

Figure 1.1.b

Table 1.2.a
RIA employing Glycine-HCl to Estimate Mab Internalization(0°C); Experiment 2
Means of duplicate tubes

TIME(min)	SAMPLE	COUNT 1	COUNT 2
5	1	19173	17428
	1c	23844	14390
30	3	30398	26070
60	5	36352	16940
90	7	54104	26707
	7c	11637	11091
120	9	42922	22458
150	11	118318	45498
	11c	12214	10066

Control sample tube: c
COUNT 1 : radioactivity counts taken prior to treatment with the glycine-HCl buffer
COUNT 2 : counts taken after buffer treatment.

LS174T Uptake/Release of 125-I-Mab After glycine-HCl Treatment (0°C)

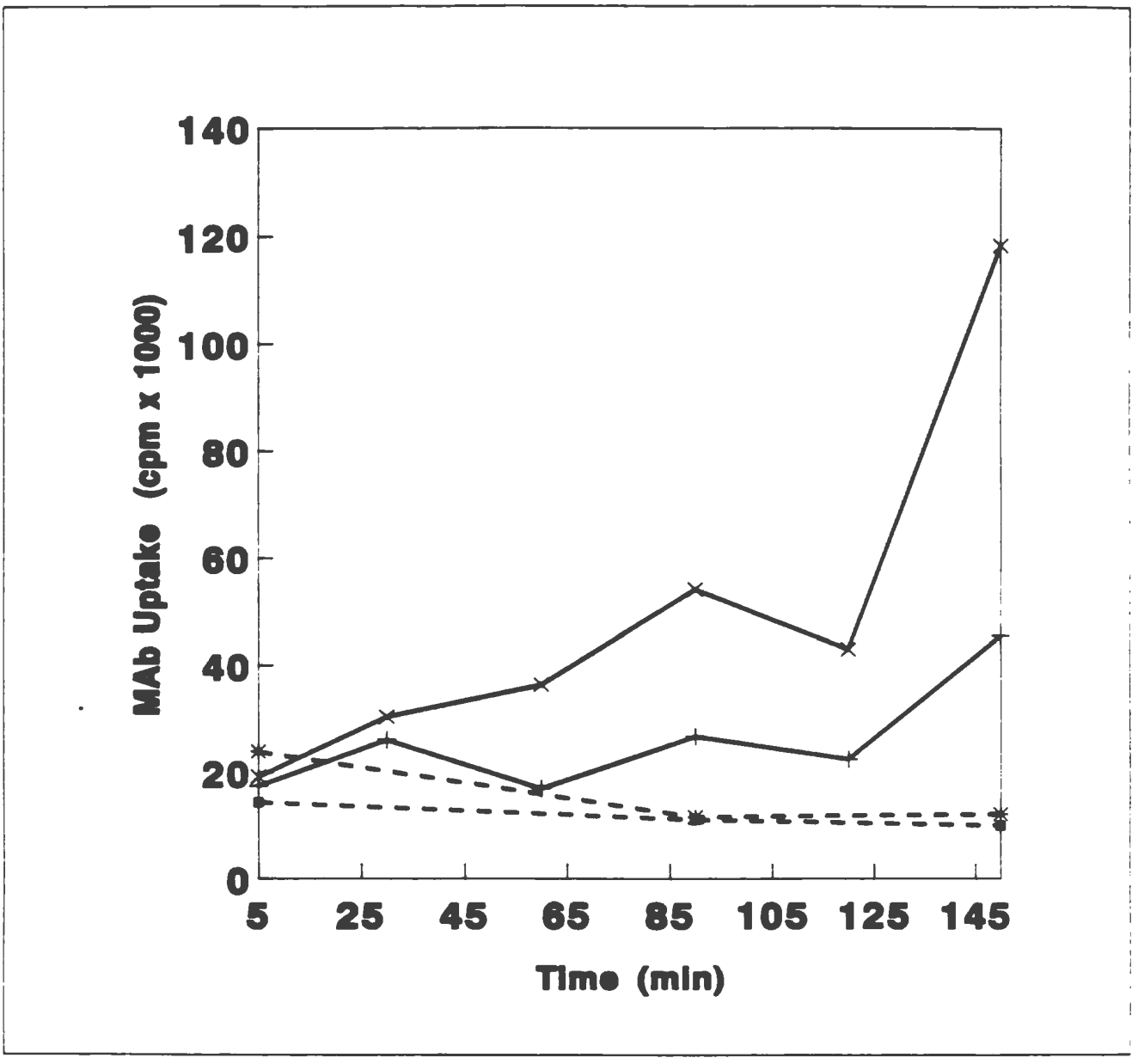


Figure 1.2.a

Table 1.2.b
RIA employing Glycine-HCl to Estimate
Mab Internalization(37°C)
Experiment 2
Means of duplicate tubes

TIME(min)	SAMPLE	COUNT 1	COUNT 2
5	2	64759	38633
	2c	54592	27142
30	4	76004	34800
60	6	83370	35587
90	8	88353	39448
	8c	25049	22408
120	10	110582	51762
150	12	150798	56525
	12c	19928	17143

Test sample tubes : a,b
Control sample tube : c
COUNT 1 : radioactivity counts taken prior to treatment with the glycine-HCl buffer.
COUNT 2 : counts taken after buffer treatment.

Table 1.2.b
RIA employing Glycine-HCl to Estimate
Mab Internalization(37°C)
Experiment 2
Means of duplicate tubes

TIME(min)	SAMPLE	COUNT 1	COUNT 2
5	2	64759	38633
	2c	54592	27142
30	4	76004	34800
60	6	83370	35587
90	8	88353	39448
	8c	25049	22408
120	10	110582	51762
150	12	150798	56525
	12c	19928	17143

Test sample tubes : a,b
Control sample tube : c
COUNT 1 : radioactivity counts taken prior to treatment with the glycine-HCl buffer.
COUNT 2 : counts taken after buffer treatment.

Table 1.3.a
RIA employing Glycine-HCl to Estimate Mab Internalization(0°C); Experiment 3
Means of duplicate tubes

TIME(min)	SAMPLE	COUNT 1	COUNT 2
5	1	38129	25870
	1c	17763	17712
30	3	41256	24177
60	5	45107	26722
90	7	42120	24090
	7c	20292	17074
120	9	42162	23410
150	11	35208	20402
	11c	19979	15704

Control sample tube : c
COUNT 1 : radioactivity counts taken prior to treatment with the glycine-HCl buffer.
COUNT 2 : counts taken after buffer treatment.

Table 1.4.a
Effect of second Mab incubation after glycine treatment
90 minute sample (0°C)

Tube Numbers	Means of counts
7a, 7b	37920
7c	26384

Immediately following the second radioactivity count 100μl cell medium with 10μl iodinated Mab were added to indicated samples and incubated at 0°C for 30 minutes. Cells were then centrifuged, washed x 3 with 1% fetal calf serum in PBS and counted. Counts from this table should be correlated to respective counts in table 1.3.a (90 min samples).

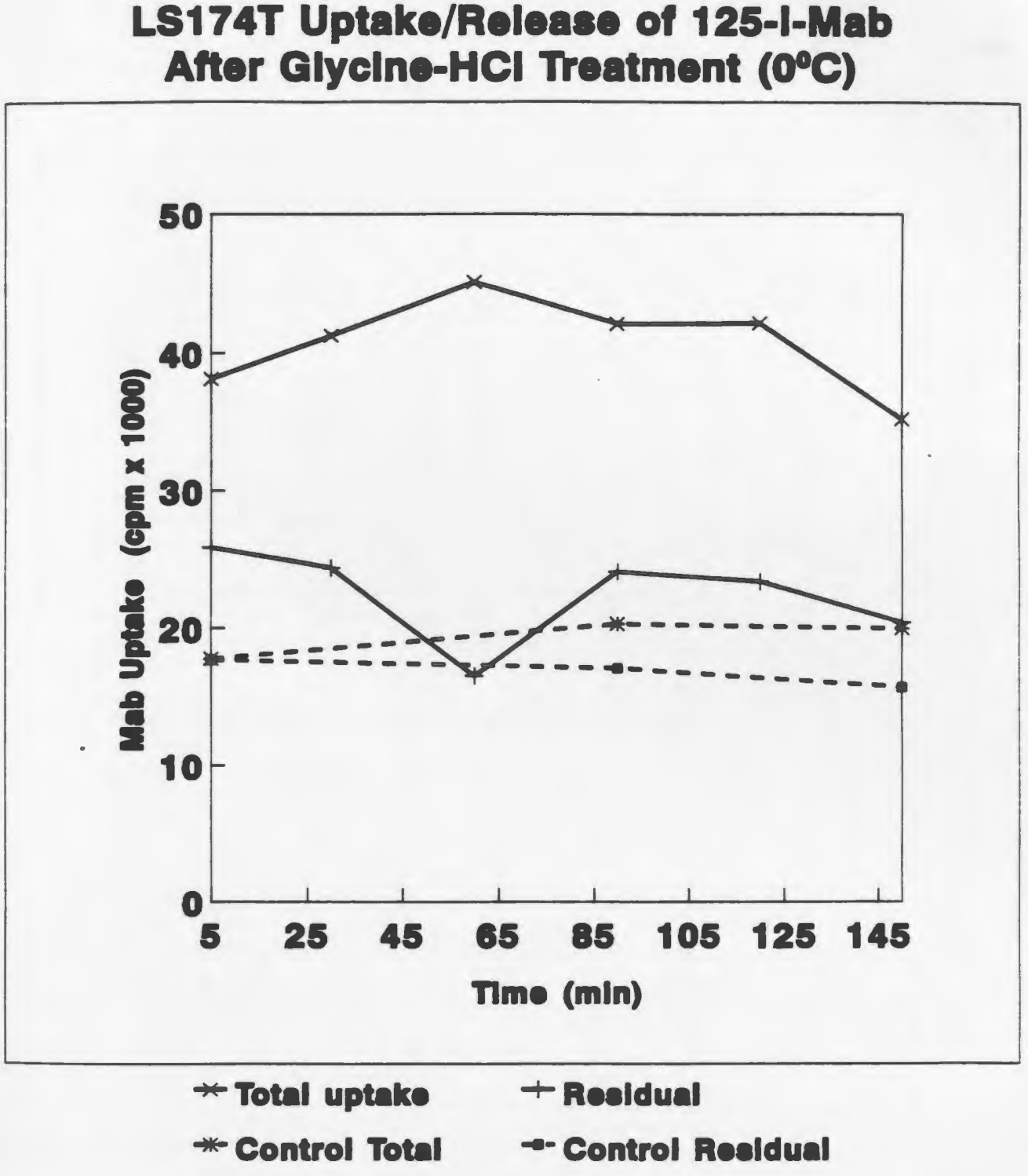

LS174T Uptake/Release of 125-I-Mab
After Glycine-HCl Treatment (0°C)
Mab Uptake (cpm x 1000)
50
40
30
20
10
0
5
25
45
65
85
105
125
145
Time (min)
Total uptake
Residual
Control Total
Control Residual

Figure 1.3.a

Table 1.3.b
RIA employing Glycine-HCl to Estimate Mab Internalization(37°C)
Experiment 3
Means of duplicate tubes

TIME(min)	SAMPLE	COUNT 1	COUNT 2
5	2	25068	16231
	2c	36682	31003
30	4	33821	19616
60	6	46968	27116
90	8	37378	20674
	8c	11011	9262
120	10	45760	25282
150	12	41136	22210
	12c	13207	11718

Control sample tubes: c
COUNT 1 : radioactivity counts taken prior to treatment with the glycine-HCl buffer.
COUNT 2 : counts taken after buffer treatment.

Table 1.4.b
Effect of second Mab incubation after glycine treatment
90 minute sample (37°C)

Tube Numbers	Means of counts
8a, 8b	30613
8c	15974

Immediately following the second radioactivity count 100μl cell medium with 10μl iodinated Mab were added to indicated samples and incubated at 0°C for 30 minutes. Cells were then centrifuged, washed x 3 with 1% fetal calf serum in PBS and counted. Counts from this table should be correlated to respective counts in table 1.3.b (90 min samples).

LS174T Uptake/Release of 125-I-Mab After Glycine-HCl Treatment (37°C)

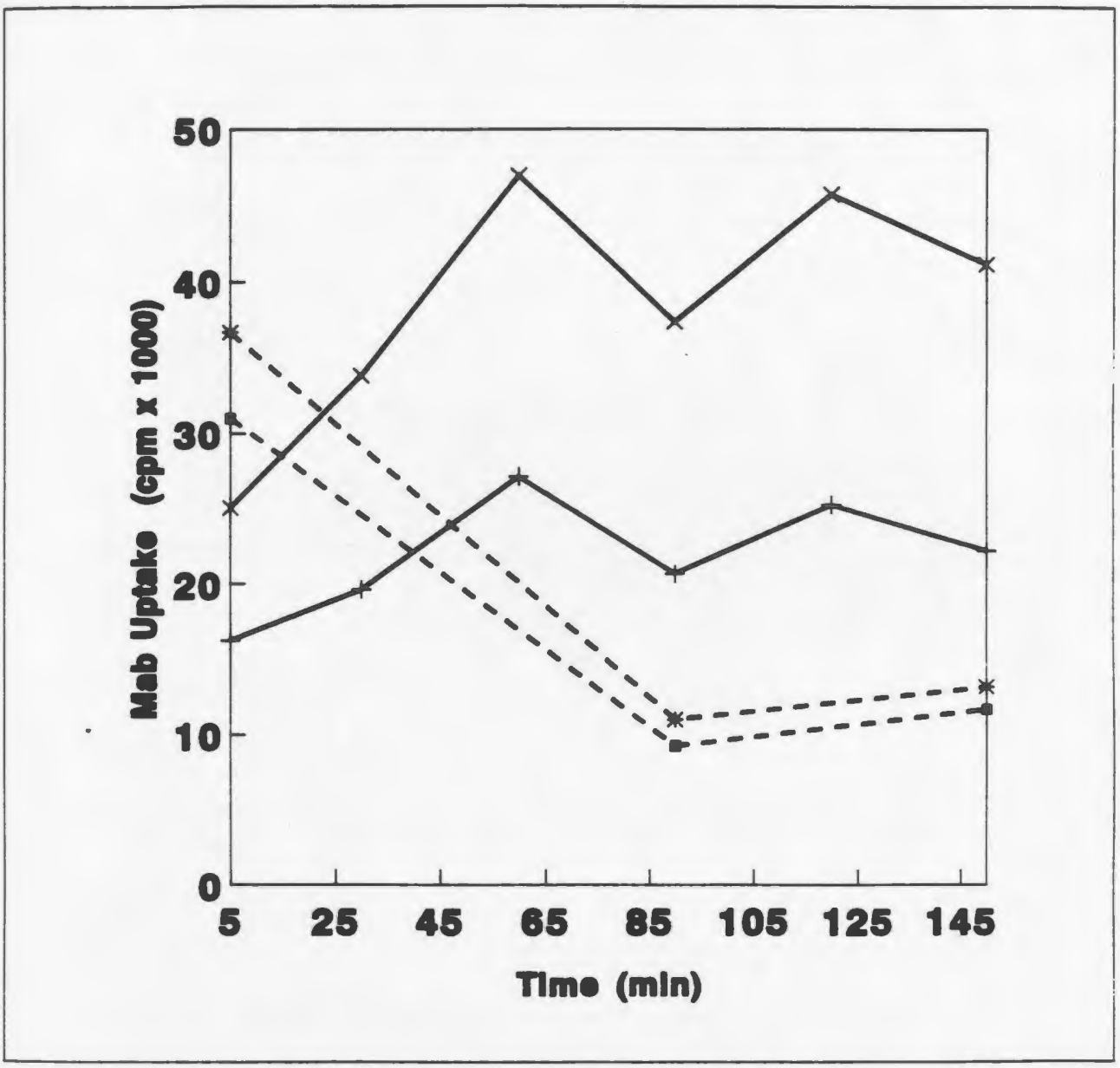


Figure 1.3.b

III. 2. Investigation of the effectiveness of the Glycine-HCl Buffer in dissociating Antigen-Antibody Bonds

Preliminary results of radioimmunoassays employing the low pH glycine-HCl buffer to dissociate antigen-antibody bonds of surface complexes (section III.1), raised some doubt as to the ability of this buffer to completely dissociate such bonds. For example, although assays 1.1 and 1.2 both showed a low to moderate level of residual activity as a percentage of total antibody uptake at 37°C, assay 1.2 also yielded low residual activity (due to internalization) at 0°C, a temperature which inhibits active internalization processes. The relatively uncomplicated nature of this assay and the fact that no other major inter-assay inconsistencies were observed, suggested the possibility of inconsistencies in the dissociating effect of the buffer, resulting in non-dissociated, surface-bound antibody being mistakenly taken as internalized antibody, leading to inaccurate internalization estimates. Therefore, before expanding this assay to study various questions of Mab internalization in our model system it was necessary to accurately establish the effectiveness of this buffer in dissociating antigen-antibody bonds. To that end, a number of immunoperoxidase and ELISA assays were performed using both fixed and live cells.

III. 2.1 Immunoperoxidase Assays Assessing the Effect of the Glycine-HCl buffer on fixed cells

Both direct and indirect immunoperoxidase assays were performed employing

the anti-CEA Mab conjugated to HRP, or unconjugated anti-CEA Mab followed by a second incubation with RAM-HRP. There was no indication of an antigen-antibody dissociation effect on LS174T and SKCO1 cells treated with 11-285-14 or 11-285-14-HRP antibodies and exposed to a 20 min incubation with the buffer as shown in Table 2.1 (summarized data).

Table 2.1

Summary of the Effect of Glycine-HCl buffer on Ag-Ab bonds on fixed cells by Immunoperoxidase			
%level of staining			
CELL LINE	ANTIBODY	Pre-buffer Treatment	Post-buffer Treatment
LS174T	11-285-14	20	20
	11-285-14-HRP	20	20
SKCO1	11-285-14	90	90
	11-285-14-HRP	95	75

Immunoperoxidase assay results (10^5 cells/slide). Antibodies used at 1 μg/slide (optimal antibody concentration determined in previous saturation assays). Control ascites and/or Ag8 or Ag8-HRP were used as controls at the same concentrations and produced no staining. Incubation time for the glycine-HCl buffer was 20 min at RT. Brown-stained cells counted in 3 fields (200 cells per field).

A reduction in staining ($\sim$20%) was observed only in the case of the conjugated antibody on the SKCO1 cells. The possibility of this effect being due to the dissociation of HRP from Mab rather than antigen-antibody bond dissociation, was then further investigated using antibody coated plates in an ELISA (see section III.2.2).

III. 2.2 ELISAs Assessing the Effect of the Glycine-HCl buffer

ELISAs were designed to investigate the dissociating effect of this buffer on fixed and live cells derived from human cancer cell lines, as well as its dissociating effect on Mab-HRP bonds and antigen-antibody bonds when purified antigen is used. Both assay results as well as summaries of averaged data are provided where appropriate.

III. 2.2.a ELISAs on Fixed Cells

In order to complement the results obtained by the immunoperoxidase method, three CEA-expressing cell lines were tested in a number of ELISAs employing methanol-fixed cells (Tables 2.2.a.1, 2.2.a.2, 2.2.a.3, 2.2.a.4 and 2.2.a.5). The dissociating effect of glycine-HCl was correlated to a reduction in absorbance readings following treatment with this buffer. To test the possibility of reduction in absorbance readings due to cells washing off, 11-285-14 Mab was reapplied on a test group after treatment with the glycine-HCl buffer. The derivation of Mab reassociation potential was also attempted using this group. The additional non-specific sticking factor due to the second antibody incubation, was eventually taken into consideration by including a control group with secondary antibody only (RAM-HRP).

