Class-Specific Monoclonal Antibodies for the Detection of O^6-alkyl-2'-deoxyguanosines

by

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Abbreviations

3'P	3'phosphorothioate
7-MedG	7-methyl-2'-deoxyguanosine
A	adenine
Ab	antibody
ABTS	2,2-azino-di-[3-ethylbenz-thiozaline sulphonic acid]
AcOH	acetic acid
Ag	antigen
APC	antigen presenting cell
BSA	bovine serum albumin
C	cytosine
CDR	complementarity determining region
CγG	chicken gamma globulin
CNBr	cyanogen bromide
CNBrAS	cyanogen bromide activated sepharose
CP	complete phosphorothioate
DAP	diaminopurine
DBU	1,8-diazobicyclo[5.4.1]undec-7-ene(1,1-5)
DCCI	N,N-dicyclohexylcarbodiimide
DCM	dichloromethane
dG	2'-deoxyguanosine
dH_2O	distilled water
DMAP	4-Dimethylaminopyridine
DMF	dimethylformamide
DMS	dimethyl sulphate
DMSO	dimethyl sulphoxide
DMTr	4,4'-Dimethoxytrityl
DNA	deoxyribonucleic acid
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N' -tetraacetic acid
ELISA	enzyme linked immunosorbent assay
eq.	equivalents
G	guanine
HGPRT	hypoxanthine-guanine phosphororibosyl transferase
HPLC	high performance liquid chromatography

hr	hours
IAC	immunoaffinity chromatography
IgG	immunoglobin G
IgM	immunoglobin M
IL-1	interleukin 1
MAb	monoclonal antibody
MeOH	methanol
MHC	major histocompatibility complex
MHMB	methyl-4(hydroxymethyl)benzoate
min	minutes
MNU	methyl nitrosourea
mRNA	messenger RNA
NAP	nucleic acid purification
NH_4OAc	ammonium acetate
O^6-alkyl dG	O^6-alkyl-2'-deoxyguanosine
O^6-CBdG	O^6-(4-carboxybenzyl)-2'-deoxyguanosine
O^6-EtdG	O^6-ethyl-2'-deoxyguanosine
O^6-EtG	O^6-ethyl guanine
O^6-hydroxyEtdG	O^6-hydroxyethyl-2'-deoxuguanosine
O^6-hydroxyEtG	O^6-hydroxyethyl guanine
O^6-MedG	O^6-methyl-2'-deoxyguanosine
O^6-MeG	O^6-methyl guanine
O^6-n-PrdG	O^6-n-propyl-2'-deoxyguanosine
O^6-n-PrG	O^6-n-propyl guanine
PBS	phosphate buffered saline
PEG	polyethylene glycol
PIAS	periodate activated sepharose
PS	phosphorothioate
sec	seconds
T	thymine
TBAF	tetrabutyl ammonium flouride
TBDMS	tetrabutyl-dimethylsilyl
TEA	triethylamine
THF	tetrahydrofuran
TIPDS	tetraisopropyldisiloxane
TLC	thin layer chromatography
TrAS	triazine activated sepharose
Tw	Tween

USERIA	ultrasensitive radioimmunoassay
UV	ultraviolet
W/C	Watson/Crick

Abstract

The reaction of alkylating reagents with DNA yields a variety of potential mutagenic and carcinogenic lesions. One such group, the O^6-alkyl-2'-deoxyguanosines (O^6-alkyl dGs) are known to induce guanine to adenine transitions, which may lead to the activation of oncogenes. Antibodies with predefined specificities can be employed in the detection and quantification of such lesions.

Four O^6-alkyl dG analogues (methyl, ethyl, n-propyl and hydroxyethyl) have been synthesised. Each was made into a suitable antigen by, i) conjugation to BSA and/or CγG and ii) incorporation into an oligonucleotide. An O^6-4(carboxybenzyl) dG derivative has also been synthesised and conjugated to BSA and CγG. All conjugates and oligonucleotides have been characterised.

Each O^6-alkyl dG antigen has been used for immunisation. Hybridoma technology was employed to give monoclonal antibody producing cell lines. Screening assays were developed for the detection of a cell line producing a monoclonal antibody specific for O^6-alkyl dG. Three monoclonal antibodies have been produced. The relative affinities of each antibody for each O^6-alkyl dG have been determined. The highest affinity antibodies have been immobilised on sepharose. Immunoaffinity columns have been constructed and employed in the extraction of O^6-alkyl dG from a pool of nucleosides.

Contents

Chapter 1. Introduction

1.1 DNA

Deoxyribonucleic acid (DNA) is the chromosomal material that contains the genetic information within the cells of living systems. It consists of billions of deoxyribonucleotides of four different types which are joined together in a sequence which is characteristic of each organism. Each deoxyribonucleotide consists of a β-D-deoxyribofuranose and one of four nitrogenous bases, adenine (A), thymine (T), guanine (G) or cytosine (C). A phosphate diester group joins the 3' and 5' positions of the sugars to create a continuous strand which forms the backbone of the molecule (fig. 1.1a). The bases pair by hydrogen bonding, which holds the two strands of the molecule together. Base-pairs consist of a purine and a pyrimidine and in the most common tautomeric form A pairs with T (two hydrogen bonds), and G with C (three hydrogen bonds), to form the bridges joining the two strands (Watson and Crick, 1953) (fig. 1.1b).

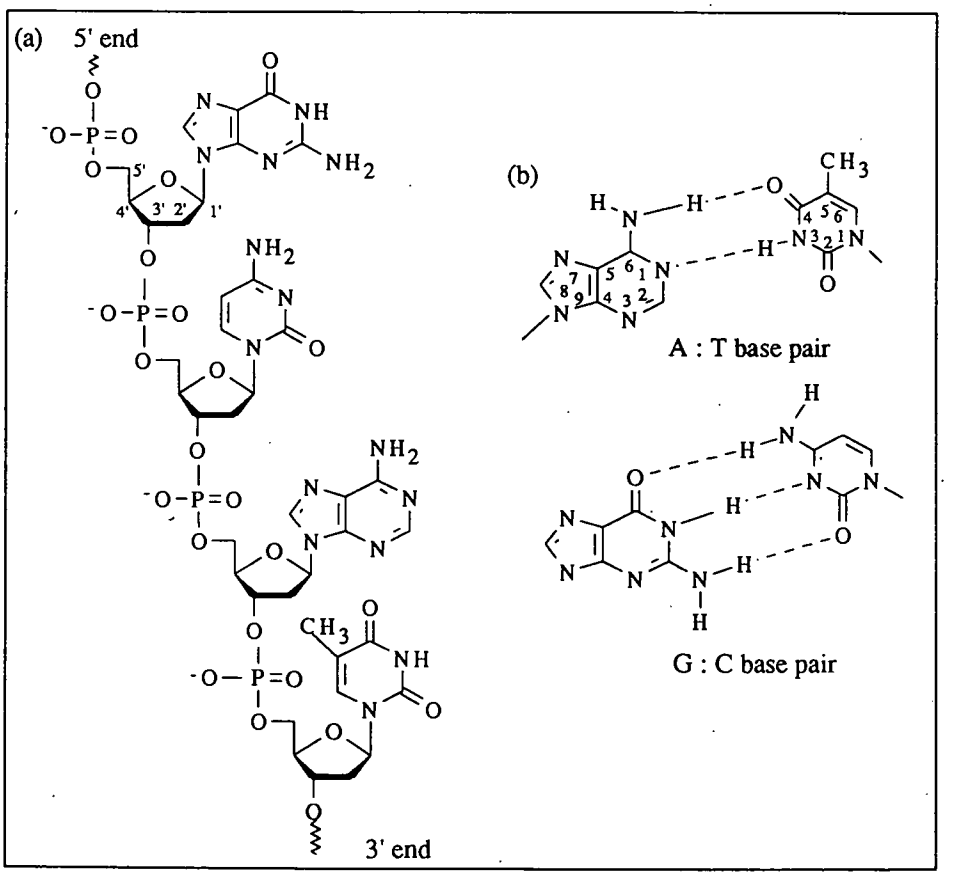


Figure 1.1 (a) Part of the polynucleotide strand of DNA showing the sugar numbering system

(b) Normal base pairing in DNA showing the base numbering system

For organisms to produce viable progeny the chromosomal material must remain almost identical over generations. When one considers that the 23 pairs of chromosomes in human cells contain 3 x 10^9 base pairs, it is apparent that transmission of this information from parent to offspring requires a highly specialised mechanism. The Watson and Crick (W/C) hypothesis for the semi-conservative replication of DNA allows an understanding of the accurate transmission of hereditary information. The parent DNA molecule unwinds, then the two strands break apart one base at a time. New identical daughter DNA is constructed from deoxyribonucleotides available in the cell, using the parent strands as templates. As A can only pair with T, and C with G the new strands are complementary to the parent strands, giving replicas of the starting molecule.

The information contained in DNA is used in the production of the proteins necessary for the survival of the organism. The genetic code is the starting point for two major events in cells, i) transcription: messenger ribonucleic acid (mRNA) synthesis and; ii) translation: protein synthesis. During transcription the enzyme DNA polymerase attaches to an area of double stranded DNA which opens up to expose one strand and this is used as a template for the synthesis of RNA (fig. 1.2). RNA is similar to DNA but has a ribose sugar, which contains a hydroxyl group in the 2' position, and uracil rather than thymine bases. The RNA is then translated as triplets of nucleotides which code for amino acids, the building blocks of proteins. The biosynthesis of these proteins utilises many complex enzyme systems which decipher and translate the genetic code.

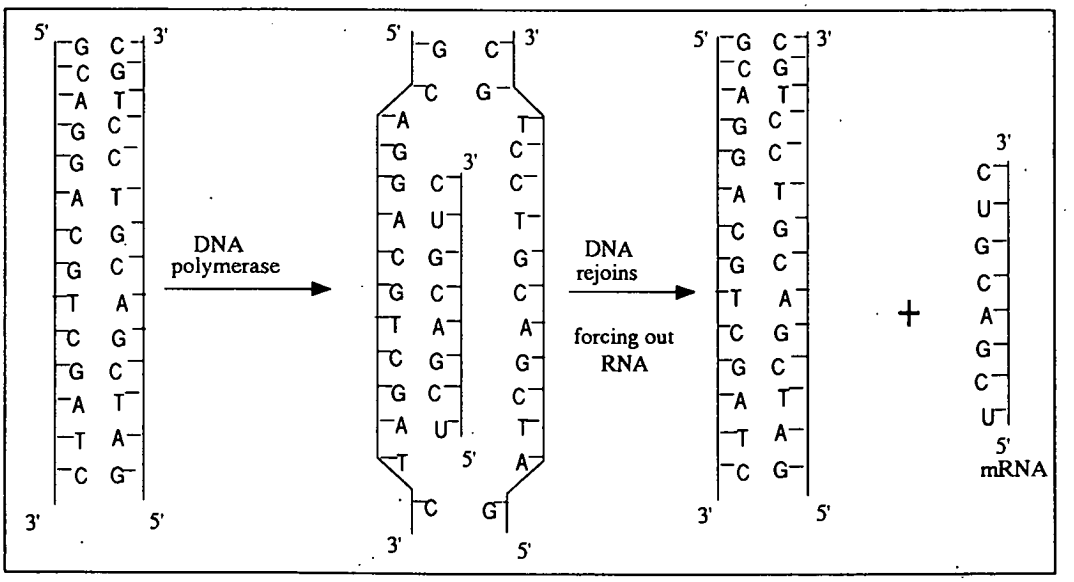


Figure 1.2. Transcription, mRNA synthesis

1.2 Damage to DNA

The existence of genetic continuity within species may imply that DNA is a stable molecule and that replication and transcription are infallible mechanisms. However, DNA is continually undergoing changes referred to as mutations. Most mutations are disadvantageous as far as the individual in which they occur is concerned. Despite the occurrence of natural mutations, the probability of DNA damage is increased manyfold when an organism is exposed to certain biological, chemical or physical environmental factors. Many mutations are repaired by specialised enzymes, for example DNA polymerases which repair strand breaks and excise incorrectly inserted deoxyribonucleotides. When mistakes are not repaired disease and heritable disorders can result.

1.2.1 Natural damage

Chromosomal DNA is constantly undergoing natural changes, referred to as spontaneous mutations. These usually arise from the mispairing of bases during DNA synthesis, with 1 in 10^7 to 10^{11} misincorporations per base-pair replicated (Drake, 1969, 1991). In addition, hydrolytic reactions which occur in DNA under physiological conditions can cause deamination, depurination and depyrimidation (Fersht and Knill-Jones, 1981). The accuracy of DNA synthesis relies on the ability of enzymes to discriminate against the insertion of an incorrect or damaged deoxyribonucleotide. Consequently, natural mutations in organisms are rare events.

1.2.2 Chemical damage

In most instances cancer is caused by mutation of DNA in cellular genes that control cell growth and cell mitosis. Only a small fraction of cells that mutate ever lead to cancer. Most defective cells have reduced survival capacity due to the loss of essential mechanisms and attack by the organism's immune system. Statistical evidence suggests that continued exposure of human beings to chemical agents including industrial chemicals, food additives, exhaust gases, dyes, flavourings, cigarette smoke and cosmetics, increases the incidence of specific types of cancer (Blackburn and Kellard 1986).

Chemicals that interact with DNA and result in damage to the molecule are referred to as mutagens. Compounds causing mutations which alter normal cell function, possibly leading to carcinogenesis are commonly referred to as carcinogens. Primary carcinogens, typically electrophilic alkylating agents react directly with DNA, whereas secondary carcinogens must undergo some form of activation prior to

their reaction with DNA. In addition, there are chemicals which are not themselves carcinogenic but their presence enhances the carcinogenicity of other compounds. DNA has an abundance of nucleophilic centres, subsequently electrophilic alkylation is a predominant modification.

1.3. Alkylation of DNA

During the last 30 years many studies have shown that alkylating agents interact with DNA to yield a variety of potential mutagenic and carcinogenic modifications (Loveless, 1969; Margison and Kleihues, 1975; Saffhill, *et al* 1985; Singer, 1986; Basu and Essigmann, 1990) . Of the chemicals which are known carcinogens to humans, approximately one fifth are alkylating agents, the most common are shown in figure 1.3. (Singer 1975).

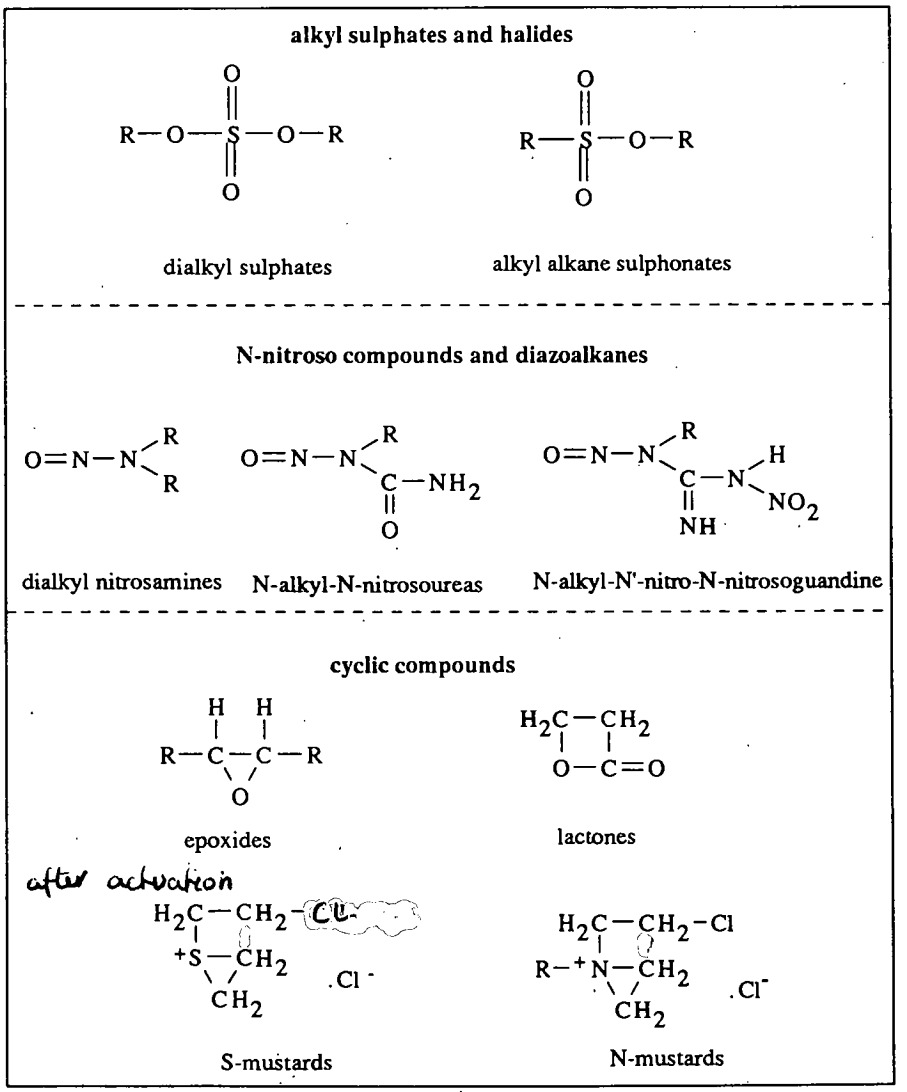


Figure 1.3. Alkylating carcinogens

Many of these compounds are found in the environment and it has been estimated that the daily human ingestion rate of dimethyl nitrosamine is 1-2μg per person (Blackburn and Kellard, 1986). Nitrosamines are believed to be the most dangerous and widespread of all alkylating compounds. They are commonly found in tobacco products as a result of the nitrosation of natural tobacco during curing and fermenting, and are released into the environment *via* mainstream tobacco smoke (Tricker *et al,* 1991). In addition, nitrosamines can be produced in the stomach by nitrosation of secondary amines. Nitrates are present in food, and following ingestion and subsequent bacterial degradation nitrites and nitrous acid are commonly produced. In the environment of the normal stomach (pH3) amines are protonated and do not react with these nitrous compounds. However, treatment for stomach ulcers often involves elevating stomach pH, creating an environment in which amines are not protonated and therefore able to react with nitrites and nitrous acid to produce nitrosamines (fig 1.4).

