THE CONSTRUCTION AND ANALYSIS OF 'BISPECIFIC' ANTIBODIES

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ABSTRACT

Three different cross-linking reagents were examined for their ability to link two antibodies together (anti-CD3 and anti-ovarian carcinoma) to produce functional 'bispecific' antibodies, that is, antibodies capable of directing cytotoxicity towards ovarian cancer cell targets.

The cross-linking of antibodies by chemical means proved to be a difficult task.

The cross-linker SMPB, was found to be impractical for use on a routine basis, and resulted in a high loss of antibody due to the number of steps involved in the process.

SPDP and O-PDM, on the other hand, did appear to produce 'bispecific' antibodies, which were the correct size by gel filtration chromatography and which also produced 'positive' fluorescence by flow cytometry. Success was also achieved in producing antibodies capable of performing some directed cytotoxicity.

This investigation, however, also revealed a problem with the specificity of the antibodies and conjugates used.

Unfortunately because of time factors, further analysis of the linked antibodies, the conjugates and the successful linkage processes, could not be undertaken and many questions were thus left unanswered.

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3. ABBREVIATIONS

MHC	Major Histocompatibility Complex
PHA	Phytohaemaglutinin
TNF	Tumour Necrosis Factor
NHS	N-Hydroxysuccinimide
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
HC	Heteroconjugate
DTNB	5,5'-dithio bis(2-nitrobenzoic acid)
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
LGL	Large granular lymphocytes
HCl	Hydrochloric acid
EDTA	Ethylene diaminetetraacetic acid
SBTI	Soya Bean Trypsin Inhibitor
TEMED	N,N,N',N'-Tetramethylethylenediamine
SDS	Sodium dodecyl sulphate
dH_2O	distilled water
PBS	Phosphate buffered saline
BSA	Bovine serum albumin
FITC	Fluorescein isothiocyanate
EBV	Epstein-Barr Virus
PAGE	Polyacrylamide Gel Electrophoresis
ELISA	Enzyme-linked immunosorbent assay

4. INTRODUCTION

4.1 IMMUNOTHERAPY IN HUMAN CANCER

The current inadequacies of the established cancer treatments (i.e. surgery, radiotherapy and chemotherapy) in controlling this devastating disease, reflects their lack of selectivity. In other words, the inability of these treatments to discriminate between normal and malignant tissues creates the serious problem of normal healthy tissues being destroyed in the process of treating the cancer. Indeed , the fact is that by the time many cancers are discovered, they have spread or metastasised to other locations in the patient's body and this makes it extremely difficult for any treatment regime to be effective in controlling or eliminating the cancer completely.

It is in this context that an immunological approach to cancer treatment provides such an attractive concept. This is because the immune system, the body's main natural defence mechanism is selective in its action, normally attacking only those agents such as bacteria and viruses for example, which it perceives as 'foreign'. As such, it has been suggested that the spontaneous disappearance of tumours which has been noted in a few patients, is evidence for the involvement of the immune system in the fight against cancer [1]. This has ultimately led to the search for immunotherapies for cancer, that is, treatments designed to enhance the ability of the patient's own immune system to react against disease.

Although immunotherapy has progressed somewhat since the early days, it should be regarded as a valuable addition to the conventional therapies, rather than as an alternative. The complexity of the immune response to tumours means that a multilateral approach to immunotherapy is required and in practice this may involve several methods.

1. Active manipulation of the immune system seeks to enhance specific and/or non-specific mechanisms of host resistance by means of vaccination. This may involve infecting the tumour with viruses, coupling haptens to tumour surface antigens or fusing the tumour with cells of a different histocompatibilty type. Additionally, with non-specific immunization, a wide variety of reagents known as biological response modifiers (BRM) are employed, and these include bacterial products, synthetic molecules, hormones and cytokines. Early attempts to stimulate the immune system used vaccines prepared from bacteria such as Bacillus Calmette Guerin (BCG) or Corynebacterium parvum. Some tumours in experimental animals were seen to shrink and in a few cases disappear entirely, and in humans the growth of certain kinds of tumours i.e. melanomas, was slowed, but the results were disappointingly inconsistent [2].

2. Adoptive immunotherapy (Cell Transfer therapy)involves the removal of immune defence cells from the cancer patient, followed by the re-introduction of these cells back into the patients bloodstream after they have undergone some sort of manipulation in vitro. This manipulation is designed to either 'educate' the cells to react against the cancer, or to enhance their intrinsic ability to kill cancer cells [1]. It was discovered that in addition to causing T-helper cells and antigen stimulated cytotoxic T-cells to replicate, Interleukin 2 (IL-2) could actually stimulate certain lymphocytes to react and kill cancer cells. These were named Lymphokine activated killer (LAK) cells. Immunotherapy based on the administration of these cells has been beneficial in some cases, but the proliferation of lymphocytes in tissues can interfere with the function of vital organs. The appearance of these and other side effects led to further research and the discovery of tumour infiltrating lymphocytes (TIL's) - cytotoxic T-lymphocytes in the tumour which appeared to have the specificity that the LAK cells did not have.

Preliminary results indicated that TIL's extracted from a patient's tumour, propagated in vitro, and then reinfused back into the patient, could home to the tumour and result in its shrinkage. Although promising results have been obtained in some instances, the approach is limited to those cases where TIL's can be found [3].

3. Passive immunotherapy involves the transfer of anti-tumour antibody to patients in order to cause tumour regression or prevent tumour recurrence.
In 1975, when a method for producing a cell line capable of secreting a single species of antibody (monoclonal antibody) with the desired specificity to antigen was described, it was thought that this would provide the solution to successful tumour immunotherapy. This so called 'magic bullet' would be able to target tumour cells , destroying cancerous tissue, whilst leaving healthy tissue unharmed. Since that date numerous attempts have been made to exploit the specificity of these reagents for cancer therapy, but they have been largely unsuccessful in producing long term therapeutic effects on their own. This is because there are a number of difficulties associated with the use of antibodies for therapy, that are not found with the conventional treatments. Firstly, the antibodies must be specific for all the neoplastic cells within tumours including the least differentiated tumour stem cells, and they must not react with any normal tissues or products within the host.

If an antibody-toxin complex is to be employed it must be lethal to the tumour cells alone, and attachment of the toxin to the antibody must be stable and not interfere with the ability of the antibody to interact with the tumour cells.

Finally, the antibody complex must be delivered to the host in such a way that it interacts effectively with all the tumour cells present but in doing so, the development of an immune response against the complex must be avoided. As such due to accessibility problems, even antibodies that do react preferentially with tumour cells, fail to reach the core of solid tumours in sufficient amounts to be effective [4].

Although the perfect antibody with absolute specificity for cancer cells has yet to be found, it has been possible to produce antibodies that are able to recognise antigens more highly expressed on malignant cells than on corresponding normal cells, and this difference could prove to be useful in the employment of monoclonal antibodies in immunotherapy. The antibodies are able to exert cytostatic effects if they bind to growth factor receptors, or interfere with their function but they tend not to be cytotoxic in their action without the aid of accessory mechanisms. The activation of secondary effector systems occurs when an intact antibody, bound to a tumour cell, binds to an Fc receptor on the surface of a macrophage, natural killer (NK) cell, or a large granular lymphocyte (LGL). This is referred to as antibody-dependent cellular cytotoxicity (ADCC) and results in an activation signal that stimulates exocytosis of cytolytic components from the effector cell onto the surface of the tumour cell. This method, however, relies on the efficient recruitment of effector cells within the host which may not be present in sufficient numbers in solid tumours, be absent from immunoprivileged sites or be compromised by disease or previous therapy.

An alternative approach, would be to use monoclonal antibodies to target agents whose activity is not dependent on the immune system of the host. For example, radionuclides, chemotherapeutic drugs and toxins all have inherent cytotoxicity but are not selective for the target cell unless directly coupled to the antibody.

Radio-immunoconjugates, which decay to release beta-particles are able to destroy malignant cells at a distance of several cell diameters from the site of cell attachment, but have the disadvantage that normal tissues will be irradiated by conjugate present in the circulation. Antibody-drug conjugates, and the more potent immunotoxins, depend upon target antigen binding and internalization, followed by the release of the agent within the cell by lysosomal digestion. This procedure is therefore limited by the need for adequate internalization pathways for the transported cytotoxic agent [5].

Whatever approach to immunotherapy is employed, it should be noted that the majority of monoclonal antibodies that have been developed are of murine origin. Although they have been found to be useful diagnostically in tumour imaging and in quantitating circulating tumour markers, they have been used with only limited success therapeutically. The main reason for this is the possibilty of the generation of anti-immune responses in the patient. This can result in the clearance of the administered antibody, thus diminishing its use as a therapeutic agent. In addition, the development of host antibodies restricts the administration of a second course of treatment and also prevents prolonged treatment.

The logical answer would therefore be to use human monoclonal antibodies for cancer therapy in the hope that they would be less immunogenic in patients, recognise more appropriate target antigens and trigger complement and antibody dependent cellular cytotoxic mechanisms more efficiently. However, the task of producing human monoclonal antibodies has proved to be a difficult one. The success of the procedure depends on an adequate supply of immune lymphocytes in an appropriate state of differentiation and proliferation, and effective methods for the immortalization, screening and cloning of these cells. This poses a problem because of the limitations of human monoclonal antibody production, with repect to donor immunization, lymphocyte availability and the suitability of fusion partners.

In most cases, the lymphocytes for immortalization have been obtained from draining lymph nodes or peripheral blood, with the immortalization procedure being carried out by cell fusion or more recently Epstein Barr Virus (EBV) transformation.

The latter procedure is that which is currently favoured, followed by fusion with either a human/mouse heteromyeloma or a mouse cell line such as SP2/0. Although antibody secreting cell lines have been generated, it is rare that the levels of specific antibody produced are sufficient to be of use.

The limitations of human monoclonal antibody production technology are thus of two kinds. Those relating to the production of the human antibodies in general, and those which are relevant to the development and therapeutic application of tumour monoclonal antibodies.

The cell lines themselves tend to be rather unstable, secreting low levels of antibody compared to murine cell lines. This could be due to a number of factors including the overgrowth by non-secreting cells, the loss of relevant regulatory and structural genes, and the inhibitory effects of contaminating micro-organisms. Even when an antibody does become available and it is highly specific for the tumour of interest, there are still a number of factors which may limit its therapeutic effectiveness. These have been summarised by Keith James [6] and are presented in Table 1.

As a result of the difficulties encountered in producing useful human monoclonal antibodies directly, considerable effort has gone into the alternative approach of 'Humanising' rodent antibodies by genetic engineering techniques.

TABLE 1 : FACTORS LIMITING THERAPEUTIC EFFECTIVENESS OF ANTIBODY

TARGET

* Does not express tumour specific or other relevant target antigens (e.g. growth factor receptors)
* Low target antigen expression
* Variation in antigen expression within and between tumours
* Modulation/shedding of target antigens
* Inaccessibility of target antigens

ANTIBODIES

* Insufficient specificity, affinity and quantity
* Lack of information on optimum dose and route of administration
* Distribution and activity becomes modified by conjugation

HOST

* Individual variability in patients
* Localisation and degradation of antibodies in lungs etc.
* Defects in host complement (C) and ADCC mechanisms
* Induction of immunosuppressive mechanisms by antibodies

A variety of antibody molecules have been constructed including antibodies in which the entire rodent variable (V) region of both heavy (H) and light (L) chains, is linked to a human constant (C) region (chimaeric antibody). These should be less immunogenic in patients, display the specificity of the original rodent antibody and show improved Fc receptor mediated effector functions.

There are also antibodies in which the polynucleotides coding for the Complimentarity Determining Region (CDR) (hypervariable region) of human molecules have been replaced by equivalent polynucleotides from a rodent myeloma cell line secreting monoclonal antibody of the desired specificity. This method, known as CDR grafting, produces a molecule which is essentially human in nature, but which may have some loss of antibody binding affinity [7,8].

Genetic engineering techniques have thus provided scientists with the means of gaining access to specific genes, which can be isolated, cloned and then expressed in an active form in bacterial or mammalian cells. The application of the Polymerase Chain Reaction (PCR) to monoclonal antibody technology has also been important in allowing multiple copies of genes to be produced from a single cell.

For example, the binding region of the light (L) chain of immunoglobulin was found to bind without the aid of its heavy (H) chain. These single domain antibodies (dAbs) can be manufactured by genetic engineering techniques and may be useful because of their small size, being able to penetrate tissue boundaries more efficiently than intact antibody molecules. They are also cleared from tissues and serum rapidly and although this may limit their use as targeting agents, it can aid the clearance of toxic drugs from the circulation [9].

4.2 BISPECIFIC ANTIBODIES

In recent years there has been a great deal of enthusiasm directed towards the use of bispecific antibodies in tumour immunotherapy. These antibodies can be created by chemical cross-linking(heteroconjugates) or by fusing hybridoma cells, and they can be designed to simultaneously recognize two moities, such as antigens on target cells, and triggering molecules on cytotoxic cells [10].

As discussed earlier, there are a number of problems associated with the use of single antibody molecules for immunotherapy. Some of these however, may be overcome by targeting the cancer cells with effector cells via appropriate bispecific antibodies. This does not present a need for internalization as does the single antibody approach, and in addition, the biological activity of the system is apparent only after specific binding of the conjugate to the target cells that have provided the immobilizing surface that is necessary for T-cell activation. This means that no systemic T-cell activation will occur and to this extent the biological effect will be confined to the tumour [11].

For T-cells to mediate this targeted lysis they must have good killing ability and this requires their activation. T-cells express a number of different surface structures which are potential targets for antibody molecules, and that which is exploited most often is the CD3/TCR (T-cell receptor) complex. For T-cells to exhibit their lytic activity, this complex must bind specifically to the target antigen fragment in association with a Class 1 MHC molecule. Most cancer patients however, do not have significant numbers of these lymphocytes which specifically react with their tumour cells and it has proved difficult to isolate and expand these cells *in vitro*.

Bispecific antibodies, however, circumvent the need for this specificity, and theoretically can be used to retarget all of the patients CTL's to destroy the cancer cells. Binding of an anti-tumour*anti-CD3 bispecific antibody to the TCR complex, results in the delivery of a signal via CD3 that activates the T-cell. This activation mimics that which occurs physiologically upon recognition of antigen by the TCR resulting in the triggering of the lytic machinery of the cytotoxic T-lymphocytes. Simultaneous binding of the tumour cell and activated T-cell results in the release of T-cell granule components close to the surface of the tumour cell. Pore-forming proteins are released producing holes in the tumour cell membrane, resulting in lysis of the tumour cell [12,13].

Investigators have been using bispecific antibodies of different types over the years in various systems, with varying success rates, and the information which has been generated from such experimentation has brought about an understanding of the mechanism by which they exert their effect, identifying the best methods of exploiting their potential.