Legend for table 2.2.a.1 and 2.2.a.2

All plates were coated with 2.5×10^5 cells ml^{-1} of each cell line and the antibody was detected by RAM-HRP as described in section II.5.3. Optimal Mab concentration was determined in previous saturation assays (not shown). A general control group testing non-specific sticking of RAM-HRP to the wells was subtracted from all values. All O.D. readings represent the average of five wells. Groups included in Table 2.2.a.1 are as follows:

Set 1: Total Mab (2 μg/well) binding to cells
Set 2: Mab (2 μg/well), followed by a 20 minute incubation with glycine-HCl; measures effect of the buffer on Ag-Mab bonds
Set 3: Mab (at 2 μg/well) followed by a 20 minute incubation with glycine-HCl, followed by a second incubation with Mab (2 μg/well)
% Res: % residual binding = (Set 2 / Set 1) x 100%
% Diss: % Ag-Mab bond dissociation = 100 - % Residual
% FB : % Final Binding (Mab binding after incubation with glycine-HCl and second Mab) = (Set 3 / Set 1) x 100% ; measures whether original Mab binding levels can be achieved again

Table 2.2.a.1
Effect of glycine-HCl on Ag-Mab bond dissociation and reassociation on fixed cells at room temperature (RT)

Assay #	Cell line	Set 1	Set 2	Set 3	% Res	% Diss	% FB
377	LS174T	.192	.074		38.5	61.5	
	SKCO1	.455	.262		57.6	42.4	
	BENN	.180	.078		43.3	56.7	
379	LS174T	.139	.061		43.9	56.1	
	SKCO1	.345	.184		53.3	46.7	
	BENN	.117	.065		55.6	44.4	
380	LS174T	.321	.047		14.6	85.4	
	SKCO1	.339	.114		33.6	66.4	
	BENN	.319	.049		15.4	84.6	
386	LS174T	.344	.343	.365	99.7	0.3	106.1
	SKCO1	.408	.334	.339	81.9	18.1	83.1
	BENN	.107	.072	.152	67.3	32.7	142
387	LS174T	.142	.092	.170	64.8	35.2	119.7
	SKCO1	.244	.142	.166	58.2	41.8	109
	BENN	.064	.03	.088	46.9	53.1	137.5
388	LS174T	.147	.097	.222	66	34	151
	SKCO1	.458	.395	.470	86	14	103
	BENN	.114	.088	.172	77.2	22.8	151
499	LS174T	.074	.037	.103	50	50	139.2
	SKCO1	.291	.131	.366	45	55	125.8
	BENN	.091	.044	.188	48.3	51.7	206.6

Table 2.2.a.2
Effect of glycine-HCl on Ag-Mab bond dissociation and reassociation on fixed cells at 37°C

Assay #	Cell line	Set 1	Set 2	Set 3	% Res	% Diss	% FB
382	LS174T	.402	.344		85.6	14.4	
	SKCO1	.441	.398		90.2	9.8	
	BENN	.273	.327		119.8	-	
386	LS174T	.315	.225	.375	71.4	28.6	119
	SKCO1	.376	.205	.271	54.5	45.5	72.1
	BENN	.103	.054	.144	52.4	47.6	140
387	LS174T	.167	.061	.172	36.5	63.5	103
	SKCO1	.308	.114	.234	37	63	76
	BENN	.054	.036	.108	66.7	33.3	200
388	LS174T	.114	.058	.153	50.9	49.1	134.2
	SKCO1	.435	.306	.433	70.3	29.7	99.5
	BENN	.114	.092	.175	80.7	19.3	153.5
499	LS174T	.056	.029	.173	51.8	48.2	308.9
	SKCO1	.165	.058	.291	35.1	64.9	176.4
	BENN	.259	.217	.424	83.8	16.2	163.7

Set 1: Total Mab (2 μg/well) binding to cells
Set 2: Mab (2 μg/well), followed by a 20 minute incubation with glycine-HCl; measures effect of the buffer on Ag-Mab bonds
Set 3: Mab (at 2 μg/well) followed by a 20 minute incubation with glycine-HCl, followed by a second incubation with Mab (2 μg/well)
% Res: % residual binding = (Set 2 / Set 1) x 100%
% Diss: % Ag-Mab bond dissociation = 100 - % Residual
% FB : % Final Binding (Mab binding after incubation with glycine-HCl and second Mab) = (Set 3 / Set 1) x 100% ; measures whether original Mab binding levels can be achieved again

Because of the greater than 100% values obtained for FB in assays 386, 387 and 388 (which could not be attributed to initial incomplete saturation of antigenic sites, as the antibody was used at a saturating concentration determined on previous titration experiments), an additional control group (Set 4), was added to monitor the effect of the second incubation with Mab without any incubation in glycine buffer (i.e. cells + Mab + Mab + RAM-HRP). This group would in effect provide the "non-specific" binding factor caused by a second incubation. A correction factor was then derived by dividing Set 1 values by Set 4 values (since Set 4 would now actually represent total Mab binding) and actual final binding values (% FB_c) would be calculated by multiplying previous Final Binding values by this correction factor.

Corrected estimates are given in Tables 2.2.a.3 and 2.2.a.4.

Table 2.2.a.3
Corrected values for the estimation of Mab reassociation (RT)

Assay#	Cell line	Set 1	Set 4	%FB	CF	%FB_c
499	LS174T	.074	.221	139.2	.335	46.6
	SKCO1	.291	.456	125.8	.638	80.3
	BENN	.091	.296	206.6	.307	63.4

Correction factor (CF) = (Set 1 / Set 4)
% Corrected Final Binding (% FB_c) = % FB x CF

Table 2.2.a.4
Corrected values for the estimation of Mab reassociation
(37°C)

Assay#	Cell line	Set 1	Set 4	%FB	CF	$\%FB_c$
499	LS174T	.056	.216	308.9	.259	80
	SKCO1	.165	.372	176.4	.443	78.2
	BENN	.259	.493	163.7	.525	86

Correction factor (CF) = (Set 1 / Set 4)
% Corrected Final Binding (% FB_c) = % FB x CF

Note: Final Binding values reflect derivations based on the actual absorbance at 405 nm (amount of Mab) detected after incubation with buffer and antibody, and not the additional absorbance at 405 nm (amount of Mab) detected after the second incubation.

If the corrected Final Binding values are now compared to the amount of residual absorbance (Mab attached after glycine-HCl treatment), any additional Mab that may have been attached after the second incubation (i.e. a reassociation value), can be estimated by subtracting Residual binding values from corrected Final Binding values. Mab reassociation estimates derived in this manner are shown in Table 2.2.a.5.

Table 2.2.a.5
Estimation of reassociation of Mab after glycine-HCl treatment and a second incubation with Mab

Cell Line	RT			37°C		
	%Res	FB_c	%Reas	%Res	FB_c	%Reas
LS174T	50	46.6	nil	51.8	80	28.2
SKCO1	55	80.3	25.3	35.1	78.2	43.1
BENN	51.7	63.4	11.7	83.8	86	2.2

%Reas = % Reassociation = FB_c - %Res

The performance of the glycine-HCl buffer in dissociating Ag-Mab bonds is summarized in Table 2.2.a.6, which contains the average dissociation values of the previously tabulated assays. Reassociation values are provided for assay #499.

Table 2.2.a.6

Summary of the effect of Glycine-HCl buffer on 11-285-14-treated methanol fixed cells*				
	%Diss		%Reas	
CELL LINE	RT	37°C	RT	37°C
LS174T	46.1 (7)	40.8 (5)	nil	28.2
SKCO1	40.6 (7)	42.6 (5)	25.3	43.1
BENN	49.4 (7)	23.3 (5)	11.7	2.2

* Numbers in parentheses indicate number of repeat assays. Reassociation estimates were derived solely from assay #499 due to lack of correction factors for the other assays. As before %Diss (% dissociation) indicates % reduction in absorbance readings after buffer treatment whereas %Reas (% reassociation) indicates relative increase in absorbance readings after buffer treatment and second antibody incubation.

If the decrease in absorbance readings is correlated to dissociation of Ag-Ab complexes due to the effect of the buffer it is obvious that the glycine-HCl buffer causes, on average, dissociation of less than 50% of Ag-Ab bonds in all three lines. Furthermore, an increase in temperature from RT to 37°C does not seem to affect Ag-Ab bond dissociation to any appreciable level (other than causing a decrease in the BENN cell line). Although values after a second incubation with antibody did not usually return to their original levels, there was a general increase in staining indicating some degree of reassociation with CEA expressed on the cell lines.

III. 2.2.b ELISAs on Live Cells

Since the characteristics of antibody uptake and dissociation from cell surface antigens may be significantly affected by fixing those cells, assays investigating the dissociating capacity of the glycine buffer were also performed on live cells. During these assays we employed both HRP-conjugated anti-CEA Mab (direct CELISA) and unconjugated anti-CEA detected by commercially available RAM-HRP (indirect CELISA). The results obtained with live cells using the same cell lines as before are shown in Tables 2.2.b.1 and 2.2.b.2.

Table 2.2.b.1
Effect of glycine-HCl buffer on Ag-Mab bonds on Live Cells at RT

Assay#	Cell line	Set1	Set2	%Diss (unc)	Set3	Set4	%Diss (con)
468	LS174T	.062	.062	0	.072	.057	20.8
	SKCO1	.355	.396	0	.691	.287	59.8
*469	SKCO1	.453	.545	0	1.267	.596	53
	BENN	.162	.085	47.5	.389	.115	70.4
472	LS174T	.274	.118	57	.286	.104	64
	LS174T	.293	.105	64.2	.278	.112	59.5
	SKCO1	.499	.350	29.9	.397	.124	68.8
522	SKCO1	.108	.143	0	ND	ND	ND
	BENN	.207	.116	44	ND	ND	ND

A control group consisting of cells incubated with PBS followed by RAM-HRP was averaged and subtracted from all other groups. Most assays also included an additional negative control which used the non-CEA specific Ab Ag8. As with test groups this antibody was also treated with the glycine-HCl buffer. In all assays values for these groups were at the levels of general (RAM-HRP) control values and were not included in the calculations.

Cell concentration: 5×10^5 cells ml^{-1}

Mab concentration : 5 μg ml^{-1} except for assay #469(*) where Mab concentration was 10 μg ml^{-1} (determined in previous saturation assays)

Set1 : Total 11-285-14 Mab binding to cell line

Set2 : Residual 11-285-14 Mab binding after Glycine-HCl treatment

%Diss(unc): (% dissociation of unconjugated Mab)
= 100 - ((Set2 / Set1) * 100 %)

Set3 : Total 11-285-14-HRP Mab binding to cells

Set4 : Residual 11-285-14-HRP Mab binding

%Diss(con): (% dissociation of HRP conjugated Mab)
= 100 - ((Set4 / Set3) * 100 %)

ND : Not Done

Table 2.2.b.2
Effect of glycine-HCl buffer on Ag-Mab bonds on Live Cells at 37°C

Assay#	Cell line	Set1	Set2	%Diss (unc)	Set3	Set4	%Diss (con)
*469	SKCO1	.719	.475	34.4	1.142	.596	52.2
	BENN	.227	.211	7	.506	.164	67.6
472	LS174T	.299	.127	57.5	.399	.076	80.9
	LS174T	.391	.154	60.5	.263	.100	62.1
	SKCO1	.499	.350	29.9	.397	.124	63.8
522	SKCO1	.334	.299	10.5	ND	ND	ND
	BENN	.242	.141	41.7	ND	ND	ND

A control group consisting of cells incubated with PBS followed by RAM-HRP was averaged and subtracted from all other groups. Most assays also included an additional negative control which used the non-CEA specific Ab Ag8. As with test groups this antibody was also treated with the glycine-HCl buffer. In all assays values for these groups were at the levels of general (RAM-HRP) control values and were not included in the calculations.

Cell concentration : 5 x 10^5 cells ml^{-1}
Mab concentration : 5 μg ml^{-1} except for assay #469(*) where the Mab concentration was doubled
Set1 : Total 11-285-14 Mab binding to cell line
Set2 : Residual 11-285-14 Mab binding after Glycine-HCl treatment
%Diss(unc): (% dissociation of unconjugated Mab)
= 100 - ((Set2 / Set1) * 100 %)
Set3 : Total 11-285-14-HRP Mab binding to cells
Set4 : Residual 11-285-14-HRP Mab binding
%Diss(con): (% dissociation of HRP conjugated Mab)
= 100 - ((Set4 / Set3) * 100 %)
ND : Not Done

A summary of the data given in Tables 2.2.b.1 and 2.2.b.2 appears in the following Table (2.2.b.3).

Table 2.2.b.3

Effect of the Glycine-HCl buffer on antigen-antibody bonds using live cells				
% reduction in absorbance readings				
Antibody		**LS174T**	**SKCO1**	**BENN**
11-285-14	**RT**	40.4 (3)	7.5 (4)	45.8 (2)
	37°C	59 (2)	24.9 (3)	24.4 (2)
11-285-14 /HRP	**RT**	48.1 (3)	60.5 (3)	70.4 (1)
	37°C	71.5 (2)	58 (2)	67.6 (1)

Percent (%) reduction in absorbance readings corresponds to % dissociation and numbers in parentheses determine the number of assays performed (numbers indicate mean values).

There was inter-assay variability due to the nature of the assay, but low background and Ag8 control values as well as low variability within test groups minimize the possibility of loss of cells, or major artifacts contributing to the results. As was the case with the fixed cell assays, the dissociating effect of the buffer, as reflected in a decrease in absorbance readings, varied from no dissociation to a maximum of approximately 60% dissociation in all cell lines tested and at both temperatures, in the case of the indirect assay using Mab and secondary Ab (RAM-HRP) (%Diss(unc), Tables 2.2.b.1 and 2.2.b.2). When HRP conjugated Mabs were used (direct CELISA), there was a stronger dissociating effect (up to 80% dissociation). There did not seem to be any

significant difference in Mab-Ag bond dissociation for the two temperatures tested. The possibility of the increase in dissociation when HRP-conjugated antibodies were used being due to a buffer-mediated disruption of Ab-HRP bonds was then investigated (following section).

III. 2.2.c Effect of the glycine-HCl buffer on Mab-HRP bonds

In order to examine a possible disruption of the Ab-HRP bond due to glycine-HCl, direct ELISAs were performed employing both laboratory HRP-conjugated antibodies (11-285-14, Ag8) and the commercially available RAM-HRP. The concentration of antibodies used was again determined in preliminary titration assays. Assay results are provided again as absorbance readings in Tables 2.2.c.1 and 2.2.c.2 and summarized results are given in Table 2.2.c.3.

All plates were coated with 100 μl of 1 μg ml^{-1} antibody per well. % Dissociation was derived as before.

Table 2.2.c.1
Effect of the glycine-HCl buffer on Ab-HRP bonds (RT)

Assay #	Antibody	Pre-buffer treatment	Post-buffer treatment	% Diss
474	11-285-HRP	1.989	1.692	15
	Ag8-HRP	1.218	.686	43.7
	RAM-HRP	.468	.336	28.2
476 (Sigma glycine)	11-285-HRP	2.612	2.209	15.4
	Ag8-HRP	1.903	1.299	31.7
	RAM-HRP	1.333	1.048	21.4
476 (Aldrich glycine)	11-285-HRP	2.447	2.066	15.6
	Ag8-HRP	1.913	.985	48.5
	RAM-HRP	1.324	.986	25.5

Table 2.2.c.2
Effect of the glycine-HCl buffer on Ab-HRP bonds (37°C)

Assay #	Antibody	Pre-buffer treatment	Post-buffer treatment	% Diss
474	11-285-HRP	1.686	.670	60.3
	Ag8-HRP	1.108	.183	83.5
	RAM-HRP	.504	.214	57.5
476 (Sigma glycine)	11-285-HRP	2.492	1.389	44.3
	Ag8-HRP	1.894	.511	73
	RAM-HRP	1.374	.660	52
476 (Aldrich glycine)	11-285-HRP	2.477	1.039	58
	Ag8-HRP	1.881	.290	84.6
	RAM-HRP	1.474	.663	45

A summary of the above assays examining the effect of the buffer on antibodies conjugated to HRP (a combination of Tables 2.2.c.1 and 2.2.c.2) is presented in the following table (Table 2.2.c.3).

Table 2.2.c.3

Effect of Glycine-HCl on HRP conjugated antibodies		
Antibodies	**% reduction in absorbance readings**	
	RT	**37°C**
11-285-14-HRP	15.3	54.2
Ag8-HRP	41.3	80.4
RAM-HRP	25	51.5

Numbers indicate the mean of three assays

The two antibodies which were conjugated to HRP in our laboratory as well as the commercially available RAM-HRP were used to initially coat ELISA plates. In all three cases there was a reduction in absorbance readings after incubation with the Glycine-HCl buffer, possibly indicating a dissociating effect on the bonds between the antibodies and HRP. This dissociating effect was consistently more prominent at higher temperatures (37°C) for all Ab-HRP conjugates. Furthermore, there seemed to be differences in Ab-HRP bond stability among the conjugates with Ag8-HRP being the most unstable (highest level of dissociation).

III. 2.2.d ELISAs on Purified CEA

In order to investigate the possibility of the cellular environment hindering the action of the buffer an ELISA assay was performed on plates coated with purified CEA.

Table 2.2.d (ELISA #528)
Effect of Glycine-HCl on purified CEA-Antibody bonds

Treatment	RT	37°C
11	.758	.594
11 + Buffer	Nil (-ve)	Nil (-ve)
11 + 11	.647	.452
11 + Buffer + 11	.472	.246
% Dissociation	100	100
% Reassociation	73	54

Plates coated with 100 μl of CEA at 2.5 μg ml^{-1} and were subsequently incubated for two hours with anti-CEA Mab or non-specific antibody at 2 μg ml^{-1} (37°C). Second incubation with Mab was performed for 2 hours at 37°C. Values represent absorbance readings after subtraction of control values (controls : CEA + RAM-HRP and CEA + Ag8). % dissociation and reassociation derived as before.
11 = 11-285-14 Mab; **Buffer** = Glycine-HCl buffer, pH 2.8.

In this case we found that glycine-HCl completely dissociated antigen-antibody bonds (absorbance readings after buffer treatment were lower than control readings). There seemed to have been complete saturation of antigenic sites after the first incubation with Mab, since the subsequent incubation did not result in increased readings. A moderate to high level of antibody reassociation with antigenic sites after buffer treatment was again observed suggesting minimal to moderate disruption of antigenic sites by this buffer. In this assay the same procedure was followed using the non-CEA specific Ag8

antibody as an additional specificity control. In this case all absorbance readings were at background levels, thus ensuring the specificity of binding for the anti-CEA Mab.

Briefly, ELISA assays performed on both fixed and live cells indicate that the average dissociating effect of this buffer on Ag-Ab bonds ranges from about 23% to 49% for fixed cells and from 8% to 59% dissociation for live cells in the three cell lines tested. Generally, comparable levels of dissociation were achieved at both RT and 37°C. Dissociation levels were slightly higher (48-72%) where direct assays were employed (antibody directly linked to HRP), but subsequent testing showed that the additional dissociation could possibly be accounted for by the disruption of the enzyme-antibody linkage of the 11-285-14-HRP, Ag8-HRP and RAM-HRP conjugates. The possibility that the addition of glycine-HCl could result in reduced absorbance readings due to its stripping the antigen or antibody coat off the plate, was also examined using CEA or 11-285-14 coated plates (data not shown). It was found that, in this case, the addition of glycine-HCl buffer did not lead to any decrease in absorbance readings relative to non-buffer treated wells.

A general summary of ELISA assay results depicting % reduction in immunostaining on both fixed and live cells after Glycine-HCl buffer treatment is provided in Table 2.2.e. As before, dissociation of antigen-antibody bonds due to the effect of this buffer is considered to be directly related to reduction in absorbance readings.

Table 2.2.e
Summary of % dissociating effect of glycine-HCl buffer on fixed and live cells (rounded assay averages)

Ab	Temp	LS174T	SKCO1	BENN	CELLS
11-285	RT	46	41	49	FIXED
	37°C	41	43	23	
11-285	RT	40	8	46	
	37°C	59	25	24	LIVE
11-285-HRP	RT	48	61	70	
	37°C	72	58	68	

III. 3. Estimation of Antibody Internalization Employing an Indirect Radioimmunoassay

Given the fact that, in our hands, the glycine-HCl buffer did not seem to have the capacity to fully dissociate Ag-Ab bonds, it became clear that the radioimmunoassay employed thus far would not provide accurate results. Consequently, a second type of RIA was employed in order to indirectly detect the amount of internalized antibody for similar incubation intervals as the ones tested above. Results of two such assays appear in figures 3.1 and 3.2, respectively. Controls consisted of cell samples incubated with the Ag8 antibody which is not specific for CEA. An extra control group (total bound radioactivity at 4°C) was employed in order to test for any possible effect of low temperature on antibody uptake, but was not used in deriving the amount of internalized antibody. In both the cases of test and control antibody, uptake (groups measuring total

radioactivity), seemed to plateau after the first 30-90 minutes of incubation, remaining relatively steady throughout the course of the experiment. Conversely, the group measuring surface activity only, showed a downward trend reflecting an increasing amount of antibody or Ag-Ab complexes being internalized. A comparison of internalization by test (11-285-14) and control (Ag8) immunoglobulins is depicted in figures 3.1.b and 3.2.b.