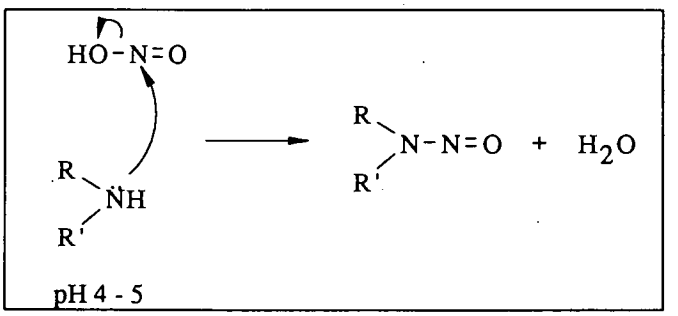


Figure 1.4. Production of nitrosamines by the reaction of nitrous acid and secondary amines in the stomach

Alkylating compounds can be sub-divided into monofunctional and bifunctional agents. The former interact with only one of any of the nucleophilic centres in DNA. The latter have the capacity to react with two sites in DNA and can consequently cross-link a base to another base or a protein. Sulphur mustard, nitrogen mustards, bis chloromethyl ether and epichlorohydrin are potential bifunctional alkylating compounds. Monofunctional alkylating agents can react at all of the oxygen and nitrogen atoms (except N^9) of the four bases. Generally, the ring nitrogens of the bases are more nucleophilic than the oxygens, although the sites of reaction of the alkylating reagents usually depend on their mechanism of action.

Pearson's "hard and soft acids and bases concept" (1967) generally applies. This states that "soft" electrophiles react with extremely nucleophilic centres in an S_N2

type reaction, and "hard" electrophiles with less nucleophilic centres in an S_N1 type reaction. The predominant sites of reaction of dimethyl sulphate (DMS) a "soft" electrophile, with DNA are at the nucleophilic N atoms of the bases. DMS reacts preferentially at the N^7 of G, then the N^1 of A, the N^3 of C and the N^3 of T, with almost no reaction at the oxygens (fig. 1.5). In comparison, the interaction of methyl nitrosourea (MNU) or nitrosamines, "hard" electrophiles, result mostly in the formation of alkylated phosphate triesters. The predominant alkylated base products are at the nitrogens but there is a greatly increased extent of modification at the oxygens, preferentially O^6 of G, followed by the O^2 of T, the O^4 of T and the O^2 of C (Blackburn and Kellard 1986).

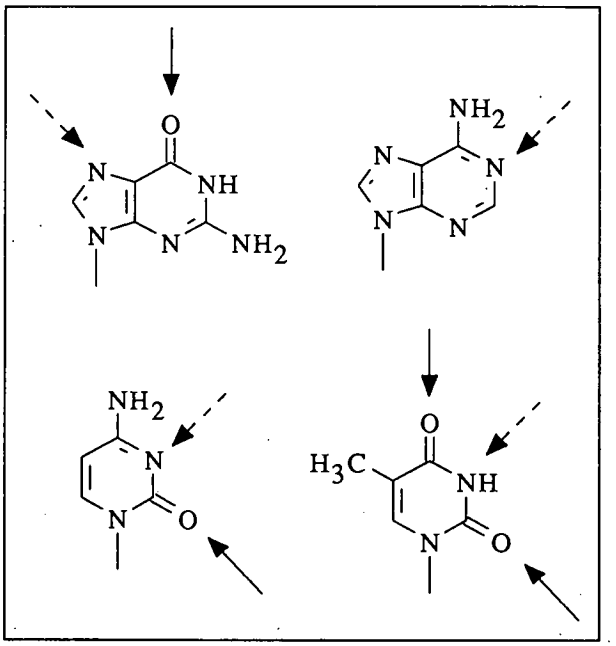


Figure 1.5. Principal targets in DNA bases for 'hard' and 'soft' electrophiles.

The sites of alkylation in DNA may also be regulated by steric factors. In the normal right handed configuration (B form) DNA has a major groove and a minor groove, where the stacked bases form a hydrophobic core with the amino and keto groups pointing out into the grooves. The N^3 of purine rings is fairly accessible in the minor groove, while the N^7 of purines and O^6 of G are easily accessible in the major groove. Occasionally, portions of the DNA molecule can adopt a left handed helical conformation referred to as Z-form (Rich *et al*, 1984) particularly at high salt concentrations (Arnott *et al*, 1980), or in DNA with continual alternating purine-pyrimidine sequences (Hamada and Kakunaga, 1982). In this conformation normal base stacking does not occur and there is one deep groove between the sugar-

phosphate backbones, which corresponds to the minor groove in normal B-DNA. This results in certain base atoms, particularly the O^6, N^7 and C^8 positions of G, being much less sterically hindered and thus more exposed to alkylating agents.

Many alkylating reagents react directly with DNA. Dialkyl sulphates react primarily at the nucleophilic N^7 position of G to produce 7-alkyl G (fig. 1.6).

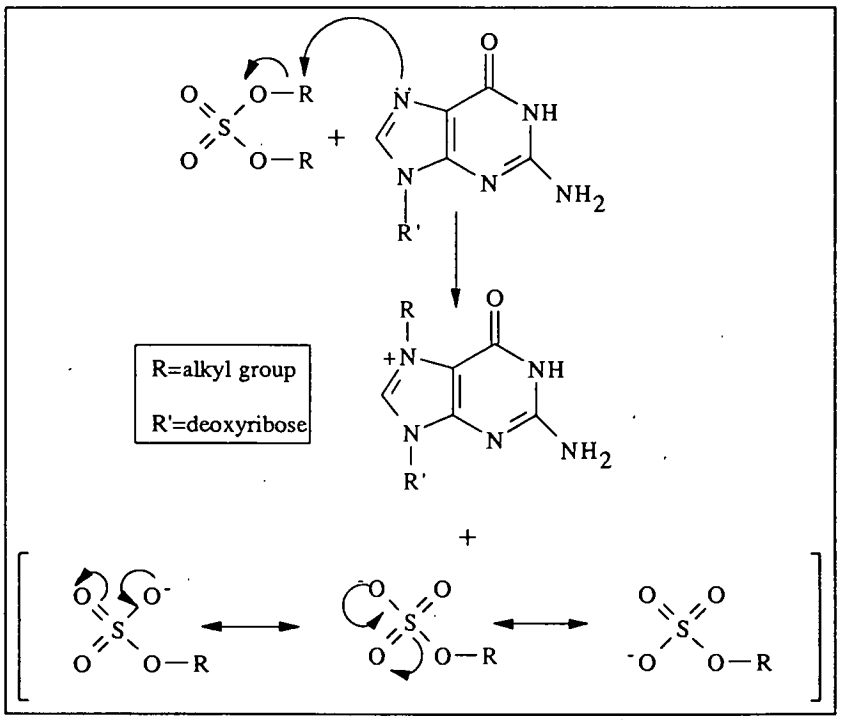


Figure 1.6. Alkylation at the N^7 position of guanine derivatives

The nitrosoureas, nitrosoguanidines and nitrosourethanes are spontaneously hydrolysed to produce alkyl diazonium hydroxides, which ultimately react with the O^6 position of G. Other compounds such as nitrosamines require enzyme catalysed chemical transformation to produce the primary carcinogen. This involves controlled oxidation by cytochrome P-450 enzymes. Dimethyl nitrosamine undergoes α-hydroxylation by these enzymes to produce N-hydroxymethyl-N-methyl-nitrosamine which spontaneously loses formaldehyde to generate methyl diazonium hydroxide, the primary carcinogen (fig 1.7). Alkyl diazonium hydroxides rearrange to give hydroxide ions and a positively charged alkyl species. In the presence of G the O^6 reacts with the positive species to generate O^6-alkylated G.

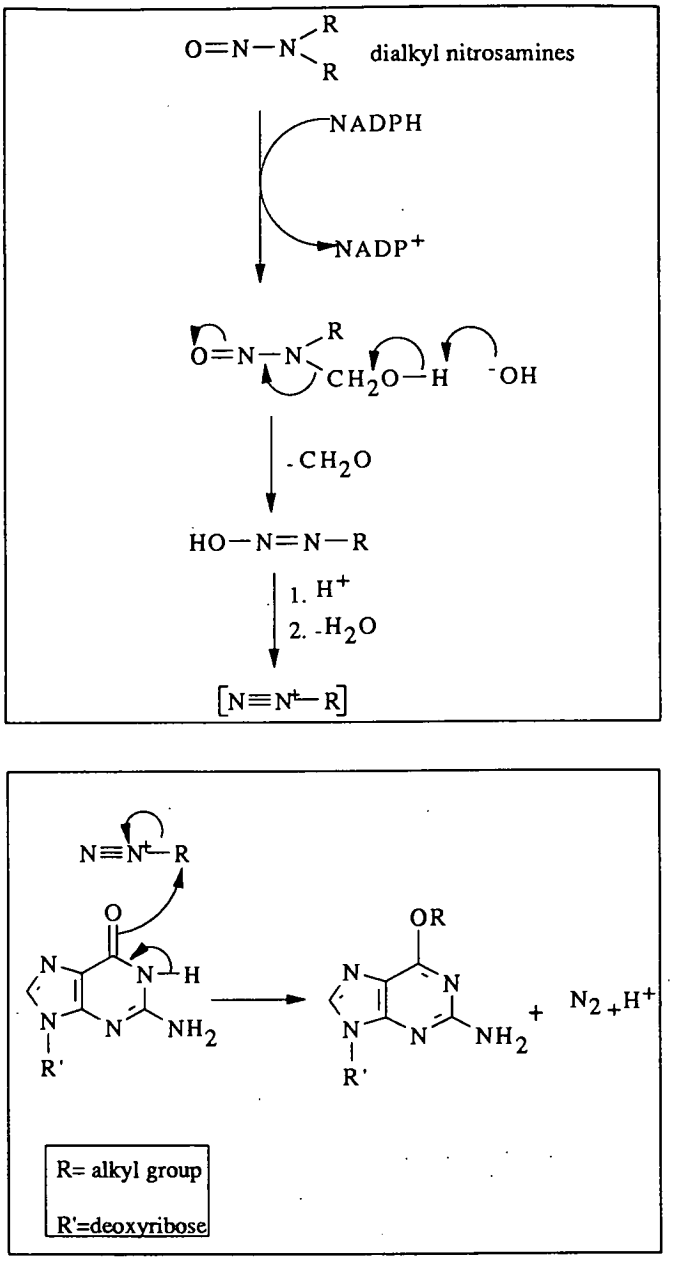


Figure 1.7 Alkylation at the O^6 position of guanine derivatives

1.3.1 O^6-alkylguanine: A pro-carcinogenic lesion

The predominant modification following a reaction of an alkylating reagent with DNA is 7-alkyl G. This modification does not appear to be directly mutagenic (Ludlum 1970). In comparison, the less common modification O^6-alkyl G has been shown to lead to point mutations in DNA following replication, (Loechler *et al*, 1984; Yamagata *et al*,1988). The molecular mechanism of this mutation is due to the enforced change in the tautomeric structure of the base causing it to mispair with T (Leonard *et al*, 1990). After replication this mispairing results in a G to A

transition which may result in the deletion or abnormal synthesis of a protein required for the survival of the cell. This transition has also been linked to the activation of the Ha-*ras* transforming oncogene (Sukumar *et al*, 1983). Activation of the *ras* gene family is associated with carcinogen-induced animal tumours. Studies have shown that a G to A transition specifically at the second base of the 12th codon of a Ha-*ras* oncogene results in its activation (Mitra *et al*, 1989).

Comparison of the structures and W/C binding sites of G, 7-akyl G and O^6-alkyl G demonstrates the disruptiveness of the O^6-alkyl derivative compared to the 7-alkyl derivative (fig. 1.8).

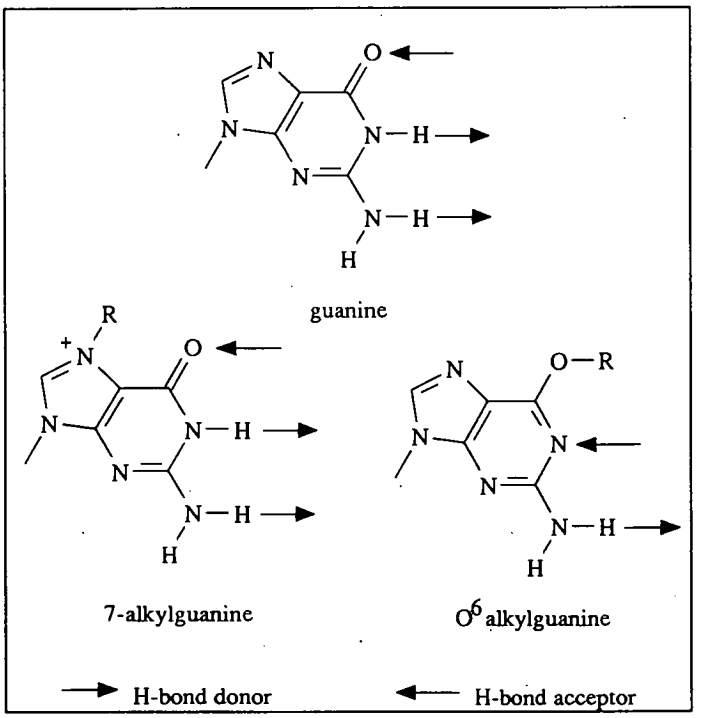


Figure 1.8 The binding sites of guanine, 7-alkylguanine and O^6-alkylguanine

The ability of 7-alkyl G to hydrogen bond to C in the usual way is not altered, since the W/C binding sites are unaffected by the addition of the alkyl group. However, alkylation at the O^6 position of G alters the position of the double bond resulting in deprotonation of the N^1 which then becomes a hydrogen bond acceptor, as it is in A, rather than a hydrogen bond donor as it should be in G. The resulting O^6-alkyl G therefore has different hydrogen bonding capacity from non-alkylated G. This alteration allows O^6-akyl G to pair with its normal base pairing partner, C; or to mispair with T.

Many researchers have addressed the problem of when and how C or T pair with O^6-alkyl G. This is complicated by the fact that the O^6-alkyl group can adopt a *syn* or *anti* conformation (relative to the N^1 of the base) (fig 1.9a). In the former the alkyl group points into the helix, potentially disrupting the normal hydrogen bonding, while in the latter the alkyl group points into the major groove. X-ray diffraction (Parthasarathey and Fridey, 1986) and NMR studies of duplex oligonucleotides containing O^6-methyl G (Patel *èt al*, 1986, and Kalnik *et al*, 1989), showed that the *syn* conformation was preferred. It was deduced that in this conformation the methyl group prevents T from base-pairing on the same plane as its partner, resulting in only a single hydrogen bond between the N^2 of G and O^4 of T (fig 1.9b). However, a G to A transition is unlikely to arise from the misincorporation of T opposite O^6-alkyl G by this mechanism as a very unstable base pair is produced (Pederson *et al*, 1988).

The original suggestion by Loveless (1969) that O^6-alkyl G adopts the *anti* conformation allowing it to pair with C or T, was supported by a recent study in which an oligonucleotide containing O^6-methyl dG (O^6-MeG) was examined by X-ray diffraction (Leonard *et al*, 1990). One hydrogen bond between N^1 of G and N^3 of T, and a second from N^2 of G and O^2 of T, were found (fig 1.9c). In this conformation the O^6-MeG : T mispair closely resembles a W/C base pair as the bases are on the same plane, and the helical parameters and torsion angles are similar to a native G : C pair. The O^6-alkyl G : C base pair has also been examined in a duplex and found to be unstable at neutral pH, but at lower pH the duplex stabilises, possibly due to protonation of the N^3 of C to form a W/C base pair (Leonard *et al*, 1990) (fig1.9d). Thus, occasionally, O^6-alkyl G may not cause a G to A transition.