Most studies have aimed to construct bispecific antibodies which are used to direct lysis of tumour target cells by effector cells, as measured by <u>in vitro</u> cytotoxicity assays. Many tumour cell lines including lymphoma, ovarian, colon, renal, melanoma, small cell lung and lung adenocarcinoma have been shown to be lysed with appropriate bispecifics and effector cells, as have freshly isolated human tumour cells from renal, colon and ovarian tumour cells, and melanomas. The majority of these studies used bispecifics linked by SPDP, in which the first antibody was directed against tumour cell surface glycoproteins, and the second against the CD3 portion of the TCR/CD3 receptor complex. Perez <u>et al</u> [14] , in fact, showed that only bispecifics containing anti-TCR components, could induce effector cells to lyse tumour cells <u>in vitro</u>, and that those containing antibody directed against other components of the T-

cell did not promote lysis, even though they promoted effector/target cell formation. In addition targeting was shown to be specific, in that only human tumour lines and fresh tumour cells were lysed, but normal cells from a variety of tissues were not i.e. substitution of the anti-CD3 portion of the bispecific with anti-class 1 MHC antibody (W6/32) or substitution of the anti-tumour portion of the bispecific with irrelevant antibody, rendered it ineffective.

Effector cell populations which have been tested have included T-cell clones, donor and patient PBLs, TILs, monocytes, LGLs and macrophages. Many studies have used cytotoxic T-lymphocytes from T-cell clones, but PBMCs from human donors have become more popular because they are much more readily available. Fresh PBL's however, are not as consistent a source of effector cells as are cultured PBL's that have been exposed to mitogens such as IL-2, PHA, anti-CD3 or combinations of anti-CD3, IL-2 and IL-4. This is because most lymphocytes in the periphery are resting cells incapable of mediating lysis. Pupa et al [15] noted that activation with PHA for 48 hours followed by 6-10 days in rIl-2 culture was most appropriate for triggering hybrid-mediated lysis, and PBL's thus stimulated, lysed 75% and 16% of ovarian carcinoma targets (in the presence and absence of hybrid bispecific antibodies) respectively. Killing by anti-CD3 alone was minimal and there was no cytotoxicity in the absence of antibody. This, and the fact that $F(ab)_2$ fragments of antibody showed as efficient lysis as that of the intact molecule, would indicate that the killing observed was not due to ADCC.

Barr et al [16] also presented similar results using fusion hybridomas of anti-CD3/anti-colon carcinoma, in that cloned human T-cells and PBL's (after activation with rIL-2) produced high levels of specific lysis, and that very little bispecific antibody was required (less than 1pg/ml).

Procedures for activating and expanding T-lymphocyte effector cells from peripheral blood of both normal and RCC patients were examined by Kerr et al [17]. Anti-CD3 and rIL-2 appeared to be the most effective combination for generating large numbers of lymphocytes, and lysis was found to be significantly augmented by increasing the length of incubation of the target and effector cells. For example, over 90% lysis was achieved at low E:T ratios by increasing the incubation period to 12 hours.

In systems which use anti-CD3 antibodies for activation of the lymphocytes, monocytes are usually required (binding to the Fc portion) in order to present the antibody to the T-cells and to provide necessary secondary signals. Serum IgG, however, was found to interfere with this binding and thus with cell activation [18]. When anti-CD3 was presented by means of an antibody heteroconjugate though, FcR involvement was avoided, and secondary signals could be supplied by anti-CD28 (which transmit progression signals in T-lymphocyte activation). Jung et al [18] showed that activation of monocyte depleted PBMC occurred with a mixture of anti-target/anti-CD3 and anti-target/anti-CD28, in the presence of melanoma target cells, and was effective at concentrations as low as 8ng/ml.

Heteroconjugate directed T-cell cytotoxic activity in vitro has been reported to be mediated by $CD8^+$T-cells, whereas in vivo cytotoxicity appeared to involve both $CD4^+$ and $CD8^+$ cells, and prior exposure to IL-2 was not as important as that in vitro [19]. Thus the mechanism of in vivo antibody mediated anti-tumour activity appears to be different from in vitro lytic mechanisms. It was shown that $CD8^-$T-cells were inactive in a four hour cytotoxicity assay, but were able to block tumour growth in mice, and this suggests that there are two distinctly different anti-tumour effects mediated by heteroconjugate antibodies. The first effect is that of direct tumour cell lysis, and requires attachment of the target and effector cells.

It is characterised by rapid anti-tumour activity (readily detected in a 4 hour Cr release assay) and does not induce lysis of bystander cells.

PBL's depleted of $CD8^+$cells are incapable of mediating this type of lysis. Tumour growth inhibition, on the other hand, is measured by long in vitro assays or in vivo models. It is thought to be due to the release of slow acting factors such as TNFa or b, and interferon , which are produced from lymphocytes targeted with bispecific antibodies, and which have been well documented to block tumour growth. Inhibition appears to be induced by receptor cross-linking per se, for example in T-cells by anti-CD3 adsorbed on to microtitre plates. Cytokine release is important in that local release at the site of the tumour could increase delivery to tumour cells while decreasing systemic toxicities. In addition, cells that are in close proximity to the target could be indirectly growth inhibited, which is important in heterogenous solid tumours where some cells may express little or no target antigen, or be sterically inaccessible to effector cells.

On further examination of the T-lymphocytes, Mosmann and collaborators [20] showed that Th clones could be divided into two classes - those secreting helper factors such as Il-4 and IL-5 (Th2 clones) and those secreting inflammatory cytokines such as lymphotoxin, TNF- , IFN- and IL-2 (Th1 clones). Targeted lymphocytes are thought to block tumour growth by secreting factors which act directly on tumour cells and it is possible that TNF- and IFN- indirectly or directly stimulate MHC non-restricted cytotoxic cells and assist in the generation of cytotoxic T-cells, both of which can lyse tumour cells. Therefore direct cell-mediated cytolysis could contribute to the blockage of tumour growth in vitro. As well as acting on tumour cells, secreted factors might also recruit and activate other anti-tumour effector cells such as Cytotoxic T-cells, NK cells, and macrophages in vivo, and may exert secondary effects such as causing blood supply to the tumour to be interrupted [19].

The other factors to be taken into consideration in this system are the type of antibodies to be used, and the choice of method for linking such antibodies.

4.3 CROSS-LINKING REAGENTS

When the coupling of two different proteins (A and B) is required, it is important that the functional integrity of both proteins is retained after cross-linking. A suitable coupling reagent should, therefore, react in a controllable manner to avoid intramolecular cross-linking and prevent the formation of homo-conjugates (A-A and B-B). There are a number of commercially available cross-linkers which have been specifically designed for this purpose. They contain at least two reactive groups, usually at opposite ends of the molecule, and can be homobifunctional and covalently couple two identical functional groups, or heterobifunctional and react with two or more different groups.

Zahn, in 1955, was the first person to use chemical cross-linking reagents to study protein structures, and one such reagent DMA(Dimethyl adipimidate) is still widely used for this purpose. Homobifunctional imidoesters such as DMA, and in particular DMS(Dimethyl superimidate) were popular throughout the 1970's, the former being used to cross-link lysine residues in bovine pancreatic ribonuclease A.

In the 1970's, NHS esters were introduced as an alternative to the imidoesters because they were reactive at physiological pH, and had long half lives in aqueous media ie. DSS (Disuccinimidyl suberate).
These cross-linkers are used in a one step reaction procedure where the compounds to be coupled are added to the same buffer and the cross-linker is added to this mixture. This can be less efficient than other methods, but may be necessary if only primary amines are available for cross-linking. This can however result in unacceptable self-conjugation of proteins.

Heterobifunctional cross-linkers however are designed to link proteins in a step-wise manner, promoting the preferential formation of heteroconjugates (A-B) [21]. Figure 1 shows the components of a heterobifunctional cross-linker.

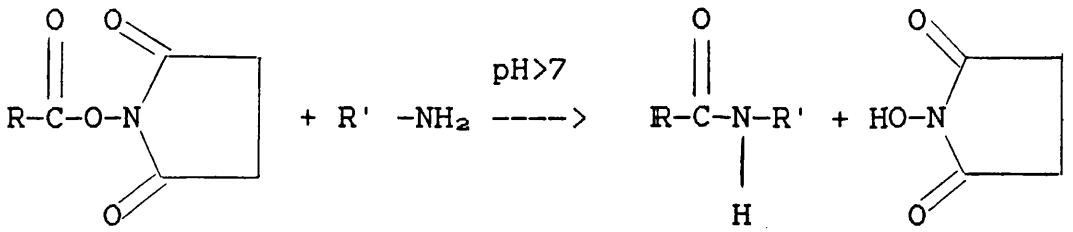
Examples of heterobifunctional cross-linkers are SPDP and SMPB. (For chemical structures see Figures 2 and 3, respectively).

SPDP(N-succinimidyl 3-(2-pyridyldithio proprionate)) is the 'classic' cross-linker, which has been used extensively over the years in the construction of heteroconjugates. Although they do form working immunotoxins *in vitro*, they have been found to be unstable *in vivo*. This is due to the breakdown of the introduced disulphide bond, and this can result in less toxin being available to destroy the targeted cells. Previous work in this laboratory has shown this to be true, and although SPDP is easy to use, the products tend to be unstable over storage and do not, therefore allow for reliable batch preparation. It was hoped to examine and to attempt to improve on this method of linking, and to compare it to other methods of interest to us.

SMPB(Succinimidyl 4-(p-maleimidophenyl)) is the extended chain length analogue of MBS(m-maleimidobenzoyl N-hydroxysuccinimide). This extension limits the interference caused by steric hindrances, and conjugates formed with SMPB have been shown to be more stable in serum than SPDP conjugates.

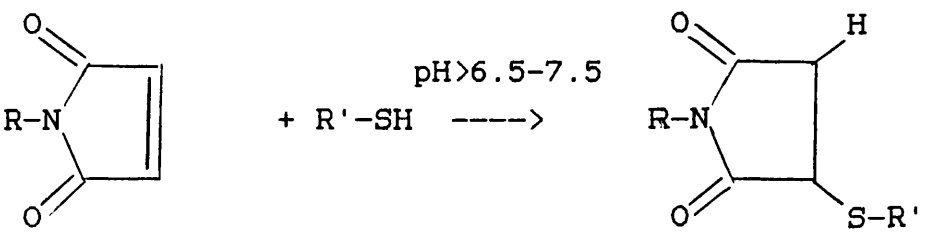
O-PDM(N,N'- O - phenylenedimaleimide) is another example of a bifunctional cross-linker and this has been used by Glennie *et al* [22] to produce functional bispecific antibodies (For chemical structure see Figure 4). The directed formation of bispecific antibodies in this case occurs by the linking of half-cysteine residues of two different Fab' species via tandem thioether bonds, to form $F(ab')_2$ bispecific molecules of approximately 110kDa.

1. A primary amine reactive group such as N-hydroxysuccinimide

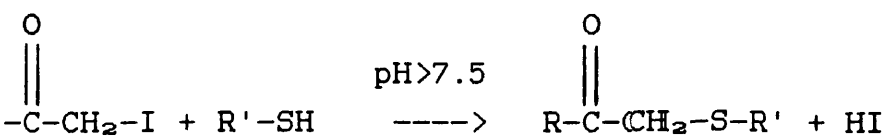


2. A thiol reactive group such as a maleimide, halogen or pyridyl disulphide group.

a. Maleimides react specifically with free sulphydryls (cysteine residues) under slightly acidic to neutral conditions (pH 6.5-7.5).



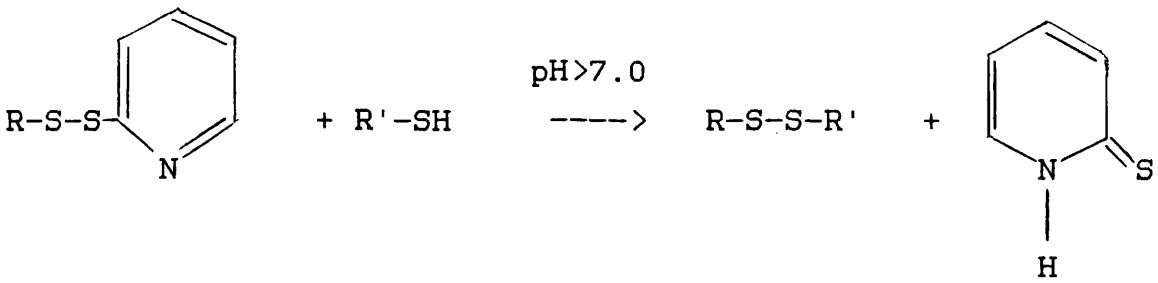
b. Halogens (iodoacetyl functions) react with SH groups at physiological pH's.



BOTH OF THESE REACTIVE GROUPS RESULT IN THE FORMATION OF STABLE THIOETHER BONDS

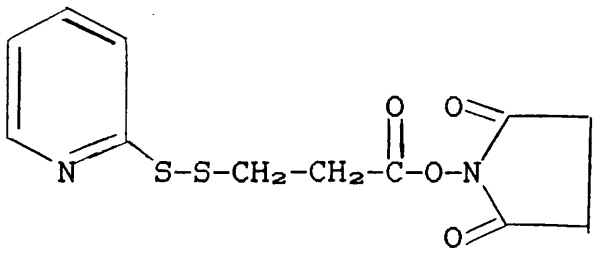
FIGURE 1: COMPONENTS OF A HETEROBIFUNCTIONAL CROSS-LINKER

c. Pyridyl disulphides react with -SH groups to form a disulphide bond, releasing pyridine-2-thione as a biproduct. Conjugates prepared by this method can be cleaved using reducing reagents.



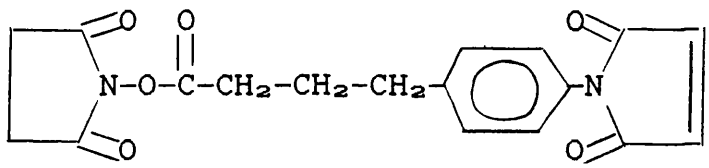
3. The third component is the spacer arm or bridge which connects the two reactive ends and thus has an effect on steric hindrances. The bridge can also affect the stability of the reactive groups for example, the cyclohexane bridge of SMCC (succinimidyl 4-(N-maleimidomethyl)cyclohexane -1-carboxylate) lends extra stability to the maleimide reactive group.

FIGURE 1 CONT/- : COMPONENTS OF A HETEROBIFUNCTIONAL CROSS-LINKER



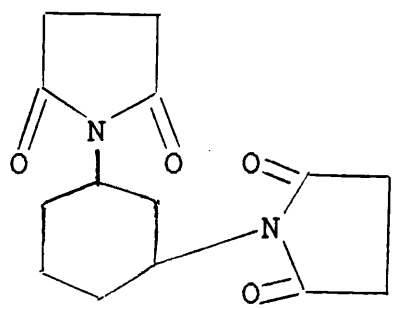
M.W. 312.4

FIGURE 2: STRUCTURE OF SPDP



M.W. 356.32

FIGURE 3: STRUCTURE OF SMPB



M.W. 268.2

FIGURE 4: STRUCTURE OF O-PDM

Functional studies have demonstrated that these bispecific heteroconjugates behave in a manner very similar to that described for bispecific IgG antibodies, but unlike the disulphide linked antibodies which can be susceptible to attack by trace amounts of thiol, the thioether linkage will ensure that these antibodies will remain intact.