Figure 3.1. Kinetics of total and surface uptake of 11-285-14 Mab (test) or Ag8 Ab (control), using the double labelling assay (experiment 1). 2.5 x 10^5 LS174T cells/sample were incubated with 2 μg of 11-285-14 or Ag8 antibody at 37°C for 30 minutes. 1 μg ^{125}I-labelled RAM was immediately added to all samples used in the assessment of total binding, whereas the same concentration of secondary antibody was added to the samples used in the assessment of surface binding at the beginning of the prespecified time intervals. Figures 3.1 (a,b) and 3.2 (a,b) correspond to two separate assays.

Figure 3.1.a. Total and surface binding of 11-285-14(solid lines) and Ag8 (dotted lines) antibodies, depicting the loss of 11-285-14 antibody from the cell surface as it becomes internalized by LS174T cells.
4C Ttotal = Total binding of test Ab (4°C)
4C Ctotal = Total binding of control Ab (4°C)
37C Ttotal = Total binding of test Ab (37°C)
37C Ctotal = Total binding of control Ab (37°C)
37C Tsurf = Surface binding of test Ab (37°C)
37C Csurf = Surface binding of control Ab (37°C)

Figure 3.1.b. Graphic representation of the kinetics of internalization of 11-285-14 Mab or Ag8 Ab by LS174T cells (total activity-surface bound activity).

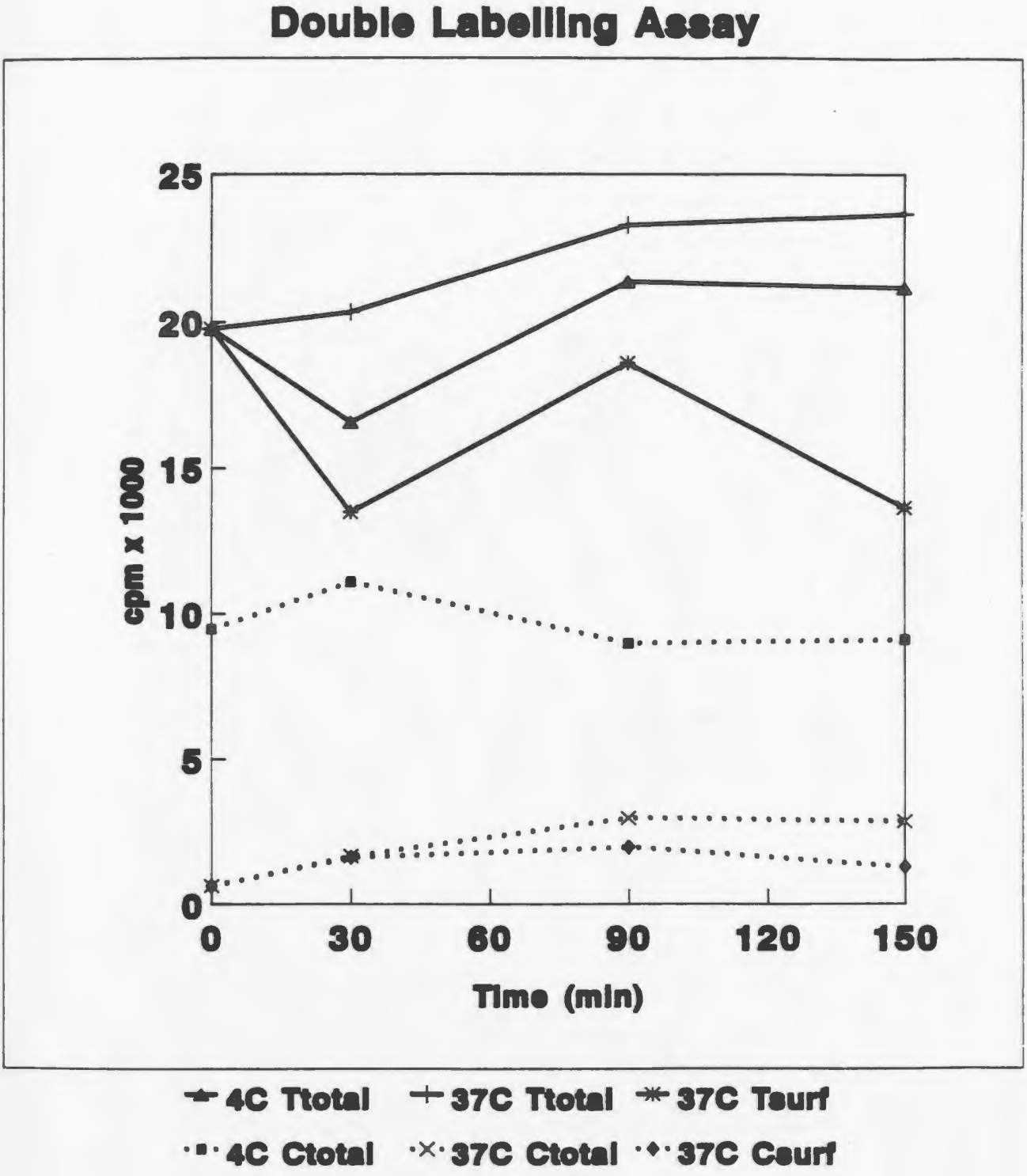

Double Labelling Assay
25
20
15
10
5
0
cpm x 1000
0
30
60
90
120
150
Time (min)
4C Ttotal
37C Ttotal
37C Tsurf
4C Ctotal
37C Ctotal
37C Csurf

Figure 3.1.a

Double Labelling Internalization
Total - surface bound activity

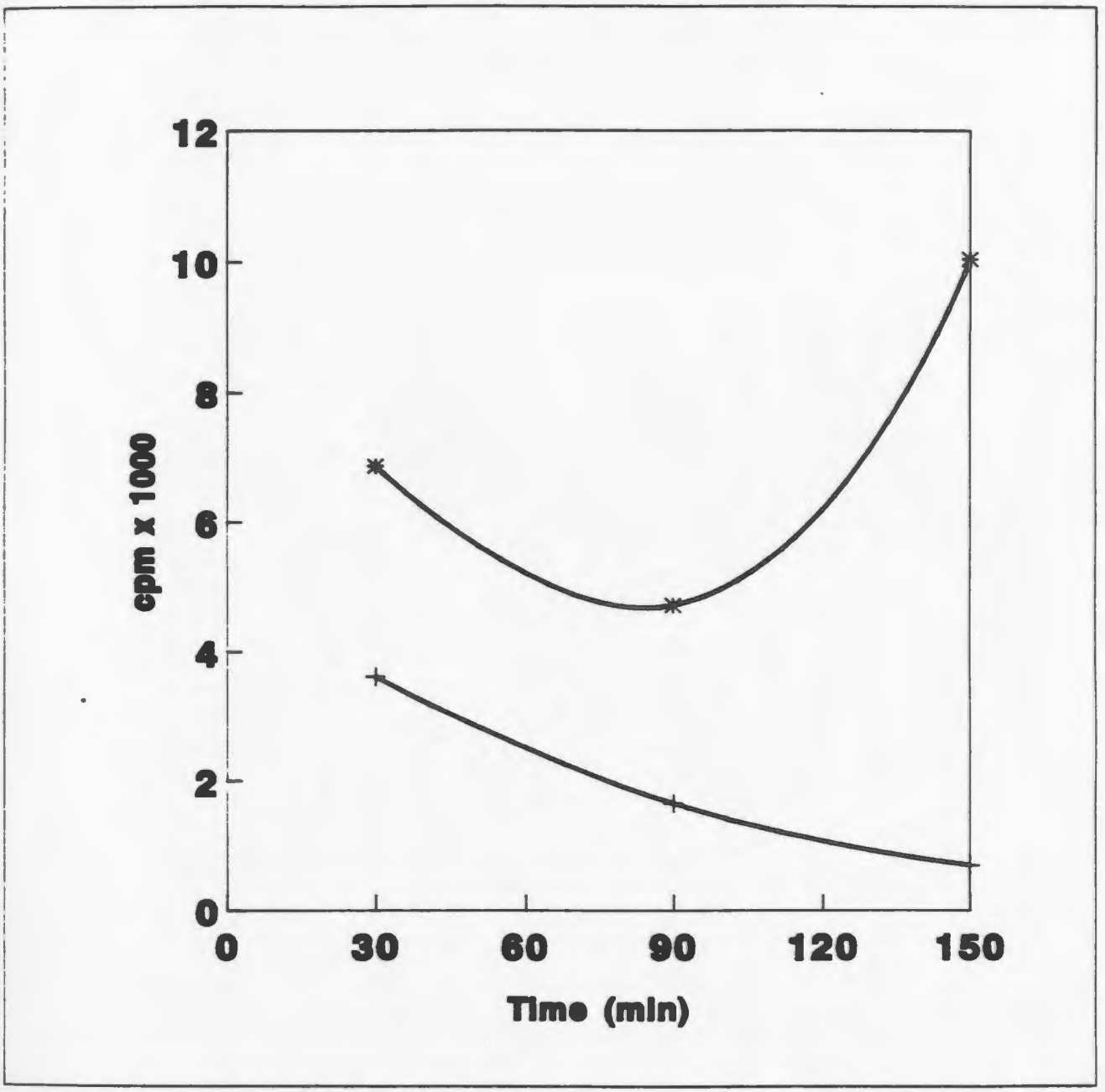


Figure 3.1.b

Figure 3.2. Kinetics of total and surface uptake of 11-285-14 Mab (test) or Ag8 Ab (control), using the double labelling assay (experiment 2). 2.5 x 10^5 LS174T cells/sample were incubated with 2 μg of 11-285-14 or Ag8 antibody at 37°C for 30 minutes. 1 μg ^{125}I-labelled RAM was immediately added to all samples used in the assessment of total binding, whereas the same concentration of secondary antibody was added to the samples used in the assessment of surface binding at the beginning of the prespecified time intervals.

Figure 3.2.a. Total and surface binding of 11-285-14(solid lines) and Ag8 (dotted lines) antibodies, depicting the loss of 11-285-14 antibody from the cell surface as it becomes internalized by LS174T cells.
4C Ttotal = Total binding of test Ab (4°C)
4C Ctotal = Total binding of control Ab (4°C)
37C Ttotal = Total binding of test Ab (37°C)
37C Ctotal = Total binding of control Ab (37°C)
37C Tsurf = Surface binding of test Ab (37°C)
37C Csurf = Surface binding of control Ab (37°C)

Figure 3.2.b. Graphic representation of the kinetics of internalization of 11-285-14 Mab or Ag8 Ab by LS174T cells (total activity-surface bound activity).

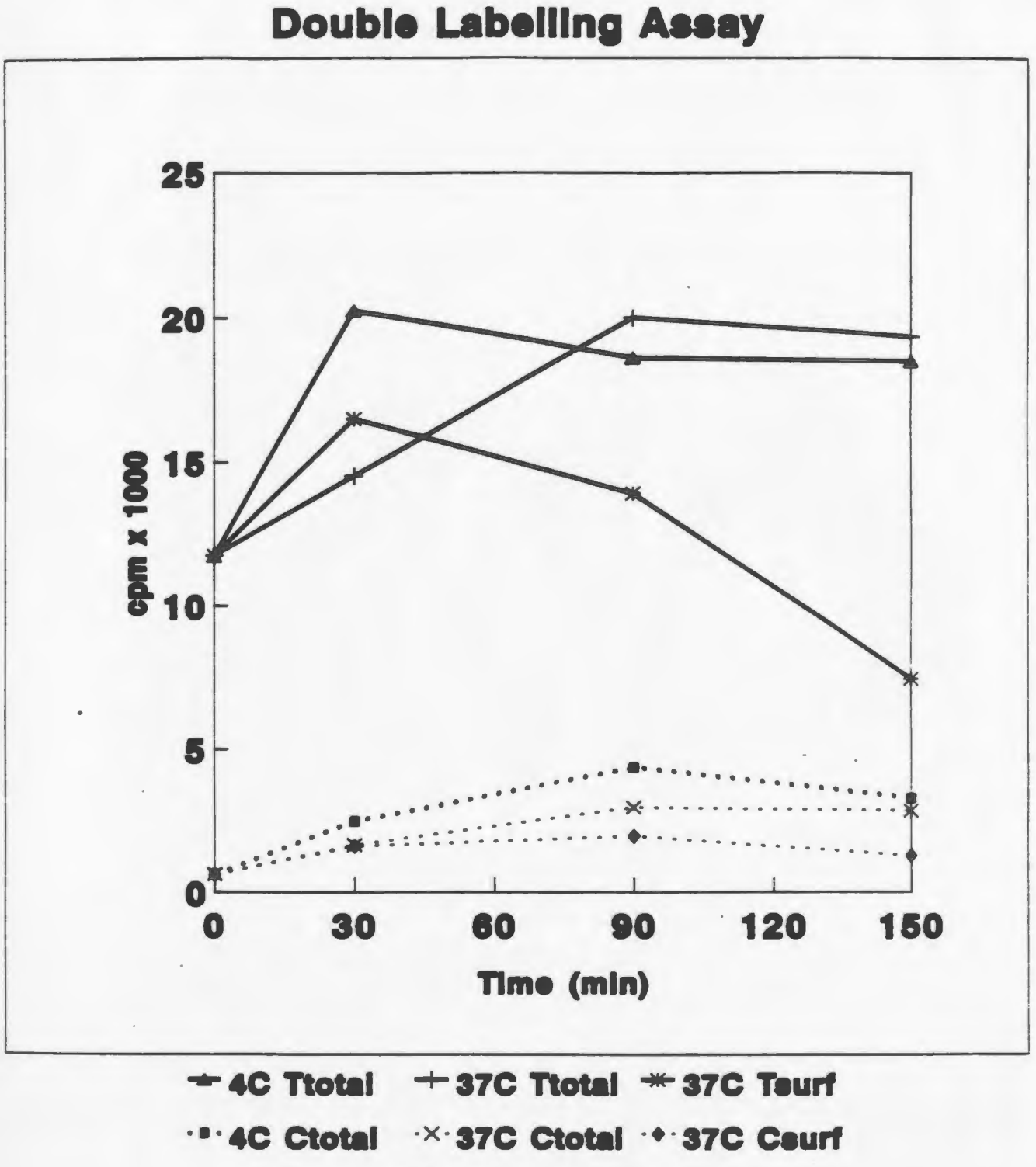

Double Labelling Assay
25
20
15
10
5
0
cpm x 1000
0
30
60
90
120
150
Time (min)
4C Ttotal
37C Ttotal
37C Tsurf
4C Ctotal
37C Ctotal
37C Csurf

Figure 3.2.a

Double Labelling Internalization
Total - surface bound activity

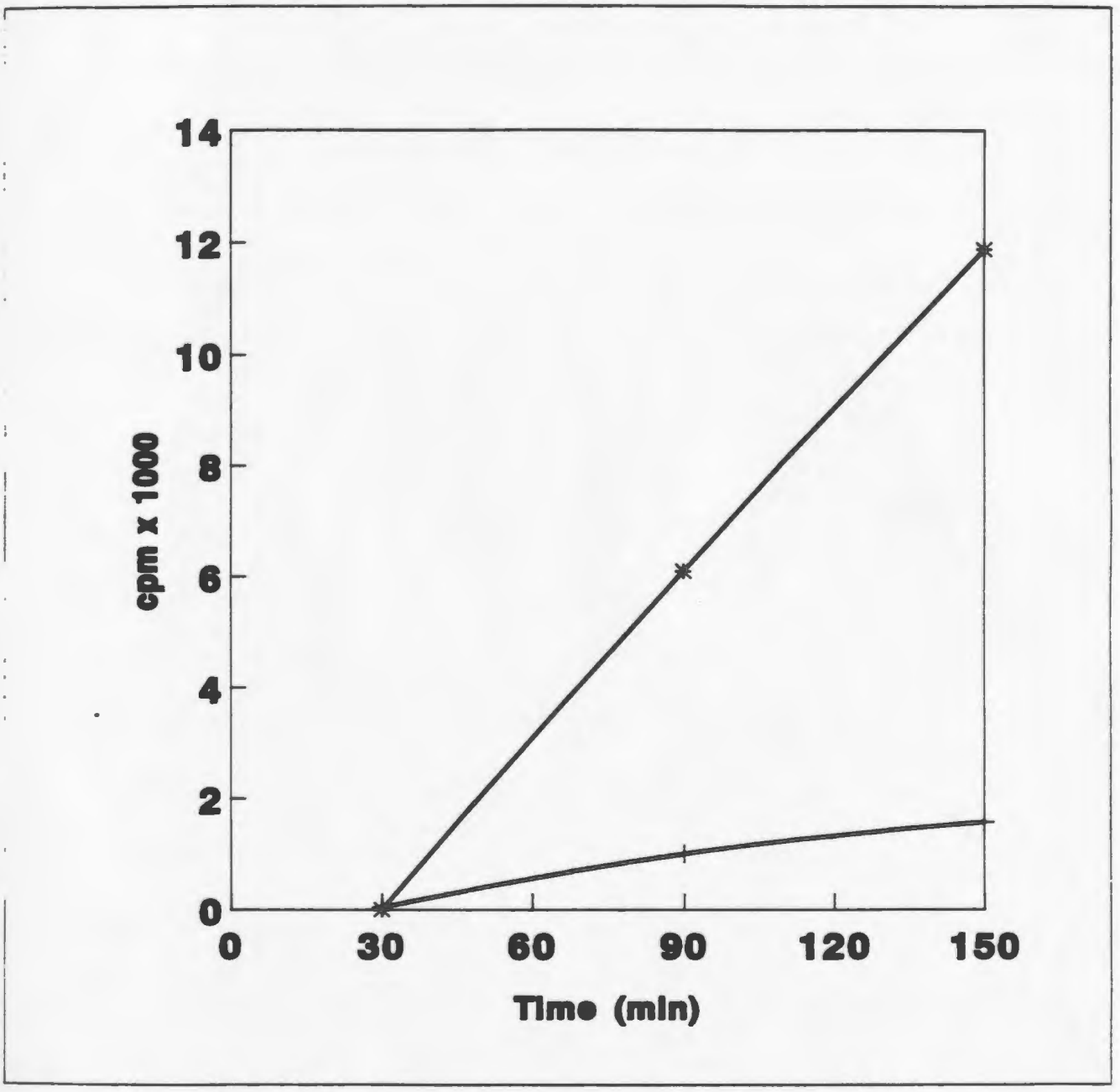


Figure 3.2.b

III. 4. Detection of Internalized anti-CEA antibody using Electron Microscopy

Figures 4.1, 4.2, 4.3 (a and b) depict test and negative control samples of SKCO1 cells incubated with CEA-specific Mab-HRP conjugate (a) or control Ag8-HRP conjugate (b). Darker areas indicate accumulation of the CEA specific conjugate both around the cell surface and in lysosomal vacuoles in the interior of the cell. In contrast, no staining is evident either on the cell surface or in the cell interior in the case of negative controls (b).

Figure 4: General legend. EM results depicting internalization of HRP labelled 11-285-14 Mab (a), but not of HRP labelled Ag8 Ab (b) by SKCO1 human cancer cells (4×10^6 cells ml^{-1}), following a two hour incubation period at 37°C.