Molecular modelling and theoretical calculations suggest that there is only a small difference in energy (< 1 kcal/mol) between the *syn* and *anti* conformations of O^6-alkyl G when paired with T in a duplex and both are likely to exist (Loechler, 1991). This theoretical analysis postulates that *anti* O^6-alkyl G forms one normal hydrogen bond between the N^2 of G and O^2 of T, and one branched hydrogen bond from the H^3 of T to both the N^1 and O^6 positions of O^6-alkyl G. Similarly *syn* O^6-alkyl G forms the normal N^2 of G, to O^2 of T bond (2.07Å) and also has bonding from H^3 of T, to N^1 and O^6 of O^6-alkyl G, but this bond is elongated (2.98Å). This is thought to occur due to the protrusion of the alkyl group into the hydrogen bonding region of the base pair causing the T to be in a different plane and thus farther from its base pairing partner.

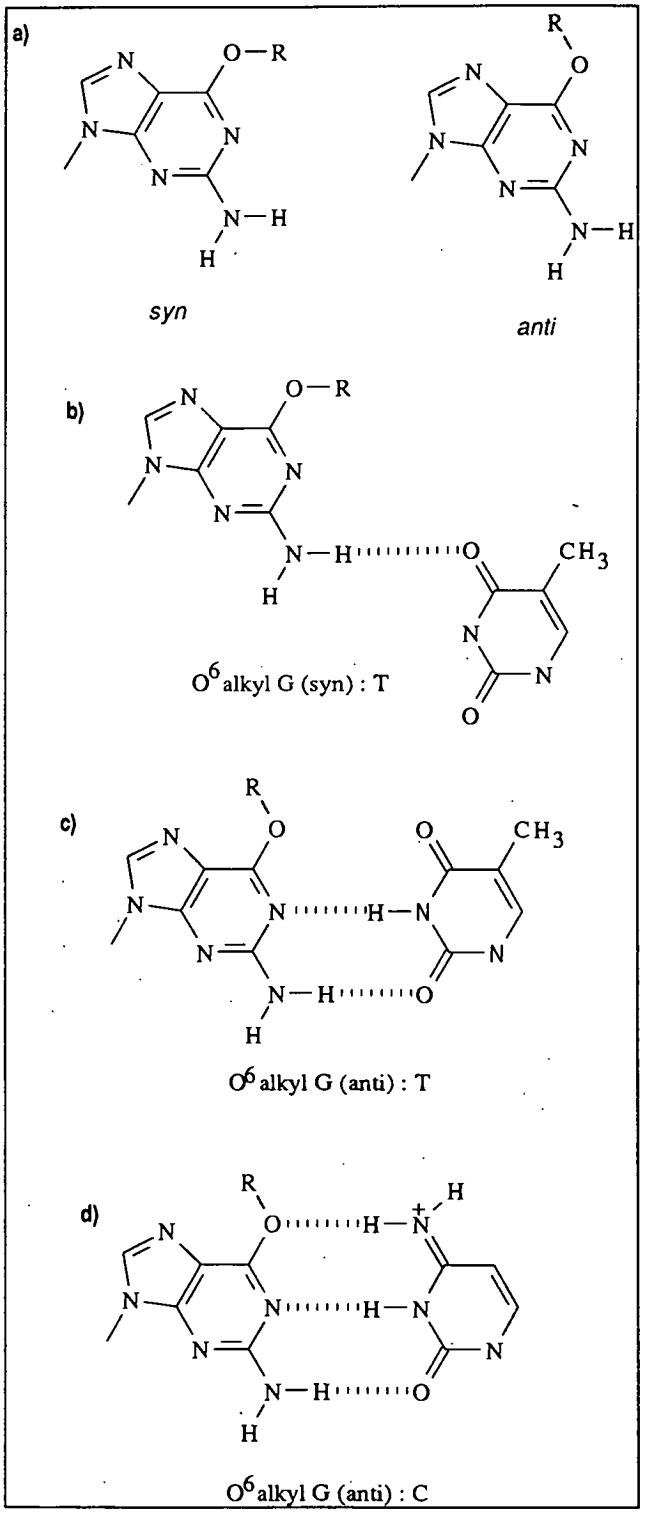


Figure 1.9. Base pairing of O^6-alkylguanine derivatives

1.3.2 Repair of O^6-alkylguanine Lesions

The action of alkylating agents has been observed in bacteria for many years. The existence of an adapted mechanism for the repair of O^6alkyl G was first suggested when researchers (Jimenez-Sanchez and Cerda-Olmedo, 1975) exposed E. coli to minute levels of alkylating agents and found that the mutation frequency increased with time of exposure but finally reached a plateau. The repair enzyme O^6-alkyl G DNA transferase (a suicide enzyme) has since been discovered (Olson and Lindahl, 1980) and the gene has been localised on human chromosomes (Zunino *et al*, 1991). The enzyme (18 000 M_r) operates by transferring the alkyl group from the O^6 of G onto an internal cysteine residue (Pegg *et al*, 1983) (fig 1.10). Subsequent to this transfer the enzyme becomes inactive. The enzyme is highly specific for methyl groups and the transfer of a methyl group to a particular cysteine (cys 69) results in the activation of a transcriptional regulator leading to a 100 fold increase of the transferase activity per cell (Teo *et al*, 1986). This regulator is only weakly activated by the transfer of higher alkyl groups, for example O^6-ethyl G (O^6EtG) is repaired 1000 times slower than O^6-MeG and O^6-isopropyl G is not repaired (Graves *et al*, 1989). Cells, therefore, have adapted a mechanism to undo the direct damage caused by alkylating agents, in particular, methylating agents. However, if the O^6-alkyl G derivatives are not repaired before replication occurs a mutation arises.

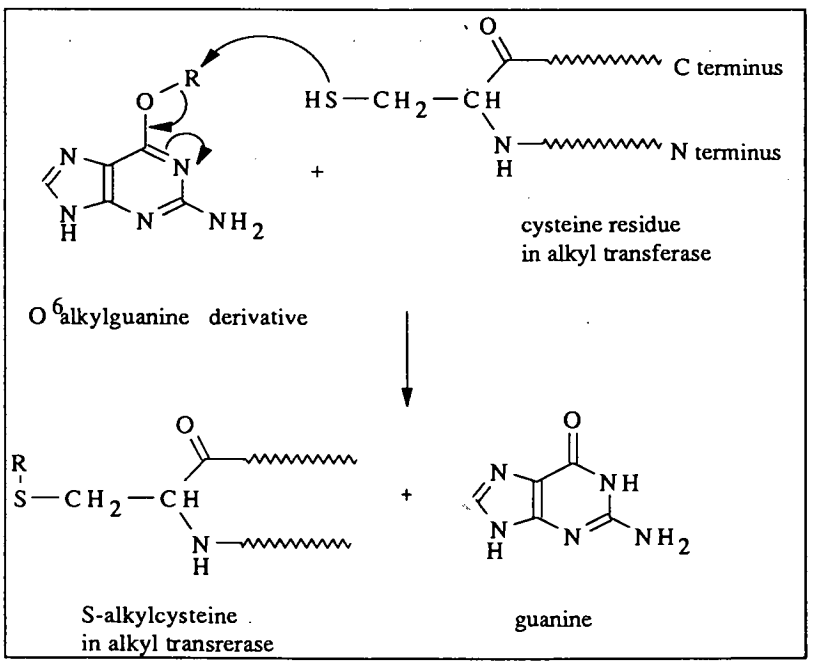


Figure 1.10. De-alkylation of O^6-alkyl G by alkyl transferase

The most common general DNA repair mechanism in cells is excision repair. This mechanism removes the mis-inserted or damaged deoxyribonucleotide and replaces it with the correct deoxyribonucleotide. Firstly, an endonuclease creates a nick in the DNA strand at the site of the undesired residue. Subsequently, an exonuclease excises the part of the DNA strand containing the wrong deoxyribonucleotide. DNA polymerase allows the correct deoxyribonucleotide to be inserted into the gap in the strand. Finally, a ligase covalently links the strand to complete the repair. This mechanism has been reported to excise O^6-alkyl G from a T : O^6-alkyl G base pair *in vivo* (Samson *et al*, 1988) and *in vitro* (Voigt *et al*, 1989), although the mechanism is believed to function less efficiently with small alkyl lesions compared to bulky lesions (Sancar and Sancar, 1988).

When O^6-alkyl G forms hydrogen bonds with T, an unstable base-pair results, but the symmetry of the sugar-phosphate backbone in the DNA sequence remains unchanged (Leonard *et al*, 1990). As the structure is similar in overall shape to a W/C base-pair enzymes cannot detect and repair the mistake. The presence of the alkyl group can also act as a steric barrier preventing the interaction of enzymes. In addition, the repair enzymes appear to discriminate in favour of the mutagenic lesion. This is due to the fact that although O^6-alkyl G can form a stable base-pair with C it is distorted and no longer resembles a W/C base-pair (Patel *et al* 1986; Leonard *et al*, 1990). Repair enzymes detect this as a mismatch, excise the correctly inserted C and allow the misinsertion of T.

Many years of research have identified the chemical agents responsible for alkylation at the O^6 position of G. In addition, the mechanism by which this creates a mutagenic lesion, which may ultimately lead to carcinogenesis, and the mechanisms of repair enzymes for this lesion have been widely investigated. Advances in the understanding of these mechanisms have led to the development of sensitive techniques to monitor and quantify the extent of damage and repair in the DNA molecule.

1.4 Biomonitoring

The knowledge that certain DNA modifications are implicated in carcinogenesis has encouraged researchers to develop techniques to monitor the effects of exposure to environmental carcinogens. Original monitoring studies estimated exposure of organisms by measuring the levels of mutagenic substances in the ambient environmental media. Exact doses received by organisms, and individual

differences, for example in adsorption, metabolism and excretion, are impossible to measure by this system. Subsequently, the development of biological monitoring has allowed the precise determination of the internal dose of a mutagenic chemical or a related metabolite in the body. Additionally, it has become possible to measure the extent of reaction of the mutagen with DNA.

The biomonitoring of carcinogen exposure in living humans presents various difficulties. Firstly, the tissues which are directly affected by the carcinogen (i.e. lungs, liver, stomach) are inaccessible, therefore surrogate tissues (i.e. white blood cells, peripheral blood lymphocytes, placental tissue) which may not have the same degree of DNA damage, are investigated. Secondly, there is large inter-individual variation, for example the P-450 isoenzymes have been recorded in humans with >1000 fold inter-individual variance, in terms of quantity, specificity and tissue distribution (Fujino *et al*, 1982). These enzymes activate many chemicals to produce carcinogenic metabolites and individuals with increased P-450 levels may be more susceptible to developing tumours than individuals with low level P-450 isoenzymes. Additionally, lowered rates of DNA repair have been recorded in certain individuals. For example O^6-alkyl-DNA alkyltransferase is found at lower levels in patients with lung cancer compared to non cancer controls (Harris, 1989). This suggests that these subjects may have developed cancer due to their low repair enzyme levels. Thirdly, humans are exposed to low doses of complex mixtures of many chemicals, including carcinogens, in the environment. This may confound the interpretation of cancer risk assessment of any one compound. These 3 factors must be considered when developing a biomonitoring system. In short, for an assay to be potentially useful it must; i) be sufficiently sensitive to allow detection of mutagenic derivatives at low levels; ii) require small samples of DNA; iii) be quantitatively related to exposure; and iv) be specific in identifying only the carcinogenic lesion of interest (Beach and Gupta, 1992).

The majority of biomonitoring techniques involve the use of biomarkers which allow quantification of biochemical, physiological, cytological, immunological or molecular changes. A knowledge of the internal interactions of the mutagen with the target molecule is necessary before choosing the appropriate biomarker. It can then be applied to directly measure mutagens in body fluids or excreta or to measure the covalent binding of the mutagen/metabolite to DNA, RNA or proteins. The former method, although employed extensively in the past, is now considered to be limited due to the rapid removal of many chemicals from the body. In comparison the

products of the latter method (i.e. altered DNA, RNA or protein) can exist in cells for weeks to a lifetime depending upon chemical stability, efficiency of repair mechanisms and processes of cell turnover. Since DNA lesions are believed to represent an early but critical step in chemical carcinogenesis, their detection may serve as a dosimeter of environmental exposure.

1.5 Detection of DNA lesions

1.5.1 Classical techniques

Over the years a number of direct and indirect assays to detect DNA lesions have been developed. There is no universal technique as many assays exhibit inherent advantages and disadvantages depending upon the system and compounds used. Generally, radio-chromatographic techniques (Baird, 1979) have been the most popular, although identification of modified bases in DNA by this method is limited. Specific activity of radiolabelled carcinogens, restriction to non-human use and limited sensitivity requiring the use of large quantities of DNA are the major disadvantages of this technique. Fluorescent techniques (Kreik *et al*, 1984) have also been explored with carcinogens which absorb in the UV visible spectrum (e.g. polyaromatic hydrocarbons and aflatoxin). DNA lesions can be directly measured by these techniques, but few human studies have been carried out.

1.5.2 Immunochemical techniques

Immunochemical detection methods which have been developed more recently rely on the principle that a specific antibody recognises a specific antigen. Antibodies produced in an immune response to a carcinogenic lesion (the antigen) are purified then employed to detect that lesion in native DNA samples. High specificity of antibodies in recognising minor alterations of molecular structure allows studies to be conducted on small samples with low damage levels in the DNA. This offers potential for studying human exposure to environmental carcinogens.

1.5.2.1 An introduction to antibodies

The interaction of an antibody with an antigen forms the basis of all immunochemical techniques. The nature of the antibody-antigen bonds, the strength of these bonds (affinity) and the stability of the complex (avidity), as well as specificity, are all important in determining the potential of an antibody for recognising an antigen. There are five classes of antibody. IgGs are the most abundant in serum after an immune response, and usually exhibit highest affinity. They are therefore the most common antibody employed in immunochemical

techniques. The IgGs have two heavy (H) peptide chains (55 000 daltons) and two light (L) peptide chains (25 000 daltons) which are joined by sulphur bridges to form a Y-like structure. The two arms of the Y; the Fab domains, each consist of an L and part of an H chain which contain a site that can bind to an antigen, while the base of the Y, the Fc domain, is concerned with immune regulation (fig. 1.11). The L and H chains can be divided into a constant region and a variable region, the latter containing approximately 110 amino acids in both chains. The variable regions of one H chain and one L chain combine to form one antigen binding site (IgGs therefore have two). Different combinations of amino acids in the variable regions provide the structural basis for the large repertoire of binding sites an antibody can exhibit to capture an antigen. The genetic basis for diversity of antibody binding sites is due to an intricate combination of recombination and mutation events (Hozumi and Tonegawa, 1976).

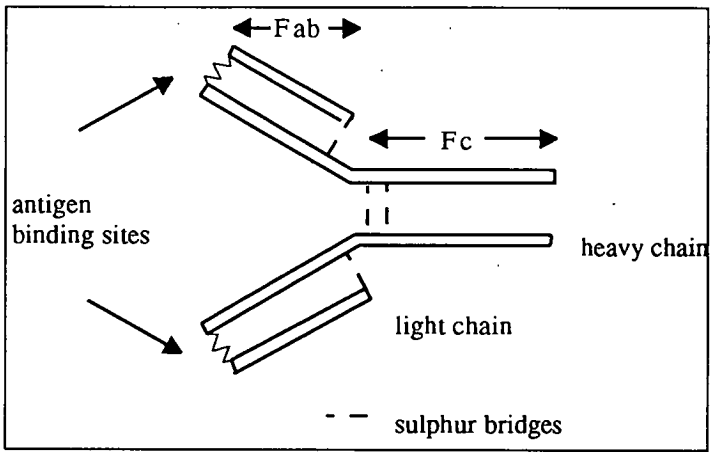


Figure 1.11. Structure of an IgG

The variable region of each chain comprises 3 areas of hypervariability referred to as complementarity determining regions (CDRs). It is these areas which bind directly to a site on the antigen referred to as the epitope. Structurally related epitopes, in molecules other than the antigen, are often recognised by an antibody. This phenomenon of specificity forms the basis of cross reactivity. The binding of an antibody-antigen complex depends on noncovalent reversible forces, including hydrogen bonds, van der Waals forces, coulombic interactions and hydrophobic bonds. The overall interaction is a balance of attractive and repulsive forces, and a small change in either the antigen or the amino acid sequence of the antibody can alter the stability of the interaction.

The basic thermodynamic principles of reversible biomolecular interaction apply to antibody-antigen binding. The strength of the binding (antibody affinity) is measured by the amount of antibody-antigen complexed at equilibrium. Compared to low-affinity antibodies, high-affinity antibodies bind larger amounts of antigen in a shorter period of time, and the dissociation rate of the complex is greater. Consequently, high affinity antibodies enhance performance in immunochemical techniques. The affinity constants measured for antibodies ranges from below 10^5 mol^{-1} to above 10^{12} mol^{-1} (Harlow and Lane, 1988). The avidity of an antibody-antigen complex refers to the overall stability, in terms of affinity, valency and geometric arrangement. Antibodies are multivalent (i.e. IgGs have 2 binding sites), and antigens can possess more than one epitope. It is therefore possible for two antibodies to bind one antigen, and for one antibody to bind two antigens. Multivalent interactions stabilise immune complexes and can allow low affinity antibodies to bind tightly to antigens. Immunochemical techniques which allow multivalent interactions can work well with low affinity antibodies, while techniques dependent on a monovalent interactions may fail to give results with these molecules.