The generation of conjugates using cross-linkers generally proceeds via a two-step reaction, as follows :

STEP 1 - The Amine Reaction

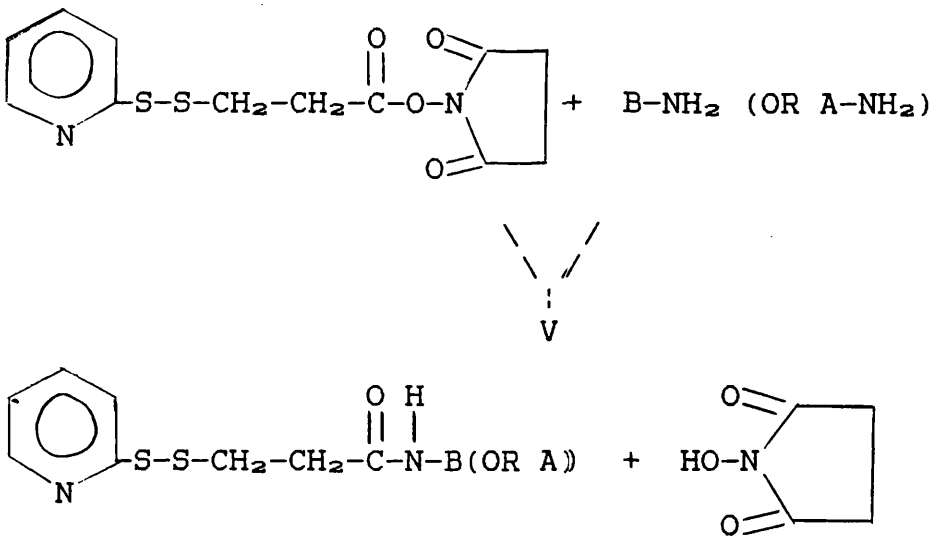
The protein chosen for reaction with the cross-linker should contain a primary amine, and should not contain free sulphydryl groups. The reaction buffer should also be free of extraneous sulphydryls and amines, and should be around pH 7.0-7.5. This prevents maleimide groups reacting with amines. NHS-ester cross-linkers have limited water solubility and have to be dissolved in a small amount of organic solvent, such as DMSO, before introducing the cross-linkers into the reaction mixture. The cross-linker/solvent forms an emulsion which allows the reaction to occur. The resulting protein becomes activated, with a sulphydryl moiety and can be isolated from the reaction mixture by gel filtration.

STEP 2 - The Sulphydryl Reaction

The protein required for the second reaction must contain a free sulphydryl group and buffers must be free of any sulphydryl containing compounds. A slightly acidic to neutral pH (6.5-7.5) is necessary for a maleimide reaction, whereas a neutral pH is sufficient for reactions involving halogens and pyridyl disulphides.

Finally, the two proteins are mixed under appropriate buffering conditions, and the resulting protein conjugate can be isolated from the reaction mixture by gel filtration (Pierce,[21]). For an example of this type of conjugation using SPDP as the cross-linker see Figure 5.

1. REACTION OF PROTEINS A AND B WITH SPDP



2. REACTION OF MODIFIED PROTEIN A WITH DTT

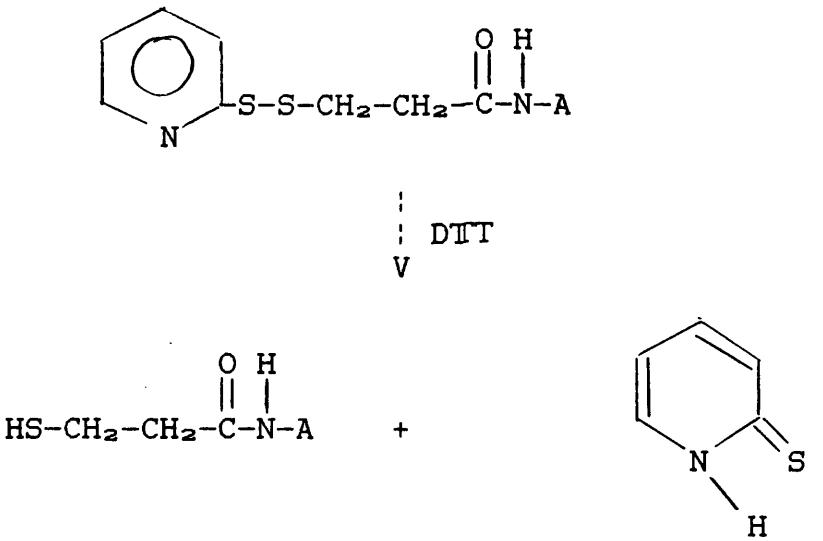


FIGURE 5: AN EXAMPLE OF A CONJUGATION USING PROTEINS A AND B

3. REACTION OF MODIFIED PROTEIN A WITH MODIFIED PROTEIN B

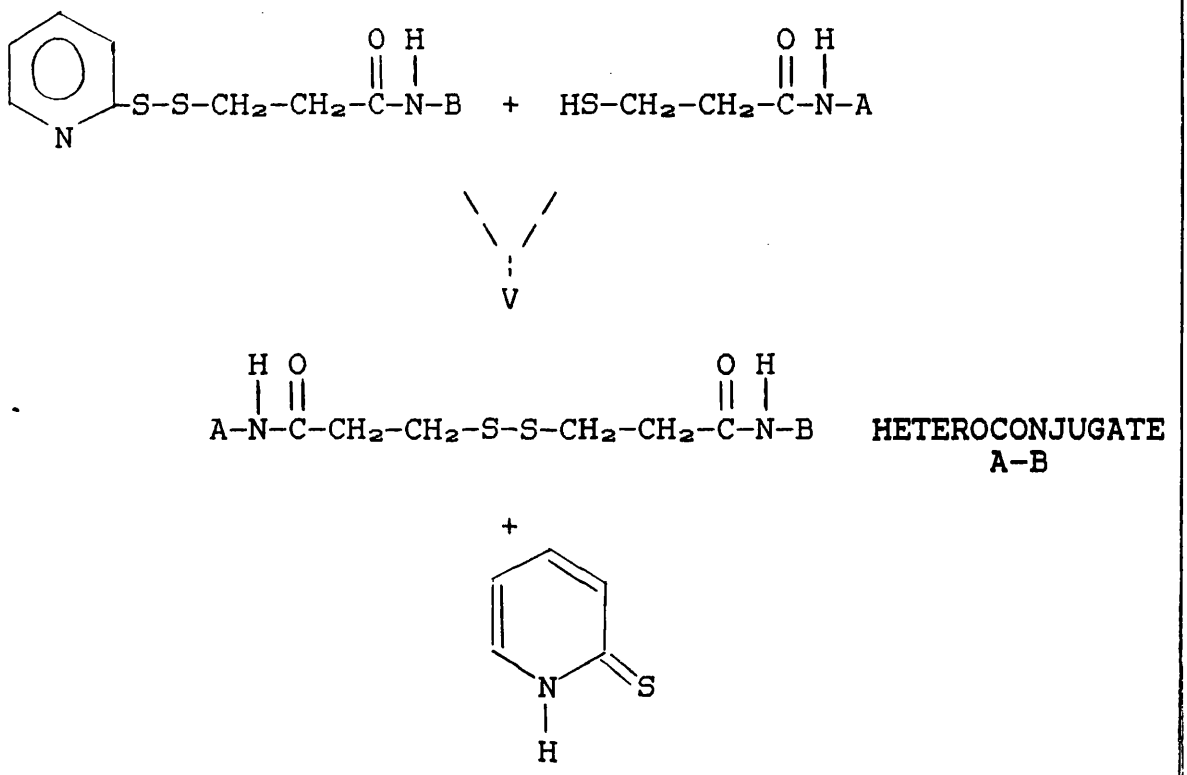


FIGURE 5 CONT/-: AN EXAMPLE OF A CONJUGATION USING PROTEINS A AND B

The major advantage in using heteroconjugates from cross-linking procedures, as opposed to the bispecific immunoglobulins secreted from hybrid hybridomas, is the ease with which they can be prepared. Cell fusion by contrast, tends to be a slow and unreliable process which has a number of limitations not least of which is the isolation of these antibodies from a heterogenous mixture of hybrid immunoglobulin molecules containing various mixtures of heavy and light chains [22].

With chemical linking, the antibodies can become aggregated producing a heterogenous population in which only some of the aggregates will be effective. The linking process may also lead to modulation of the TCR complex, rendering the T-cells useless. With the hybrid hybridoma technique, antibodies are homogenous, have a normal half life in vivo and are expected to induce little or no modulation of antigens. Because they retain their native configuration, they are theoretically more desirable for clinical applications than the chemical linkages but, only a small fraction of hybrids produce the desired hybrid antibody due to preferential isotypic assortment, low frequency of specific spleen cells and chromosome losses.

Mezzanzanica et al [23] compared SPDP-linked HC's and hybrids of anti-target/anti-CD3 antibody using MOV18 antigen (reactive with human ovarian carcinoma). The binding reactivity of the HC and the hybrids was shown to be similar to that of the parental antibody, but maximum binding levels differed between the two. The latter had a lower level of binding and a lower percentage lysis, but exhibited a longer shelf life than the HC, due to the reduction of the disulphide bridge between the two monoclonal antibodies in the chemical pairing. The HC also had a low yield (approximately 5% of original).

Canevari et al [24] also looked at SPDP-linked (divalent) and hybrid bispecifics (monovalent), of anti CD3/MOV18 which also showed similar patterns of reactivity to that of the native antibodies. Both promoted lysis by CTL's from T-cell clones or from activated PBl's, and there was no observed lysis with CTL's alone, or with uncoupled antibodies, and cytotoxicity was seen to be antibody concentration related.

Hybrid monoclonal antibodies tended to be superior to HC's as far as purification recovery was concerned, the HC yield again being 5% of the initial volume, with cytolytic induction being partially lost about 20 days after coupling. This problem might be alleviated by the use of different linking reagents that do not link through these chemical groups.

HC however, appeared to be more efficient at promoting lysis ie. 50% lysis of OVCA line by 0.1pm HC and 6.6pm hybrid bispecific in the presence of CTL from T-cell clones. This lower efficiency in the latter example could be as a result of its monovalency or could also be due to the presence of parental antibody.
Hybrid antibodies tend to be more stable in storage and therefore are likely to be more stable in vivo, however, both methods are time and labour intensive techniques and need to be more efficient to produce large quantities for trials.

In addition to SPDP, Nitta et al [25] used DTNB as a cross-linker, and combinations of SAMSA and SPDP to produce different conjugates of 7S antibody.

A. DTNB - $(F(ab)'_2)$ 100-110kDa (Monomer) Yield 70-80%

B. SAMSA/SPDP - Monomer, dimer, trimer
220kDA (Dimer) Low yields

C. SPDP - Various polymeric forms of Fab
Main antibody 330kDa (Trimer)

The monomeric form (A) appeared to be the most efficient at inducing cytolysis. The lower activity seen in the polymeric forms could be due to steric hindrances on antigen-antibody interaction.
Dimers and polymers of 7s antibody could be removed by FcR+RES cells such as NK cells and monocytes, and lost from the circulation.

Therefore, removal of the Fc region is necessary to ensure that the antibody is not immediately cleared, but remains bound and available on the target/effector cell surface until such time as the two surfaces are juxtaposed [22]. In addition, the non-specific killing of FcR+cells is avoided.

$F(ab)'_2$ fragments have proved more useful than their intact IgG counterparts because as small, high affinity molecules, they can cross capillary membranes and diffuse into tissue spaces more readily. Conjugates are also easier to prepare and appear to be more stable and less immunogenic in humans and so multiple courses of treatment can be considered if appropriate. In addition, their specific activity will be higher because more of the mass of each HC will be associated with antigen binding components, and studies have shown that Fc-free hybrids are only slightly less effective than their intact IgG counterparts [26].

The model system for immunotherapy would therefore appear to be to use $F(ab')_2$ fragments of antibodies (anti-target/anti-CD3 and anti-target/anti-CD28) which have been chemically cross-linked, and use these to target effector cells of CTL from T-cell clones or PBMC (activated by rIL-2) towards the tumour target. This is the system we wished to examine.

5. MATERIALS AND METHODS

5.1 Cell Lines

OWmM1 - Human ovarian cancer cell line growing as adherent cells. Cells are maintained by pouring off the old medium and replacing with fresh medium. Cells are split by incubating with 0.2% EDTA (in PBS) to detach them from the flask, spinning down and re-seeding at the required count. This cell line has previously been shown to have characteristics associated with primary human ovarian tumours (Gallagher et al, [27]).

OVAN-4 - Human ovarian cancer cell line growing as adherent cells and cultured as above.

F14A/14C1 - B-cells from the lymph nodes of patients with ovarian cancer, immortalized by EBV. They grow in suspension and secrete antibody (IgG) against human ovarian cancer into the medium (F14A and 14C1 antibodies). When splitting or feeding cells, the suspension is spun down, the supernatant is retained and the cell pellet is resuspended in fresh medium at the required count.

OKT3 - Mouse hybridoma cells producing monoclonal antibody against the CD3 component of the T-cell (mouse anti-human antibody). Cell line grows in suspension and antibody is secreted into the medium and retained as above.

COLO-320 - Human colon cancer cell line, growing 50% suspension, 50% adherent. When feeding, the cells in suspension are spun down and fresh medium is added, and they are incubated with PBS to remove them from the flask when splitting the cells.

All cell lines were maintained in Hams F-10 with 10%(v/v) Foetal calf serum, penicillin/streptomycin and 2mM L-glutamine.

5.2 Antibodies

Rat CD3 : SHL45.6 bivalent CD3 antibody generated as a biproduct of the purification of monovalent CD3. FPLC purified. Supplied by Mike Clark (Cambridge)

W6/32 : Anti-class 1 MHC antibody

595 : Anti-human ovarian cancer antibody(Supplied by M.Pimm, Nottingham)

505 : Anti-colonic cancer antibody(Supplied by M.Pimm, Nottingham)

5.3 Preparation of IgG and $F(ab')_2$ fragments

IgG from human serum or cell supernatants was isolated by adsorption to a Protein A sepharose column.

$F(ab')_2$ fragments were prepared by digestion of the IgG (at approximately 10mg/ml) in 0.1M sodium acetate (pH 4.2) with pepsin at 0.3mg/ml, for 18 hours at 37°C. The $F(ab')_2$ was then separated from the digest mixture by gel filtration on a column of Ultrogel Aca34 equilibrated in 0.2M Tris/HCl(pH 8.0) containing 10mM EDTA.
Alternatively, fragments could be purified by protein A affinity chromatography and a Centricon-30 microconcentrator.

5.4 Bicinchoninic Acid (BCA) Assay

Solution A - contains bicinchoninic acid, sodium carbonate, sodium tartrate and sodium bicarbonate in 0.1 N sodium hydroxide (pH 11.25)

Solution B - Copper (II) sulphate pentahydrate 4% solution containing 4% (w/v) cupric sulphate.$5H_2O$.