4.1.a,b Ab-HRP Mab concentration: 25 $\mu g\ ml^{-1}$ (2.5 μg/well)

4.2.a,b Ab-HRP Mab concentration: 50 $\mu g\ ml^{-1}$ (5 μg/well)

4.3.a,b Ab-HRP Mab concentration: 100 $\mu g\ ml^{-1}$ (10 μg/well)

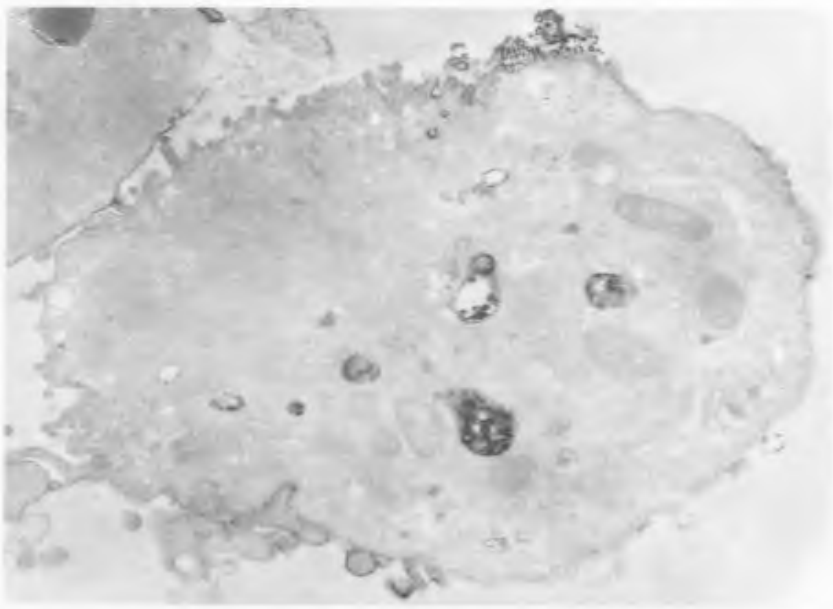


Figure 4.1.a

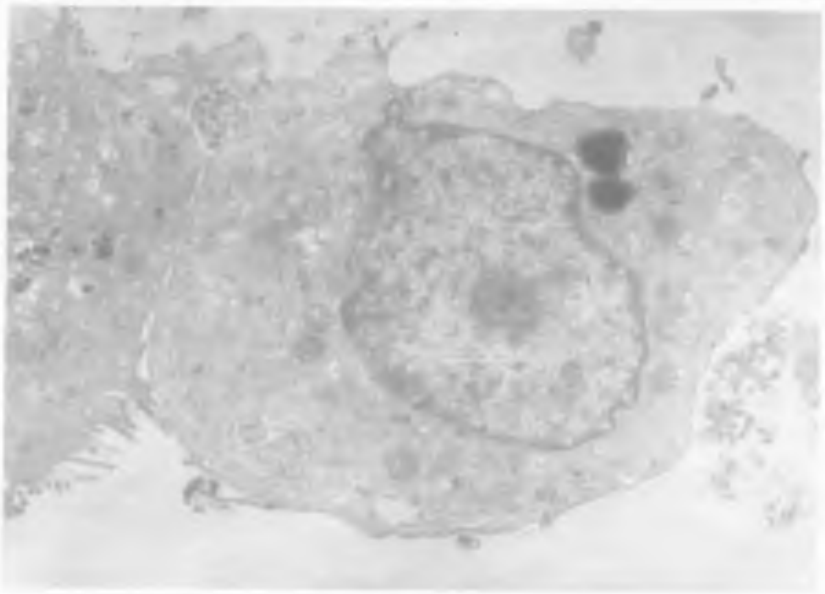


Figure 4.1.b

Figure 4.1 EM sections of SKCO1 cells depicting uptake and internalization of HRP conjugated antibodies at a concentration of 25 $\mu g\ ml^{-1}$.
4.1.a : 11-285-14-HRP anti-CEA Mab
4.1.b : Ag8-HRP (non-CEA specific Ab)

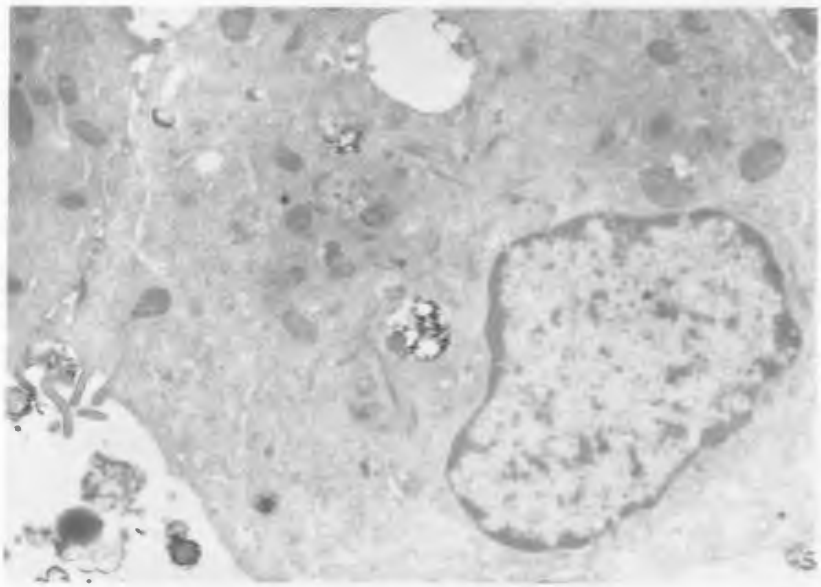


Figure 4.2.a

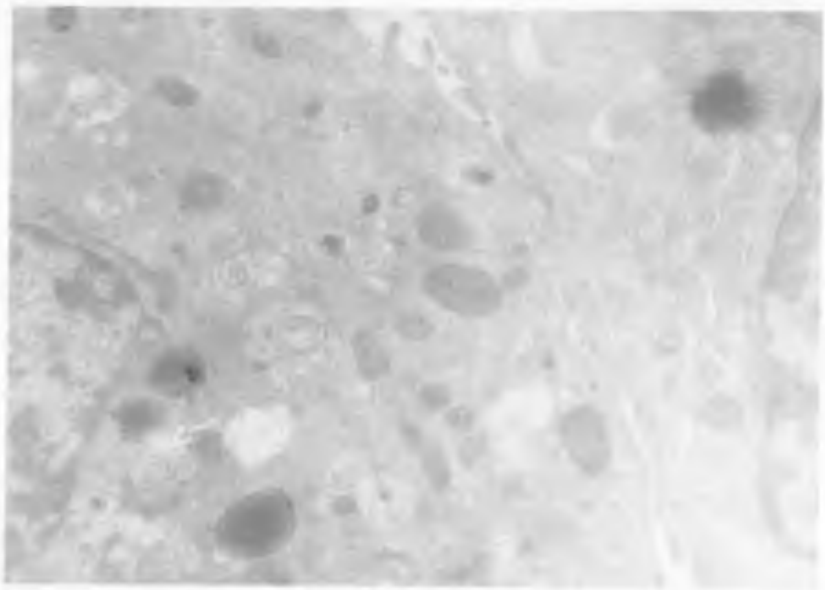


Figure 4.2.b

Figure 4.2 EM sections of SKCO1 cells depicting uptake and internalization of HRP conjugated antibodies at a concentration of 50 $\mu g\ ml^{-1}$.
4.2.a : 11-285-14-HRP anti-CEA Mab
4.2.b : Ag8-HRP (non-CEA specific Ab)

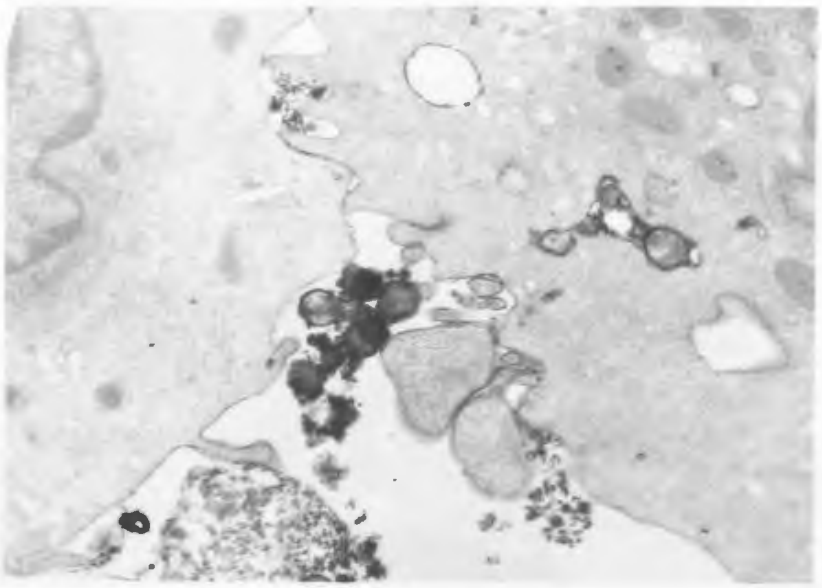


Figure 4.3.a

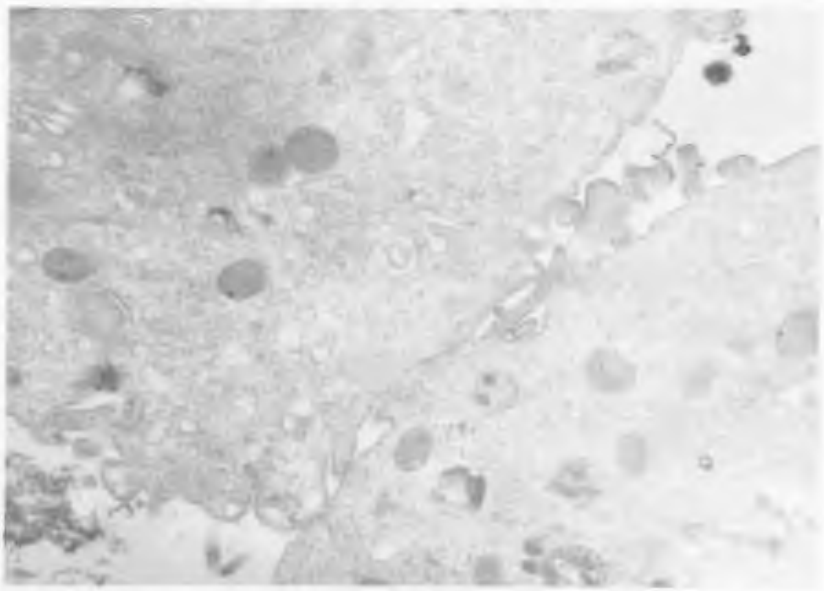


Figure 4.3.b

Figure 4.3 EM sections of SKCO1 cells depicting uptake and internalization of HRP conjugated antibodies at a concentration of 100 $\mu g\ ml^{-1}$.
4.3.a : 11-285-14-HRP anti-CEA Mab
4.3.b : Ag8-HRP (non-CEA specific Ab)

III. 5. SDS/PAGE Analysis of Internalization

We attempted to confirm the progress of antibody internalization by examining both the membrane and cytosolic components of cells incubated with the anti-CEA Mab or control Ag8 for increasing time intervals (section II.8). Approximately 5-6 repeats of the assay were performed for each cell line examined, but due to space considerations one representative Western blot per cell line is presented in this section. Figures 5a, 5b and 5c show the results of Western blots performed on membrane and cytosolic samples for three different CEA expressing cancer lines. In all cases, immunostaining for the primary anti-CEA antibody shows the presence of this Mab in both the membrane and cytosolic portions of the cells from a very early stage (after about 5 minutes of incubation).

In Figure 5a anti-CEA bands are evident throughout the first 60 minutes of incubation (part A, lanes b,d,f,h,j,l) whereas non-specific control staining is only evident in the 60-minute cytosolic component (part A, lane k), and may indicate that beyond a 60-min incubation interval non-specific accumulation in the cytosol may occur. In part B of the same figure evidence for accumulation of non-specific antibody is again evident (lanes c,e,g,l). As shown by the controls in these gels (lanes a, h) intensity of staining is generally much higher. Even under these conditions however anti-CEA cytosolic samples corresponding to 90-minute and 120-minute incubations (lanes b, f) have the strongest intensity bands. In Figure 5b (SKCO1 cells), there is strong evidence of increasing accumulation of anti-CEA Mab in the cytosol from the 5 min to the 60 min

incubation intervals (part A, lanes b, f, j). The highest anti-CEA intracellular accumulation furthermore, is noted at the longest incubation time of 180 min (part B, lane j). For this cell line only minimal accumulation of non-specific control antibody is evident in one case (part B, lane g).

In the case of the human lung carcinoma line (BENN, figure 5c), similar levels of intracellular anti-CEA Mab are obtained throughout the incubation intervals examined (part A, lanes b, f, j; part B, lanes b, f), although part B seems weaker due to much fainter staining in the case of this gel. Also uptake of non-specific antibody is much higher in the case of this cell line (part A, lanes c, g, k) (see Discussion).

An additional antigen specificity control is shown in figure 5d. In this case the cell line used shows very low levels of binding of 11-285-14, presumably due to its very low expression of CEA. There is only very weak evidence of anti-CEA antibody membrane binding (part A, lanes i, m), as is its intracellular accumulation (part A, lanes b, k; part B, lanes a, e). Conversely there is evidence of surface and cytosolic accumulation of non-specific antibody (part A, lanes g, j, l, n).

Figure 5. Detection of anti-CEA Mab in membrane and cytosolic portions of solubilized cancer cells using Western blots.
1×10^8 cell samples from four human cancer cell lines were incubated with 200 μg of 11-285-14 or Ag8 antibody from 5 minutes to 3 hr at half hour to one hour intervals at 37°C. Cells were then solubilized and membrane and cytosolic samples were electrophoresed on SDS gels which were then Western blotted. 11-285-14 Mab at 50 $\mu g\ ml^{-1}$ was used as a positive control. Due to space considerations blots were combined in groups of two. Where appropriate, positive controls are shown for each blot in order to directly assess possible differences in background staining for each blot. For ease of reading lane assignment is provided in tabulated form.
(cyt = cytosolic; mem = membrane).
Note: The control Mab (11-285-14) is partially purified, hence more than a single band is evident on the SDS/PAGE gels.

Figure 5.a legend
Description of Western blots of solubilized LS174T cells

LANE ASSIGNMENT

		11-285-14		Ag8	
TIME		cyt	mem	cyt	mem
5	A	b	d	c	e
30		f	h	g	i
60		j	l	k	m
90	B	b	d	c	e
120		f	i	g	j
180		k	m	l	n

11-285-14 Controls: Lanes A(a), B(a), B(h)

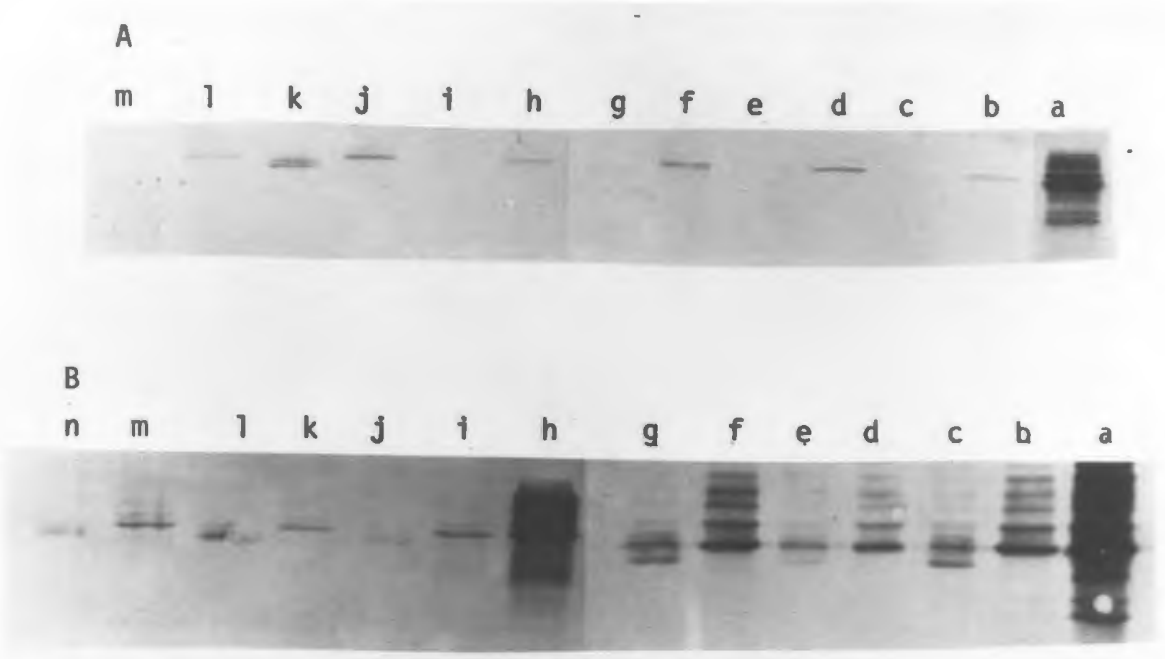


Figure 5.a

Western blots derived from time course incubations of solubilized LS174T cells incubated with 200 μg of anti-CEA (11-285-14) or non-CEA specific (Ag8) antibodies.

Figure 5.b legend

Description of Western blots of solubilized SKCO1 cells

LANE ASSIGNMENT

		11-285-14		Ag8	
TIME		cyt	mem	cyt	mem
5	A	b	d	c	e
30		f	h	g	i
60		j	l	k	m
90	B	b	d	c	e
120		f	h	g	i
180		j	NS	k	NS

NS = No sample

11-285-14 Controls: Lanes A(a), B(a)

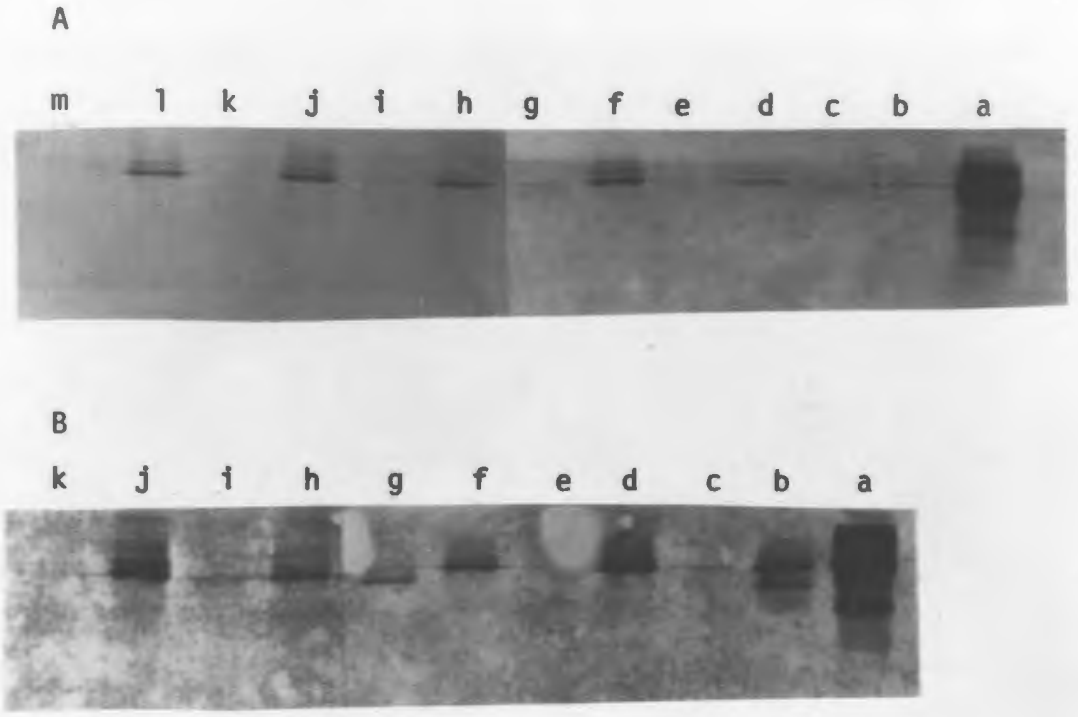


Figure 5.b

Western blots derived from time course incubations of solubilized SKCO1 cells incubated with 200 μg of anti-CEA (11-285-14) or non-CEA specific (Ag8) antibodies.