1.5.2.2 Antibodies to DNA

The disease systemic lupus erythematosus (SLE) is characterised by the production of anti-DNA antibodies (reviewed by Avrameas and Trenynck, 1993). It is therefore known that the immune system has the capacity to produce antibodies to DNA. As early as the 1960's and 1970's researchers had begun to investigate the possibility of eliciting an immune response with natural complexes containing DNA. During this time antibodies were raised to ribonucleotides and ribonucleosides (Erlanger and Beiser, 1964), purines (Butler *et al,* 1962), pyrimidines (Garro *et al,* 1971), nucleic acids (Wallace *et al,* 1971), and denatured DNA (Plescia *et al,* 1964). The development of antibodies to carcinogen modified DNA occurred during the 1980's, creating the potential for quantification of DNA lesions. Since this time researchers have aimed to raise antibodies to a variety of DNA modifications so that immunological probes for the detection of potentially dangerous mutations can be developed. Specific antibodies to a variety of modified bases in DNA have now been developed and characterised (Briscoe *et al,* 1978; Muller and Rajewsky 1980; Saffhill *et al,* 1982).

There are several practical problems of inducing antibodies to carcinogen-DNA derivatives which are normally too small to elicit an immune response. This will be

discussed in further detail in chapter 2. The major shortcomings of immunological techniques are largely concerned with antibodies which cross react with more than one antigen. Generally antibody sensitivity is greatest for the original antigen, although many antibodies also recognise structurally related compounds (Santella, 1988). More recently this cross-reactivity has been used to an advantage to allow the production of class-specific antibodies, eliminating the requirement of a single antibody for every possible DNA lesion.

There are a number of different immunological techniques which can be applied in the identification of DNA lesions. Competitive radioimmunoassay (RIA); competitive or non-competitive enzyme linked immunosorbent assay (ELISA); and ultrasensitive radioimmunoassay (USERIA) are most commonly used. RIA involves mixing a tracer of radiolabelled antigen with an antibody and increasing concentrations of the unlabelled antigen requiring quantification (i.e. O^6-alkyl G). This generates a standard curve of inhibition of the antibody binding to the radiolabelled tracer. Precipitation of the antibody-antigen complex allows determination of the amount of bound tracer. Low levels of bound tracer indicate the presence of high levels of O^6-alkyl G, as this has competed with tracer for binding to the antibody. In ELISA the antigen is fixed to a plate and a standard curve of inhibition of antibody binding is constructed by adding a mixture of antibody and decreasing concentrations of the antigen requiring quantification (i.e. O^6-alkyl G). Binding of antibody to the plate is detected with a second antibody conjugated to an enzyme which produces a colour reaction on the addition of the appropriate substrate. Low levels of antibody binding to the antigen on the plate suggests that there are high levels of antigen in the mixture being investigated. USERIA is similar to ELISA except that the substrate is radiolabelled. These techniques can theoretically allow the detection of lesions at levels of 1 in 10^8 bases in 25-50μg of DNA (Santella, 1988), but more sensitive techniques have been developed (see below).

1.5.3. ^{32}P-Postlabelling techniques

The ^{32}P-postlabelling technique (Randerath K,*et al*, 1981) can be used to detect 1 lesion in 10^{10} bases in 1-10μg of DNA. Additionally, this technique is not lesion specific and can therefore detect multiple lesions in complex mixtures of DNA. The assay, which enzymatically incorporates radiolabel into the DNA constituents, consists of 5 biochemical reactions involving, i) digestion of the DNA containing the lesions into nucleotides, ii) enrichment of the lesions by eliminating normal

nucleotides, iii) attachment of a ^{32}P label, iv) separation of the lesions by high resolution TLC and detection by autoradiography then, v) measurement of radioactivity to quantify lesions.

Enrichment of the lesion is a crucial step in the assay. The principle is to eliminate normal nucleosides without eliminating or modifying the lesion, so that ultimately only the lesion becomes ^{32}P labelled. There are several versions of the assay each varying in the enrichment step, for example the standard (Randerath K. *et al* 1981) and ATP deficient (Randerath E. *et al*, 1985) assays do not involve this step. This decreases sensitivity to the detection of 1 lesion in 10^7 to 10^8. The two most commonly used enrichment techniques are butanol (Gupta, 1985) and nuclease P_1/S_1 (Reddy, 1986 and 1991) enrichment. The former involves an aqueous/organic solvent separation, where aromatic or hydrophobic lesions are partitioned into the organic solvent (butanol), while normal nucleotides remain in the aqueous phase. The P_1/S_1 technique utilises the P_1 and/or S_1 nuclease enzymes to hydrolyse normal 3'-nucleoside monophosphates to nucleosides, which cannot be ^{32}P labelled due to the absence of the phosphate group. The structure of many lesions renders them resistant to hydrolysis by these enzymes and they therefore continue to possess a phosphate group and become selectively ^{32}P labelled.

The enrichment techniques differ greatly in lesion selectivity (Beach and Gupta, 1992). For example dibenz[a,h]anthrancene diolepoxide DNA derivatives are recovered equally well by the butanol and nuclease procedures, while aceanthrylene derivatives are recovered at levels 2-fold higher in the nuclease P_1, compared to the butanol procedure. Additionally, aromatic amine derivatives are almost non-recoverable by butanol extraction, but a large number are isolated by the nuclease P_1 procedure. Unknown samples can be tested by ^{32}P postlabelling, but absolute quantification of all lesions present may be impossible. Firstly, some may be eliminated by the enrichment procedure used, and secondly the efficiency of ^{32}P labelling differs between lesions. In many cases it is therefore necessary to know the exact characteristics of the lesions of interest.

DNA derivatives which result from alkylation have been detected by this technique, but the efficiency of detection has been poor as these derivatives cannot be extracted from normal DNA digest mixtures by the butanol or nuclease P_1/S_1 enrichment techniques. If the enrichment step is not carried out normal nucleotides will be present during the TLC separation step. TLC does not allow good separation

between alkylated and normal DNA constituents. The development of an efficient enrichment step for the extraction of alkylated nucleotides therefore appears to be an important requirement to allow the detection of quantities as small as 1 in 10^{10} of these derivatives.

1.5.4 Immunoaffinity purification as an enrichment technique

Immunoaffinity chromatography (IAC) is a separation method based on specific and reversible interactions between an antigen and an antibody immobilised on a solid support. Generally, the antibodies are chemically bound to beads which are then used to construct a column. The digested DNA containing the lesion to be quantified is applied to the top of the column. Immobilised antibody binds the lesion retaining it on the column, while the normal DNA is unretained. The pure lesion is eluted using an appropriate eluant. The coupling of antibodies to solid supports, loading, washing and elution strategies, and variability in column capacity, are discussed in detail in chapter 4. The major requirement for this enrichment technique is an antibody with a fairly high affinity for the lesion of interest. Recently, antibodies specific for O^6-MeG were immobilised on sepharose and used in immunoaffinity mode to enrich O^6-MeG (Cooper *et al*, 1992). This derivative was subsequently quantified by ^{32}P postlabelling and two dimensional TLC. The detection limit was determined to be 1 O^6-MeG in 10^8 normal deoxyguanosines, and 0.38, 0.39 and 0.45 μmol of O^6-MeG per mol of dG were found in three human samples respectively.

1.6 Aims

The present study aimed to produce antigenic compounds containing the carcinogenic O^6-alkyl dG lesions and subsequently produce a class specific antibody capable of recognising a range of O^6-alkyl dG derivatives. It was hoped that the synthesis of a variety of O^6-alkyl dG derivatives, their conjugation to proteins and their incorporation into oligonucleotides would lead to an immune response following immunisation. One antibody which recognises a class of compounds eliminates the need for a single antibody for each derivative, allowing an initial speedy identification of the amount of damage caused by a class of compounds. The project aimed to identify an O^6-alkyl dG class specific antibody by developing appropriate ELISA experiments, and it was proposed that such an antibody would be taken to monoclonality. The ultimate purpose of the study was to construct immunoaffinity columns to allow the enrichment of O^6-alkyl dG from normal DNA for the ^{32}P postlabelling assay.

This approach requires the chemical synthesis of O^6-alkyl G derivatives and their conjugation to a protein or incorporation into oligonucleotides, the details of which are discussed in chapter 2. Production and isolation of a class specific antibody requires extensive immunisation and screening strategies, which are described in chapter 3. Finally, chapter 4 outlines the problems in immobilising antibodies on solid supports and the application of immunoaffinity chromatography.

Chapter 2. The Synthesis and Characterisation of Antigens Containing O^6-alkylguanine

2.1. Introduction

Alkylation of guanine in the 6-position by methylating and ethylating agents has received a great deal of attention during the last decade. The mutagenic properties of O^6-MeG and O^6-EtG have been demonstrated by, i) replicating synthetic DNA templates (Saffhill *et al*, 1985), ii) exposing cells to alkylating agents then sequencing the cellular DNA (Richardson, *et al* 1987) and iii) site directed mutagenisis (Chambers *et al*, 1985). The rate of methylation at the O^6 position of G (*in vivo* and *in vitro*) is generally faster than that of ethylation (Singer 1975). The existence of repair mechanisms which have become specifically adapted to the repair of O^6-MeG, results in relatively low numbers of this lesion in DNA. In comparison, O^6-EtG is repaired at a low level, therefore a fairly high number are present in DNA despite the lower reactivity of ethylating agents.

The mutagenic activity of higher O^6-alkyl G derivatives has received less attention, although di-n-butylnitrosamine has been shown to cause mutations (Spiegelhalder and Preussmann, 1983) and di-n-propylnitrosamine has been shown to induce liver tumours in rats (Druckrey, 1967). Nitroso compounds with larger alkyl chains have been found to produce a higher O^6-alkyl G : 7-alkyl G ratio than methylating and ethylating agents (i.e. a ratio of 0.7 compared to 0.1) (Saffhill *et al*, 1985). The larger O^6-alkyl G derivatives are often considered to be less mutagenic as they are more easily detected and repaired by enzymes (Basu and Essigmann, 1990). This should result in a reduction in mutagenicity as the size of the alkyl group increases. This was supported by a later study which reported mutation frequencies of 8-11% with O^6-MeG and O^6-EtG compared to 0.9% with O^6-n-propyl and O^6-n-butyl G (Chambers, 1991). In contrast, mutation frequencies of 2-3% were found with n-propyl and n-butyl derivatives (Baumgart *et al*, 1993). An O^6-n-octyl dG was also investigated and found to be equally as mutagenic as the smaller O^6-alkyl G derivatives, suggesting that larger alkyl groups are not less mutagenic. Large O^6-alkyl Gs are thought to be repaired by excision repair mechanisms rather than by alkyl transferases (Mackay *et al*, 1992). This may explain the discrepancies in the findings of these researchers. In addition, the sequences surrounding the lesion are believed to influence the efficiency of repair (Liem *et al*, 1993).

In addition to alkylating compounds, hydroxyalkylating agents have been investigated and found to react at the O^6 position of G, (Kohda *et al*, 1987). In comparison with ethylating agents a hydroxyethylating agent was shown to induce cancer in mice at a similar incidence, and with a shorter latency period (Swenson *et al*, 1979). Hydroxyethylating agents are widespread in the environment from industrial pollution and as metabolites of nitrosamines (von Hofe *et al*, 1986). In addition, ethene and ethylene oxide, which are used extensively in the chemical industry, have been shown to produce hydroxyethylated DNA derivatives (Segerback, 1983).

2.1.1 Requirements of an O^6-alkyl G antigen

The production and selection of a class specific antibody for the detection of these potential carcinogenic lesions requires immunisation and screening with a variety of O^6-alkyl G derivatives. The two limiting steps in the production of a suitable O^6-alkyl G antigen are, i) the availability of O^6-alkyl G and ii) its conversion into a compound capable of eliciting an immune response. Generally, O^6-alkyl G derivatives are not commercially available and have to be produced chemically in the laboratory or extracted from cells treated with alkylating compounds. In addition, substances with molecular weight less than 1000 do not normally elicit an immune response. Therefore antibodies to single bases, deoxynucleosides or deoxynucleotides are induced by immunisation with complexes made up of the low molecular weight substance conjugated to proteins or incorporated into synthetic immunogenic molecules. Bases, nucleosides and nucleotides can be conjugated to proteins. Antibodies raised to deoxynucleoside or deoxynucleotide conjugates are likely to have higher recognition for native DNA than antibodies to free bases (Erlanger and Beiser, 1964).

In the present study we therefore used O^6-alkyl-2'-deoxyguanosines (O^6-alkyl dG) and related derivatives. The specific alkyl groups investigated were methyl, ethyl and n-propyl. The difference in structure between these three groups is small (fig 2.1) so an antibody to any one derivative may have the potential to cross react with another. In addition, O^6-hydroxyethyl dG (O^6-hydroxyEt dG) was investigated. This derivative differs from O^6-ethyl dG (O^6-EtdG) by the addition of a single hydroxyl group, but has an overall similar shape (fig 2.1). An antibody with specificity relying on shape may cross react with the O^6alkyl dG derivatives and O^6-hydroxyEtdG. An O^6-(4-carboxybenzyl) dG derivative (O^6-CBdG) was also used. This group produces an O-CH_2 bond at the O^6 position of dG (fig 2.1).

Immunisation with this compound may produce antibodies which recognise the O^6 position of dG linked to a CH_2 group plus any small group.

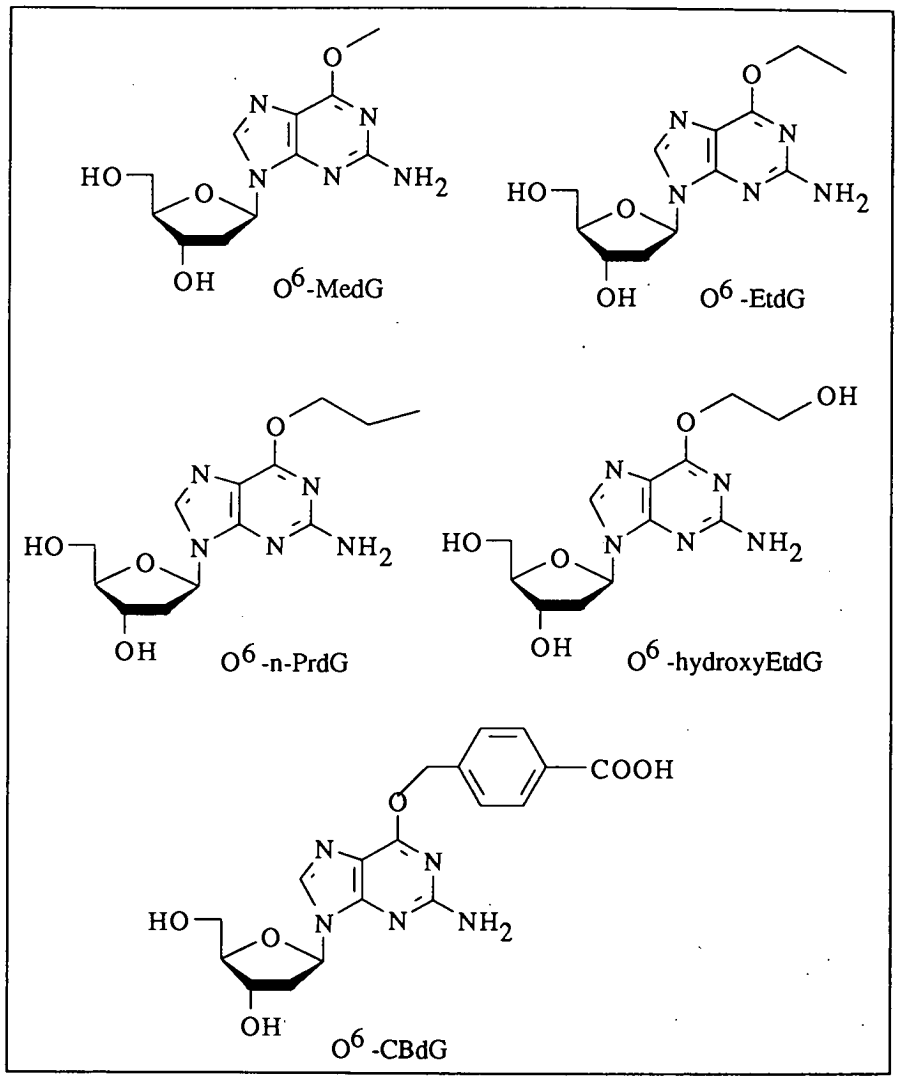


Figure 2.1. O^6-alkyl dG and related derivatives

These O^6 modified dG derivatives were converted into antigens by conjugation to proteins. In addition, some of the derivatives were incorporated into oligonucleotides to investigate a novel approach for producing antibodies to modified deoxynucleosides.