Protein determination reagent (1ml) consisting of 1 part solution B to 50 parts solution A was added to 0.05ml sample in a test-tube and incubated for 30 minutes at 37°C. The tubes were then cooled to room temperature and three aliquots of 0.1ml from each were measured for absorbance at 562nm. The absorbance values were compared to those of a standard curve (series of doubling dilutions of BSA, starting at 10mg/ml) and the concentration of protein in each sample was determined.

5.5 Preparation of Bispecific antibodies using O-PDM

Bispecific antibodies were prepared according to the method described by Glennie et al(1987).

$F(ab')_2$ (see procedure 2.3) from the two antibodies of interest at approximately 10mg/ml in 0.2M Tris/HCl buffer (pH 8.0) containing 10mM EDTA (Buffer A), was reduced by the addition of 20mM 2-mercaptoethanol (2-ME), for 30 minutes at 30°C. Both samples of reduced Fab' (Fab'_{SH}) were then chilled to 4°C, a temperature which was maintained throughout the remainder of the procedure, before running through Sephadex G-25, equilibrated in 50mM sodium acetate (pH 5.3) containing 0.5mM EDTA (Buffer B). A half volume of 12mM O-PDM in chilled dimethylformamide was then added to one of the antibodies for 30 minutes after which the maleimidated Fab' (Fab'_{MAL}) was separated from other solutes by passage through Sephadex G-25 equilibrated in Buffer B. The product was then added immediately to the other antibody and concentrated using a Centriprep-30 device.

After an incubation period of 18 hours the pH of the reaction mixture was adjusted to 8.0 with 1M Tris/HCl (pH 8.0) before reducing with 2-ME at a final concentration of 20mM for 30 minutes at 30°C, and alkylating with 25mM iodoacetamide.
Finally, the bispecific $F(ab')_2$ was separated from other products of the reaction mixture by passage through Ultrogel Aca34, equilibrated in Buffer A.

NOTE

1. After maleimidation of one of the antibodies, no free sulphydryl groups should be present, indicating that the reaction with O-PDM has been completed. This can be confirmed by reacting with Ellman's reagent, which produces a yellowing reaction in the presence of sulphydryl groups (See determination of sulphydryl content).

2. The final reduction and alkylation step is designed to remove any residual products, including $F(ab')_2$ monomers which may have formed.

3. The major product of interest, the bispecific $F(ab')_2$ should elute at approximately 110kDa molecular weight.

5.6 Preparation of Bispecific antibodies using SPDP

Bispecific antibodies were prepared according to the method described by Karpovsky et al [28]. The detailed pocedure for one preparation was as follows :

Intact Rat OKT3 antibody (4ml, 1mg/ml in PBS) and Mouse anti-ovarian antibody (1.3ml, 3mg/ml in PBS) were each concentrated to approximately 0.1ml and then made up to 1ml with 0.1M sodium phosphate 0.1M sodium chloride (pH 7.5) [Coupling Buffer]. Both antibodies were then incubated separately with 3-fold molar excess of SPDP (0.1ml of 0.24mg/ml solution of SPDP in ethanol was added to each sample) for 30 minutes at room temperature.
The anti-ovarian antibody (595) was passed through a G-25 column to allow exchange into coupling buffer to occur.
The rat OKT3 antibody was passed through a G-25 column equilibrated in 0.1M sodium acetate/0.1M sodium chloride (pH 4.5), before adding dithiothreitol (DTT) to a final concentration of 0.02M. After 30 minutes at room temperature, the antibody was passed throught a G-25 column equilibrated with coupling buffer and immediately added to the 595 antibody.

After incubating overnight at room temperature, iodoacetamide was added to the reaction mixture before it was passed through a column of Ultrogel Aca34 equilibrated in PBS.

NOTE

The major product of interest, bispecific $(IgG)_2$ should elute at approximately 300kDa molecular weight for intact IgG and 220kDa for $(F(ab')_2)_2$ bispecifics.

5.7 PREPARATION OF BISPECIFIC ANTIBODIES USING SMPB

Bispecific antibodies were prepared according to the method described by Pierce [21].

This cross-linker can be used to link intact antibodies together, as well as $F(ab')_2$ fragments of antibodies.
Antibody A was dialysed against 0.05M phosphate buffer (pH 8.0) whilst Antibody B was dialysed against 0.05M phosphate buffer (pH 7.0).
SMPB dissolved in DMSO was added to Antibody A at a molar ratio of 1:40 SMPB, and stirred for 30 minutes at room temperature. The antibody was then passed through a G-25 column, and added to Antibody B, for incubation with stirring at room temperature for approximately 3 hours. The mixture was then concentrated using a Centriprep-30 device and passed through a column of Ultrogel Aca34, equilibrated in PBS.

NOTE

In this reaction scheme, the heterobifunctional cross-linker reacts with a primary amine in the first step (Reaction with Antibody A), and a free sulphydryl group in the second (Reaction of cross-linker-A with Antibody B).
Sulphydryl groups may be generated from primary amines on the antibody molecule using a protein modification reagent such as SATA (See generation of sulphydryl groups).

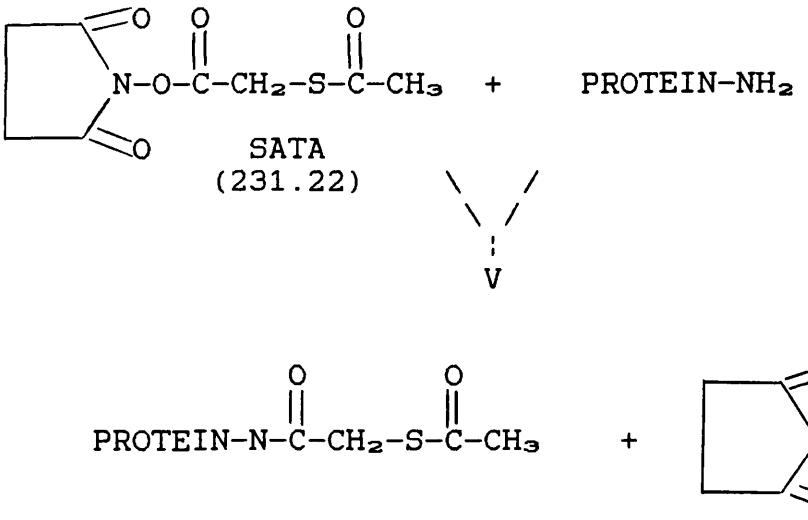
5.8 GENERATION OF SULPHYDRYL GROUPS USING SATA (N-succinimidyl S-Acetyl thioacetate)

Immediately before the reaction, SATA(13-15mg) was dissolved in 1ml DMSO. The IgG to be derivatized should be in 50mM sodium phosphate (pH 7.5) containing 1mM EDTA, at a concentration of 60uM, before reacting with the SATA solution (0.01ml SATA/1ml IgG) for 30 minutes at room temperature. The reaction mixture was then passed through a G-25 column to recover the IgG fraction. The sulphydryl groups are introduced in a protected form and may be made free by reacting the antibody with deacetylation solution (50mM sodium phosphate, 25mM EDTA, 0.5M Hydroxylamine hydrochloride (pH 7.5)(0.1ml/ml IgG)) for two hours at room temperature. The protein was then passed through a G-25 column to remove unreacted and hydrolysed SATA, along with hydroxylamine and its biproduct. The buffer used should contain 1mM EDTA to help protect the free sulphydryl groups. (For the reaction between SATA and a protein see Figure 6).

5.9 DETERMINATION OF -SH CONTENT WITH ELLMAN'S REAGENT (DTNB)

Samples can be assayed for sulphydryl content by reacting with Ellman's reagent in 0.1M sodium phosphate (pH 8.0). A sample of the protein (0.1ml) and 0.1ml of Ellman's reagent solution was added to 5.0 ml of 0.1M sodium phosphate buffer (pH 8.0) and allowed to react at room temperature for 15 minutes. A yellow colour is formed in the presence of free sulphydryl groups. The sulphydryl content can be determined by preparing a standard curve using a series of concentrations of cysteine hydrochloride monohydrate which are treated in the same manner as the protein samples. The absorbance at 412nm of each protein sample is measured and the sulphydryl content calculated via the standard curve. By comparing the absorbance at 280nm of the protein solution to its extinction co-efficient, the number of moles of sulphydryl per mole of protein can be determined.

1. REACTION OF SATA WITH A PROTEIN PRIMARY AMINE, FORMING AN ACETYLTHIOACETYLATED PROTEIN AND N-HYDROXYSUCCINIMIDE



2. DEPROTECTION OF THE SATA MODIFIED PROTEIN WITH HYDROXYLAMINE, FORMING A PROTEIN WITH A FREE SULPHYDRYL AND ACETYL HYDROXYLAMINE.

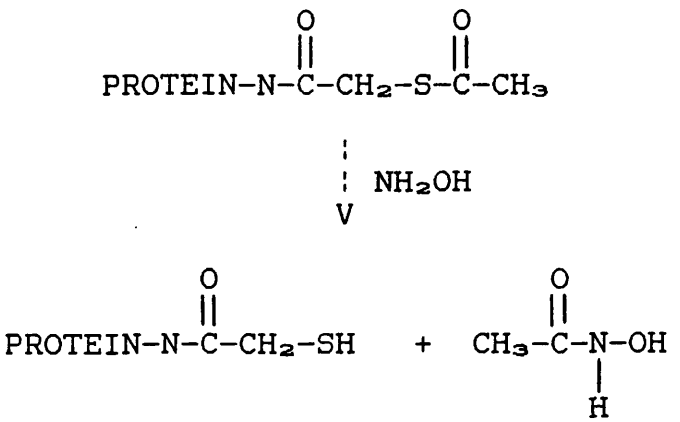


FIGURE 6: REACTION SCHEME OF SATA

5.10 GEL FILTRATION CHROMATOGRAPHY

Sephacryl S-200 or Ultrogel Aca34 columns were used to separate reaction mixtures depending upon the estimated size of molecule to be isolated.

Sephacryl is a rigid gel capable of good resolution at comparatively high flow rates, and each bead consists of a mixture of agarose and acrylamide. The gel used in this instance excludes molecules above 200kDa molecular weight.

Ultrogel is composed of a homogenous network of polyacrylamide and agarose in bead form and large columns should be run at low flow rates for maximum resolution.
All buffers used contained a small amount of azide to prevent column degradation.

Before use, the columns were calibrated using a series of known molecular weight standards, allowing at least one void volume of PBS to pass through between each sample. Standards were dissolved in PBS containing a small amount of glycerol to increase the density (see Table 2).

Molecular weight determinations of the unknown proteins were made by comparing the ratio of Ve/Vo for the protein to that of the standards (Ve - elution volume of sample, Vo - void volume (volume of effluent required for the elution of Blue Dextran)).

A calibration curve was then prepared by plotting the logarithms of the known molecular weight standards versus their respective Ve/Vo values.

TABLE 2 : MOLECULAR WEIGHT STANDARDS

STANDARD	MOLECULAR WEIGHT
Blue Dextran	$2*10^6$ K
Urease	270 K
B-amylase	200 K
Alcohol dehydrogenase	180 K
Bovine serum albumin	66 K
Carbonic anhydrase	29 K
SBTI	20.1 K
Cytochrome c	12.4 K

5.11 SDS-PAGE

This system uses TEMED and ammonium persulphate to initiate polymerization of the acrylamide gels, and SDS to dissociate all proteins into their individual polypeptide sub-units. A discontinuous buffer system is used to give better resolution than a continuous buffer system and involves loading the sample onto a large pore 'stacking' gel polymerized on top of a small pore 'resolving' gel.

Gels of 0.75 mm thickness and 10%(with respect to the acrylamide monomer) were prepared in an LKB 'Midget' casting apparatus, and stored at 4°C in electrophoresis buffer (1*) until required. Samples at 0.2mg/ml in loading buffer (without mercaptoethanol) were heated at approximately 60°C for 1 minute, cooled, and either stored at -20°C or loaded onto a gel (15ul aliquots). Molecular weight markers were also included for reference purposes (see 2.12).

Each gel was run at 10mA, until the bromophenol blue dye front was just off the bottom of the gel. The gels were then removed from the gel plates and processed as required.

5.12 SDS-MOLECULAR WEIGHT MARKERS

A vial containing 3.0mg of a lyophilised mixture of proteins (SIGMA)(See Table 3) was reconstituted in 1ml Sample buffer (made up from sodium phosphate monobasic(0.34g), sodium phosphate dibasic(1.02g), SDS(1.00g), 2-Me(1.00ml), Bromophenol blue(0.015g), Urea (36.00g) in 100ml distilled water.

TABLE 3 : SDS-MOLECULAR WEIGHT MARKERS

PROTEINS	MOLECULAR WEIGHT(Da)
Myosin	205 K
B-galactosidase	116 K
Phosphorylase b	97.4 K
Bovine serum albumin	66 K
Egg albumin	45 K
Carbonic anhydrase	29 K

5.13 COOMASSIE BLUE STAINING

The gel was placed in staining solution (0.625g bromophenol blue, 40%(v/v) methanol, 7%(v/v) acetic acid, dH_2O to 250ml) overnight. The solution was then removed and replaced with destaining solution (30%(v/v) methanol, 10%(v/v) acetic acid) with agitation. This was changed at regular intervals until the desired level of staining was obtained.

5.14 SILVER STAINING USING THE WRAY METHOD

After fixing the gel in 50% reagent grade methanol (with 0.1ml formaldehyde/100ml methanol), for at least one hour, the silver stain (prepared by adding 0.4g silver nitrate (in 2.0ml water) dropwise to a solution of ammonia, [11ml of 0.36% sodium hydroxide, 1.0ml 14.8M ammonium hydroxide], with constant vortexing and increasing to 50 ml volume with distilled water) was added for 15 minutes with constant gentle agitation. The stain was then removed and the gel washed twice in dH_2O for 10 minutes each wash. The gel was then developed by immersion in citric acid(1ml)/formaldehyde(0.1ml) solution in 200ml water. The bands appeared in not less than 10 minutes and not more than 15 minutes, after which the gel was washed in water and placed in 50% methanol overnight, and then stored in dH_2O.

5.15 SILVER STAINING USING ALTERNATIVE METHOD

After prefixing the gel in 50% methanol/10% acetic acid for 30 minutes, followed by 30 minutes in 5% methanol/7% acetic acid, fixing was achieved by soaking the gel in 5% gluteraldehyde solution for 30 minutes. The gel was then either washed overnight in dH_2O, (or washed six times at 15 minutes each wash in dH_2O), followed by 30 minutes in DTT(5ug/ml). Without rinsing, this solution was poured off and 0.1% silver nitrate solution was added for 30 minutes. A small amount of dH_2O was used to wash the gel once, and then developer (0.05ml formaldehyde in 100ml sodium carbonate (3%)) was used to wash the gel twice The gel was stained to the desired level with developer, and the reaction was stopped with 5ml of 2.3M citric acid, agitated for 10 minutes and then rinsed and stored in dH_2O.