Figure 5.c legend

Description of Western blots of solubilized BENN cells

LANE ASSIGNMENT

		11-285-14		Ag8	
TIME		cyt	mem	cyt	mem
5	A	b	d	c	e
30		f	h	g	i
60		j	l	k	m
90	B	b	d	c	e
120		f	h	g	i

11-285-14 Controls: Lanes A(a), B(a)

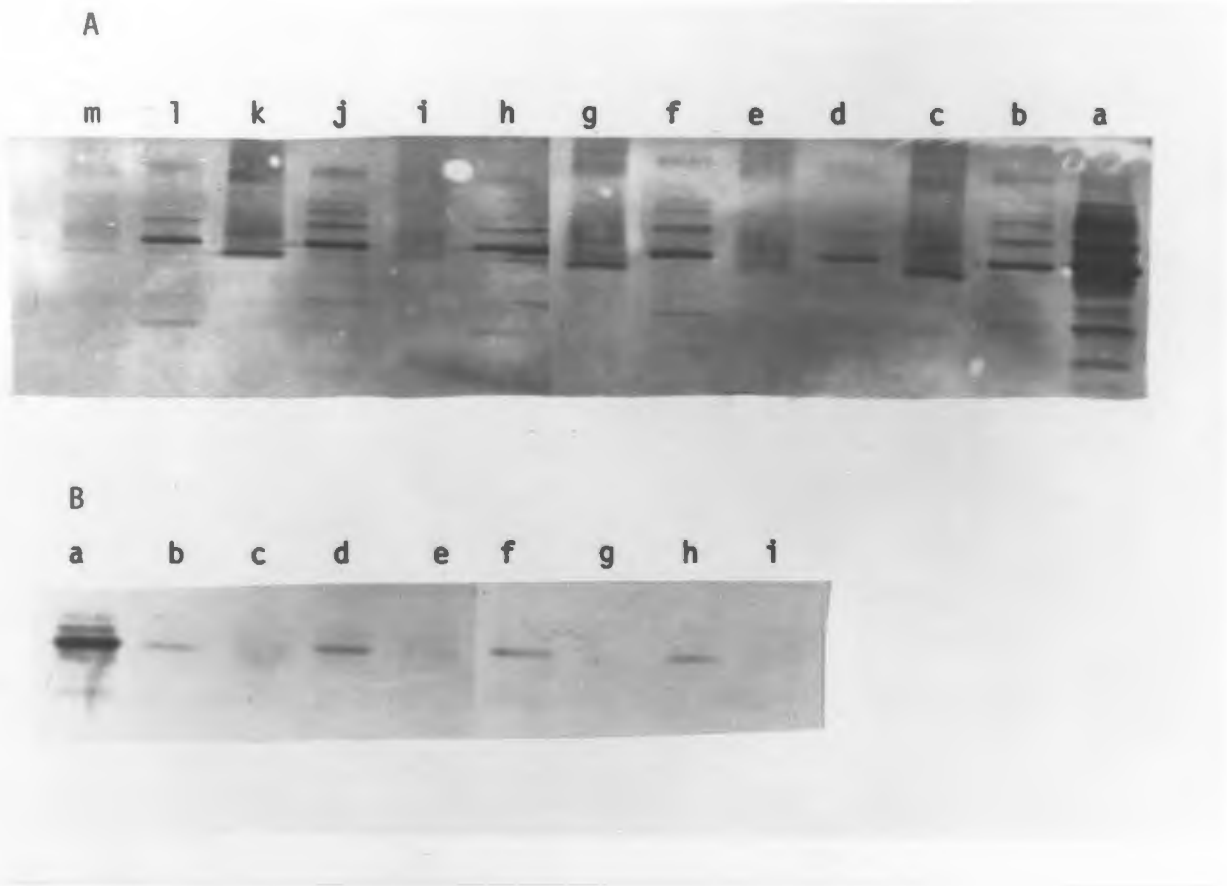


Figure 5.c

Western blots derived from time course incubations of solubilized BENN cells incubated with 200 μg of anti-CEA (11-285-14) or non-CEA specific (Ag8) antibodies.

Figure 5.d legend

Description of Western blots of solubilized COLO320 cells

LANE ASSIGNMENT

		11-285-14		Ag8	
TIME		cyt	mem	cyt	mem
5	A	b	d	c	e
30		f	i	g	j
60		k	m	l	n
90	B	a	c	b	d
120		e	i	f	j
180		k	m	l	n

11-285-14 Controls: Lanes A(a), A(h), B(h), B(g)

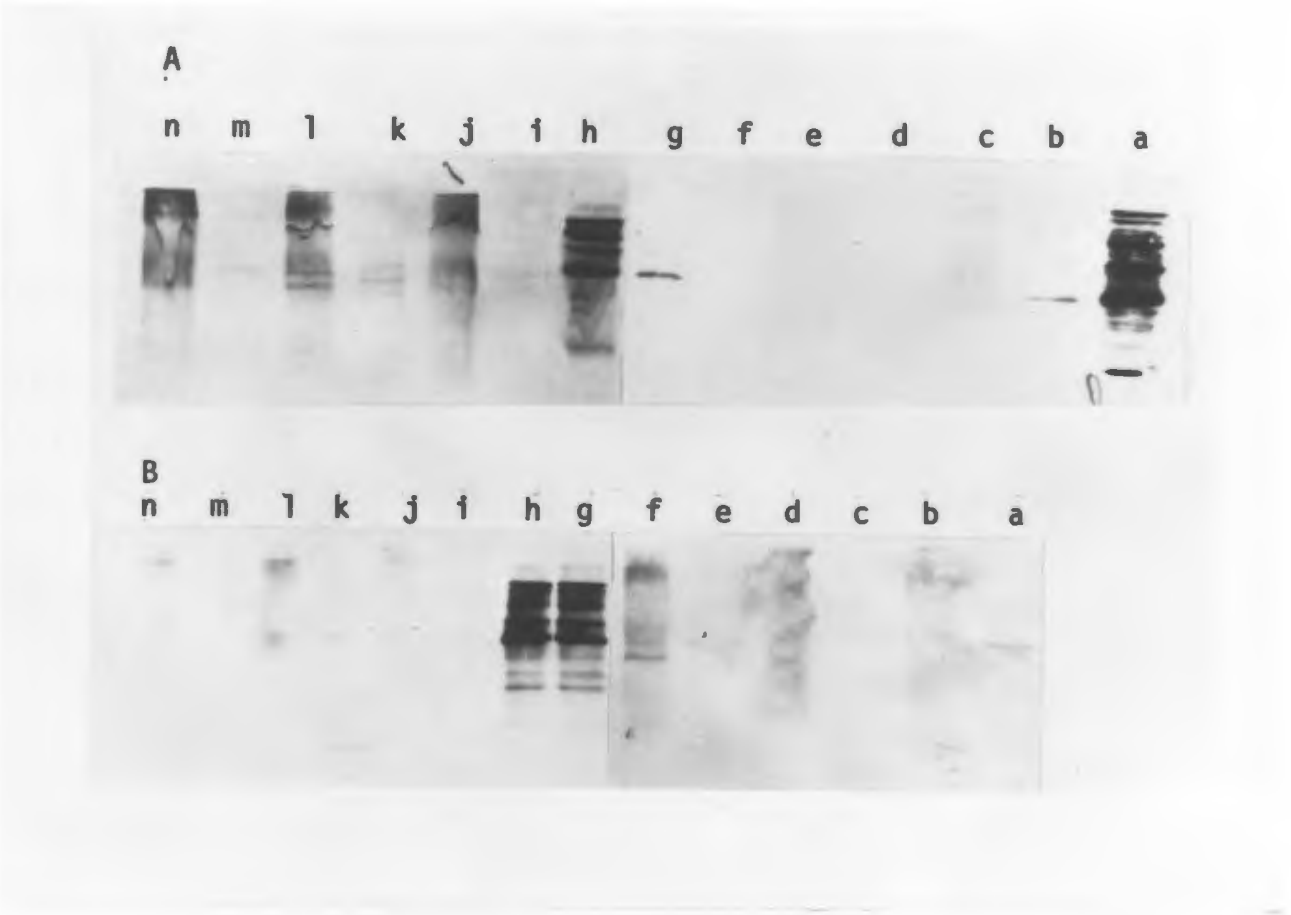


Figure 5.d

Western blots derived from time course incubations of solubilized COLO320 cells incubated with 200 μg of anti-CEA (11-285-14) or non-CEA specific (Ag8) antibodies.

III. 6. Comparison of SDS/PAGE and Flow Cytometric analysis in the depiction of anti-CEA Mab internalization

In an effort to develop a more convenient assay for detecting antibody internalization, we attempted to make use of flow cytometry. The principle of this assay is that surface FITC-labelled antibody can be detected by anti-FITC antibodies which will "quench" surface fluorescence, while internalized FITC-labelled antibody will escape such detection and the subsequent quenching. In the case of an internalizing antibody therefore, initial (time 0) fluorescence should be minimal (with quenching effect being maximal), since the primary antibody would still be on the cell membrane and would not have been internalized yet. However, with increasing incubation intervals, fluorescence should increase progressively, as increasing amounts of internalizing antibody escape anti-FITC-mediated quenching.

Table 6 provides a summary of the arithmetic estimation of the degree of anti-CEA antibody internalization per time interval, for each of the three cell lines studied (values are means of results from six or seven repeat assays for each line). Internalization is expressed as a percentage of the positive control (% antibody internalization = test sample fluorescence reading / positive control fluorescence reading x 100). There was some degree of variability in terms of antibody uptake (positive controls) for each cell line, which is also inevitably reflected in the test samples. This variability however, does not exceed differences in cell line characteristics normally observed with passaging of cell lines.

Table 6
Estimation of anti-CEA Mab internalization by human cancer cell lines using data from flow cytometric assays

Cell Line	Incubation time (min)	Positive control	Test	% Intern.
LS174T (7 assays)	0	27.5 (12)	10.7 (6.8)	38.2 (11.9)
	30	26.5 (11.4)	13.7 (8.5)	50.9 (15.3)
	90	25.5 (8.2)	14.5 (6)	58.6 (16.2)
SKCO1 (6 assays)	0	69 (22.1)	11.1 (5.6)	15.4 (4.8)
	30	59.4 (11.9)	18.1 (7)	30.5 (12.3)
	90	61 (7)	17.3 (6.2)	28 (7.9)
COLO320 (6 assays)	0	1.7 (0.9)	1.8 (0.9)	NA
	30	1.5 (0.7)	2.3 (1.7)	NA
	90	2.1 (1.2)	1.8 (0.9)	NA

Numbers correspond to % fluorescence emitted by the respective cell samples. Control ascites was used as a negative control. All values are means derived from the repeat assays indicated below the cell line. Numbers in parentheses indicate standard deviations for the respective groups.

Positive control : fluorescence emitted by cell samples incubated with FITC-labelled anti-CEA Mab only.

Test : fluorescence emitted by cell samples incubated with FITC-labelled anti-CEA Mab, followed by incubation with anti-FITC antibody.

% Intern (% Internalization) : $\frac{\text{test}}{\text{control}} \times 100\%$

NA : Not Applicable (due to background fluorescence values for all samples of this cell line).

Studies on the progress of internalization using flow cytometry are depicted in Figures 6.1.a, 6.2.a and 6.3.a. The negative control employed in these assays consisted of a sample of the tumour cells studied, incubated with fluoresceinated control ascites, ie. antibodies which do not bind to CEA. This negative control is represented in the FACS image by the clear leftmost curve and is the point of reference for positive controls and test samples. The clear rightmost curve depicts positive controls, ie. cancer cell samples which have been incubated with FITC-conjugated anti-CEA antibody for different time intervals, but have not been subsequently treated with anti-FITC, so that no surface fluorescence quenching has occurred. These samples therefore represent total fluorescence of both surface and internalized antibody at each time interval (positive control). The middle (shaded) curve represents cancer cell samples incubated with FITC-conjugated anti-CEA antibody for the same time intervals, but which have subsequently been treated with anti-FITC to quench surface antibody fluorescence. Thus, at each time interval the only fluorescence detectable from these cell samples should be fluorescence associated with internalized antibody. Actual FACS outputs corresponding to one assay for each cell line are shown in figures 6.1.a, 6.2.a and 6.3.a. As incubation times of LS174T (legend to Figure 6.1.a) and SKCO1 (Figure 6.2.a and legend) cells with fluoresceinated anti-CEA antibody increased, there also occured an increasing right shift of the curve representing test samples. This shift represents increased depiction of fluorescence due to the fact that surface antibody is being progressively internalized and is no more subject to the anti-FITC quenching activity. However, as seen in figure 6.3.a,

when COLO320 cells (which express very low levels of CEA) are used, both positive controls and test samples exhibit a fluorescence level which is very close to that of the negative control, throughout the time intervals studied, reflecting the inability of the anti-CEA antibody to adhere onto the cell surface. In order to ensure consistency with the previously performed cell solubilization assays (section III.5), such flow cytometric assays were coupled with Western blots, which were derived using membrane and cytosolic samples of solubilized cells from the same lines as before and were treated in parallel and under the same experimental protocol as the cells used for FACS analysis. Results of these SDS/PAGE assays are presented below their flow cytometric counterparts (Figures 6.1.b, 6.2.b, 6.3.b).

In Figure 6.1.b we saw evidence (bands corresponding to the cytosolic portion of the sample) of anti-CEA antibody internalization by LS174T cells at all time intervals studied whereas for the most part no bands are visible on the lanes where cells were incubated with control ascites. Similar results were obtained for the SKCO1 cell line (figure 6.2.b), whereas in the case of the COLO320 cells (very low CEA expressor; figure 6.3.b), no bands were visible either for test or for negative control samples (with the exception of a very light band for the 30-minute cytosolic test sample), which was confirmed by the FACS results. Lack of densitometric data prevent quantitative correlations of the results from the two sets of assays, however a large number of such assays with solubilized cells (Tsaltas et al., 1992) has provided evidence of increased antibody internalization through the time intervals studied for the same cell lines.

Figure 6.1.a. Detection of anti-CEA antibody internalized by LS174T cells via FACS analysis (comparison with SDS/PAGE analysis). Samples of LS174T cells (1 x 10^6 cells/sample), were incubated with: 0.1 μg of FITC-labelled control ascites only (leftmost curve -negative control; this curve is overshadowed by the middle (test) curve; since it was not possible to provide clear coloured curves LS174T results are better understood through the arithmetic data provided in this figure's legend or by comparing to figure 6.2); 0.1 μg of FITC-labelled 11-285-14 Mab followed by a 30 minute incubation at 0°C with anti-FITC (middle, shaded curve -test); or 0.1 μg of FITC-labelled 11-285-14 followed by a 30 minute incubation at 0°C with PBS (positive control). To follow internalization, incubations with the anti-CEA-FITC-labelled Mab were performed for 0 minutes (i), 30 minutes (ii), or 90 minutes (iii) at 37°C, and the results were as follows: (Groups as per table 6, where summarized FACS results appear)

Estimation of anti-CEA Mab internalization by LS174T cells via FACS analysis

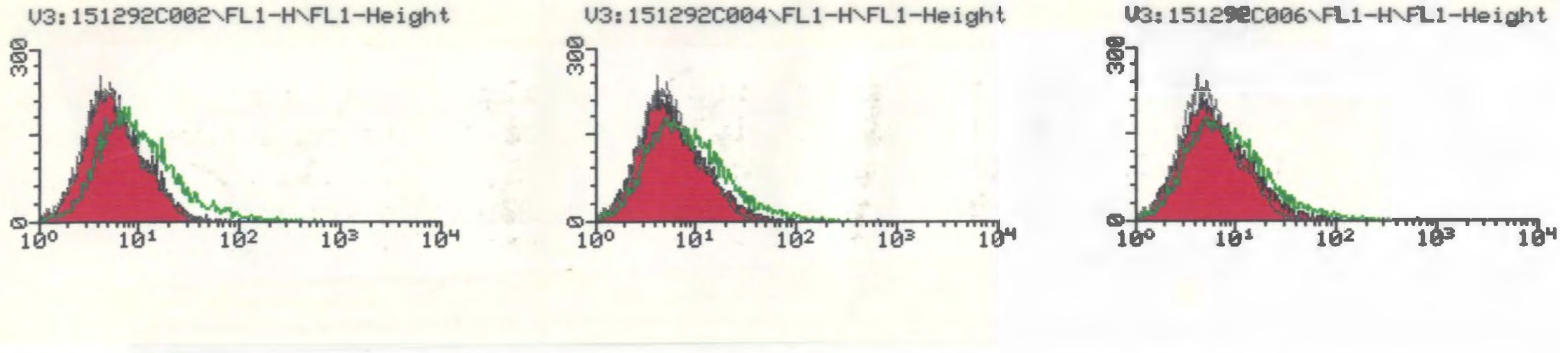
Incubation time	Positive control	Test	% Intern.
0	39.71	18.09	45.5
30	40.73	20.25	49.7
90	34.67	23.67	68.3

Figure 6.1.b. Detection of anti-CEA Mab in membrane and cytosolic portions of solubilized LS174T cells run in parallel with samples for FACS analysis. LS174T samples of 1 x 10^8 cells each, were incubated with 200 μg of 11-285-14 Mab, or control ascites for 0, 30 or 90 minutes at 37°C. Two blots were combined at a time to accomodate all samples of the cell line, with positive controls provided in each blot. For ease of reading, lane assignment is tabulated (**test** = incubated with 11-285-14; **control** = incubated with control ascites).

Western blots of solubilized LS174T cells(Lane Assignment)

Incubation Time	Membrane portion		Cytosolic portion	
	Test	Control	Test	Control
0	c	d	e	f
30	g	h	j	k
90	l	m	n	o

11-285-14 positive controls : lanes a,b,i.



i ii iii

Figure 6.1.a

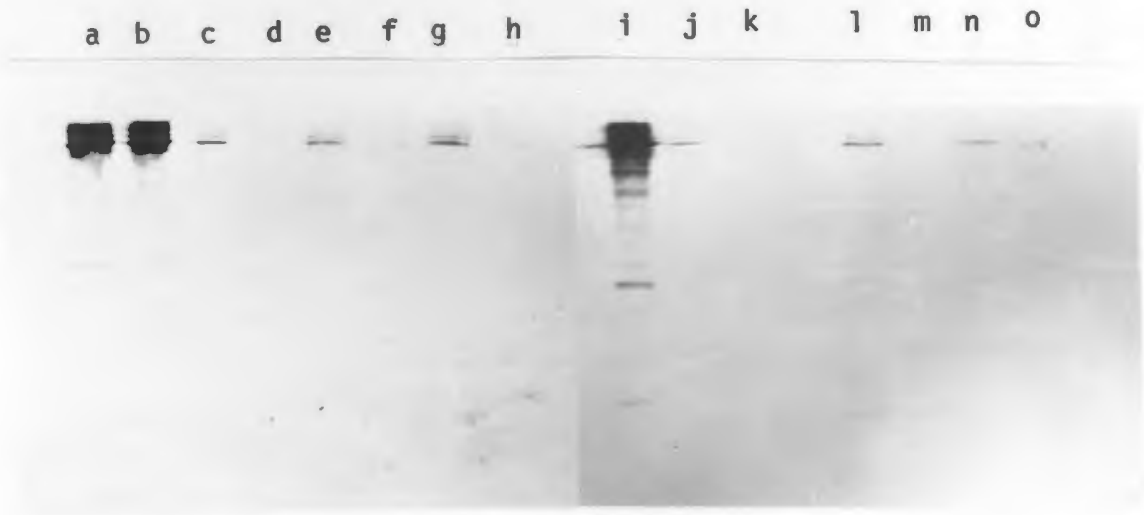


Figure 6.1.b

Figure 6.1(a,b). Methodological comparison in the detection of internalized anti-CEA Mab by LS174T cells, using flow cytometry (6.1.a), or Western blots of solubilized cells (6.1.b). (Legend provided on opposite page)

Figure 6.2.a. Detection of anti-CEA antibody internalized by SKCO1 cells via FACS analysis (comparison with SDS/PAGE analysis). Samples of SKCO1 cells (1 x 10^6 cells/sample), were incubated with: 0.1 μg of FITC-labelled control ascites only (leftmost curve -negative control); 0.1 μg of FITC-labelled 11-285-14 Mab followed by a 30 minute incubation at 0°C with anti-FITC (middle, shaded curve -test); or 0.1 μg of FITC-labelled 11-285-14 followed by a 30 minute incubation at 0°C with PBS. To follow internalization, incubations with the anti-CEA-FITC-labelled Mab were performed for 0 minutes (i), 30 minutes (ii), or 90 minutes (iii) at 37°C and the results were as shown in the following table (Groups as per table 6, where summarized FACS results appear)

Estimation of anti-CEA Mab internalization by SKCO1 cells via FACS analysis

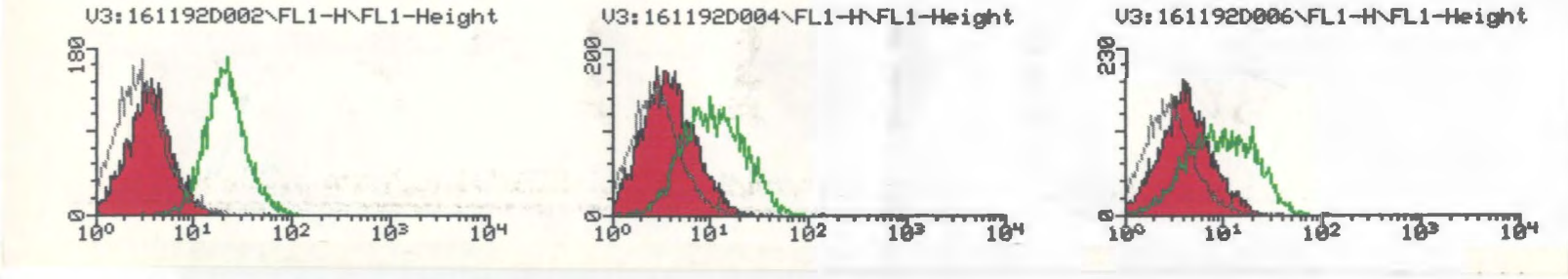
Incubation time	Positive control	Test	% Intern.
0	95.48	17.03	17.8
30	63.34	17	26.8
90	59.07	20.76	35.1

Figure 6.2.b. Detection of anti-CEA Mab in membrane and cytosolic portions of solubilized SKCO1 cells run in parallel with samples for FACS analysis. SKCO1 samples of 1 x 10^8 cells each were incubated with 200 μg of 11-285-14 Mab, or control ascites for 0, 30 or 90 minutes at 37°C. 11-285-14 Mab is used as a positive control at 50 μg ml^{-1}. Two blots were combined at a time to accomodate all samples of the cell line with positive controls provided in each blot. For ease of reading, lane assignment is tabulated (**test** = incubated with 11-285-14; **control** = incubated with control ascites).