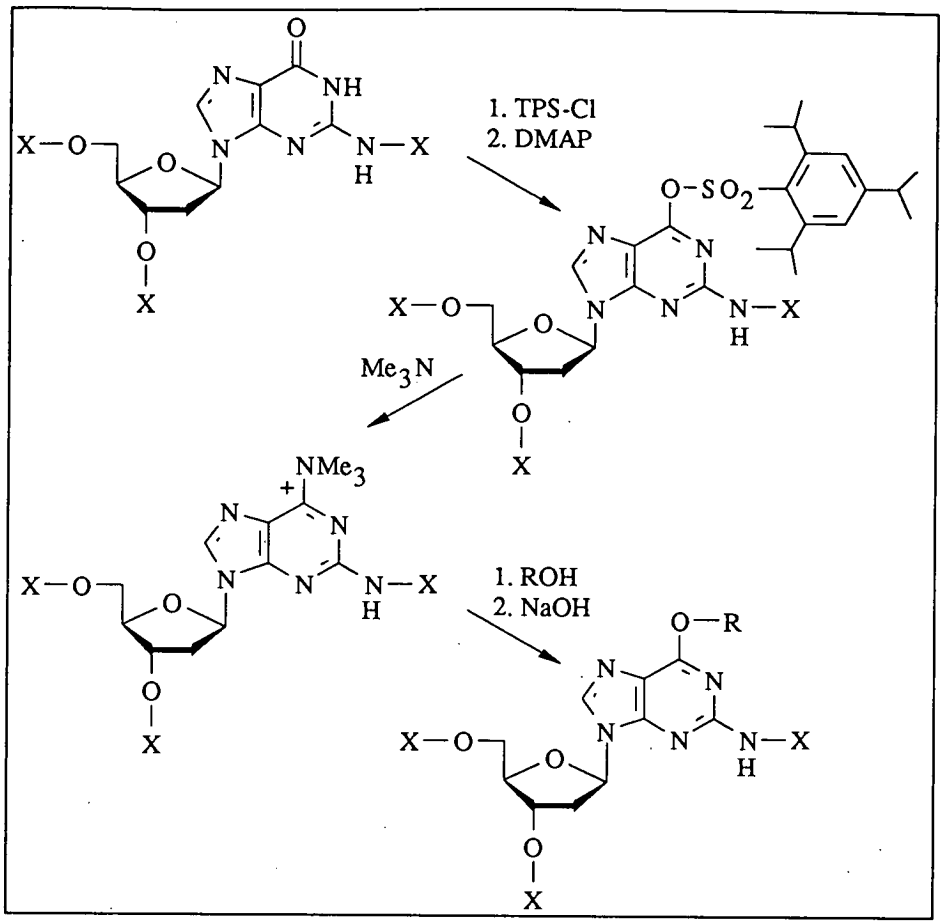
2.1.1.1.Synthesis of O^6-alkyl dG

General strategies for the chemical synthesis of modified bases have been outlined. Initially O^6-alkyl G was synthesised by attaching a thiol group to the O^6 position, then displacing it with chlorine. The O^6-chloro G derivative was converted to O^6-

alkyl G after reaction with an excess of sodium alkoxide in the corresponding alcohol (Balsinger and Montgomery, 1960). This procedure is not applicable to the synthesis of O^6alkyl dG. The most commonly used procedure for the synthesis of the modified nucleoside is that of Farmer *et al* (1973) in which an ethereal solution of diazoalkane is reacted with the nucleoside. The predominant product is 7-alkyl dG (42%), followed by O^6-alkyl dG (24%), and a variety of additional modifications including N^1-alkyl and N^2,O^6-dialkyl dG. Low yields due to non-selectivity of the site of alkylation of diazoalkanes makes this an undesirable method for large scale synthesis of O^6-alkyl dGs.

Biological procedures for the production of O^6-alkyl dGs have been employed (Schendel and Robins, 1978; Hochleitner *et al*, 1991). Cells grown in the presence of alkylating agents are killed by adding ice-cold medium. The membranes are lysed and DNA and RNA obtained by phenol-chloroform extraction. After digestion by RNase enzymes, the desired modified products are selected by chromatography. These purification procedures are lengthy as many modified derivatives are produced.

A practical route for the synthesis of O^6-alkyl dGs, which avoids direct use of alkylating reagents, was outlined by Gaffney and Jones (1982). Triisopropylbenzenesulphonyl chloride, is attached to the O^6 position then displaced by a nitrogen nucleophile (trimethylamine), which is displaced by alkoxide ions (fig. 2.2). A similar route was followed by Li and Swann (1989) but the protecting groups were adapted to allow the synthesis of O^6-alkyl dG for incorporation into oligonucleotides. We therefore followed this procedure for the synthesis of the O^6 modified dG derivatives in this study.



TPS= triisopropylbenzenesulphonyl; DMAP= dimethylaminopyridine; R= alkyl group; X= an appropriate protecting group

Figure 2.2. Modification of the O^6 position of dG (Gaffney and Jones, 1982)

2.1.2 Conjugation of modified nucleosides to protein carriers

The immunochemistry of low molecular weight compounds began in 1917 when Landsteiner established that the original specificity of an antibody towards a protein was changed by introducing a small group referred to as a hapten. The antibodies recognised the small groups, although they were not themselves immunogenic.

Complexing DNA with methylated protein has allowed the production of anti-DNA antibodies (Butler *et al*, 1962; Tanenbaum and Beiser, 1963; Erlanger and Beiser, 1964; Poirier *et al*, 1980). The complex results because the methyl groups convert the carboxylic acid groups of the protein to methyl esters. At acidic pH the positively charged amino groups of the protein couple with the negatively charged phosphate backbone of DNA. Free nucleosides and nucleotides require covalent coupling to proteins. Serum albumins, immunoglobulins, ovalbumin and haemocyanin are the most commonly used proteins. Linking procedures have taken

advantage of carboxyl groups of aspartic and glutamic acid, amino groups of lysine, the imidazole function of histidine, the phenolic function of tyrosine and sulphydryl groups of cysteine residues in proteins. The most commonly applied techniques are summarised in table 2.1.

Table 2.1. Methods For The Conjugation of Nucleotides and Nucleosides to Proteins

Method	Reference
Periodate oxidation of the ribose moiety of ribonucleosides or 5' nucleotides followed by linkage to amino groups of proteins	Erlanger and Beiser 1964
Succinylation of a hydroxy group followed by linkage of the free succinyl carboxyl group to amino groups of a protein using a water soluble diimide	Steiner *et al* 1969/1972
Use of water soluble carbodiimides to conjugate nucleotides to proteins *via* a phosphoramide linkage	Halloran and Parker 1964, 1966
Oxidation of the 5'OH group of nucleosides to a carboxyl group followed by use of a carbodiimide to link the COOH to an amino group on the protein	Sela *et al* 1964
The formation of diazotisable derivatives of an azoprotein	Garbar *et al* 1968
Linkage *via* the C^6 atom of a purine to produce a purin-6-oyl protein conjugate	Beiser *et al*, 1968

The most widely applied technique for ribosides is that of Erlanger and Beiser, in which the 2' and 3' C atoms of the *cis* diol moiety of ribose are oxidised resulting in ring opening and the production of two aldehyde functions. Free amino groups on proteins react spontaneously with these groups, and after treatment with sodium borohydride a morpholine ring system is formed. This procedure cannot be applied to deoxyribonucleosides which do not possess adjacent hydroxyl groups and therefore cannot undergo the periodate reaction. The most common coupling

procedure for deoxynucleosides is succinylation of the deoxyribose hydroxyl group followed by coupling of the resulting carboxylic acid group to an amino group on a protein using a carbodiimide (Steiner *et al*, 1969). Carbodiimides are ideal for use with modified DNA as they function in very mild conditions therefore chemical alteration of the modified DNA is avoided. Carbodiimides are also employed to link deoxynucleosides to proteins *via* the 5' hydroxyl group after oxidation to a carboxyl group (Sela *et al*, 1964), and to link deoxyribonucleotides *via* the phosphate group to form a phosphoramide linkage (Halloran and Parker, 1966). The remaining methods described in Table 2.1 are not commonly used with modified nucleosides and nucleotides because of possibile destruction or alteration of the overall structure of the base.

In this study O^6-MedG, O^6-EtdG, O^6-n-PrdG and O^6-hydroxyEt dG were conjugated to bovine serum albumin (BSA) and chicken gamma globulin (CγG) by succinylating the 3' hydroxyl groups of the nucleosides followed by linkage to amine groups of proteins using a carbodiimide (Steiner, 1969). The carboxyl group of O^6-(4-carboxybenzyl) dG was also activated by a carbodiimide and conjugated to amine groups of BSA and CγG.

2.1.3. Oligonucleotide synthesis

There are two solid phase oligonucleotide synthesis methods in general use, i) the phosphotriester method (Letsinger and Mahadevan, 1965) and ii) the more commonly used phosphoramidite method (Beaucage and Caruthers, 1981). The development of the phosphoramidite method has recently been reviewed by Beaucage and Iyer (1992) and the practical synthesis and purification of oligonucleotides by this method has been described by Brown and Brown (1991).

In short, solid phase synthesis involves the assembly of an oligonucleotide on an inert solid support. A cycle of events in which the required reagents are delivered to the growing molecule is repeated for the addition of each new nucleotide. The entire reaction and work up (filtering off excess reagents and by-products, then washing before the next step) takes place in one reaction vessel. At the end of the synthesis the oligonucleotide is cleaved from the solid support and purified. The required reagents and nucleotides (which must be appropriately protected) are commercially available, adding to the ease of synthesis.

In the past synthetic oligonucleotides were not considered immunogenic unless coupled to a protein carrier. Most immunisations carried out with oligonucleotides-protein conjugates used short (10-20 nucleotides), low molecular weight molecules. Advancements in automated solid phase DNA synthesis means that oligonucloetides of up to 150 residues can be easily synthesised. In addition, the development of procedures for modifying the phosphate backbone of DNA allows the synthesis of enzymatically stable compounds. O^6-MedG, O^6-EtdG, O^6-n-PrdG and O^6-hydroxyEt dG were therefore incorporated into oligonucleotides to investigate the possibility of producing antibodies to O^6 modified dG by immunising with these compounds.

An O^6-alkyl dG moiety suitable for incorporation into an oligonucleotide must possess the following characteristics:
i) a 4',4"-dimethoxytrityl temporary protecting group on the 5' hydroxyl position. This group prevents self polymerisation of the nucleotide, and its removal by trichloroacetic acid during oligonucleotide synthesis allows the efficiency of the synthesis to be monitored.
ii) a 2-cyanoethyl diisopropyl phosphoramidite linking group on the 3' hydroxyl - this is an essential group containing the phosphate (which is protected) necessary for oligonucleotide synthesis.
iii) a suitable protecting group on the amino group of the base . This must remain stable during the course of the synthesis, yet be labile for removal after the synthesis.

During the synthesis of oligonucleotides containing O^6-alkyl dG there are a significant number of side-products (Borowy-Borowski and Chambers, 1987). Many result from exposure of the oligonucleotide to the harsh conditions necessary for the removal of the protecting group from the base, e.g. benzoyl and isobutyryl deprotection of the base has been reported to take 72 hr in ammonia at 65°C. The predominant impurity is a 2,6-diaminopurine (DAP), which can result during the ammonia deprotection. Nucleophilic attack by the ammonia at the O^6 position results in displacement of the O^6-alkyl group by an amino group. The presence of a DAP in oligonucloetides has been shown to increase stability of duplexes as it can form three hydrogen bonds (Howard *et al*, 1966; Scheit & Rackwitz, 1982). In addition, this molecule has similarities with with both adenine and guanine. Therefore, a DAP in an oligonucleotide has the potential to mispair with other

nucleosides (Krieg, 1963). For this study DAP is an undesirable side product, since it will reduce the number of O^6-alkyl dG residues in the oligonucleotide.
There are two possible solutions to this problem, i) to deprotect with an alternative reagent or, ii) to use a more labile N^2 protecting group which can be removed under less harsh conditions. The removal of an isobutyrl protecting group with methoxide ions has been reported (Gaffney and Jones, 1982). This procedure is lengthy (72hr) and is not applicable to O^6 modified dG derivatives other than O^6-MedG, as methoxide ions can exchange with the larger O^6-alkyl groups. A phenylacetyl group has been used to protect the base. Removal of this group after 50 min in ammonia at room temperature has been reported (Li and Swann, 1989). At the end of normal oligonucleotide synthesis the product is dissolved in a concentrated aqueous ammonia solution, which also removes the cyanoethyl protection on the phosphates. Phenylacetyl protection was chosen for the present study as it requires straightforward, mild deprotection procedures.

2.2. Results and discussion

The conversion of 2'-dG to an O^6-alkyl dG derivative suitable for i) conjugation to a protein, and ii) incorporation into an oligonucleotide, involved a number of reactions. The general procedure followed (Li and Swann, 1989) required slight variations depending on i) whether the final product was to be conjugated to a protein or incorporated into an oligonucleotide and ii) the nature of the alkyl group being attached.

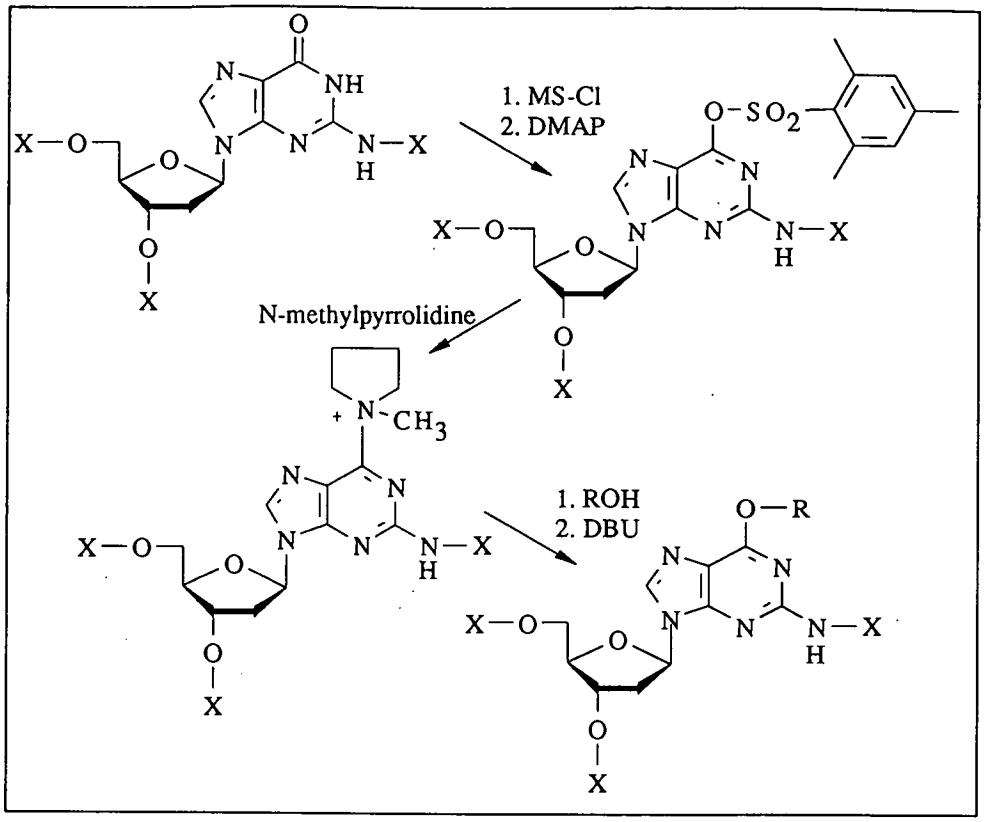
2.2.1. Selective alkylation of the O^6 position of dG

Mesitylene sulphonyl chloride was used to sulphonate the O^6 position of dG to form the O^6-sulphonated derivative [a]. The sulphonyl group was displaced by N-methylpyrrolidine to form derivative [b]. This extremely nucleophilic group easily displaces the sulphonyl group in addition to being readily displaced itself by alkoxide ions allowing the O^6-alkyl derivative [c] to be formed (fig. 2.3). The entire three step reaction was carried out in 90-240min. (depending upon the alkyl group being added) in a one flask procedure. A strong base was required to produce the alkoxide ions which react at the O^6 position of dG. As most nucleophiles react at the O^6 position as well as converting the alcohol to alkoxide ions, 1,8-diazabicyclo[4.5.0.]undec-7-ene (DBU), a strong base was used. The bicyclic structure of this compound prevents its reaction at the O^6 position, probably due to steric hindrance.

Methanol, ethanol and n-propanol were sucessfully converted to the alkoxide derivatives which subsequently reacted at the O^6 position. The reaction time for longer alkyl chains was slower and the yields lower (table 2.2). The lower yields were in part due to difficulties in purifying the higher O^6-alkyl dGs by flash chromatography.