5.16 CYTOTOXICITY ASSAY

Preparation of Target cells

Tumour target cells were removed from culture flasks by incubating for a short time with 0.2% EDTA (in PBS) and washed in RPMI-1640 medium. The cell pellet was incubated with 3MBq Cr^{51} for approximately 1 hour at 37°C, washed (2*10 minutes) and resuspended in 10ml RPMI-1640. After a second hour incubation period, the cells were washed (2*10 minutes), resuspended in 2ml medium and counted.

Preparation of Effector cells

Human peripheral blood mononuclear cells isolated from heparinized blood by density gradient centrifugation, were washed, resuspended in RPMI-1640 and counted.

Both cell volumes were adjusted accordingly before adding to the test plate.

Target cells were added to all test wells in a 96-well U-bottomed microtitre plate, together with combinations of antibody conjugate and effector cells. The plates were incubated at 37°C (with 5% CO_2) for 4 hours and 24 hours. Supernatants (0.1ml) were collected and the amount of Cr^{51} was measured. Percent specific cytotoxicity was calculated by the standard formula -

$$\% \text{ CYTOTOXICITY} = \frac{\text{cpm Exp} - \text{cpm Spon}}{\text{cpm Max} - \text{cpm Spon}} * 100$$

The maximum release values (cpm Max) were obtained by adding an equal volume of TritonX-100 to the target cells.

Spontaneous release values (cpm Spon) were determined by measuring the radioactivity released during incubation of target cells in media alone.

Tests were also included that contained PBS instead of medium as another control.

Triplicate samples for each data point were measured and the mean values calculated (cpm Exp).

5.17 PREPARATION OF PBMC FOR FLOW CYTOMETRY

Human PBMC were isolated from normal healthy heparinized blood by Ficoll-Hypaque density centrifugation. The cells were then washed twice with PBS (with 0.2% BSA), resuspended in 5mls PBS, and counted.

5.18 FLOW CYTOMETRY

Binding of the antibody conjugates (or control molecules) to the target cells (and to PBMC) was monitored by Flow Cytometry using the EPICS Profile II.

Cells or PBMC (at approximately 1 million per test) were washed with PBS (containing 0.2% BSA) and exposed to 0.1ml of appropriate antibody sample (or control) for 30 minutes on ice. The cells (or PBMC) were then washed with PBS (2*10 minutes) and treated with 0.1ml of the appropriate labelled secondary antibody (For example, $F(ab')_2$ Rabbit Anti-mouse IgG FITC conjugate), for 30 minutes on ice. After a final washing stage (2*10 minutes) to remove any unbound reagents, the samples were resuspended in 0.3ml wash buffer before examining for fluorescence.

All labelled antibodies were supplied by Serotec or Sigma.

6. RESULTS AND DISCUSSION

This project was proposed to examine the best and most convenient methods of chemically linking two different species of monoclonal antibody, using bifunctional reagents, and subsequently to compare the conjugates for storage stability, ease of preparation, and yield and activity of final product.

The two monoclonal antibodies which we were interested in joining initially were OKT3 and 14C1 or F14A. The former antibody reacts with the CD3/TCR complex on T-lymphocytes, as described earlier, whilst the latter are human monoclonals which have been reported as showing histological specificity towards human ovarian epithelial carcinomas, being the result of EBV-transformation of lymphocytes obtained from the involved lymph nodes of ovarian cancer patients [29]. It was thought that conjugates of this type (anti-CD3 * anti-14C1/F14A) could be potentially useful in the immunological targeting of ovarian cancer, because of the highly localised nature of the disease (i.e. the metastases usually remain within the peritoneal cavity). In fact an ovarian cancer associated membrane antigen, 14C1, has been defined using the same named human monoclonal antibody. The expression of the antigen was highly restricted to ovarian epithelial tumours, particularly of the clear cell carcinoma and serous or mucinous cystadenocarcinoma, of which 88% were positive, whereas a wide range of other normal and malignant tissues did not express the 14C1 antigen [30]. This antigen may therefore represent a potent target for both active and passive immunotherapy in the post surgical treatment of ovarian cancer.

The cell-lines OWmM1, previously identified as bearing the antigen recognised by F14A, and OVAN-4, which has a greater expression of the F14A antigen, were chosen as the 'target' cell-lines for our system, and were maintained as described in method 5.1.

The first stage of the procedure was concerned with obtaining and purifying the chosen antibodies. The antibodies described above were secreted in cell culture from continuous cell-lines, maintained within the laboratory, and were purified as described in the methodology.
Quantitation of the purified antibodies was carried out by a BCA assay according to method 5.4, which measured protein content (it was assumed that the antibody was the only protein present). This method was rapid and very straight forward producing colour changes in the presence of protein.
Because cross-linking procedures operate more efficiently in concentrated protein solutions, it was necessary to concentrate the antibody solutions using Centriprep-10 devices (Amicon). Each device was able to concentrate a maximum of 15mls of solution to approximately 1ml volume, operating by centrifugation (N.B. Centricon-10 devices could be used to concentrate smaller volumes (i.e. 1mls) to approximately 0.1ml volume). The '10' indicates the cut-off point - anything smaller than 10KDa molecular weight passed through the membrane, leaving larger molecules in the concentrate.

The 14C1 and F14A antibody cell line supernatants were normally combined and produced approximately 0.1mg/ml IgG per flask every four days. The OKT3 antibody had a lower yield producing approximately 0.001mg/ml per flask every four days. Therefore, accumulating sufficient amounts of antibody to be of use in experiments could sometimes take a number of weeks.

Before attempting the cross-linking reactions with the antibodies of interest, it was necessary to follow the procedure through with irrelevant antibodies.

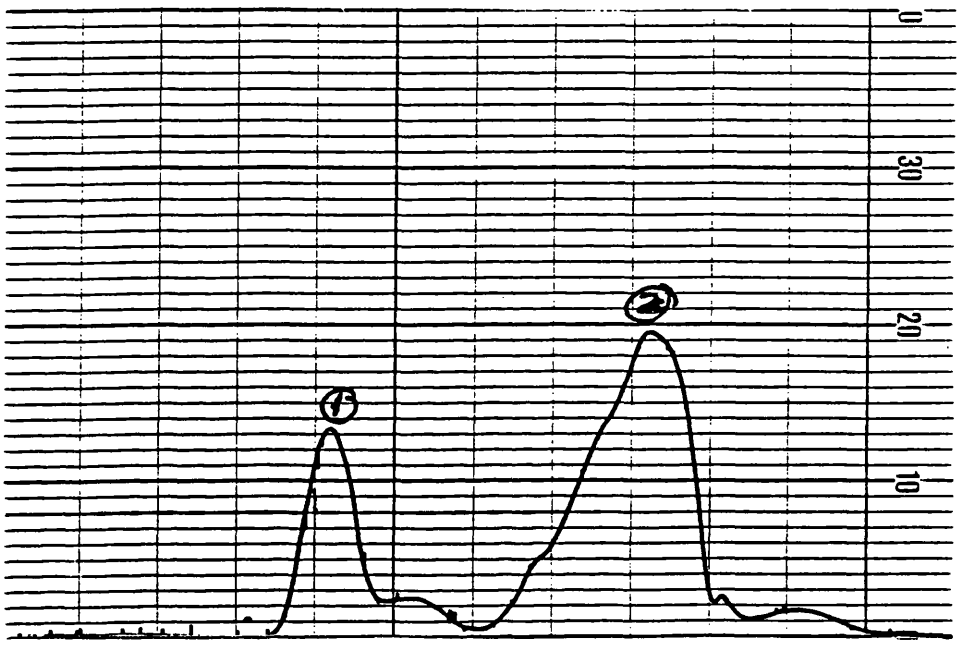
CROSS-LINKING WITH O-PDM

The first cross-linker to be examined was O-PDM. Sheep and human IgG's were used in the reaction according to the method described in 5.5 , as this method has been successfully carried out by Glennie et al [22] and is carefully detailed. The final digestion mixture was passed through a column of Sephacryl (S-200), linked to a monitor and chart recorder, which produced a trace of the products as they passed out of the column. The volume at which each peak reached its maximum was recorded (Ve). The ratio of Ve/Vo (void volume) was then read from the standard curve (see method 5.10) to obtain an estimate of the molecular weight of the recovered product. In this instance the products were of very low molecular weight i.e. 2,000 Daltons, suggesting that the complex had either degraded, perhaps due to too rapid a passage through the column or an incorrect pH of the elution buffer, or the antibodies had not conjugated initially. At various stages in the linkage procedure, samples of the products were taken for SDS-PAGE (methods 5.11 and 5.12), to provide a visual assessment of their molecular weights. This would give an indication of the size of the complex throughout the experiment. Unfortunately, the silver staining method (Wray, method 5.14) which had previously been carried out successfully did not produce good staining. Changes were made in the protocol to use bottled water instead of distilled water as the latter was thought to contain chloride ions which may have interfered with the staining procedure. This did not make any difference and an alternative method (5.15) was attempted which gave slightly better staining but the gels could still not be read accurately. Coomassie Blue was then employed to stain the gels (according to method 5.13)(results not shown). It was less sensitive than the silver nitrate method, but any bands present were easily read against control molecular weight markers. This confirmed the earlier results that the molecular weights of the products were very low and therefore also confirmed that the linkage had not been successful.

CROSS-LINKING WITH SMPB

Consequently, another cross-linker, SMPB, was used to link the $F(ab')_2$ fragments (see method 5.3 for preparation) of human IgG (5mg total) and sheep IgG (10mg total), (according to method 5.7). The resulting product, after passage through sephacryl S-200, produced a major peak corresponding to approximately 180KDa. This was slightly less than was expected for this type of linkage (i.e. $F(ab')_2$* $F(ab')_2$ should be about 220KDa) but it is possible that the light chain of one of the antibodies may have become detached at some stage in the linkage process.
This procedure was therefore carried out with the mouse OKT3 and human 14C1/F14A antibodies. The resulting mixture produced the trace observed in Figure 7 after S-200 column chromatography. The first product (Peak 1)approximated to 230KDa molecular weight and the second (Peak2)to 5KDa, suggesting that the former could be a bispecific antibody according to its size. The products were then run on SDS- polyacrylamide gels and stained with coomassie blue (results not shown). This appeared to confirm the molecular weight of the product as approximately 230KDa (Designated Product A) and the linkage was repeated with similar results (Product B), but it was necesssary to confirm the composition of the products by other methods.

Martin Glennie et al [22] examined their bispecific products by double diffusion analysis in agar (Ouchterlony and Nilsson, 1973) to visualize the individual components, and this was attempted with the SMPB bispecific products A and B.
Four 1.5% agar plates were set up according to Figure 8 and incubated in a 'moist box', until precipitin lines were observed. In plates 3 and 4 there were no precipitin lines formed between product B and either of the expected components (i.e. anti-human IgG and anti-mouse IgG), and whilst in plates 1 and 2, product A did produce precipitin lines with both anti-human IgG and anti-mouse IgG, the 14C1/F14A and OKT3 antibodies reacted with their own antigens and with the opposite antigens (i.e.human ovarian antibody reacted with anti-mouse IgG, and mouse OKT3 reacted with anti-human IgG).

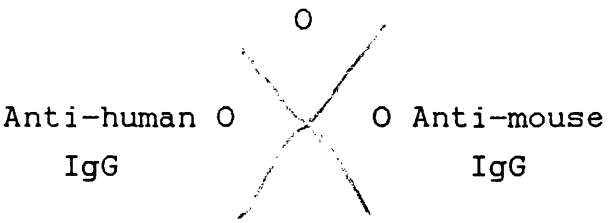


PEAK 1 PEAK 2

FIGURE 7: TRACE OF SMPB LINKAGE PRODUCTS AFTER PASSAGE THROUGH S-200

PLATE 1

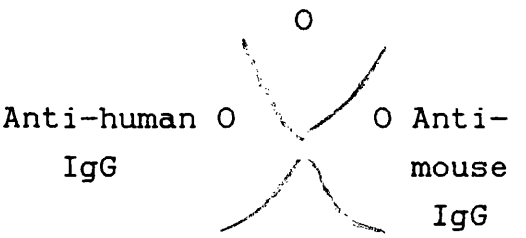
14C1/F14A



PRODUCT A

PLATE 2

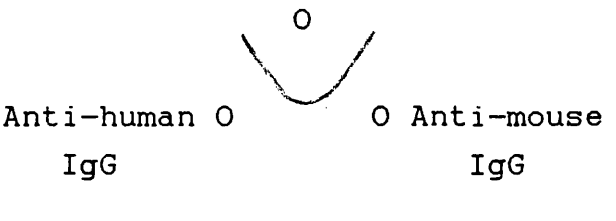
OKT3



PRODUCT A

PLATE 3

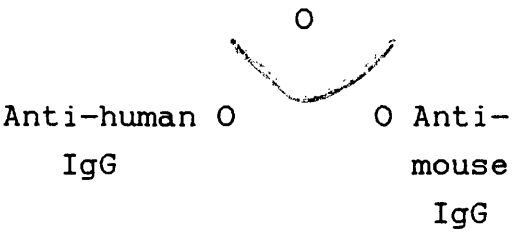
14C1/F14A



PRODUCT B

PLATE 4

OKT3



PRODUCT B

O - Agar wells

FIGURE 8: OUCHTERLONY REACTION

This observation led us to believe that the cell lines had become cross-contaminated. This was , however, ruled out by ELISA testing of the cell-line supernatants, and therefore the reaction in the ouchterlony was probably due to cross reactivity between antigens and antibodies, and no definite conclusions about the components of the 'bispecific' antibodies could be made.

It was therefore decided to employ Western Blotting in an attempt to detect the presence of both human and mouse components in the 'bispecific' products. Products A and B, were subjected to SDS-PAGE and then blotted onto nitrocellulose. The mouse OKT3 and human 14C1/F14A antibodies were also put on individually to ensure that the conjugates were specific and active for each component. The gel was stained to ensure complete transfer of all proteins, and the nitrocellulose then probed with anti-mouse Fab' conjugate. This procedure was repeated with another gel and the nitrocellulose probed with anti-human Fab' conjugate. The latter conjugate resulted in the appearance of a band in the lane in which the 'bispecific' products had run and in the lane containing anti-ovarian antibody as expected. However, the anti-mouse conjugate also produced these results and there was no band apparent in the lane in which the OKT3 antibody was present. Again there would appear to be the problem of cross-reactivity, and this method was therefore considered unsuitable for examining any 'bispecific' products.

The products of both linkages however, were combined and concentrated (1.7mg total) and retained for use in later experiments (Designated SMPB A-B).

For the directed formation of bispecific antibodies using SMPB, the first antibody in the reaction should contain a primary amine for reaction with the cross-linker, and the second antibody should contain free sulphydryl groups. This was not the case on our previous attempts at linking the antibodies and therefore it is unlikely that the product recovered was in fact a 'bispecific' antibody.

The molecular weight observed initially which had led us to believe that the linkage had worked successfully, may perhaps have been due to a $F(ab')_3$ molecule of one of the antibody species.