Western blots of solubilized SKCO1 cells
Lane Assignment

Incubation Time	Membrane portion		Cytosolic portion	
	Test	Control	Test	Control
0	c	d	e	f
30	g	h	j	k
90	l	m	n	o

11-285-14 positive controls : lanes a,b,i.



i ii iii

Figure 6.2.a

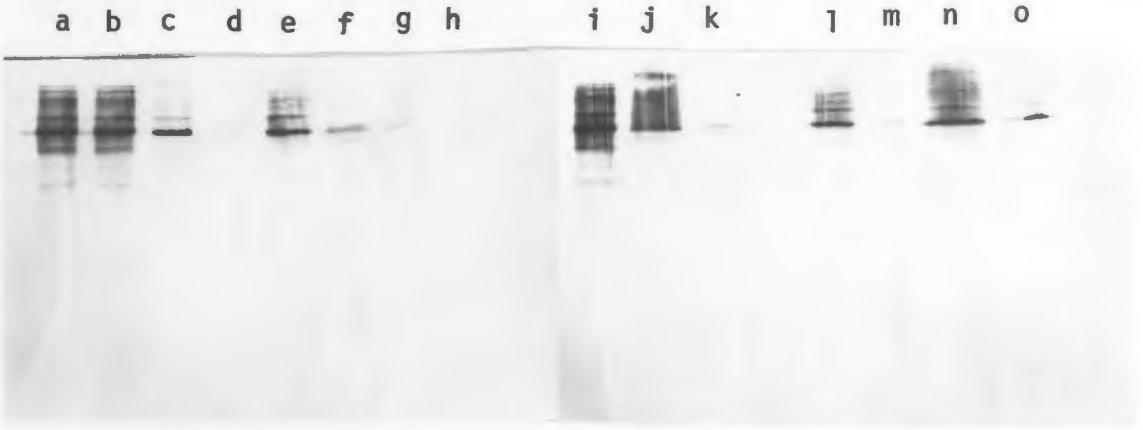


Figure 6.2.b

Figure 6.2(a,b). Methodological comparison in the detection of internalized anti-CEA Mab by SKCO1 cells, using flow cytometry (6.2.a), or Western blots of solubilized cells (6.2.b). (Legend provided on opposite page)

Figure 6.3.a. Detection of anti-CEA antibody internalized by COLO320 cells via FACS analysis (comparison with SDS/PAGE analysis). Samples of COLO320 cells (1 x 10^6 cells/sample), were incubated with: 0.1 μg of FITC-labelled control ascites only (leftmost curve -negative control); 0.1 μg of FITC-labelled 11-285-14 Mab followed by a 30 minute incubation at 0°C with anti-FITC (middle, shaded curve -test); or 0.1 μg of FITC-labelled 11-285-14 followed by a 30 minute incubation at 0°C with PBS. To follow internalization, incubations with the anti-CEA-FITC-labelled Mab were performed for 0 minutes (i), 30 minutes (ii), or 90 minutes (iii) at 37°C and results were as shown in the following table (Groups as per table 6, where summarized FACS results appear)

Estimation of anti-CEA Mab internalization by COLO320 cells via FACS analysis

Incubation time	Positive control	Test	% Intern.
0	1.53	1.59	NA
30	1.89	5.34	NA
90	1.53	1.88	NA

Figure 6.3.b. Detection of anti-CEA Mab in membrane and cytosolic portions of solubilized COLO320 cells run in parallel with samples for FACS analysis. COLO320 samples of
1 x 10^8 cells each were incubated with 200 μg of 11-285-14 Mab, or control ascites for 0, 30 or 90 minutes at 37°C. 11-285-14 Mab is used as a positive control at 50 μg ml^{-1}. Two blots were combined at a time to accomodate each cell line with positive controls provided in each blot. For ease of reading, lane assignment is tabulated (**test** = incubated with 11-285-14; **control** = incubated with control ascites).

Western blots of solubilized COLO320 cells
Lane Assignment

Incubation Time	Membrane portion		Cytosolic portion	
	Test	Control	Test	Control
0	c	d	e	f
30	g	i	k	l
90	m	n	o	p

11-285-14 positive controls : lanes a,b,j.

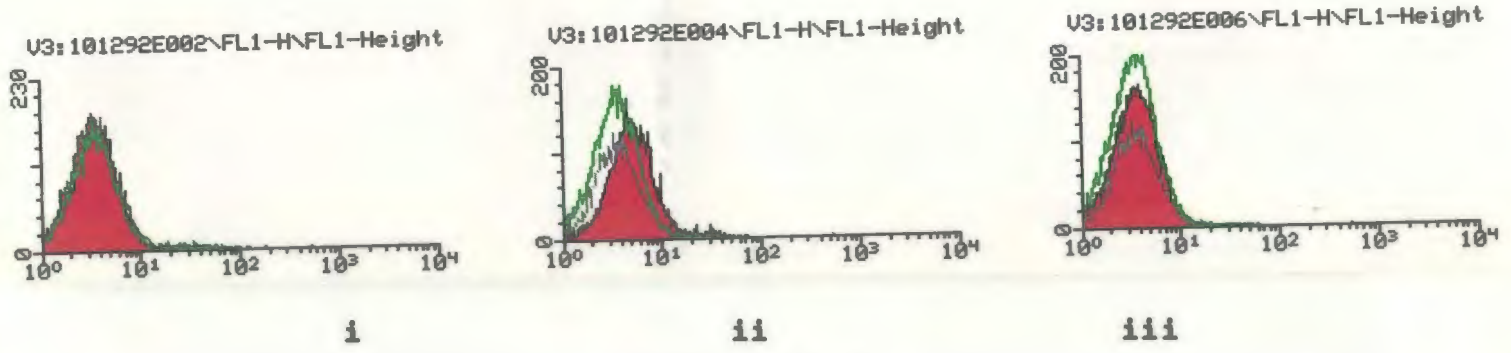


Figure 6.3.a

Figure 6.3.b

Figure 6.3(a,b). Methodological comparison in the detection of internalized anti-CEA Mab by COLO320 cells, using flow cytometry (6.3.a), or Western blots of solubilized cells (6.3.b). (Legend provided on opposite page)

III. 7. Observation of the effect of hypertonic medium on anti-CEA Mab uptake and internalization, using flow cytometry.

The involvement of clathrin in the uptake and internalization of the anti-CEA Mab was examined through cell incubation in hypertonic medium, a method which was recently shown to inhibit clathrin-mediated endocytosis by preventing clathrin and adaptors from interacting (Hansen et al., 1993). Antibody was again detected through the use of flow cytometry as described in the two previous sections. This assay involved the use of only one high CEA-expressor, the colorectal cancer cell line (SKCO1), chosen mostly for the large number of CEA antigenic sites it expresses. The time course incubations at 37°C, which allowed for internalization of surface adherent anti-CEA antibody, again involved the three time intervals previously examined, namely 0, 30 and 90 minutes. Assays were performed both after pre-incubating cells in hypertonic medium for 30 minutes, prior to the beginning of the experiment (Table 7.a) and by incubation of test cell samples in hypertonic medium thereafter. Alternatively, as a further test, test cell samples were exposed to hypertonic medium through the course of the experiment only (no prior pre-incubation in hypertonic medium (Table 7.b)).

From the two alternative treatments it became obvious that the decreased overall anti-CEA uptake by cells bathed in hypertonic medium, necessitated extensive (30 minute) pre-incubation of the test samples in this medium. When the hypertonic medium was introduced following incubation of cells with the primary antibody (anti-CEA-FITC), uptake values were similar between the hypertonic-medium treated and regular-medium

treated groups.

Overall, the use of hypertonic media seemed to have a sizeable and consistent effect in diminishing primary anti-CEA-FITC antibody uptake (see Table 7.a, samples (c) versus (e)) when pre-incubation of the cells in this medium took place however this medium did not seem to inhibit antibody internalization (as measured by this assay), to any appreciable degree (Table 7.a, (g) versus (h)). Lack of pre-incubation in hypertonic medium resulted in no change in anti-CEA-FITC uptake (Table 7.b, samples (c) versus (e)) and in no inhibition of its internalization (Table 7.b, samples (g) versus (h)). Interestingly, relative internalization in cells which were exposed to hypertonic medium during the course of the assay without being pre-incubated in this medium, seems to exceed internalization rates of cells which had been pre-incubated in such medium. However, more assays would have to be performed in order to ascertain that such a trend is not due to inter-assay variability. Representative FACS outputs from two such assays are presented in figures 7.a and 7.b.

Table 7.a
Effect of hypertonic medium on Mab Internalization
with 30 min. preincubation

SAMPLES	Average values (3 assays)		
	0(min)	30(min)	90(min)
(a) $medium_R$	1.2 (0.6)	1.8 (0.7)	1.8 (0.3)
(b) $medium_H$	1.3 (0.8)	0.7 (0.2)	1.0 (0.3)
(c) 11-285-$FITC_R$	72.3 (21.1)	61.3 (17.7)	56.9 (23.9)
(d) 11-285-FITC + anti-$FITC_R$	25.5 (20.5)	11 (6.3)	20.7 (15)
(e) 11-285-$FITC_H$	38.9 (15.1)	30.4 (14.8)	23.5 (6.5)
(f) 11-285-FITC + anti-$FITC_H$	3.8 (5.3)	4.7 (4.2)	6.1 (4.8)
(g) %$Internal._R$	33 (22.2)	17.3 (5.7)	33.3 (12.1)
(h) %$Internal._H$	8.3 (9.9)	13.6 (6.8)	26.6 (21)

1 x 10^6 SKCO1 cells were preincubated in hypertonic medium containing 0.45M sucrose for 30 min. at 37°C. All subsequent incubations were performed in respective (regular or hypertonic) media.
The subscripts $_{R, H}$ signify regular and hypertonic medium respectively.
Samples (a) to (h) are explained in the table.
%Internal. = Internalized (non-anti-FITC quenched) primary antibody, as compared to its respective positive control i.e. sample (d) / sample (c) for regular medium and sample (f) / sample (e) for hypertonic medium (11-285-14-FITC, abbreviated in the table as 11-285-FITC).
All % fluorescence values were read against a common negative control represented by sample (a).
All data represent mean values of the respective groups from the combined (3) assays. Numbers in parentheses denote standard deviations.

Table 7.b
Effect of hypertonic medium on Mab Internalization
<u>without</u> 30 min. preincubation

SAMPLES	Average values (3 assays)		
	0(min)	30(min)	90(min)
(a) $medium_R$	1.1 (0.5)	1.0 (0.1)	1.2 (0.4)
(b) $medium_H$	0.2 (0.3)	0.3 (0.4)	0.3 (0.2)
(c) 11-285-$FITC_R$	58.0 (11.2)	50.9 (22.7)	45.3 (19.7)
(d) 11-285-FITC + anti-$FITC_R$	6.5 (5.2)	6 (3)	11.1 (5.3)
(e) 11-285-$FITC_H$	60.9 (24.4)	44.3 (25.7)	50 (23.8)
(f) 11-285-FITC + anti-$FITC_H$	12 (10.3)	14.8 (8)	20.1 (10.7)
(g) %$Internal._R$	10.8 (7.7)	11.7 (1.7)	24.1 (4.6)
(h) %$Internal._H$	17.3 (11.4)	37.8 (27.7)	41.3 (22.7)

SKCO1 cell samples were not subjected to pre-incubation in hypertonic medium (0.45M sucrose) this time, but all other incubations were performed on respective media as before.
Again the subscripts $_{R, H}$ signify regular and hypertonic medium respectively.
Samples (a) to (h) are explained in the table.
%Internal. = Internalized (non-anti-FITC quenched) primary antibody, as compared to its respective positive control i.e. sample (d) / sample (c) for regular medium and sample (f) / sample (e) for hypertonic medium (11-285-14-FITC, abbreviated in the table as 11-285-FITC).
All % fluorescence values were read against a common negative control represented by sample (a).
All data represent mean values of the respective groups from the combined (3) assays. Numbers in parentheses denote standard deviations.

Figures 7.a and 7.b combine FACS results of one run with 0, 30 and 90 minute anti-CEA Mab incubation intervals at lanes i, ii and iii respectively. Table legends are provided in tabulated form opposite the figures for ease of reading.

Legend for Figure 7.a
Effect of hypertonic medium on Mab Internalization
with 30 min. preincubation

SAMPLES	Date of run: 20/ 5/ 1994		
	0(min)	30(min)	90(min)
(a) $medium_R$	1.69	2.59	1.32
(b) $medium_H$	1.20	0.92	0.96
(c) 11-285-$FITC_R$	86.48	76.62	50.24
(d) 11-285-FITC + anti-$FITC_R$	20.12	18.21	17.42
(e) 11-285-$FITC_H$	51.20	45.18	28.31
(f) 11-285-FITC + anti-$FITC_H$	9.87	9.40	9.01
(g) %$Internal._R$	23.26	23.77	34.67
(h) %$Internal._H$	19.28	20.80	31.83

1 x 10^6 SKCO1 cells were preincubated in hypertonic medium containing 0.45M sucrose for 30 min. at 37°C. All subsequent incubations were performed in respective (regular or hypertonic) media.
The subscripts $_{R, H}$ signify regular and hypertonic medium respectively.
Samples (a) to (h) are explained in the table.
%Internal. = Internalized (non-anti-FITC quenched) primary antibody, as compared to its respective positive control i.e. sample (d) / sample (c) for regular medium and sample (f) / sample (e) for hypertonic medium (11-285-14-FITC, abbreviated in the table as 11-285-FITC).
All % fluorescence values were read against a common negative control represented by sample (a).

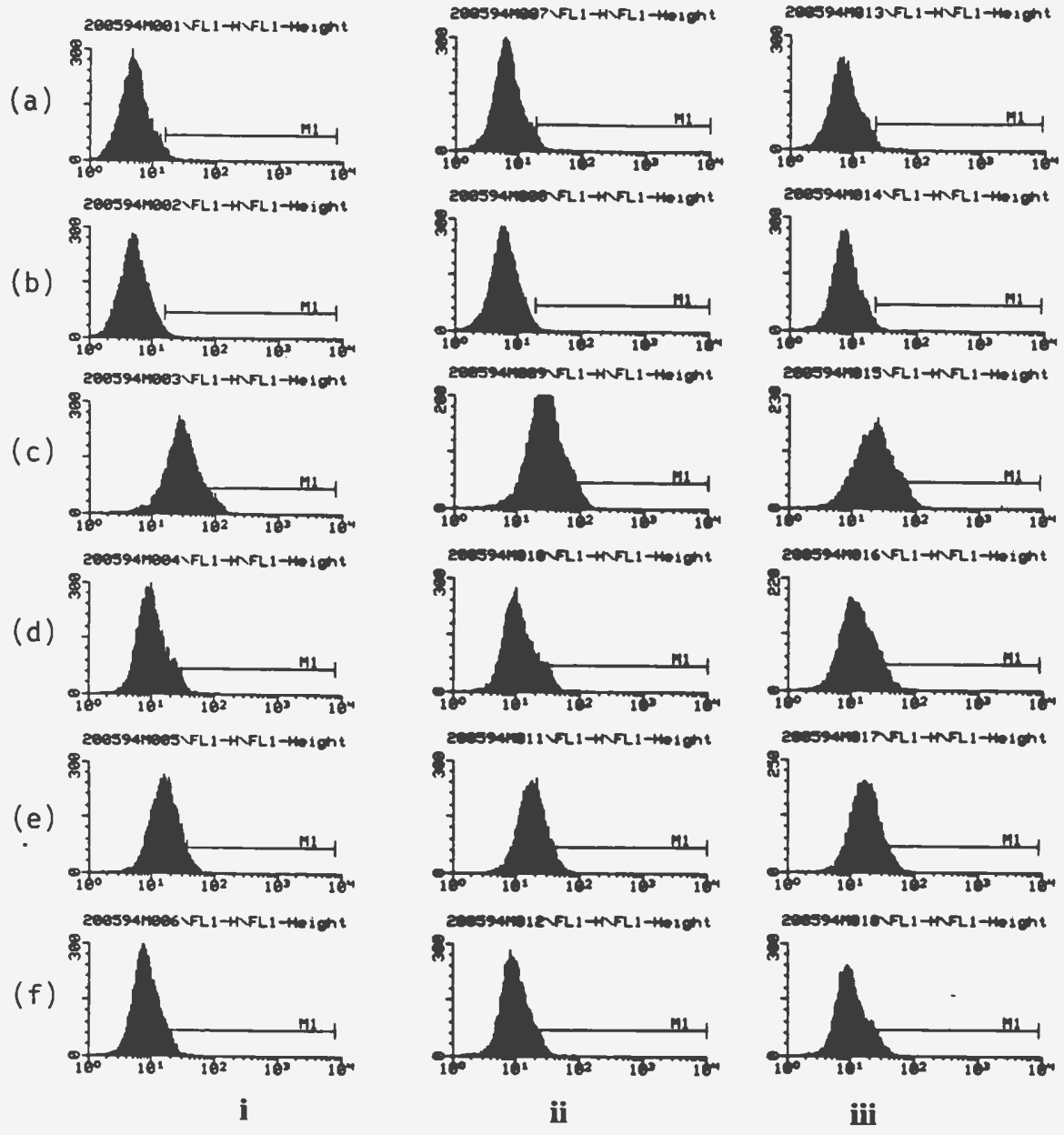


Figure 7.a

FACS results of samples treated (both prior to beginning and throughout the assay) with hypertonic medium in order to examine the possibility of inhibition of antibody internalization via the clathrin-mediated pathway

(i) Cells incubated with medium only at 0°C.

(ii) Cells incubated with 11-285-14 Mab for 30 min. at 37°C.

(iii) Cells incubated with 11-285-14 Mab for 90 min. at 37°C.

Legend for Figure 7.b
Effect of hypertonic medium on Mab Internalization
<u>without</u> 30 min. preincubation

SAMPLES	Date of run: 2/ 5/ 1994		
	0(min)	30(min)	90(min)
(a) $medium_R$	1.61	1.04	1.37
(b) $medium_H$	0.50	0.73	0.45
(c) 11-285-$FITC_R$	50.49	74.57	61.87
(d) 11-285-FITC + anti-$FITC_R$	7.38	9.34	13.37
(e) 11-285-$FITC_H$	73.20	73.92	77.14
(f) 11-285-FITC + anti-$FITC_H$	10.62	19.28	25.60
(g) $\%Internal._R$	14.62	12.52	21.61
(h) $\%Internal._H$	14.51	26.08	33.18

SKCO1 cell samples were not subjected to pre-incubation in hypertonic medium (0.45M sucrose) this time, but all other incubations were performed on respective media as before.
Again the subscripts $_{R, H}$ signify regular and hypertonic medium respectively.
Samples (a) to (h) are explained in the table.
%Internal. = Internalized (non-anti-FITC quenched) primary antibody, as compared to its respective positive control i.e. sample (d) / sample (c) for regular medium and sample (f) / sample (e) for hypertonic medium (11-285-14-FITC, abbreviated in the table as 11-285-FITC).
All % fluorescence values were read against a common negative control represented by sample (a).