Table 2.2. Efficiency of the alkylation reaction

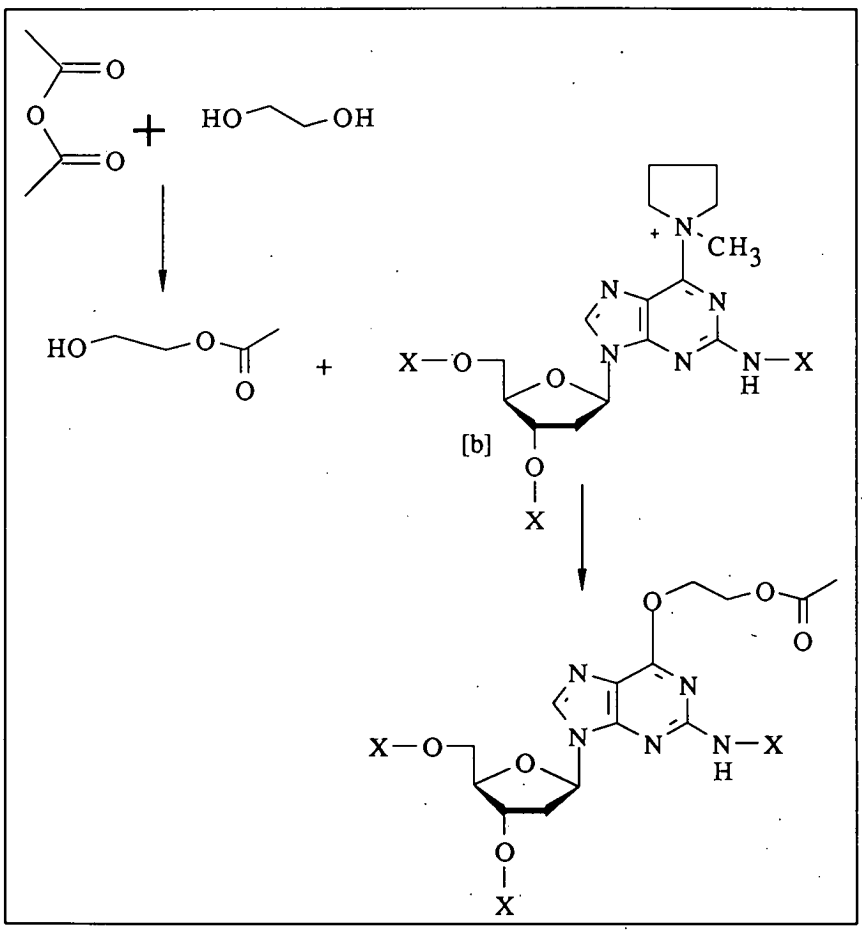
Alkyl Group	Reaction Time	Yield%
methyl	40min	77
ethyl	2hr	65
n-propyl	4hr	55
hydroxyethyl	4hr	33
4-carboxybenzyl	2hr	28



MS= mesitylenesulphonyl; DBU= 1,8-diazabicyclo[4.5.0]undec-7-ene; TEA= triethylamine: DMAP= dimethylaminopyridine; R= alkyl; X= an appropriate protecting group

Figure 2.3. Modification of the O^6 position of dG (Li and Swann, 1989)

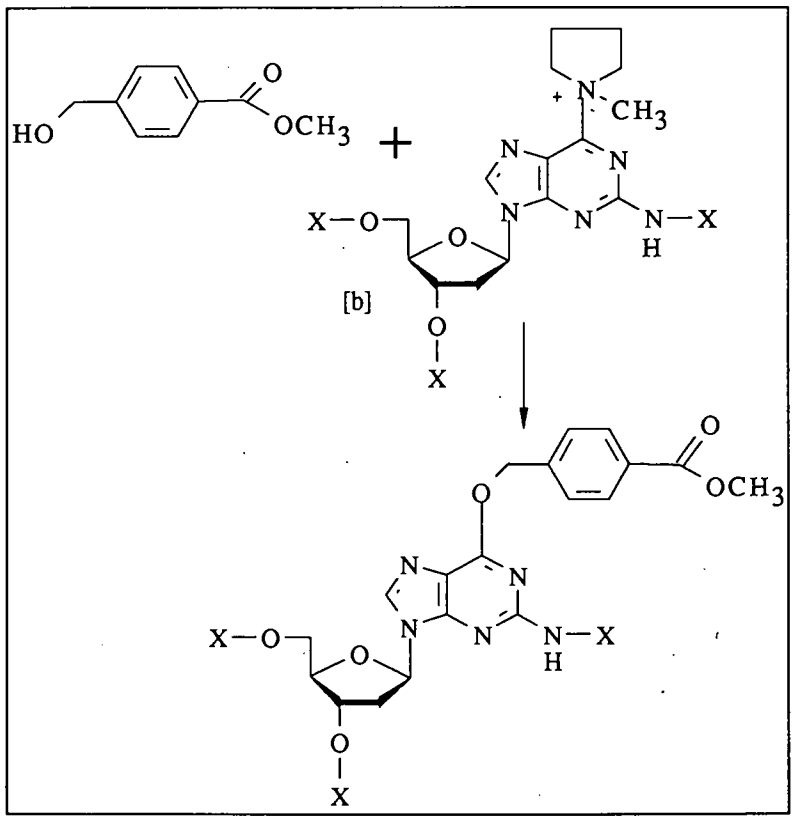
Ethylene glycol was used to add a hydroxyethyl group to dG. Acetic anhydride was reacted with an excess of ethylene glycol to produce monoacetyl ethylene glycol. This avoided the activation of both hydroxyl groups of ethylene glycol by DBU. This protected compound was sucessfully activated and added to the O^6 position of dG (fig. 2.4).



X = an appropriate protecting group

Figure 2.4. Production of O^6-hydroxyEtdG

O^6-CBdG was synthesised by reacting methyl-4-(hydroxymethyl)benzoate (MHMB) with DBU then with protected dG (fig 2.5). Only the hydroxymethyl function of this compound was activated by DBU. The ester methyl group, which acts as a protecting group during the O^6 substitution reaction was later removed to produce O^6-CBdG.



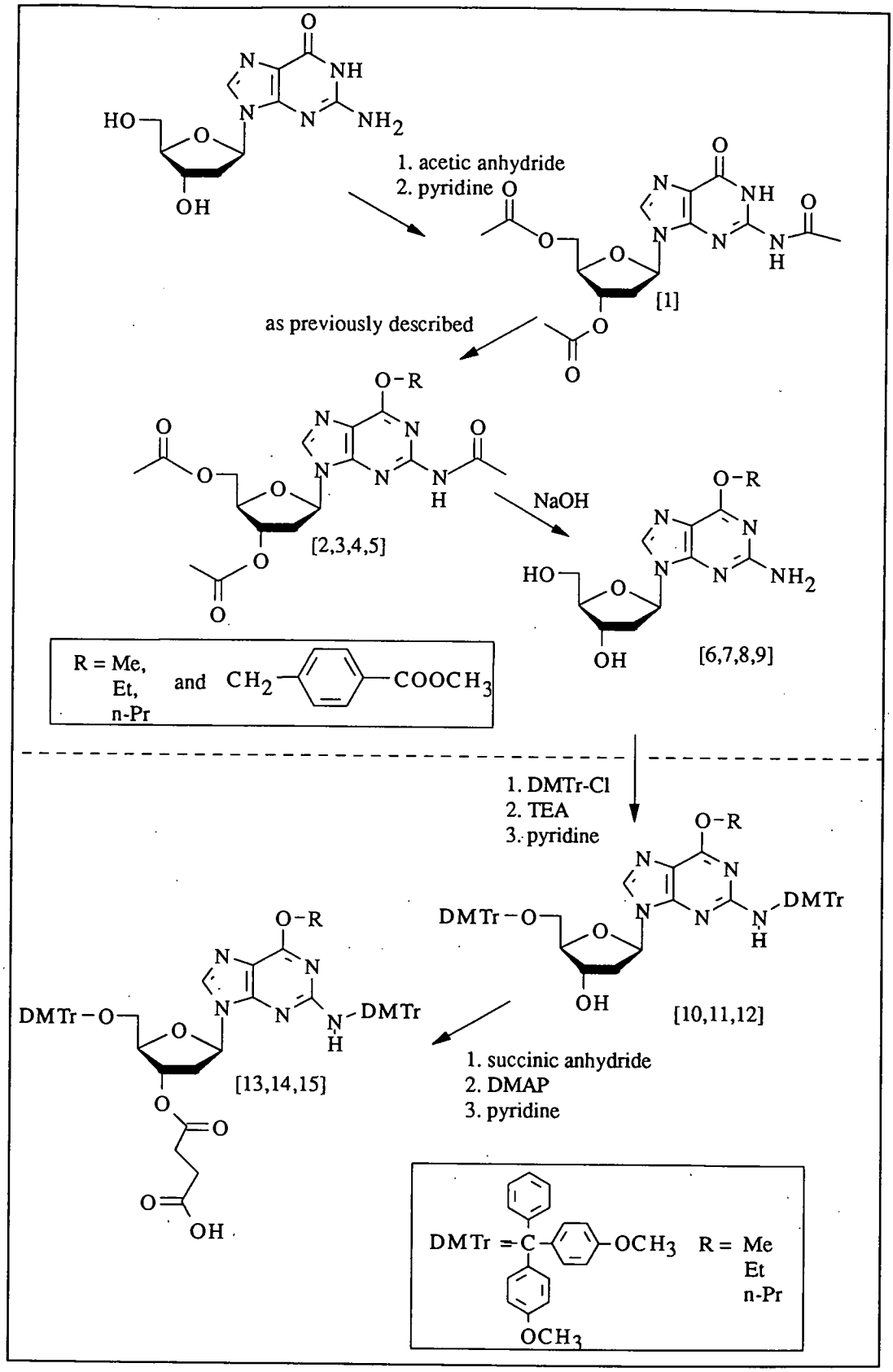
X = an appropriate protecting group

Figure 2.5. Production of O^6-CBdG

2.2.2 Synthesis of O^6-MedG, O^6-EtdG O^6-n-PrdG and O^6-CBdG for conjugation to a protein carrier (scheme 1)

The hydroxyl groups of the sugar and the 2-amino group of the guanine base were protected with acetyl groups by refluxing dG with acetic anhydride, to form 3',5',N^2-(triacetyl)-2'-dG [1]. The protected nucleoside was then modified at the O^6 position with methanol, ethanol, n-propanol and MHMB, as previously discussed, to give [2,3,4 and 5] respectively. The protecting groups were removed by the addition of 2.5M NaOH to give compounds [6,7,8 and 9]. The acetyl groups protecting the 3' and 5' hydroxyl positions were removed within 5 min at room temperature. The removal of the acetyl groups protecting the NH_2 position of the heterocyclic base required 24-48 hr.

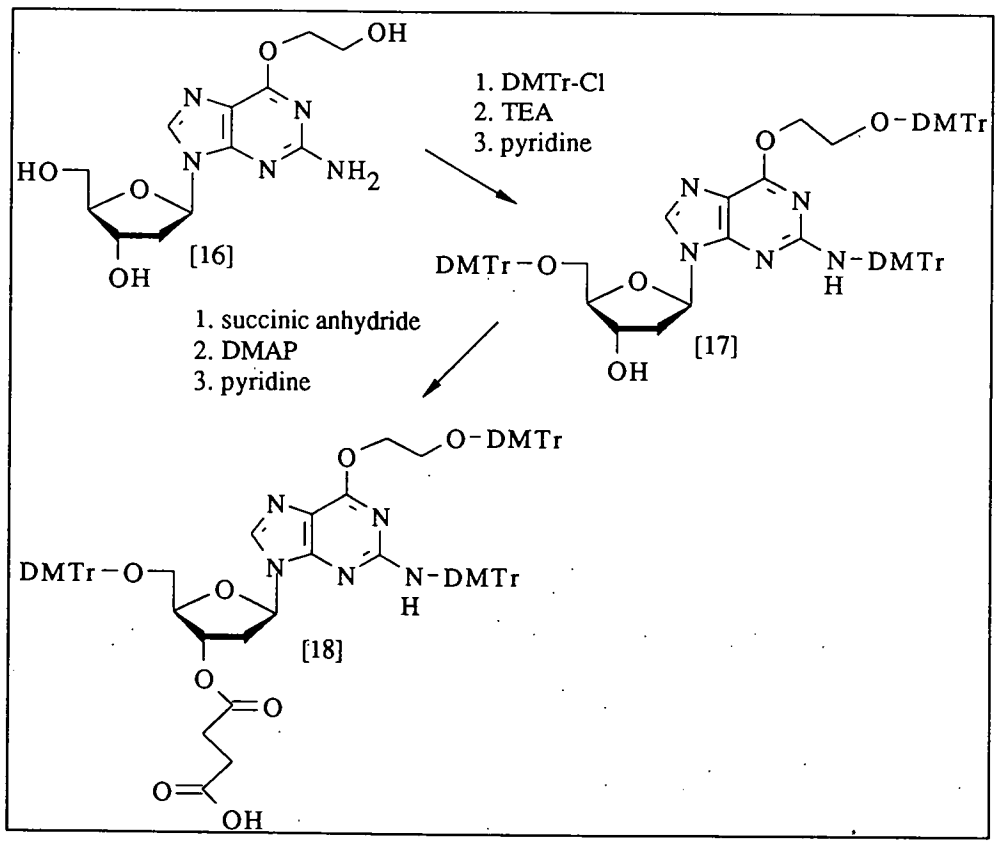
The O^6-(4-carboxybenzyl) derivative [9] was now in a suitable form for conjugation to a protein. The O^6-methyl, ethyl and n-propyl derivatives required the addition of a succinyl linker which was attached to the 3' hydroxyl group using succinic anhydride and DMAP as a catalyst. Succinic anhydride also reacts at the 5' hydroxyl group of the deoxyribose sugar and therefore this position was protected with a dimethoxytrityl (DMTr) group. DMTr chloride reacted primarily with the 5' primary hydroxyl group and minimal tritylation occured at the 3' secondary hydroxyl position. In addition to reacting at the 5'-OH, DMTr chloride also reacted with the NH_2 position of the base, and gave compounds [10,11,12], but this was not disadvantageous as in addition to providing a suitable protecting group DMTr provided a method to monitor the extent of nucleoside-protein conjugation. It was necessary to maintain alkaline conditions throughout the synthesis and purification of DMTr compounds, as neutral-acidic environments result in cleavage of the DMTr group. After the addition of the DMTr groups and a succinyl linker, O^6-methyl, ethyl and n-propyl dG, derivatives [13,14 and 15 respectively] were suitable for protein conjugation.



Scheme 1. Synthesis of O^6-alkyl dG for conjugation to a protein carrier

2.2.3. Synthesis of O^6-hydroxyEtdG for conjugation to a protein carrier (scheme 2)

O^6-hydroxyEt dG was synthesised using protecting groups suited to oligonucleotide synthesis and these will be discussed later. After modification of the O^6 position as previously discussed, all protecting groups were removed to obtain O^6-hydroxyEt dG [16]. Again it was necessary to add a succinyl linker before protein conjugation was possible. The reactive positions of O^6-hydroxyethyl dG (5' OH, 2-amino and hydroxyethyl OH) were therefore protected with DMTr groups and the succinyl linker added, as previously described for the O^6-alkyl dG derivatives, to give compounds [17] and [18] respectively.