Sulphydryl groups can however, be introduced into proteins by the agent SATA, according to the method described in 5.8. The sulphydryl group is introduced in a protected form allowing it to be stored for long periods of time without degradation of the groups. Deprotection is then achieved in a subsequent reaction with Hydroxylamine,Hydrochloride. This was attempted using sheep and human immunoglobulins, which were first digested to produce $F(ab')_2$ molecules. The sheep IgG (10mg/ml) was then modified with SATA, the human IgG (4mg/ml) was reacted with the cross-linker SMPB, and the antibodies were linked as described earlier (Method 5.7). After S-200 column chromatography, a peak was observed at approximately 100kDa (which corresponds to a Fab' sized molecule) followed by a smaller peak at approximately 5kDa (probably breakdown products). It would appear then, that the linkage had either not been successful, or it is possible that any conjugated products, being fairly large, could have broken down on the journey through the column. The deprotection stage is designed to free the sulphydryl groups, and the success of this reaction can be judged by a yellowing of the reaction mixture using Ellman's reagent (according to method 5.9) which detects the presence of SH groups. This was performed on a sample from this stage of the procedure and only a very slight yellowing of the sample was observed. It is probable then, that there were not enough -SH groups available for the reaction to proceed and this prevented the conjugation of the two antibodies from occurring.

The introduction of -SH groups to proteins was therefore attempted as an exercise on its own using lyophilized human IgG, but this was never achieved even though the instructions were followed to the letter, and time factors prevented the matter being pursued further. This method of linking using the SATA modification folowed by SMPB conjugation involved a number of manipulations which may have adversely affected the antibodies being used, and also caused the antibodies to be diluted at each stage and this is especially important when the initial concentration of the antibodies is low. It was decided, therefore, that this method was not practical for the efficient preparation of 'bispecific' antibodies.

A second attempt was made to link the chosen antibodies with O-PDM, but the molecular weight of the product was very low (i.e. approximately 2KDa) and this would suggest that the sample had degraded. The samples were examined at each stage for sulphydryl content, to check if the reaction was proceeding as normal. When the cross-linker was added, the maleimide groups should have used up the -SH groups available, and therefore no such groups should be detected if the reaction had occurred successfully. In this case, however, SH groups were detected at this point in the reaction, suggesting that the cross-linker was not functioning as it should and it was decided to obtain a new batch.

THE USE OF FLOW CYTOMETRY IN THE ANALYSIS OF BISPECIFIC PRODUCTS

Flow Cytometry was chosen to analyse the bispecific products and therefore initial experiments to establish the optimum and efficient running of the system had to be performed, with regards to incubation times and conjugates to be used.
OWmM1 cells were used as the target cells for the analysis of the binding of the anti-ovarian antibodies(F14A/14C1) and the previously prepared SMPB (A-B) product (see earlier text).

The ovarian cancer antigen-specific component (F14A/14C1) would bind to the target cells leaving the mouse component free to be detected by an anti-mouse fluorescent conjugate.
Fluorescence would therefore only be observed if a bispecific product was present. For example, if the human component was present alone then the anti-mouse conjugate would not bind to this and fluoresce, and similarly, if the mouse component was present alone, it would not be able to bind to the target cells initially and no fluorescence would be observed.

The following combinations of antibody and conjugates (whole IgG molecule) were analysed for fluorescence according to method 5.18.

1. OWmM1 alone
2. OWmM1 + F14A/14C1 + anti-human FITC conjugate(HFITC)
3. OWmM1 + anti-human FITC conjugate
4. OWmM1 + IgG + anti-human FITC conjugate
5. OWmM1 + SMPB 'bispecific' + anti-mouse FITC conjugate(MFITC)
6. OWmM1 + anti-mouse FITC conjugate

All combinations, with the exception of the target cells alone (which had a positive population of 1%), produced levels of fluorescence in excess of 95% (data not shown). It was observed that the conjugates alone were binding to the target cells, and were therefore not suitable for any analysis work. As an alternative, conjugates were acquired which lacked the Fc portion of the molecule i.e. F(ab)'$_2$ molecule conjugates, and these were used in the analysis as before, and the results shown in Table 4.
On this occasion, the F(ab)'$_2$ conjugates did not bind with any significance to the target cells suggesting that the cell line might bear Fc receptors (FcR), since no positive fluorescence was observed when the Fc portion of the antibodies were absent. It was hoped to test this hypothesis by using the antibodies CD16, CD32 and CD64 which would bind to FcRIII, FcRII and FcRI respectively,but unfortunately these were not available at the time.

TABLE 4: FLOW CYTOMETRY 1

TEST	% POSITIVE
OWmM1	0.5
OWmM1 + 14C1/F14A + HFITC	3.3
OWmM1 + HFITC	0.5
OWmM1 + IgG + HFITC	38.1
OWmM1 + SMPB BISP + MFITC	1.6
OWmM1 + MFITC	1.4

This analysis also demonstrated that neither the SMPB 'bispecific' product, or the anti-ovarian antibodies alone, bound to the target cells with any significance, whilst the irrelevant human IgG antibody at the same concentration (isolated from serum by protein A affinity chromatography) did. This analysis was repeated with similar results (not shown) and it was clear that the antibody we were interested in studying, did not bind to the target cells as previously reported. It is possible that the characteristics of the target cell line had changed over a period of time and were no longer expressing antigen recognizable by the antibody, or the cell lines producing the antibody had become altered in some way, through continuous culture. This may have been caused by contamination with Mycoplasma, which is known to contaminate a large number of cell-lines throuhgout the world. This has been ignored by many investigators, but mycoplasma has been shown to alter the cell in terms of macromolecular synthesis, stability of genetic material, and other parameters including sensitivity to drugs and viruses [31]. The cell lines used in this investigation were tested and shown to be mycoplasma positive, and this may have been the reason for the observed results.

As a result of the non-binding of the anti-ovarian cancer specific antibodies, it was necessary to check the specificity of the OKT3 antibody, with regards to its target antigen - the CD3/TCR complex on T-cells, before proceeding any further. The PMBC's were prepared according to method 5.17 and the results of a number of different tests are shown in Table 5.

The OKT3 antibody bound to the PBMCells as expected, and the W6/32 antibody (anti-MHC class 1) produced over 90% fluorescence with both PBMC and OWmM1 target cell lines. This antibody was included in the system as a control to ensure that the conjugates were binding and fluorescing as they should be (a profile of the binding of W6/32 to OWmM1 can be seen in Figure 9).

TABLE 5: FLOW CYTOMETRY 2

TEST	% POSITIVE
PBMC	0.3
PBMC + MFITC	0.4
PBMC + OKT3 + MFITC	49.5
PBMC + W6/32 + MFITC	98.2
OWmM1	0.4
OWmM1 + W6/32 + MFITC	99.9
OWmM1 + IgG + MFITC	1.8
OWmM1 + IgG + W6/32 + MFITC	81.1
OWmM1 + MFITC	1.9

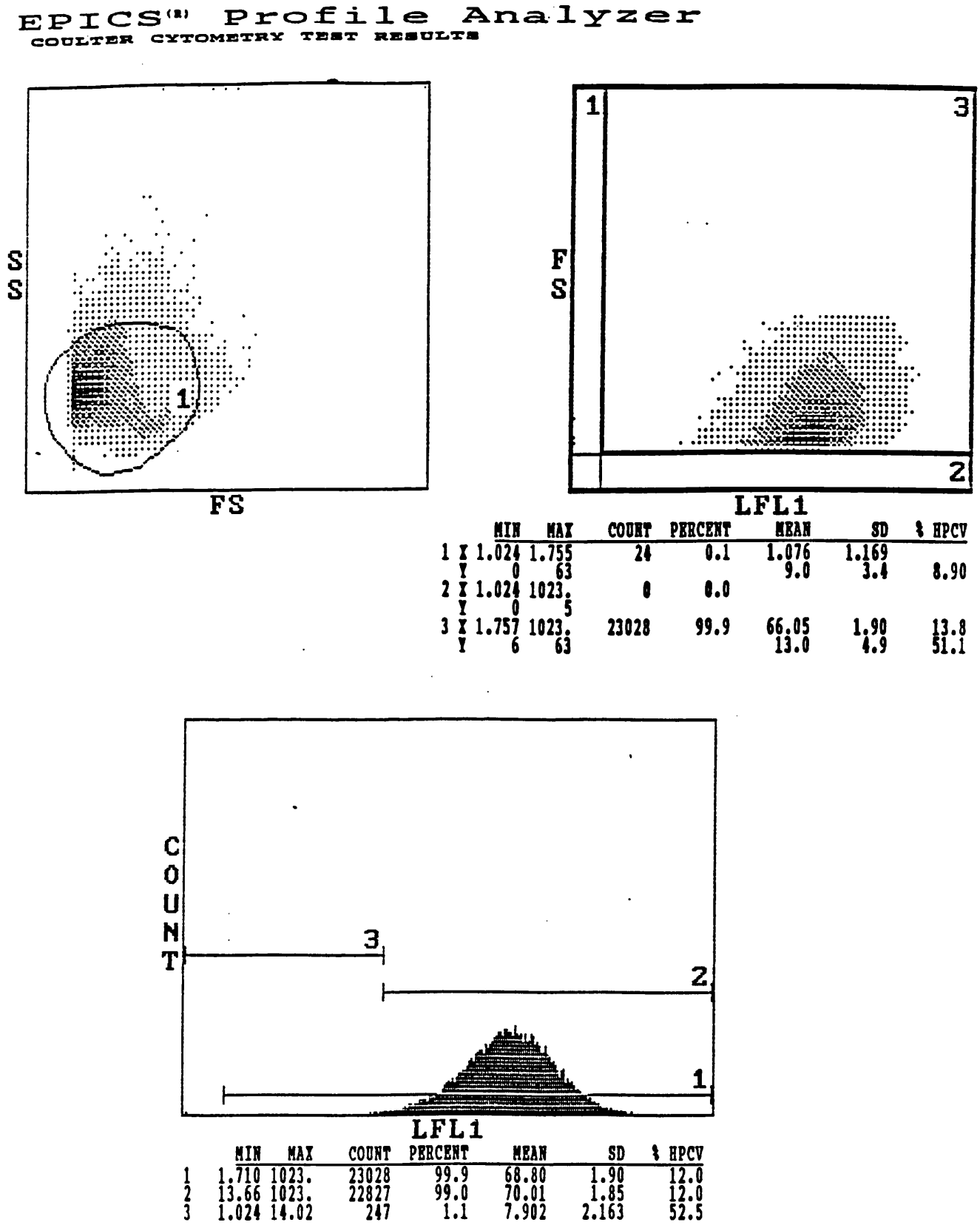


FIGURE 9: PROFILE OF W6/32 ANTIBODY BINDING TO OWmM1 CELLS

It was therefore possible to examine the SMPB 'bispecific' product by binding the OKT3 component to PBMC's, which would leave the anti-ovarian component free to be detected by an anti-human FITC conjugate. This was attempted but no measurable fluorescence was detected, and therefore it was concluded by flow cytometry that this SMPB product did not constitute a Bispecific antibody.

In a previous analysis (Table 4) human IgG was shown to bind to the OWmM1 target cells, but it was not known which receptors were involved. Consequently, experiments were performed with human IgG to obtain more information on the nature of the binding. Target cells were pre-incubated with human IgG to see if this would affect the subsequent binding of W6/32 since this produces strong fluorescence.(See Table 5 for results).

There was a slight reduction in the positive population when IgG was included in the test(approximately 10%), compared to that observed with the W6/32 antibody alone, but this was not greatly significant, and it is therefore likely that the IgG was binding to a different receptor than the W6/32 antibody. This analysis was repeated with similar results (not shown).

Another irrelevant antibody was also tested in the system - A mouse anti-toxoplasma antibody which was incubated with the target cells (OWmM1) alone, and also after pre-incubation with human IgG. The positive populations observed were 53% and 47% respectively (incubation of the conjugate and target cells alone being 2.6%). This antibody appeared to bind quite strongly to the cell line, but again it was not known which receptors were involved but they seemed to be different from those binding the human IgG since the pre-incubation stage did not affect the binding with any significance. They could, however, also be binding through their Fc-components if there were a large number of Fc receptors on the target cell surface.

To test this hypothesis of binding through the FcR, the binding of the $F(ab')_2$ fragment of human IgG was compared to that of the intact molecule, producing positive fluorescence of 12.6% and 58.6% respectively.
This would appear to suggest that the Fc component does have a positive role in the binding of the antibody since its removal resulted in a decrease in the fluorescence observed.

It would have been useful to test the anti-toxoplasma antibody in this manner, to see how the removal of the Fc component affected its binding to the target cells, but there was no time available to do so.

As alternatives to the anti-ovarian antibodies F14A/14C1, an anti-colonic carcinoma antibody (505), and an anti-ovarian carcinoma antibody (595), were obtained for use in the conjugations with the OKT3 antibody.

These were analysed against the target cell lines OWmM1, OVAN-4 and COLO-320, and the results are presented in Table 6. The antibodies bound to their respective cell lines as expected - 595 to OWmM1 and OVAN-4, and 505 to COLO-320, the latter showing a very high positive fluorescence. Antibody 595 also bound to the COLO-320 cell line but with a lower percentage which was probably not significant.

A new anti-CD3 antibody (Rat derived) was also acquired because the laboratory cell lines were not producing adequate amounts for our purposes. This was subjected to flow cytometry against PBMCs and fluoresced with a positive population of 72%, which was greater than the previous antibody and could therefore be used in this investigation.

TABLE 6: FLOW CYTOMETRY 3

TEST	% POSITIVE
OWmM1	0.4
OWmM1 + MFITC	0.9
OWmM1 + 595 + MFITC	53.0
OWmM1 + 505 + MFITC	3.0
OVAN-4	0.2
OVAN-4 + MFITC	0.3
OVAN-4 + 595 + MFITC	60.0
OVAN-4 + 505 + MFITC	1.4
COLO-320	0.1
COLO-320 + MFITC	0.2
COLO-320 + 595 + MFITC	14.1
COLO-320 + 505 + MFITC	98.0

CROSS-LINKING WITH SPDP

A third cross-linker - SPDP, was chosen to link the new antibodies. Rat anti-CD3 antibody (4mg total) and the 595 antibody (4.2mg total) were concentrated to 0.1ml before the addition of 0.9ml coupling buffer to each. Intact antibodies were used in this instance, to keep to a minimum, the number of manipulations carried out . The reaction continued as described in method 5.6, and the resulting product was passed through a column of Ultrogel AcA34. This was used because it has an exclusion limit of 300KDa, and this was approximately the size of the desired 'bispecific' product. The first peak observed corresponded to a molecular weight of approximately 250Kda, whilst the second approximated to a very small molecular weight. The first product was slightly less in terms of molecular weight than was expected for a 'bispecific' product, but this may have been due to the disassociation of one of the light chains from the complex. The product was then concentrated to approximately 1ml in PBS (0.4mg/ml) and stored for further experimentation. The recovery of the product was approximately 5% of the initial antibody available.

Analysis of this product before (SPDP B) and after (SPDP A) column chromatography was carried out by flow cytometry and produced the results shown in Table 7.

Initial binding studies then appeared to demonstrate that a 'bispecific' product had been produced, since the positive fluorescence was quite significant compared to the control tests. The reduced fluorescence observed in the linkage after it had been subjected to column chromatography is consistent with the digestion mixture being 'cleaned up', leaving only one major product.
The products were also analysed against PBMCs to see if the conjugation process had affected the binding site of the other antibody, and the results are presented in Table 8.