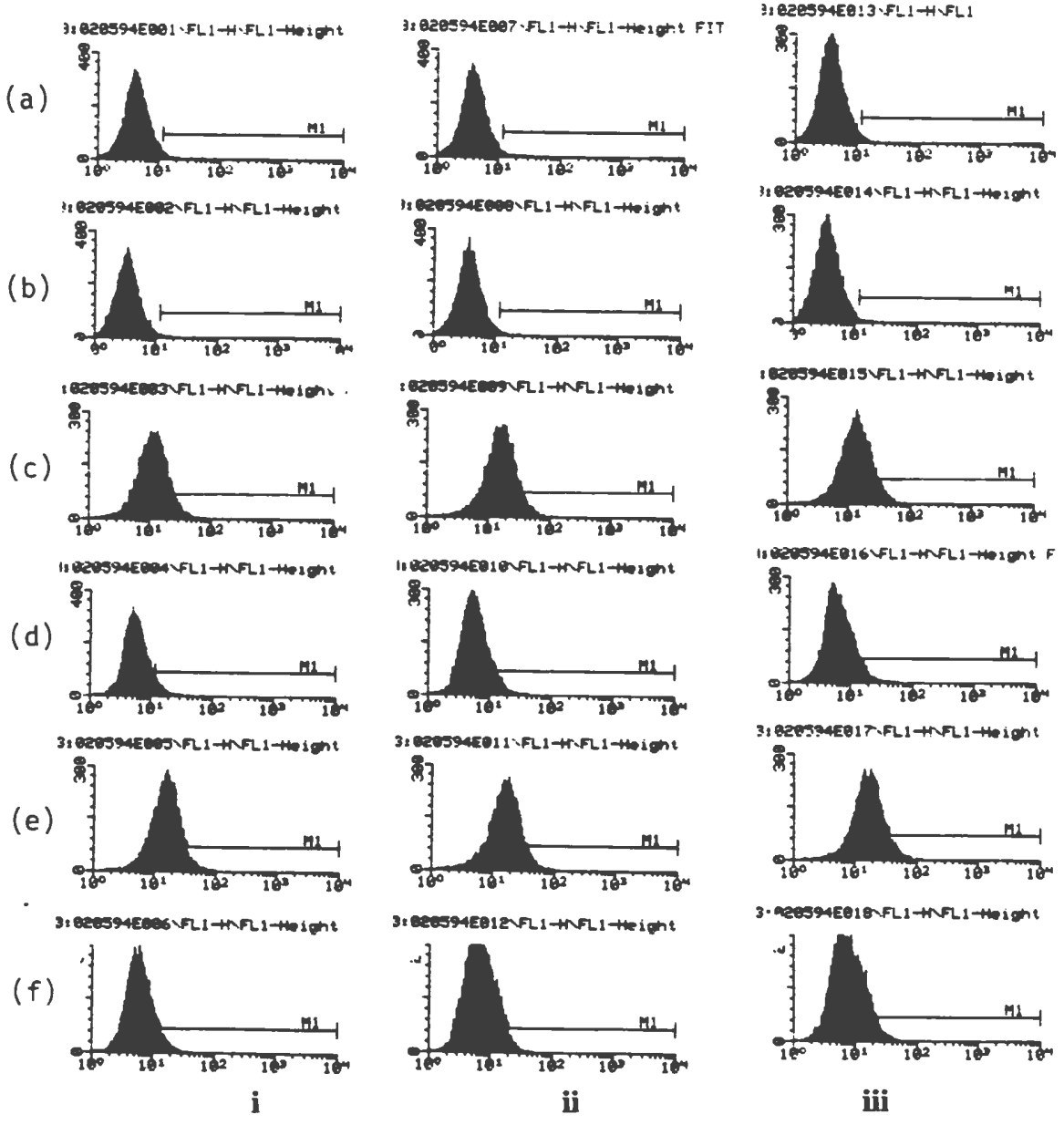


Figure 7.b

FACS results of samples treated (during the assay only) with hypertonic medium in order to examine the possibility of inhibition of antibody internalization via the clathrin-mediated pathway

(i) Cells incubated with medium only at 0°C.

(ii) Cells incubated with 11-285-14 Mab for 30 min. at 37°C.

(iii) Cells incubated with 11-285-14 Mab for 90 min. at 37°C.

CHAPTER IV

DISCUSSION

IV. 1. Immunoconjugate internalization: Mode and Significance

The effective destruction of tumour cells by any of the major classes of cytotoxic immunoconjugates currently in experimental or clinical use, has been shown on a number of occasions to correlate with the ability of the targeting antibody to be internalized by the targeted cancer cell.

Antibody internalizing ability has been found particularly essential in the case of immunotoxins (IT's), where internalization of the toxin is generally necessary for cytotoxic action. In fact, due to their extremely high cytotoxic potential, IT's have been some of the most widely studied "magic bullets". A general background on IT's has been given briefly in Chapter I, while a number of reviews on immunotoxins have appeared in the literature in the last 15 years (Vitetta et al., 1987; Olsnes et al., 1989; Lord et al., 1985; Byers et al., 1992). The structure of toxins and method of IT preparation will not be repeated in this section, however the action of IT's on the protein synthesis machinery will be elaborated on briefly.

Most bacterial toxins (such as diphtheria toxin and *Pseudomonas aeruginosa* exotoxin A) inactivate elongation factor 2, an enzyme required for the translocation of growing peptide chains from the A-site to the P-site of the ribosomes. Elongation factor 2 modified by these toxins cannot properly interact with the ribosomes, resulting in blocking of protein synthesis (Olsnes et al., 1989). Most plant toxins inactivate the large ribosomal subunit, by modifying a loop in the 28S RNA of the subunit. Although toxins

are very effective cell killers, their cytotoxic efficacy depends directly on their binding, uptake and intracellular routing, in other words their ability to selectively internalize. For that reason immunotoxins have been the major class of immunoconjugates for which internalization potential has been studied in parallel with the development of new IT constructs.

Some initial experimental evidence on this subject raised a number of questions, when it was found that IT killing of human melanoma cells (Casellas et al., 1982; Godal et al., 1986), was increased by ammonium chloride. This agent, which was thought to inhibit internalization, turned out to increase IT cytotoxicity on tumour cells that expressed high levels of the antigen being targeted (p97) (Casellas et al., 1982). The authors speculated that ammonium chloride (a lysosomotropic amine), inhibited the degradation of the IT by inactivating lysosomal enzymes through raising intralysosomal pH. The role of this agent and monensin (a carboxylic ionophore) was immediately investigated in an examination of the endocytosis of an antibody ricin-A conjugate (Carrière et al., 1985). In this study it was concluded that such agents markedly slowed down the speed of IT transportation through lysosomal compartments, thus increasing cytotoxic efficacy, probably both by lengthening the period the IT remains in a non-lysosomal compartment, as well as by lowering intralysosomal pH. Notably, these agents did not interfere with the initial IT internalization process, which was thought to occur mostly through receptor-mediated endocytosis.

Alternative experimental approaches however, such as subjecting cells to

hypotonic shock followed by incubation in potassium (K^+)-free medium, showed at the time that human fibroblasts can arrest their coated pit formation and therefore arrest receptor-mediated endocytosis in the case of low density lipoprotein (Larkin et al., 1983). When Hep-2 cells were subjected to a similar treatment, it was shown that although cytotoxicity mediated by diphtheria toxin was blocked, the one mediated by ricin toxin was not (Moya et al., 1985), suggesting a non-receptor mediated internalization pathway for the unconjugated ricin toxin. However, a subsequent comparison of three anti-CEA ricin A ITs (Levin et al., 1987), suggested modulation of the antigen-antibody complex from the cell surface.

While the debate on the endocytic pathway followed by toxins continued, the above study also suggested that cytotoxic efficacy of the ITs studied was not related to the affinity of the monoclonal antibody carriers to the CEA target antigen. This point however was also debated since it was later shown that the cytotoxic ability of an anti-CEA IT containing ricin A (228-RTA), was potentiated by Mabs recognizing different epitopes, suggesting that enhanced antibody affinity leads to increased endocytosis of bound immunoconjugate and potentiates cytotoxicity (Byers et al., 1988).

The one factor that seems to be more important than antibody affinity in terms of immunotoxin efficacy however, is the rate of IT internalization. Specifically, it was shown with human melanoma and small lung carcinoma cell lines using an anti-ganglioside GD2 Mab linked to ricin-A toxin, that the rate of IT internalization correlated with cytotoxic activity against human tumour cells (Wargalla et al., 1989). Furthermore,

an analysis of the rate of endocytosis of another extremely potent ricin-A Mab against sarcomas and colon and ovarian cancer cells (Byers et al., 1991), showed that this particular IT internalized very slowly (possibly via smooth pits), and that in these cases cytotoxicity was not affected by potentiators such as ammonium chloride.

In turn, the importance of internalization rate as the major variable influencing IT potency, has also been questioned. Specifically, it was determined using ITs constructed against different epitopes of murine B cells, that neither cross-linking nor rate of internalization could account for the degree of difference in potencies between various ITs (May et al., 1991). However, in the same study, the type of intracellular routing promoted by the choice of target antigen was found to have a profound effect on IT potency. When the respective effects of the intracellular compartments (namely endosomes versus lysosomes), were made relatively similar due to the pH-raising action of the lysosomotropic amine chloroquine, the less potent IT became 100 times more potent, whereas the other IT's potency was unaffected. This outcome was thought to reflect differential compartmentalization of the two ITs during intracellular routing. The role of another major compartment in intracellular routing, namely the Golgi region, has also been extensively studied mostly through the use of brefeldin A (BFA), which affects the Golgi network by causing a redistribution of its proteins into the ER (Wood et al., 1991). Such disruption of the Golgi apparatus has been shown to inhibit the cytotoxicity of ricin, modeccin and *Pseudomonas* toxin, (Yoshida et al., 1991), while more recently such Golgi-dependent inhibition of cytotoxic action was observed for cholera toxin

(Nambiar et al., 1993), although it differs considerably in structure and mode of action.

Irrespective of what the point of greatest importance in the process of IT internalization is, one thing that seems to have been made rather clear is that IT's require that their specific endocytic mechanism be intact for maximizing, or for that matter, potentiating their cytotoxic action. To further illustrate the point, it has been shown that conjugation of an IT to a non-internalizing antibody completely abrogates any specific conjugate cytotoxicity (Cogliati et al., 1991). Such observations, along with the recent progress in the preparation of recombinant IT constructs whose internalizing profiles have yet to be elucidated (Brinkmann et al., 1993; Better et al., 1993; Friedman et al., 1993), make this a subject of urgent study.

The mode of action of chemotherapeutic drugs has been, for the most part, less clearly defined in terms of membrane or intracellular targets, and studies correlating between immunoconjugate internalization and drug efficacy have been rather limited. Initial studies on Mab-methotrexate (Mab-MTX) conjugate uptake and internalization by melanoma and teratocarcinoma cell lines (Uadia et al., 1985; Shen et al., 1986), established some links between immunoconjugate efficacy and its degree of internalization and lysosomal degradation. A more detailed study on the nature of linkage and mode of action of methotrexate conjugated with Mabs against a mouse mammary tumour, or irrelevant antibody (Endo et al., 1988), suggested that amide-bond linked MTX is taken up by endocytosis, whereas a substantial portion of MTX linked by an ester or other less stable bonds is released extracellularly, and may then enter cells by

the MTX active transport system. In a recent effort to increase the levels of expression of the human folate receptor (hFR) using transfectants of human MCF-7 breast cancer cells (Chung et al., 1993), it was also shown that increased levels of hFR resulted in increased MTX uptake, internalization and cytotoxicity. Interestingly, some evidence derived from *in vivo* studies of the antitumour activity of a non-internalizing Mab-*vinca* alkaloid immunoconjugate (Starling et al., 1991), suggested that non-internalizing ICs may still have significant antitumour activity. Conversely, adriamycin hydrazone (ADM-Hzn) immunoconjugates, were shown to be more cytotoxic against lymphoma tumour xenografts, when linked to internalizing rather than non-internalizing Mabs (Braslawsky et al., 1991). Furthermore, a recent *in vitro* study of the internalization of a doxorubicin anti-CEA Mab immunoconjugate, constructed using an aminodextran carrier (Shih et al., 1994), showed that both the unconjugated Mab and the IC were internalized and distributed mostly in the cytoplasm of LoVo cells, whereas unconjugated doxorubicin was quickly absorbed by the cells and concentrated in the nucleus of those cells. This immunoconjugate had previously shown a better therapeutic effect than either unconjugated doxorubicin or an irrelevant antibody conjugate (Shih et al., 1991). Unfortunately, due to the diverse mode of action of different chemotherapeutic drugs as compared to toxins and a paucity of information on IC internalizing potential and efficacy, it is impossible to clarify this relationship at the present stage.

Internalization of radioimmunoconjugates has attracted interest (Mariani et al., 1990; Scheinberg et al., 1991) as the potential of Auger emitters such as the commonly

used Iodine-125 (with an effective radius of only 20 to 30 A°) is greatly enhanced when the energy derived from the radionuclide is highly localised. This is a point of great interest since it has been shown that radiotherapy of small lesions necessitates nuclides with low energy radiation due to diffusion of long range radiation (Griffith et al., 1988). A number of studies on immunodiagnosis have also shown that retention time of radioactivity is a combination of labelling technique and the ability to internalize (Mariani et al., 1990).

The problem inherent to most of these studies however, has been the lack of an adequate and consistent experimental model studying the process of internalization for each system under consideration. Methodologies which have been used to study antibody or immunoconjugate internalization, initially involved mostly indirect methodologies, such as attempting to detach radiolabelled cell-surface bound antibody via the use of low pH buffers (Matzku et al., 1986; Matzku et al., 1990; Starling et al., 1991; Tagliabue et al., 1991; Casalini et al., 1991) or using secondary radiolabelled antibody (Rosenthal et al., 1980) to detect and quantify any remaining surface primary antibody. Direct methods of visualization have primarily involved electron microscopy for the detection of internalized antibody conjugated to different extracellular markers such as horseradish peroxidase (HRP), cationized ferritin (CF) (Bäck et al., 1993) or colloidal gold (Weltzin et al., 1989). Fluorescence microscopy has been a preferred method for the direct detection of cell surface phenomena, such as the capping of antibodies tagged by fluorescein isothiocyanate (FITC) (Pulczynski et al., 1993).

Recently, direct or indirect methods have made use of flow cytometry, for faster quantitative analyses of internalized antibody (Pulczynski et al., 1993; Garrigues et al., 1993; Hopper et al., 1990). Furthermore, the question of lysosomal degradation of internalized antibodies has been addressed by estimating the amount of remaining intact radiolabelled antibody which is precipitable with trichloroacetic acid (TCA) (Kyriakos et al., 1992). A method which allows visual detection of internalized antibody involves solubilization of cells previously incubated with antibody, followed by sodium dodecyl sulfate/ polyacrylamide gel electrophoresis (SDS/PAGE). This approach was developed as part of this project in order to verify quantitative internalization evidence derived from previously used assays (see section III.5 and Tsaltas et al., 1992), and has recently been used by another group for the detection of non-degraded internalized antibody (Garrigues et al., 1993).

The present study attempts to establish a reliable and consistent approach to the study of internalization of a well characterised anti-CEA Mab by different human cancer lines, by providing a basis for comparison of results obtained by a number of direct and indirect techniques.

IV. 2. Direct radioimmunoassay for the detection of internalized antibody.

This method was chosen due to its widespread use at the time the study was initiated, as well as its ability to generate quantitative results in a relatively uncomplicated manner. However, as described in the literature, this assay did incorporate a number of experimental shortcomings. For example, no time was allowed for the initial binding of antibody to antigen, hence the increasing binding values for all the time intervals studied. Additionally, since dead cells are permeable to Ig molecules, cell viability should have been determined as part of the assay procedure, as this would contribute to increased estimates for internalization. Nevertheless, we decided to initially perform the assay as described and our initial assays (presented in section III.1), were generally indicative of moderate internalization of the particular antibody, at the 30% to 40% level of total antibody uptake (although the fraction of antibody internalized varied according to incubation time). Also, there was consistently no evidence for any internalization at least during the first 30 minutes of incubation, despite the fact that general internalization profiles for most antibodies that follow the endocytic pathway seemed to suggest that the process usually begins much earlier on (during the first 5 minutes of antibody incubation), and that the 20 minute incubation interval with glycine-HCl would have allowed some antibody to be internalized.

Results were contradictory in terms of peak internalization and saturation of antibody uptake, since there was no consistent plateau observed on the respective binding curves. Furthermore, one of the assays (figure 1.1) showed a slight decrease in

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IV. 3. Use of low pH glycine-HCl buffer in dissociating antigen-antibody bonds.

The low pH (2.5 - 2.8) Glycine-HCl buffer has been used extensively in acid elution of antibodies from immunoadsorbents. More recently (during the last decade), assays employing the same buffer for the dissociation of bonds between antigen-antibody complexes have extended the use of this buffer and have established methodologies which are being used in studies involving internalization of antigen-antibody complexes (Matzku et al., 1986; Imamura et al., 1987; Matzku et al., 1990; Starling et al., 1991).

The general outline of this method, as applied to internalization experiments, includes the following steps.

1) Incubation of cells with antibodies specific to surface antigens for various time intervals, in order to allow for internalization of antibody or antigen-antibody complexes.

2) Measurement of the total cell-associated antibody (comprising both surface-bound and internalized) on samples from each time interval.

3) Application of the glycine-HCL buffer to the sample in order to dissociate surface bound Ag-Ab complexes.

4) Estimation of the amount of antibody remaining in the target cell assuming that all surface bound complexes have been dissociated.

5) Equating the amount of remaining radioactivity with the amount of internalized complexes.

Recently, the question of incomplete Ag-Ab bond dissociation by this buffer has

also been addressed by another laboratory (Matzku et al., 1991), which had been instrumental in establishing this technique. However, no data have been provided to illustrate the nature and extent of this observation, and to our knowledge, there has been no attempt to date to analyze the degree of error that this experimental approach might introduce. This confidence in the effectiveness of the dissociating action of the glycine-HCl buffer seemed peculiar in view of the repeated cautioning in the literature (Absolom et al., 1986; Helmerhorst et al., 1982; van Oss et al., 1981) suggesting the need for improvements in the methodology used to reverse attractive forces in antigen-antibody complexes. For example, a thorough analysis of the nature of such bonds (Absolom et al., 1986) strongly suggests that low pH conditions alone would generally prove ineffective in fully dissociating them. This review stresses the fact that antigen-antibody interactions are usually the combination of two types of attractive forces, namely van der Waals' and electrostatic (Coulombic) forces. Lowering the surface tension of the aqueous medium by adding organic solvents such as ethylene glycol or dimethyl sulfoxide, results in reversing the attractive van der Waals' force into a repulsive one, whereas extreme (either high or low) pH conditions are bound to reduce or even reverse the electrostatic attractive component. Therefore, although bond dissociation in other systems may be possible solely by pH adjustment, as is the case in the dissociation of DNA-anti-DNA complexes at high pH (de Groot et al., 1980), both pH adjusting and lowering of the surface tension of the surrounding aqueous medium are required in order to effectively reverse antigen-antibody binding (Helmerhorst et al., 1982; van Oss et al., 1981).

Based on the above, a thorough examination of the role of this buffer on both

fixed and live cells expressing CEA was thought to be necessary. Both immunocytochemical assays and enzyme-linked immunosorbent assays (ELISAs) were employed to this end. In addition to the question of efficacy of the buffer in breaking antigen-antibody bonds, we also briefly investigated its possible role in the shedding of antigen-antibody complexes or in dissociating antibody-HRP bonds. Information on the latter would be useful in cases where such antibody conjugates are used in a direct assay.