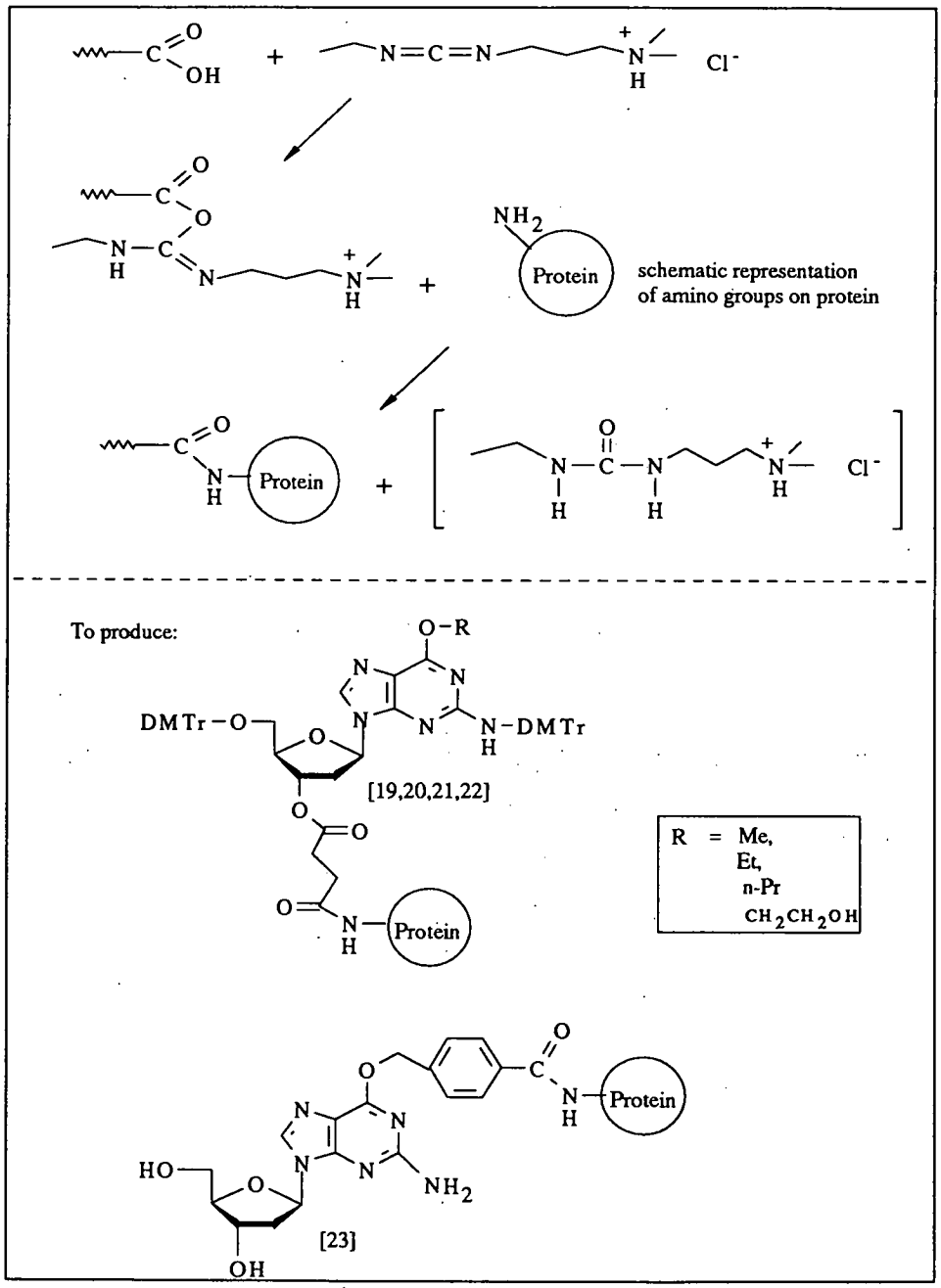


Scheme 2. Synthesis of O^6-hydroxyEt dG for conjugation to a protein

2.2.4. Protein Conjugation (scheme 3)

The succinylated O^6-MedG, O^6-EtdG, O^6-n-PrdG and O^6-hydroxyEt dG derivatives and O^6-CBdG, were treated with 1-ethyl-3-(3-dimethylamino)carbodiimide (EDC) to activate the carboxylic acid group of the succinyl linker and the carboxybenzyl group of O^6-CBdG. The carbodiimide reacted with the carboxyl group to produce an O-acyl isourea. This reacted readily

with amine groups on the lysine residues of the protein producing a protein-nucleoside conjugate and a urea salt. Each O^6 modified dG derivative was conjugated to BSA to produce [19,20,21,22,23]. BSA contains a proportionately high number of lysine residues (59) and dissolves readily in water. Conjugation to this protein was expected to yield highly substituted soluble conjugates. Conjugation to two proteins was necessary for antibody screening, therefore each O^6 modified dG derivative was also linked to CγG.



Scheme 3. Protein conjugation.

It was necessary to find a suitable solvent in which to conduct the conjugation reactions. In general, proteins are soluble in water but relatively insoluble in organic solvents. O^6-CBdG dissolved readily in water and was conjugated to both proteins without problems. O^6-MedG, O^6-EtdG, O^6-n-PrdG and O^6-hydroxyEt dG were insoluble in water and it was hoped that the use of a water miscible solvent would allow sufficient contact between the protein and the nucleoside for conjugation to occur. The solubility of BSA, CγG and each O^6 modified dG derivative was tested in a variety of 50:50 organic solvent/water systems. It was thought that the removal of the DMTr groups may make the modified nucleosides more water soluble, therefore a small sample of each was detritylated and also tested for solubility.

The solubilities of each O^6 modified dG derivative were similar. BSA was the more soluble protein (table 2.3). The proteins dissolved most efficiently in 50% aqueous pyridine. The tritylated O^6 modified dG derivatives dissolved readily in most solvent system investigated. Detritylation made the O^6 modified derivatives more water soluble, but they became less soluble in organic solvents (apart from DMF).

Table 2.3. Test of the solubility of BSA, CγG and tritylated and de-tritylated O^6 modified dG derivatives

	Test Compounds			
Solvent 50%aqueous soln	BSA	CγG	tritylated O^6 modified dG derivative	detritylated O^6 modified dG derivative
acetonitrile	*	*	O	O
DCM	**	*	****	O
1,4 dioxane	**	*	****	O
DMF	***	**	***	****
DMSO	***	**	O	O
pyridine	****	***	****	O

O= insoluble, *= slightly soluble, to ****=readily soluble

Conjugations were therefore carried out with tritylated nucleosides in a 50:50 solution of pyridine and water. Typically 5-10mgs of protein were dissolved in 0.4mls of water, and 0.1ml of pyridine was added. This introduced the protein

gradually to the solvent environment. Likewise, 10mgs of tritylated O^6 modified dG was dissolved in 0.4mls of pyridine and 0.1 ml of water was added. Water soluble carbodiimide (EDC) was added to this solution. The presence of the small amount of water allowed the carbodiimide to dissolve. Mixing of the samples often resulted in precipitation of the nucleosides and/or the proteins. For example, the first few drops of nucleoside solution added to the protein solution were in a very high concentration of water and therefore precipitated. The precipitates rarely redissolved on the addition of more solvent. Repeated experiments showed that rapid addition of one solution to the other resulted in the components remaining in solution.

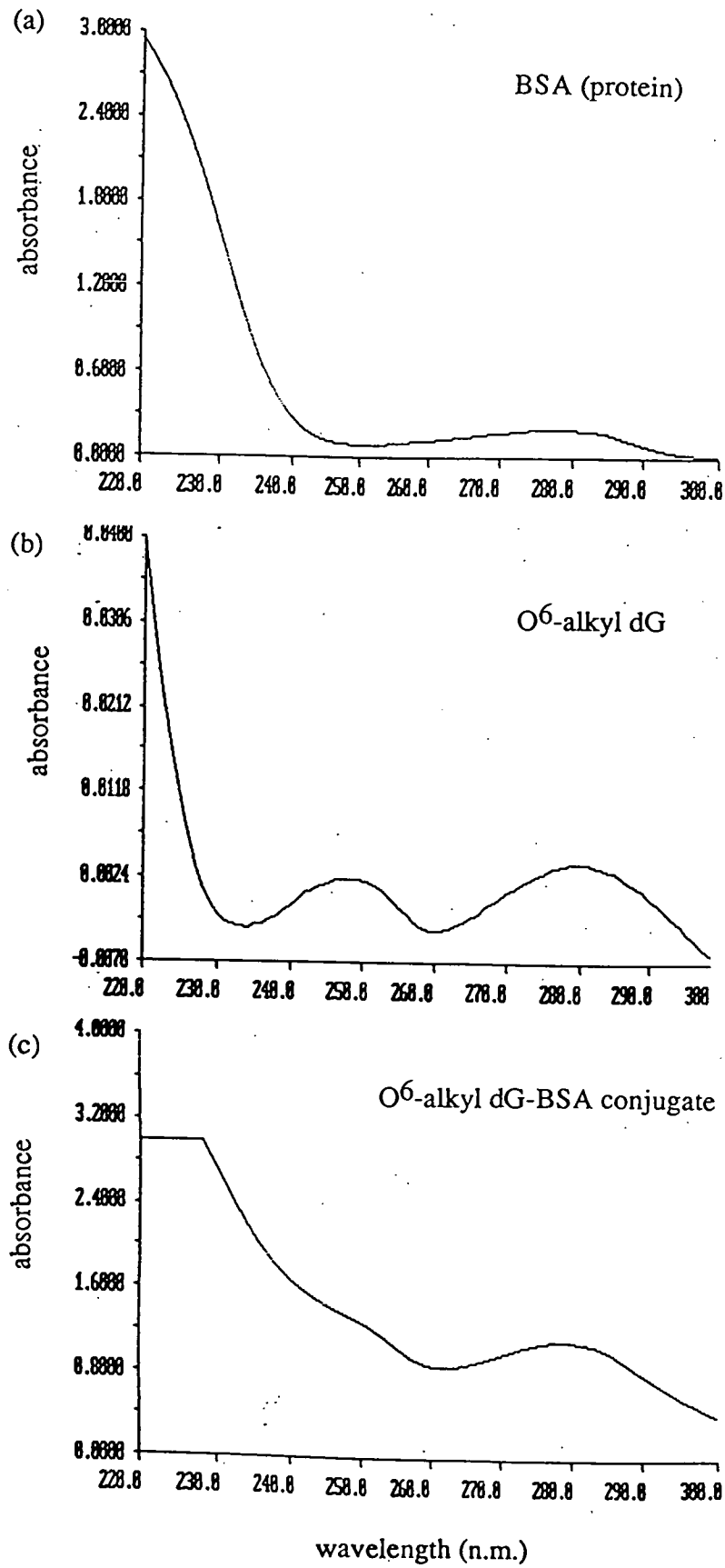
The conjugated products were not soluble in 100% water. This was attributed to the hydrophobicity of O^6 modified dG and its effect on the protein conjugate. Purification by gel filtration to remove the unconjugated nucleoside and excess carbodiimide was carried out in 50:50 ethanol/water. A small amount of each conjugate was kept aside to allow the extent of nucleoside-protein conjugation to be calculated. The remainder was detritylated using acetic acid and the products were purified by gel filtration.

2.2.5. Characterisation of conjugates

Substitution of 10 haptens per protein molecule have been reported to give a superior immune response subsequent to immunisation (Landsteiner,1945). A more recent investigation by Stollar (1980) which examined the work carried out to that date suggested that 8-25 haptens per protein led to the production of suitable antibodies. Despite these findings some researchers have produced excellent antibodies with as few as 5 haptens per protein (Klaus and Cross, 1974), although efficiency of antibody production is probably hapten dependent.

The extent of nucleoside substitution is usually determined from the ultraviolet (UV) absorbance spectrum. The protein contribution to the A_{260} and A_{280} of the conjugate is determined from the known spectrum of unmodified protein for a known protein concentration. Proteins have a weak absorbance around 260n.m. (fig 2.6a) whereas most nucleosides have a maximumat this point. The difference between the total A_{260} of the conjugate and the A_{260} of the protein is therefore largely contributable to the nucleoside. The concentration of nucleoside is then estimated by comparing the A_{260} to A_{280} ratio of the conjugate with non-conjugated protein at a similar concentration.

Figure 2.6 UV scans of a) BSA, b) O^6-alkyl dG and c) an O^6-alkyl dG-BSA conjugate



2.2.5.1. Calculating hapten substitution of O^6-MedG, O^6-EtdG O^6-n-PrdG and O^6-hydroxyEtdG conjugates

UV scans of succinylated O^6-MedG, O^6-EtdG, O^6-n-PrdG and O^6-hydroxyEtdG showed that these derivatives have a minimum absorbance at 260n.m. and maximum absorbance at 245 and 285n.m (fig. 2.6b). Therefore a direct comparison of A_{260}:A_{280} is not an accurate index of the extent of hapten substitution. The degree of conjugation was therefore estimated by comparing the A_{245} where the modified nucleoside has maximal absorbance, with the A_{260} where the protein has minimal absorbance. For non-conjugated protein the A_{245}:A_{260} was approximately 0.6:1 (table 2.4).

Table 2.4. Absorbance of 1mg of each conjugate

		Absorbance at:			Ratio
Protein	**O^6modified dG**	A_{280}	A_{260}	A_{245}	A_{245}:A_{260}
BSA	-	0.28	0.20	0.13	0.65
CγG	-	0.34	0.23	0.14	0.61
BSA	O^6-MedG	38.7	28.2	47.4	1.68
BSA	O^6-EtdG	39.3	26.9	39.5	1.47
BSA	O^6-n-PrdG	39.2	29.7	42.3	1.42
BSA	O^6-hydroxy Et dG	38.2	33.5	41.3	1.23
CγG	O^6-MedG	40.3	27.0	44.8	1.66
CγG	O^6-EtdG	37.2	27.2	45.6	1.68
CγG	O^6-n-PrdG	36.8	30.6	38.2	1.25
CγG	O^6-hydroxy Et dG	38.8	26.3	40.8	1.55

The UV scans of the conjugates showed an increased absorbance at 245n.m. relative to protein (fig 2.6c). The A_{245}:A_{260} ratios for all conjugations carried out were between 1.2 and 1.7 indicating that a significant degree of conjugation had occurred (table 2.4). These absorbance values could not be accurately translated into quantitative data because the absorbance measured at A_{260} was attributable to both the nucleoside and the protein. Consequently, at every wavelength the protein was

obscured by the nucleoside and therefore traditional protein conjugation calculations could not be conducted on O^6-alkyl dG conjugates.

Additional information on the extent of conjugation was obtained by measuring the orange colour produced during the removal of the DMTr groups from the conjugates. This colour was measured at 504n.m. and the number of DMTr molecules present, hence the number of nucleoside molecules present, was calculated using the extinction coefficient. DMTr analysis was carried out on aliquots of O^6-EtdG-BSA and O^6-n-PrdG-BSA conjugates which had been reserved for this experiment.

O^6-EtdG-BSA
A_{504} for 5µg of conjugate = 62.4

O^6-n-PrdG-BSA
A_{504} for 5µg of conjugate = 71.7

Using these values and the extinction coefficient for DMT at 504n.m (76µM/ml) the number of DMTr groups attached to 5µg of conjugate was calculated.

$c = \frac{\text{Absorbance (at 504n.m)}}{(E \times d)}$

E = extinction coefficient = 76µM/ml
d = pathlength, = 0.5

O^6-EtdG-BSA
c = 1.68µM/ml
= 1.68×10^{-9}mol/5µg of BSA
= 1.68×10^{-9}mol/7.7×10^{-11}molBSA
= 168 trityl groups per 7.7 BSAs

O^6-n-PrdG-BSA
c = 1.89µM/ml
= 1.89×10^{-9}mol/5µg of BSA
= 1.89×10^{-9}mol/7.7×10^{-11}molBSA
=189 trityl groups per 7.7 BSAs

Since there are 2 trityl molecules for each nucleoside the final results are:

O^6-EtdG-BSA
84 nucleosides per 7.7 BSAs
approx. 11 nucleosides/BSA

O^6-n-PrdG-BSA
95 nucleosides per 7.7 BSAs
approx. 12 nucleosides/BSA

These amounts were within the average range of 8-25 haptens per protein, found in other studies. It was assumed that the extent of hapten substitution for all O^6-alkyl and hydroxyEtdG conjugates in this study was similar to that of O^6-EtdG-BSA and O^6-n-PrdG-BSA, as the shape of the UV scans and A_{245}:A_{260} ratios were similar.

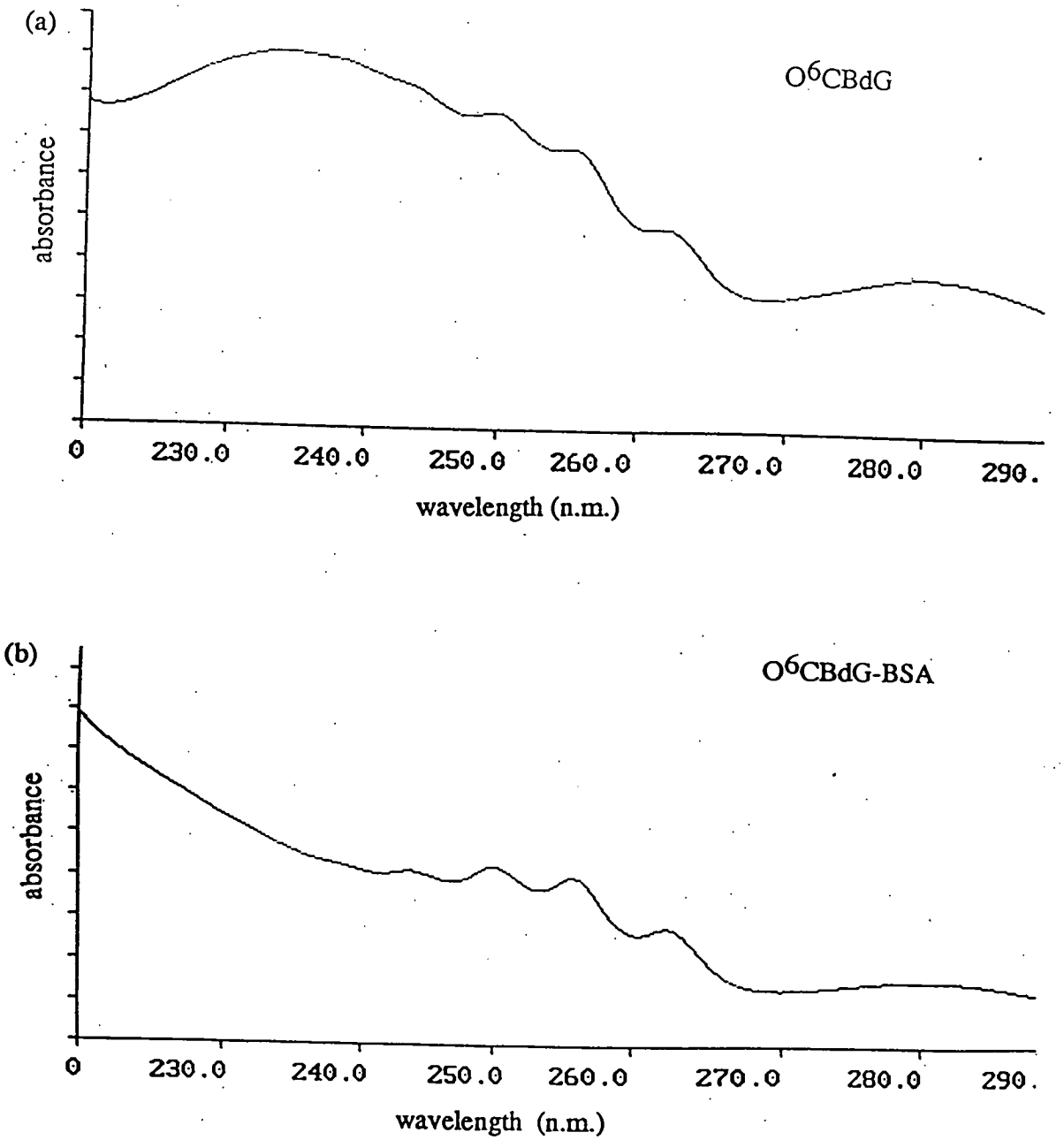
2.2.5.2. Calculating hapten substitution for O^6-CBdG conjugates

The extent of conjugation of O^6-CBdG was estimated by UV scan analysis only, as no DMTr groups were added to this derivative. Maximum absorbance for O^6-CBdG occured at 235n.m. and the aromatic ring was detected by the presence of 3 characteristic peaks betwen 245 and 265n.m (fig. 2.7a). UV scans of the conjugates (fig. 2.7b) closely resembled the modified nucleoside, therefore it was assumed that conjugation had occurred. The A_{245}:A_{260} ratio was not considered as the nucleoside has high absorbance at 260n.m. (table 2.5) due to the benzene ring.