TABLE 7: FLOW CYTOMETRY 4

TEST	% POSITIVE
OVAN-4	0.1
OVAN-4 + RFITC	8.5
OVAN-4 + SPDP B + RFITC	65.0
OVAN-4 + SPDP A + RFITC	34.0

TABLE 8: FLOW CYTOMETRY 5

TEST	% POSITIVE
PBMC	0.1
PBMC + MFITC	2.7
PBMC + SPDP B + MFITC	50.1
PBMC + SPDP A + MFITC	77.2
PBMC + W6/32 + MFITC	99.3

Again the 'bispecific'products showed a high positive fluorescence compared to the controls, but this time the greater percentage was observed in the link that had been subjected to column chromatography.

Analysis of the products with the OVAN-4 target cell-lines was repeated and the results are shown in Table 9. This presented the question of the specificity of the anti-rat conjugate, which bound to the mouse 595 antibody with approximately the same efficiency as the anti-mouse conjugate, but cross-reactivity between rat and mouse components is to be expected.

Because of the design of the test, if specific fluorescent conjugates are used for the different antibody components involved, then any fluorescence observed with 'bispecific' products usually confirms that the linkage has been successful.
In this case, fluorescence was indeed observed with the 'bispecific' products, but one of the fluorescent conjugates used was shown to bind to both antibody components. However, the fluorescence observed with the 'bispecific' products was greater than that observed with the conjugates and single antibody alone, and therefore the product probably was in fact a 'bispecific' antibody.
If this is true then the 'bispecific' antibody, should be able to direct effector cells to ovarian cancer target cells in a cytotoxicity assay and lead to their destruction. Method 5.16 describes how the assay was set up.

OVAN-4 cells (Target - T) were used in the assay in combination with the 'bispecific' antibody (SPDP - A) at various concentrations, and effector cells (PBMC - E) at two effector:target ratios. Four plates were set up. Two plates were incubated with non-activated PBMC's for 4 and 24 hours, and two were incubated with activated PBMC's (cultured with 500units/ml IL-2 for 48 hours) for 4 and 24 hours, and the results of specific cytotoxicity(%) are presented in tables 10-13.

TABLE 9: FLOW CYTOMETRY 6

TEST	% POSITIVE
OVAN-4	0.1
OVAN-4 + RFITC	3.2
OVAN-4 + MFITC	2.8
OVAN-4 + SPDP B + RFITC	79.7
OVAN-4 + SPDP A + RFITC	62.2
OVAN-4 + 595 + MFITC	52.6
OVAN-4 + 595 + RFITC	49.6
OVAN-4 + W6/32 + MFITC	91.0

In the assay, each test, apart from those containing PBS (as stated in the tables) was performed in medium(MED).
The PBS controls were included as a comparison, and demonstrated that on average, the medium did not have an adverse on the cells.

In Table 10, the tests containing the neat and 1:2 antibody dilution of the 'bispecific' antibodies did not produce any measurable chromium release at all, and therefore, did not appear to have directed any specific cytotoxicity towards the target cells. The test with the 1:4 antibody dilution did produce a small amount of cytotoxicity but this was less than that of the control without antibody present (in MED) and was not regarded as significant.

When the radioactivity was measured after an incubation period of 24 hours, and the percentage cytotoxicity calculated, the figures were much higher in most cases (Table 11), compared to the respective tests after 4 hours. Again, the neat and 1:2 antibody dilution tests produced little or no cytotoxicity towards the target cells, whereas the 1:4 antibody dilution did. This was higher than that observed in the test sample without antibody (in MED) by approximately 50% at both effector:target cell ratios.

This could therefore be an indication of the presence of 'bispecific' antibody, able to direct the specific killing of target cells.

In Tables 12 and 13, activated effector cells were used in the assay and similar results were obtained, that is, in those tests where cytotoxicity was observed the figures were greater after 24 hours than after 4 hours. In addition, the values were much higher than those observed with the non-activated effector cells.

TABLE 10: CYTOTOXICITY ASSAY 1

NON-ACTIVATED PBMC : AFTER 4 HOURS

TEST	% CYTOTOXICITY
T + MED + E 40:1	28.0
T + MED + E 20:1	9.9
T + PBS + E 40:1	11.1
T + PBS + E 20:1	6.7
T + SPDP A(N) + E 40:1	0.0
T + SPDP A(N) + E 20:1	0.0
T + SPDP A(1/2) + E 40:1	0.0
T + SPDP A(1/2) + E 20:1	0.0
T + SPDP A(1/4) + E 40:1	10.5
T + SPDP A(1/4) + E 20:1	2.3

TABLE 11: CYTOTOXICITY ASSAY 2

NON-ACTIVATED PBMC : AFTER 24 HOURS

TEST	% CYTOTOXICITY
T + MED + E 40:1	29.0
T + MED + E 20:1	20.6
T + PBS + E 40:1	55.0
T + PBS + E 20:1	33.6
T + SPDP A(N) + E 40:1	0.0
T + SPDP A(N) + E 20:1	11.8
T + SPDP A(1/2) + E 40:1	0.0
T + SPDP A(1/2) + E 20:1	0.0
T + SPDP A(1/4) + E 40:1	48.5
T + SPDP A(1/4) + E 20:1	40.2

TABLE 12 : CYTOTOXICITY ASSAY 3

ACTIVATED PBMC : AFTER 4 HOURS

TEST	% CYTOTOXICITY
T + MED + E 40:1	49.0
T + MED + E 20:1	33.2
T + PBS + E 40:1	43.8
T + PBS + E 20:1	34.0
T + SPDP A(N) + E 40:1	0.0
T + SPDP A(N) + E 20:1	0.0

TABLE 13 : CYTOTOXICITY ASSAY 4

ACTIVATED PBMC : AFTER 24 HOURS

TEST	% CYTOTOXICITY
T + MED + E 40:1	79.0
T + MED + E 20:1	61.4
T + PBS + E 40:1	74.0
T + PBS + E 20:1	60.6
T + SPDP A(N) + E 40:1	74.6
T + SPDP A(N) + E 20:1	79.0

The neat antibody did not increase the cytotoxic effect of the effector cells alone after 24 hours, and no chromium was released at all after 4 hours, but this was consistent with the results obtained from the same tests using non-activated PBMC's. If antibody at the different dilutions had been included in this assay, they may also have resulted in the increased cytotoxicity observed in Table 11, since it is possible that antibody at too high a concentration leads to cross-linking, preventing the effector cells from binding and exerting a cytotoxic effect.

Therefore, non-activated PBMCs did appear to have directed some cytotoxicity against the target cells and this was enhanced when the antibody (1:4) was included in the assay, suggesting that it was 'bispecific' in nature.

ANALYSIS OF PRODUCTS USING SDS-PAGE

SDS-PAGE was performed on samples from the various stages of this SPDP-linkage to obtain an estimate of their molecular weights, and this would give an indication as to whether the linkage had been carried out successfully. The following samples were subjected to SDS-PAGE and the resulting pattern of bands can be seen in Figure 10 .

Lane 1. Molecular weight markers
Lane 2. Human IgG
Lane 3. 595 antibody
Lane 4. Rat CD3 antibody
Lane 5. Bispecific before chromatography (SPDP B)
Lane 6. Bispecific after passage through Ultrogel (SPDP A)
Lane 7. Bispecific after passage through S-200
Lane 8. Molecular weight markers

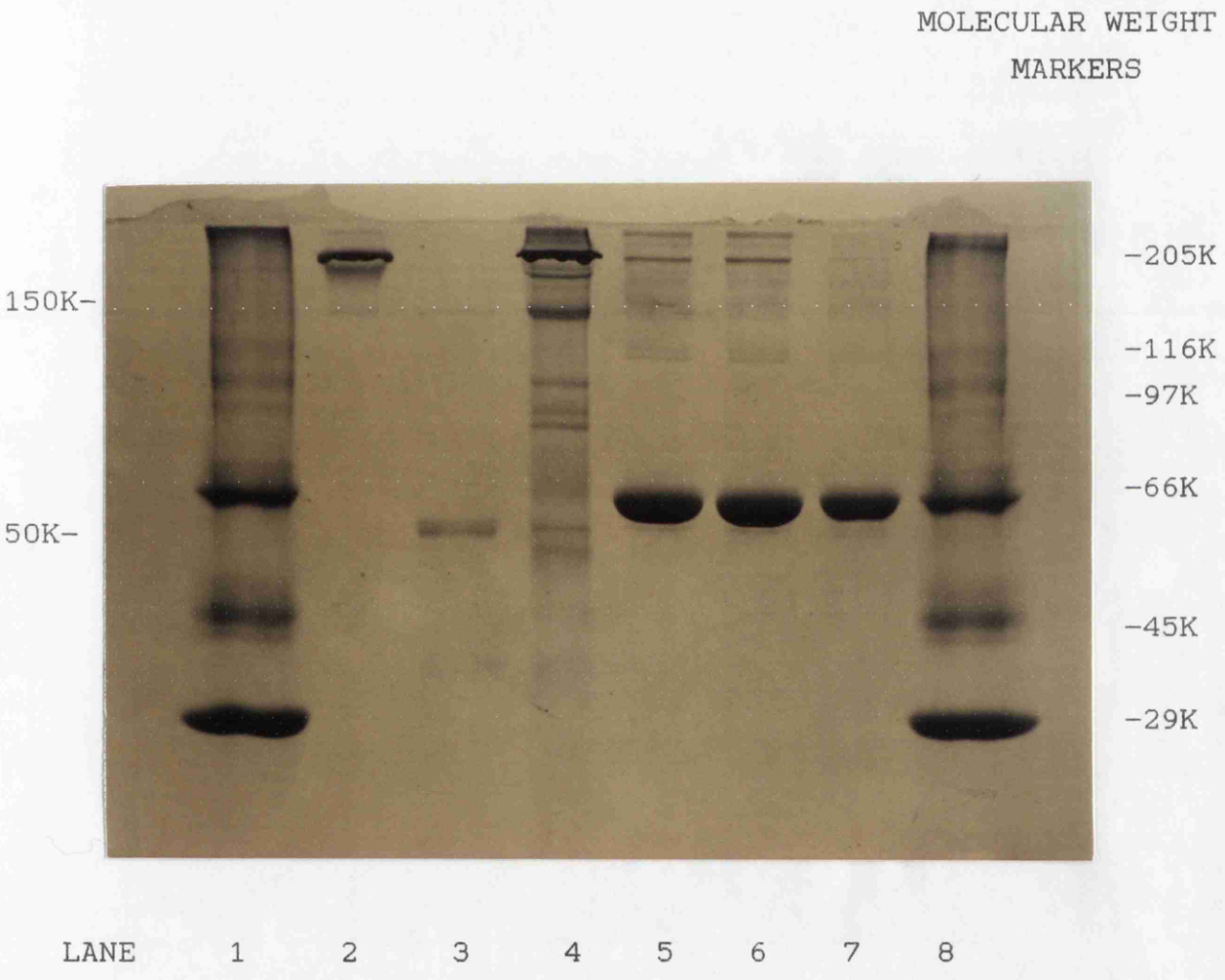


FIGURE 10: SDS-POLYACRYLAMIDE GEL STAINED WITH COOMASSIE BLUE

Human IgG produced a band at approximately 150KDa as expected, but the 595 antibody produced a band at 50 KDa. This is the size of the heavy chain of IgG antibody but it is possible that the antibody light chains had dissociated during the preparation stages for SDS-PAGE. The literature suggests that samples should be boiled with a reducing agent to dissociate the proteins into individual polypeptides which bind to the SDS. The proteins become negatively charged and they will migrate through the gel according to their molecular weight.

We however, wished to obtain an estimate of the molecular weight of the intact structure of each antibody and therefore did not use a reducing agent in the sample buffer and only heated the samples to approximately 60°C. The effect of heating the samples beyond this to boiling can be seen in Figure 11.

Lane 1. Molecular weight markers
Lane 2. Human IgG
Lane 3. 595 antibody
Lane 4. Rat anti-CD3 antibody
Lane 5. SPDP B prepared as normal
Lane 6. SPDP B prepared at R.T.
Lane 7. SPDP B prepared by boiling
Lane 8. Molecular weight markers

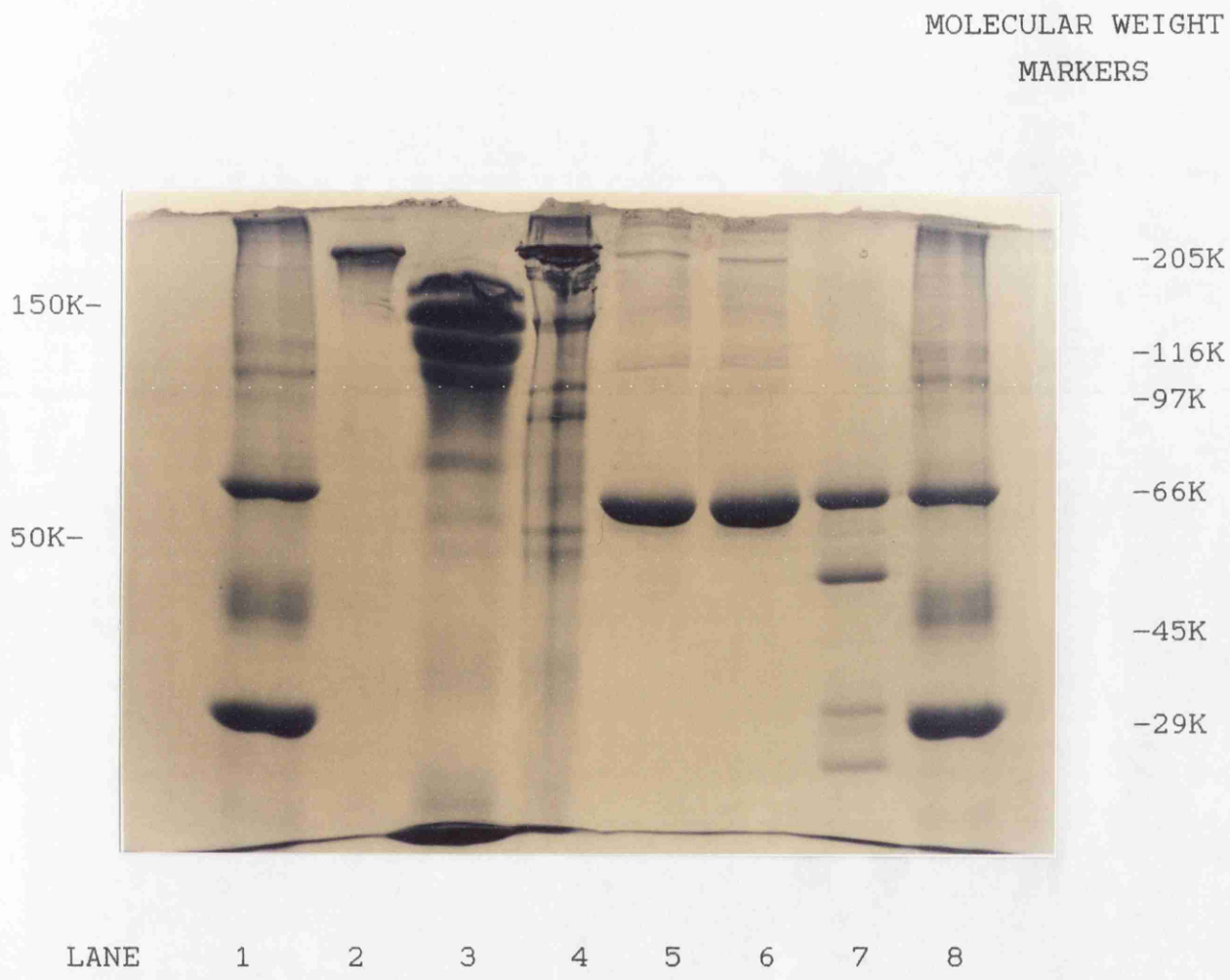


FIGURE 11: SDS-POLYACRYLAMIDE GEL STAINED WITH COOMASSIE BLUE

This clearly demonstrated that boiling samples in loading buffer was not appropriate for our purposes since this resulted in the dissociation of the antibody into its individual components (as seen in lane 7).
The large band present in lanes 5,6 and 7 of both gels is due to the presence of BSA which was added to stabilize the products. Also in these lanes, bands can be seen just entering the resolving gel, and these may represent high molecular weight products such as the bispecific antibodies. The Rat anti-CD3 antibody produced a band at approximately 150Kda as expected (lane 4), but there appeared to have been an excess of 595 antibody loaded onto the gel producing the smear seen in lane 3.