Initial immunoperoxidase results on fixed cells provided no evidence of dissociation except in the case of the direct assay using HRP-conjugated antibodies. Similar ELISAs on fixed cells did show evidence of partial dissociation at both room temperature and at 37°C, but in only one occasion was a high level of original binding shown to be lost (85 % dissociation). After averaging the dissociation values derived from a number of these assays (Tables 2.2.a.1 and 2.2.a.2), it was shown that this buffer's dissociating effect did not exceed (on average) a 50 % reduction in binding (Table 2.2.e), and that this effect was very similar for the three cell lines used. However, this dissociation effect did show some variability among the assays performed, while there was very little variability among wells belonging to the same group (usually 5 wells per group) in any particular assay. Although readings of individual plates were not generally presented in the results, for the sake of brevity and in order to avoid confusion, part of this variability seemed to be due to variation in the level of antibody uptake in each particular assay. This difference in uptake could be due to some variation in cell characteristics in terms of antigen expression after multiple passaging of cells. Such variations were commonly observed with most of the cell lines used in this project, as

documented in the FACS analysis of internalization (Table 6, standard deviations for means of antibody uptake values). In general, a higher overall uptake of antibody seemed to reduce the dissociating effect of the glycine-HCl buffer, a phenomenon which might be explained by an increased antibody avidity due to either level of antigen expression or conformational changes in the way this antigen was expressed. However, some exceptions did exist which would not support such a hypothesis (for example, in Table 2.2.a.1, assay #386, low dissociating levels were observed despite rather high original antibody uptake by the two higher CEA expressor cell lines). Dissociation did not seem to be particularly favoured by one of the two temperatures during incubation with the glycine-HCl buffer. Furthermore, use of glycine obtained from different companies or slight increases in buffer incubation times, did not seem to have any effect either.

Initial estimations of any distorting effect that the glycine-HCl buffer might have on antigenic sites (through evaluating the amount of antibody that can re-attach to those sites after treatment with this buffer), seemed to indicate that full reassociation could be achieved, suggesting intact antigen (%FB in tables 2.2.a.1 and 2.2.a.2). However the implementation of a correction factor (as shown in tables 2.2.a.3 and 2.2.a.4), resulted in deriving a more accurate estimation of reassociation values shown in table 2.2.a.5. According to this derivation, reassociation of antibody to cells previously treated with the glycine-HCl buffer is quite low, indicating distortion of antigenic sites.

Results tended to be more variable in the case of ELISAs employing live cells, probably due to the nature of the assay, but again dissociation levels rarely exceeded 50%. However, when direct assays were performed using HRP-conjugated antibodies

there was a two to three fold increase in dissociation observed (tables 2.2.b.1 and 2.2.b.2). This increase may have been attributed to antibody-HRP bond dissociation rather than CEA anti-CEA bond dissociation as shown in tables 2.2.c.1 and 2.2.c.2. In similar immunocytochemical assays (table 2.1) the low levels of dissociation observed only in the case where the HRP conjugate was used might be entirely attributable to dissociation of the enzyme. Results also indicated that the high dissociating effect on antibody-HRP bonds, seems to depend both on strength of bond (conjugation) and temperature conditions during incubation with the buffer (table 2.2.c). In general, our results indicate that use of the glycine-HCl buffer is particularly inappropriate in cases where such conjugates are used in direct assays, since dissociation levels do not only reflect dissociation between antigen-antibody complexes, but also the dissociation of enzyme from the antibody.

In contrast to the above results, when purified antigen was used in similar assays there was complete dissociation of CEA anti-CEA bonds after a 20 minute incubation with this buffer (table 2.2.d). One possibility accounting for the difference between assays involving cells versus purified CEA, would be that cellular conformation might deny the buffer access to the desired epitope. This view is in contrast to a study involving different modes of binding and internalization of Mabs to human melanoma cell lines (Matzku et al., 1986). Although, according to this report, antigens expressed on the cell surface and complexed with antibody show no resistance to the dissociating effect of this buffer, the same investigator has more recently alluded to the fact that there may be incomplete desorption of high affinity antibodies when this buffer is used (Matzku et

al., 1990; Matzku et al., 1991). The systematic production of evidence shown in this thesis has certainly substantiated such fears. Furthermore, since it seems likely that degree of antibody desorption by this buffer is not uniform across a panel of antibodies, it is not feasible to introduce a uniform correction factor accounting for these differences. Realisation of this shortcoming has recently led this group to the evaluation of alternative ways of dissociating antigen-antibody complexes from cell surfaces (Matzku et al., 1991). Although there has been no such systematic study that we are aware of to date supporting the acknowledgment that this buffer may not be desorbing "very high" affinity antibodies (Matzku et al., 1990), alternative desorption procedures such as the one employing phospholipase C to detach CEA from its phosphotidylinositol bond (Matzku et al., 1991) have been suggested. Stripping of the antigen from the surface of the cell however, might be considered a rather drastic alternative, since it would make it impossible to further study the fate of the antigen (for example in cases where recycling potential is of interest).

In conclusion, the consistency of our results across different assays involving high CEA-expressing cell lines, strongly indicates that routine use of glycine-HCl buffer for the purpose of dissociating cell-bound antigen-antibody complexes can introduce major inaccuracies in the analysis of antibody internalization experiments and its use in such experiments should be reconsidered. Alternatively, if this internalization assay is to be used in the future, the utmost care should be taken to ensure that the action of the buffer in question (or variants of it) is thoroughly investigated prior to use.

IV. 4. Use of an indirect radioimmunoassay in estimating antibody internalization

The alternative indirect double radiolabelling assay, consistently showed relatively high retention of radioactivity correlating with an upward trend in antibody internalization. Both uptake and internalization seem to be specific to the anti-CEA antibody, since the nonspecific control antibody did not exhibit any significant uptake or residual activity. However, there were some notable differences between results derived from the direct versus the indirect RIA, the most obvious being the much lower activity registered in the case of the indirect assay, which at times reached 8-10 fold radioactivity decreases, as compared to respective groups in the direct assay. This was probably due to the fact that cell samples were pre-incubated with primary antibody as described in the literature (i.e. at 37°C and not at 0°C) for 30 minutes in order to establish initial binding. During this time it is certain that some internalization would have occurred, leading to decreased levels of surface-bound activity detectable by the secondary antibody. This pre-incubation of cell samples at 37°C also led to another major difference between the two assays, namely that the groups used to test for differences in antibody uptake at 4°C versus 37°C did not show any appreciable differences in uptake when the indirect RIA was used, whereas a 50% decrease in uptake was noted at 4°C in the case of the direct (previous) RIA. Furthermore, there was no peak in internalization profile through the time intervals examined using the indirect RIA, but rather a steady upward trend suggesting continued internalization at almost the same rate up until the 150-minute interval. This rate of internalization was definitely much higher than the one noted with

the direct assay, however the most likely explanation for this is that cross-linking between primary and secondary antibodies may have strongly influence (increased) internalization activity.

In general, although this method offered more consistent results that suggested vigorous and sustained internalization, it was subject to a number of theoretical shortcomings the most serious of which involved the augmenting effect that cross-linking caused by the secondary antibody would have on internalization. From a technical point of view the pre-incubation of samples with primary antibody should be performed at 0°C, a larger number of samples per group should be included and cell viability should be determined for every step of the assay. Additionally, this assay followed a relatively complicated protocol, which made the study of short incubation time intervals quite difficult. Furthermore, both of the RIAs did not allow direct visualization of a possible internalization process. In view of all the above, it was decided that the tworadioimmunoassays should be abandoned without introducing any of the necessary modifications and that a new approach should be developed, which would be free of theoretical and technical inadequacies. As a first step, we attempted to visualize the surface-bound and internalized antibody emplying a quantitative electron-microscopy approach.

IV. 5. Visualizing internalized antibody through Electron Microscopy.

Labelling the test and control antibodies with HRP and incubating for a two hour time interval, resulted in cell sections which under EM analysis showed that our CEA-specific Mab both accumulated on the cell surface of a high CEA expressor cancer line, and was internalized into lysosomal vacuoles (figures 4.1.a, 4.2.a, 4.3.a). In contrast no antibody uptake or internalization were evident with the HRP-labelled, non-CEA specific antibody at antibody concentrations of 25 and 50 μg ml^{-1} (figures 4.1.b, 4.2.b). Increasing the concentration to 100 μg ml^{-1}, led to much stronger staining but also a very low level of non-specific uptake as indicated by faint staining along the cell contour (figure 4.3.b). This profile was evident on all samples examined (approximately 5 per group). Overall, this methodology provided very strong qualitative evidence for antibody internalization, although it did not allow for quantitation of surface-bound or internalized antibody.

IV. 6. Detection of internalized antibody using SDS/PAGE.

Strong evidence for internalization was also provided by Western blots of samples from membrane and cytosolic portions of cells incubated with test and control antibodies, which was developed in order to better define changes in surface-bound and internalized antibody through time. Although this method had not been used in the literature for the study of internalization at the time we initiated this study, we found that it generally has the potential of assessing internalization in a more direct way. All three CEA expressor

lines tested showed definite evidence of CEA-specific Mab accumulation in the cytosolic portion of the cells, with mostly minor background accumulation of the control antibody. The high accumulation of non-specific antibody in the case of the BENN cell line may be due to a higher number of Fc receptors on the surface of BENN cells, although we have no direct evidence for this at present. Although, as stated before, all our test lines (LS174T, SKCO1 and BENN) showed uptake and internalization of the anti-CEA Mab, it was interesting to note characteristics specific to each cell line. For example, strong evidence for time-dependent increasing intracellular accumulation of anti-CEA Mab which was CEA-specific (extremely low control antibody levels evident on blots) was obtained in the case of the SKCO1 line, whereas this accumulation was relatively uniform in the case of LS174T and BENN cell lines. Factors such as levels of antigen re-expression (turnover rate) and surface antigenic distribution particular to each cell line may be affecting the degree and rate of Mab internalization. Furthermore, some of the internalized antibody may be degraded intracellularly to a point where it cannot be detected by the secondary antibody employed in this assay. Although there is no evidence supporting the notion that differences in cell line characteristics (other than the type and level of receptors they express), may seriously affect intracellular routing and level of lysosomal degradation, this still remains a possibility. In the case of an additional specificity control provided by a very low CEA expressor line, we saw evidence of some non-specific antibody accumulation in our cell samples, whereas there was little evidence of CEA-specific antibody internalization other than in the cytosol after a 30 minute incubation (Figure 5d.A, lane g). We have no explanation for this at present and it is

possible that this band may be attributable to an artefact. Again, Fc receptor status of this, and the other lines, is not known at present.

Although this assay provided clear and consistent results, it was also recognized that it was burdened with shortcomings, such as that it contained multiple steps, therefore increasing the probability of introducing errors at each step, that it was lengthy, and that it required large numbers of cells and amounts of antibody. The requirement for high cell numbers per sample, in addition to the practical difficulties in cell culture, also presented potential problems of cell aggregation and increased cell loss. Another problem was that, using this assay, we were not able to quantitatively define internalization and that the level of possible contamination of cytosolic components by membrane components was again, not quantifiable. Furthermore, although this assay is ideal for estimating non-degraded antibody, it may not detect all possible antibody fragments. Given that in most circumstances internalization is rapidly followed by sequestration of internalized material into lysosomal vacuoles and degradation, a more generalized approach would have to be followed for a more accurate estimation of internalized antibody levels.

IV. 7. Detection of internalized antibody using flow cytometry.

Internalization assays using flow cytometry developed in this laboratory (Hopper et al., 1990; Osborne, 1992) generated concise and reproducible internalization data. Characteristics particular to each line were again observed, as was the case with the SDS/PAGE analysis. For example the lines LS174T and SKCO1, despite both being high CEA expressors, exhibited variable potential for anti-CEA internalization with SKCO1

having the highest antibody uptake (consistently higher positive controls), and LS174T being the most avid "internalizer" (higher relative test curve shifts) (Table 6). In both lines there was strong evidence for very early internalization activity, as was noted with previous assays. Internalization seemed to plateau on average by the 30 minute interval incubation in the case of the SKCO1 cell line (Table 6), whereas no decrease in internalization rate was observed in the case of the LS174T line. The COLO320 line provided evidence for the specificity of the process, since no anti-CEA antibody uptake was evident at any time (section III.6, Tsaltas et al., 1992; Osborne, 1992).

In an effort to establish the reliability of internalization data produced using flow cytometry, SDS/PAGE assays similar to the ones presented in the previous section and flow cytometric analyses were compared. Indeed, when samples were run in parallel for FACS and SDS/PAGE analyses and treated in an identical manner, the same pattern emerged. Uptake and internalization (bands on membrane and cytosolic sample lanes) were clearly visible for all time intervals for both lines, with SKCO1 sample bands being stronger (presumably higher overall antibody uptake). In certain cases some nonspecific uptake of control antibody was visible (LS174T membrane 30 min, SKCO1 cytosol 0 min), which we have no explanation for at present. No such non-specific uptake was noticed in the case of the COLO320 line. In general, results from flow cytometric analyses correlated well with those from Western blots. FACS results indicated highest antibody uptake in the case of the SKCO1 cells, the cell line for which the strongest bands were also obtained for membrane samples. Similar results were obtained for internalized antibody (cytosolic bands and absolute numbers for test samples during the

FACS analysis), although relative curve shifts (percentage of antibody internalized) seemed to be highest for LS174T cells (although, due to moderate antibody uptake absolute amounts of internalized antibody for this cell line were relatively low in both procedures). Based on these observations, it appears that internalization profiles can be affected by characteristics particular to the cell line. The cell line used as a specificity control (COLO320) showed no antibody uptake and consequently no internalization following either procedure. Interestingly, both assays showed evidence of very early internalization activity (0 minute samples). Presumably, procedures followed during both assays (i.e. length of initial washes following antibody application, or inability to completely remove primary antibody) allow for some internalization activity in the first minutes of exposure to antibody. Although some additional intracellular antibody accumulation is discernible with both assays as incubation intervals increase, it seems that in this model internalization activity is a very early phenomenon, with internalization rates remaining rather stationary thereafter. However, it is rather difficult to comment on the incremental nature (if any), of the progress of internalization based on the blots since there were no densitometric data available, although earlier results (previous section), provided some evidence of increasing internalized antibody with prolonged incubations. Slight procedural modifications of the Western blot assay (for example increased number of washes and resuspensions) which were necessary in order to make it possible to directly compare the two assays, may have also affected the degree of sensitivity of the Western blot assay. Based on those results it could be concluded that the assay for the detection of internalized antibody using flow cytometry compares well

with other reliable internalization assays and has the added advantages of being considerably faster, less cumbersome and requiring much lower amounts of the necessary cells and antibodies. A possible extension of this assay might involve the inclusion of control, glutaraldehyde-fixed cells for comparison with cell samples incubated at 0°C. In this way the inhibition of endocytosis during washes or removal of supernatants would be verified.

IV. 8. CEA-anti-CEA complexes and clathrin-mediated endocytosis.

Endocytosis mediated by clathrin-coated vesicles is known to be perturbed by different mechanisms, such as cytosol acidification, potassium depletion and use of hypertonic media (Hansen et al., 1993) (discussed in section I.6.5.c). Cytosol acidification interferes with budding of clathrin-coated vesicles from the plasma membrane, whereas potassium depletion and hypertonic media are believed to act by preventing clathrin and adaptors from interacting. Other methods of inhibiting endocytosis include pH-clamping of cells at neutrality with nigericin, swelling cells with hypotonic media and sticking cells to the surface of a culture dish with polylysine (Heuser, 1989). In an effort to examine whether internalization of monoclonal anti-CEA antibody proceeds via the clathrin-mediated pathway, cells were exposed to hypertonic medium containing a high concentration of sucrose. Results indicate that only prolonged incubation in hypertonic medium inhibits absolute amounts of antibody internalized due to a much reduced overall antibody uptake by cells treated in this manner (figure 7.a).

This inhibition does not take place when cells are exposed to hypertonic media throughout the assay but have not been pre-treated in such media (figure 7.b). These results suggest that disruption of clathrin-coated membrane domains partially abrogates internalization in our model, solely by reducing antibody uptake. The fact that uptake is only partially reduced (50% reduction in treated versus untreated samples), while internalization relative to uptake is either unchanged or increases, suggests that some CEA-anti-CEA complexes may also be internalized via an alternative pathway not involving clathrin. The profile of internalization in clathrin-inhibited samples, is quite similar to previous ones, inasmuch as the rate of internalization seems to be highest in the very initial stages of incubation with antibody (probably first few minutes). Since non-specific internalization through fluid-phase endocytosis is a much slower process than active endocytosis, it is very likely that this alternative endocytic pathway may involve a pathway similar to the recently demonstrated non-clathrin coated vesicle pathway (see section I.6).

IV. 9. Conclusions and future.

Based on the above data, we conclude that the anti-CEA monoclonal antibody, 11-285-14 does get specifically internalized by CEA expressing cancer cell lines. This information is essential to further construction of immunoconjugates, since the strong possibility that the cytotoxic moiety will be allowed to enter the cell may well influence the mode of drug conjugation so that it would become easier for the drug to detach from

its antibody carrier in order to exert its action. Furthermore, comparison of experimental results across the different assays used, suggests that the direct internalization assays involving the examination of solubilized membrane and cytosolic components of cells, as well as flow cytometry assays, provide a good indication of amount of antibody internalized. In comparison, the indirect internalization assays employed proved to be cumbersome and provided less conclusive results in our hands.

The incorporation of results on the inaccuracy of the assay involving the low pH glycine-HCl buffer in the general body of knowledge on internalization seems to be particularly relevant, since this assay is still used to study internalization. It is interesting to note that even recently produced humanized Mabs, are still being characterized in terms of internalizing potential through the use of this assay (Caron et al., 1992). Since the production capacity of monoclonal antibodies is rapidly expanding with the use of different repertoires (Hoogenboom et al., 1992; Burton, 1993; Winter et al., 1991), their characterization in terms of endocytosis should be based on reliable and uniform procedures.

Indeed, establishing a rapid and reliable internalization assay is central to the study of the dynamics of any antigen-antibody system, before it is attempted to evaluate the cytotoxic action of targeted immunoconjugates on cancer cells. Through use of SDS/PAGE on solubilized cells and flow cytometric assays, internalized antibody has been detected in all the CEA-positive cell lines examined. Furthermore, depiction of internalized antibody was possible without the use of radioactive probes, and was rapid

and consistent when flow cytometry was used. Given that flow cytometric techniques have been rapidly developing in recent years with the use of multiple cell markers (Stewart, 1992) for the detection of an ever increasing number of surface and intracellular proteins (Melamed et al., 1990), this approach seems to provide an excellent alternative to existing methods for the detection of internalized antibody.

Eventually this method will be modified to accomodate detection of both drug and antibody, once satisfactory immunoconjugates have been produced in this laboratory. Initial attempts to detect doxorubicin separately via its natural fluorescence have not been successful due to an extensive overlap in the emission spectra of FITC and doxorubicin (Osborne, 1992) (emission peaks differing by no more than 20 nm).

Another future project of great interest would involve the further examination of endocytic mechanisms of CEA-anti-CEA complexes. To date (to our knowledge), there has been no report on the mode of uptake and intracellular routing of this receptor molecule and its complexes. Preliminary work in this project has suggested that the endocytosis of this molecule proceeds via an active pathway possibly involving both clathrin-coated pits and non-clathrin-coated vesicles. The recent discovery of markers for non-clathrin-coated vesicles and related adaptors (Carter et al., 1993; Maholtra et al., 1989; Orci et al., 1986; Serafini et al., 1991), makes it possible to address the question of co-localization of these markers and markers of the CEA-anti-CEA complex. Given that CEA does not fall into the general category of transmembrane proteins that internalize following the recognition of signal peptides, the study of any further

similarities or differences in the mode of endocytosis of CEA versus such transmembrane proteins might shed some light both on the process of internalization and on particular characteristics of this elusive molecule.

Another point of interest in terms of immunoconjugate internalization and processing, would involve the place and degree of their degradation (if any), and the effect of this degradation on their efficacy as cytotoxic agents. Such studies might involve the inhibition of various intralysosomal compartments (such as endosomes and lysosomes) through the use of agents that may disrupt intracellular trafficking, neutralize intralysosomal pH or inhibit lysosomal enzymes (such as lysosomotropic amines and carboxylic ionophores (Press et al., 1990)), coupled to estimation of levels of degraded antibody and degree of cytotoxic activity of respective immunoconjugates.

It is entirely possible that the key to turning immunoconjugates into the "magic bullets" envisioned by Ehrlich, lies in deciphering the effect that a number of different factors have on the processing of these agents. We believe that their internalization and intracellular fate makes up an essential component of their potency and hope that work on this subject will be ongoing.

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