Because high molecular weight products were observed on both gels the bispecific products were analysed once more against the OVAN-4 cell line, and the results presented in Table 14. SPDP-B and SPDP-A produced high levels of fluorescence as before.
However, when the mouse 595 antibody was probed with the anti-rat fluorescent conjugate (RFITC), and the rat anti-CD3 antibody was probed with the anti-mouse conjugate high levels of fluorescence were also observed. Once again, cross-reactivity would appear to be the problem and it was therefore not possible to conclude that the product was a 'bispecific' antibody by flow cytometry using these conjugates.

In this test the anti-CD3 antibody was also shown to bind to the OVAN-4 target cells which was not expected, and this test was repeated with similar results (not shown). This test also included PBMC's as the target cells and compared the binding of the CD3 antibody using the anti-mouse and anti-rat conjugates as probes. In this case higher levels of fluorescence were observed with the anti-mouse conjugate than with the specific anti-rat conjugate(81.6% and 37.7% respectively). Again, these conjugates could not be used to reliably analyse the 'bispecific' conjugates prepared.

TABLE 14: FLOW CYTOMETRY 7

TEST	% POSITIVE
OVAN-4	0.2
OVAN-4 + RFITC	2.6
OVAN-4 + MFITC	3.7
OVAN-4 + SPDP B + RFITC	95.7
OVAN-4 + SPDP A + RFITC	94.8
OVAN-4 + 595 + MFITC	96.6
OVAN-4 + 595 + RFITC	80.4
OVAN-4 + CD3 + RFITC	93.0
OVAN-4 + CD3 + MFITC	87.8

It was proposed that another linkage be attempted using a new batch of O-PDM. Irrelevant antibodies were again used in the initial experiments to preserve stocks of specific antibodies. Human IgG was digested with pepsin to produce $F(ab')_2$ antibody and then reduced with 2-ME to produce two Fab' components. One of the Fab' components was reacted with the cross-linker dissolved in DMSO (the protocol according to Glennie et al [22] used dimethylformamide, but this caused precipitation of the antibody solution in this case and was therefore not suitable) and was then added to the unreacted Fab' component. After concentration and incubation overnight at 4°C, the mixture was subjected to column chromatography(S-200) and this produced a large peak approximating to 80KDa and a smaller peak corresponding to low molecular weight products.

The reaction was followed using Ellmans reagent to check for the presence of sulphydryl groups and this suggested that the reaction had been carried out successfully. The products of the linkage were subjected to SDS-PAGE and the results are presented in Figure 12.

Lane 1. Molecular weight markers
Lane 2. Human IgG
Lane 3. Human IgG $F(ab')_2$ molecule
Lane 4. $F(ab')_2$ + 2-ME
Lane 5. Bispecific after S-200 (peak 1)
Lane 6. Bispecific after S-200 (peak 2)
Lane 7. Molecular weight markers

Human IgG produced a band at approximately 150KDa, and the $F(ab')_2$ molecule appeared to be lower down at approximately 120KDa, confirming that the pepsin digestion had been successful. The $F(ab')_2$ molecule was then subjected to reduction by 2-ME in the linking procedure and a band was seen at 50KDa in lane 4, which was the size of the expected product.

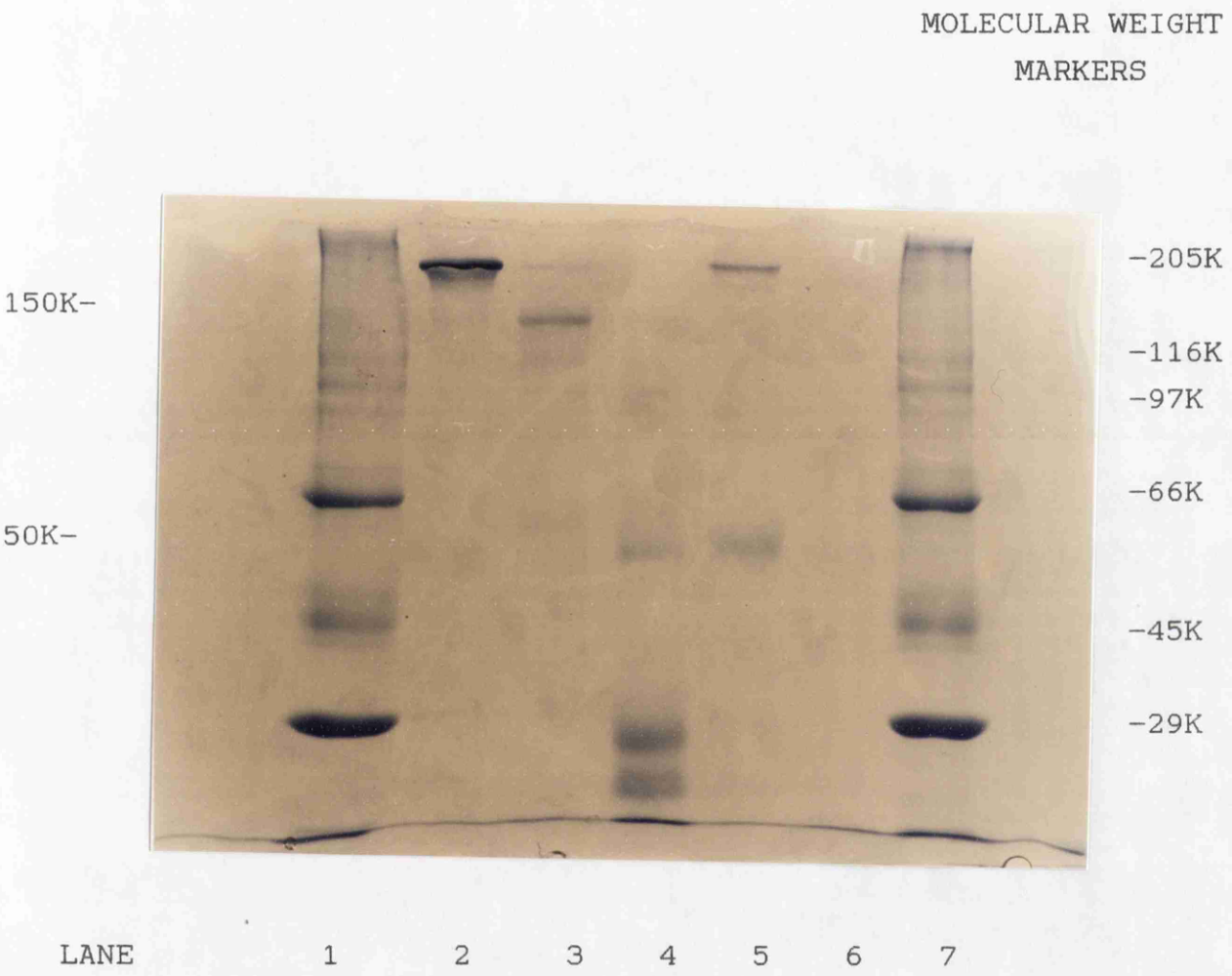


FIGURE 12 : SDS-POLYACRYLAMIDE GEL STAINED WITH COOMASSIE BLUE

The resulting linkage mixture after passage through sephacryl S-200 produced two peaks (run in lanes 5 and 6). There did not appear to be any bands in lane 6 (peak 2) but the products were estimated to be of low molecular weights than the standards and had probably run off the gel. In lane 5, a band can be seen at the 50KDa level (size of a Fab' molecule) and at the 150KDa level, and this suggested that the antibodies had conjugated. The O-PDM cross-linker then, appeared to be able to link antibodies successfully and was therefore considered suitable for further investigation.

Before any attempts were made to link the antibodies of interest it was again necessary to check their specificity with respect to their target cell lines. In a previous test (Table 14), anti-CD3 antibody had been shown to bind to the OVAN-4 cell line with a high positive population, This test was therefore repeated with different combinations of anti-CD3 and human IgG to see how this affected its binding to the cell line. The results in Table 15 indicate that pre-incubating the cell line with anti-CD3 before incubating with IgG, reduced the binding of the IgG by only a small percentage. Controls were included to ensure that any values of binding observed, would be attributed to the antibody in question and not to conjugates binding to cell lines, or to antibodies used in pre-incubating stages binding to the conjugates. Human IgG would therefore appear to bind to a different site than that used by the anti-CD3. In a similar test, the cells were incubated with human IgG as the primary antibody followed by antiCD3 and the anti-rat conjugate, producing a positive fluorescence of 55.4%. This test run however did not include the CD3 incubated with the cells alone for a comparison. The above test was therefore repeated to include this control and the results presented in Table 16.

TABLE 15: FLOW CYTOMETRY 8

TEST	% POSITIVE
OVAN-4	0.1
OVAN-4 + HFITC	2.6
OVAN-4 + IgG + HFITC	51.3
OVAN-4 + CD3 + IgG + HFITC	43.2
OVAN-4 + CD3 + HFITC	6.2
OVAN-4 + RFITC	2.7
OVAN-4 + IgG + RFITC	1.5
OVAN-4 + IgG + CD3 + RFITC	55.4

TABLE 16: FLOW CYTOMETRY 9

TEST	% POSITIVE
OVAN-4	0.1
OVAN-4 + HFITC	3.2
OVAN-4 + IgG + HFITC	21.4
OVAN-4 + CD3 + IgG + HFITC	20.1
OVAN-4 + CD3 + HFITC	3.4
OVAN-4 + RFITC	3.2
OVAN-4 + IgG + RFITC	4.8
OVAN-4 + CD3 + RFITC	52.0
OVAN-4 + IgG +CD3 + RFITC	19.9

The results were similar to that observed previously except that the percentages were lower but this was probably due to the fact that fewer target cells were present initially. However, the inclusion of the anti-CD3 antibody alone appeared to indicate that pre-incubation of the cells with IgG did reduce the binding of the CD3 to the cell line and this would suggest a similar binding site, such as the Fc receptor on the tumour cell surface.

On the other hand, if a high concentration of IgG antibody had been present, cross-linking might have occurred which may have obscured other binding sites and prevented the anti-CD3 from attaching. The fact that this antibody was binding to the target cell line at all meant that any bispecific products produced containing this antibody could not be analysed as before. This is because, to use Flow Cytometry to check for the presence of 'bispecific' antibodies, each antibody must be specific for a different target to allow specific conjugates to bind and fluoresce. Further characterization of this cell line and the binding sites involved could not be undertaken because of limited time and many questions were thus left unanswered.

SUMMARY

This investigation was designed to look at the construction of bispecific antibodies using three different cross-linkers - SMPB, SPDP and O-PDM.

Conjugation with SMPB involved a number of manipulations and was not considered to be practical for routine use.

SPDP produced a molecule which was the correct size for a bispecific antibody and flow cytometry analysis demonstrated its binding to the target cells. The fluorescent conjugates available however, were discovered not to be specific for the antibodies in question and the fluorescence observed could not be taken as being solely due to the presence of 'bispecific' antibodies. Further cytotoxicity testing of the product did however produce some evidence that the antibody was in fact 'bispecific' in nature.

The third cross-linker, O-PDM, also appeared to be successful, cross-linking two human IgG Fab' fragments but unfortunately time factors prevented the process being repeated with the antibodies of interest. If time had been available, and 'bispecifics' of 595/OKT3 had been successfully produced, these would have been analysed by Flow Cytometry and cytotoxicity assays to see if they had lost any specificity or activity as a result of the linking process.

This investigation also raised a number of questions on the specificity of the antibodies involved. The previously defined antibody 14C1 no longer bound to the ovarian target cell line, while irrelevant antibodies did bind with relatively high percentages of fluorescence. This binding however, was thought to be due to the presence of Fc receptors on the surface of the target cell lines, since antibodies lacking the Fc component did not bind as well as the intact molecules.

The linking procedures with SMPB and O-PDM proved to be time consuming, labour intensive and difficult to perform on a routine basis. The SPDP process was fairly straight forward, and out of the three would be the method of choice if linking had to be carried out regularly.

The lack of antibody also meant that conjugation procedures were not being carried out at their optimum levels and if more antibody had been available the linkages might have been carried out more successfully.

Other investigators have, successfully linked different antibodies together by chemical means. Glennie et al, [22] in particular has produced bispecific antibodies without much trouble, linking mouse/mouse antibodies and mouse/rabbit antibodies. In this investigation however, we were trying to link mouse/rat and mouse/human antibodies and this may have been the reason for the difficulties encountered.

Glennie and collaborators [30] have even gone a step further in terms of chemical linkages with the production of Trispecific antibodies. Tumour targeting with antibody derivatives requires activated effector cells, since even with the appropriate bispecific antibody, unprimed T-cells and monocytes are only minimally cytotoxic to tumour targets. Activation with IL-2 causes problems, because in vivo it acts locally and its systemic use in patients can be inefficient. Used at the levels required for activation it can lead to severe and life threatening side effects. The alternative, it is suggested, is to use trispecific antibodies which can activate T-cells using co-operative signalling via two Fab' arms and retarget through the third arm. They are completely stable to reduction and are near IgG size, mediating highly efficient lysis of tumour cells using fresh unprimed PBMC.

To produce the trispecific version, bispecific antibodies are prepared according to the O-PDM protocol , and then linked to a FAb'(MAL) from a third antibody, and these have been suggested for use as a more suitable alternative to the much studied 'bispecific' antibodies.

Whether 'bispecific' or 'trispecific' antibodies fulfil their potential as alternatives to the conventional therapies has yet to be seen, but it does seem likely that their influence on immunotherapy will not be as much as had been hoped, for the reasons stated in this investigation.

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