Table 2.5. O^6-(4-carboxybenzyl) dG conjugates

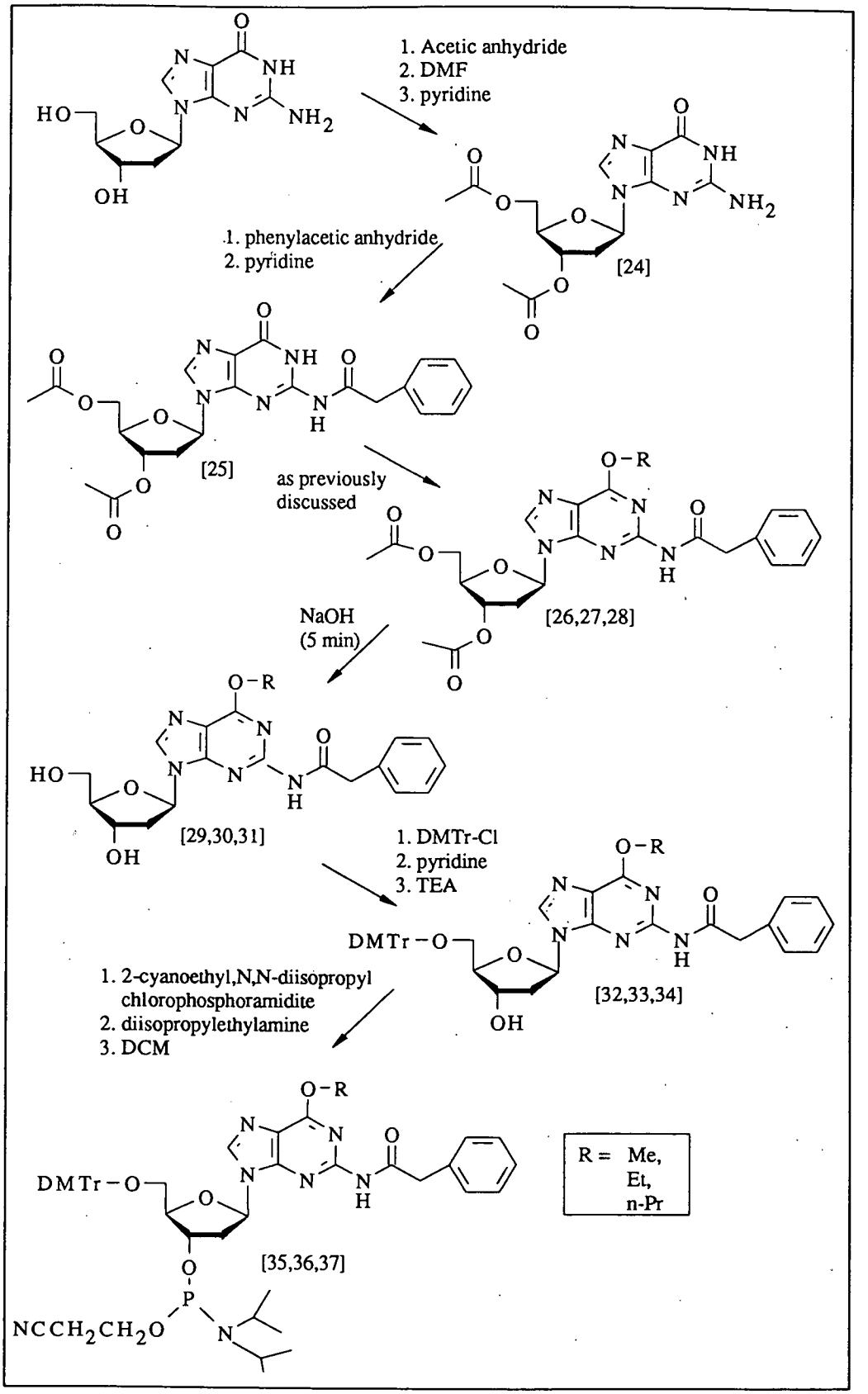
	Absorbance at:		
Conjugate	A_{280}	A_{260}	A_{245}
O^6-CB dG-BSA	38.6	59.3	98.0
O^6-CB dG-CγG	37.7	70.5	106.7

Figure 2. 7 UV scans of a) O^6CBdG and b) O^6CBdG-BSA conjugate



2.2.6 Synthesis of O^6-MedG, O^6-EtdG and O^6-n-PrdG for incorporation into an oligonucleotide (scheme 4)

Phenylacetic anhydride was chosen as an appropriate protecting group for the 2-amino position of the heterocyclic base. The 3' and 5' sugar hydroxyl groups were firstly protected by mixing acetic anhydride with dG at room temperature for 48 hours. Under these conditions the hydroxyl groups were selectively acetylated, to produce 3',5'-bis(O-acetyl)-2'-dG [24]. The 2-amino group of the base was then protected by refluxing [24] with phenylacetic anhydride forming 3',5'-bis(O-acetyl)-N^2-(phenylacetyl)-2'-dG [25]. The O^6 position was modified as previously described by methanol, ethanol and n-propanol, giving compounds [26,27,28]. On addition of an aqueous 2.5M solution of NaOH the acetyl groups were displaced, giving O^6-alkyl dG protected only at the base 2-amino position [29,30,31]. This reaction was neutralised with Dowex after exactly 5 min to avoid prolonged exposure of the nucleosides to extremely basic conditions, which would result in partial removal of the N^2-phenylacetyl protecting group. The 5'-OH was tritylated, giving compounds [32,33,34] and the 3'-OH, was selectively phosphitylated by 2-cyanoethyl,N,N-diisopropylphosphoramidite, producing compounds [35,36,37].

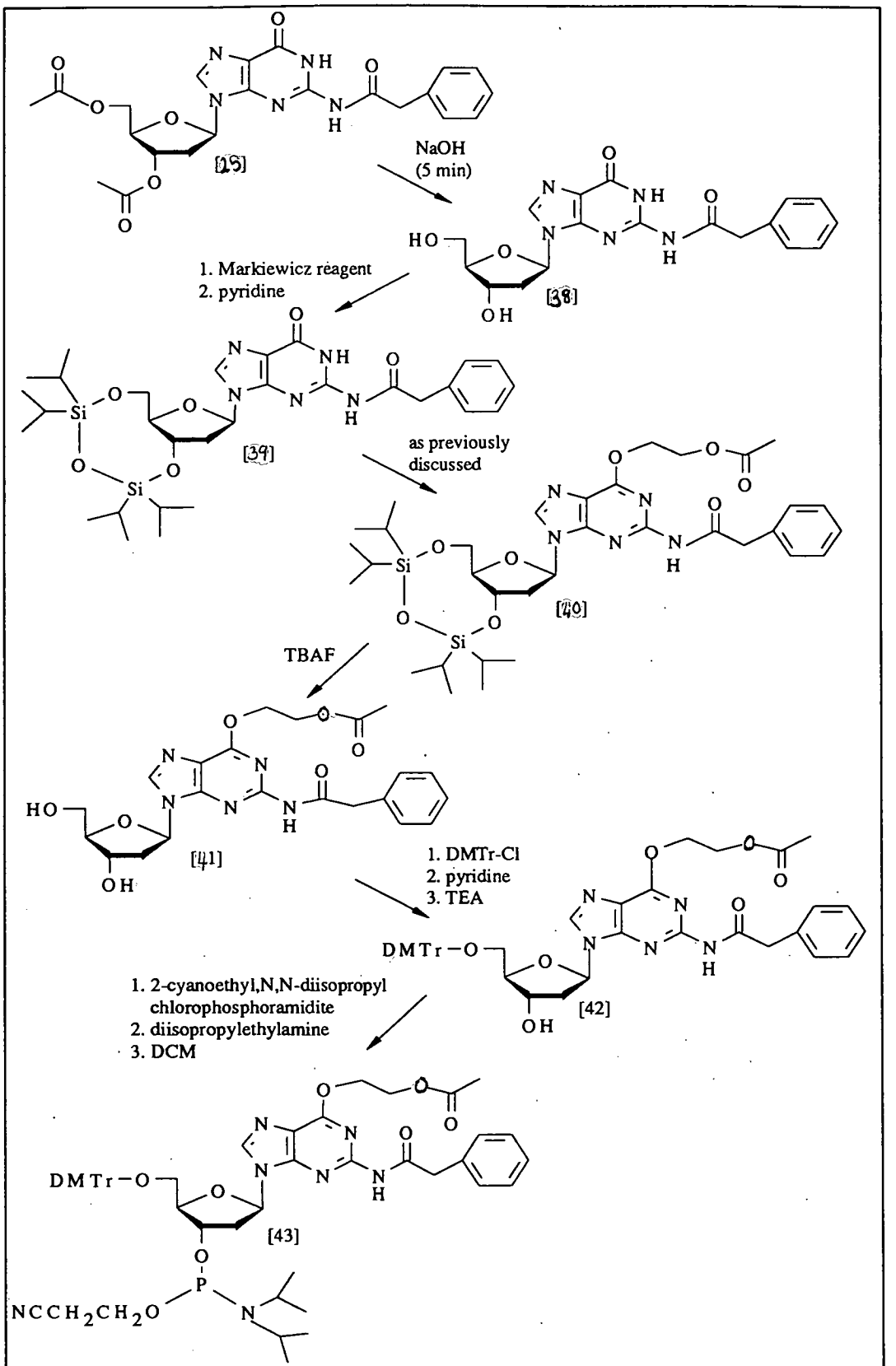


Scheme 4.Synthesis of O^6-alkyl dG for incorporation into an oligonucleotide

2.2.6.1. Synthesis of O^6-hydroxyEt dG for incorporation into an oligonucleotide (scheme 5)

The addition of ethylene glycol to the O^6 position of dG was carried out as previously described using monoacetyl ethylene glycol. For oligonucleotide synthesis it was necessary for the O^6hydroxyEt group to remain acetyl protected. It was therefore not possible to use acetyl protecting groups on the 3' and 5' sugar hydroxyl positions, as deprotection to remove the 3' and 5' sugar protecting groups would also result in some loss of the O^6-monoacetyl group from the ethylene glycol moiety. The hydroxyl groups of dG were therefore protected with a tetraisopropyldisiloxane (TIPDS) group using Markiewicz reagent. This group was removed by the addition of tetrabutyl ammonium flouride (TBAF), leaving the guanine 2-amino and the O^6-hydroxyEt protecting groups intact.

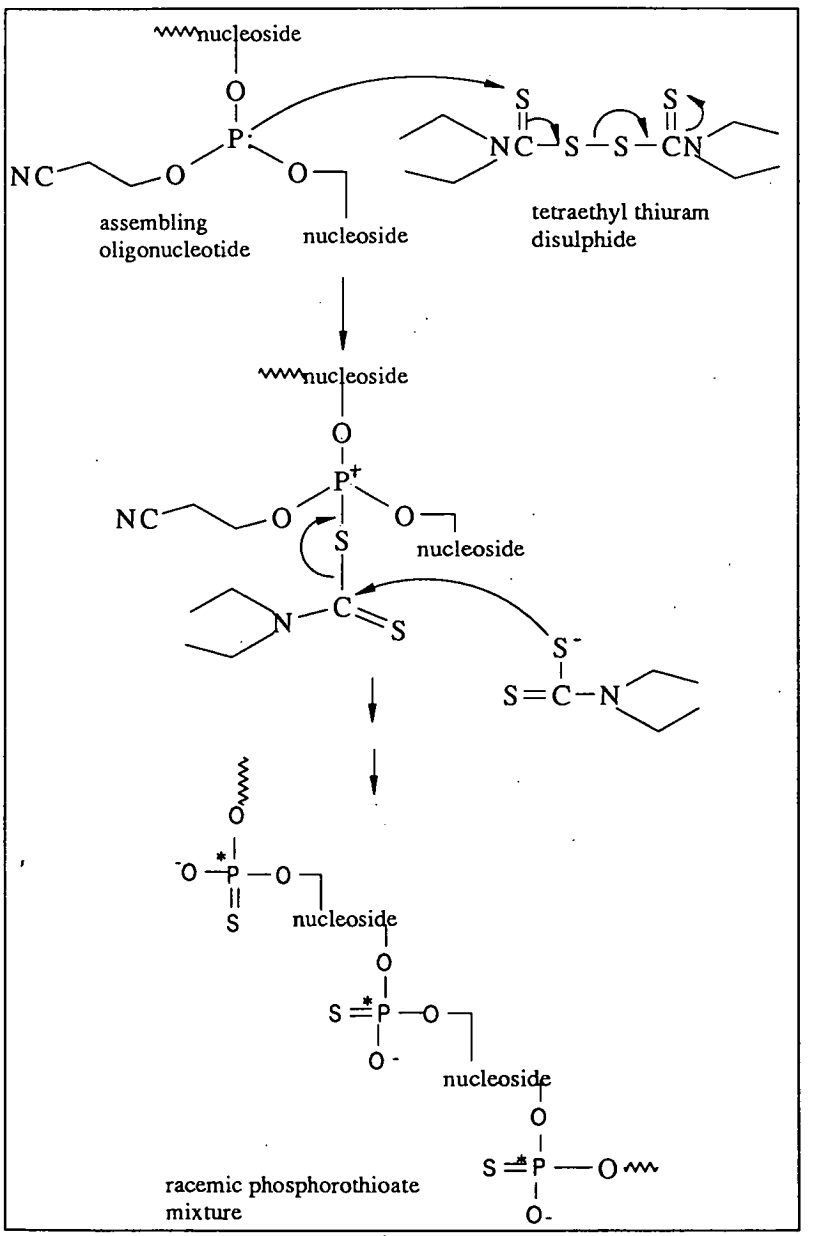
The 3' and 5' OH groups of 3', 5'-bis(O-acetyl)-N^2-(phenylacetyl)-dG [25] were deprotected to give N^2-(phenylacetyl)-dG [38] and the TIPDS group added giving [39]. After O^6 modification giving [40] the TIPDS protection was selectively removed by the addition of TBAF to give compound [41]. 5' DMTr and 3' phosphoramidite groups were added to give [42] then the O^6-hydroxyethyl dG oligonucleotide monomer [43].



Scheme 5. Synthesis of O(6)hydroxyEt dG for incorporation into an oligonucleotide

2.2.7. The synthesis of oligonucleotides containing O^6-MedG, O^6-EtdG, O^6-n-PrdG and O^6-hydroxyEtdG

To prevent enzymatic degradation of the oligonucleotides subsequent to immunisation, phosphorothioate (PS) linkages were incorporated. This was carried out using tetraethyl thiuram disulphide during oligonucleotide synthesis (Vu and Hirschbein, 1991) to allow the addition of a sulphur atom to the phosphate backbone (fig 2.8).



* = chiral phosphorus racemic mixtures

Figure 2.8. Phosphorothioates

A racemic phosphorothioate mixture is produced and this is degraded very slowly by enzymes. PS linkages added to the 3' end of oligonucleotides have been shown to reduce degradation by the abundant 3' to 5' exonucleases (Putney *et al*, 1981). Therefore the PS linkages were added to the 3' end of the oligonucleotides synthesised in this study.

Oligonucleotides incorporating O^6-MedG, O^6-EtdG and O^6-n-PrdG were synthesised. Each consisted of 8 modified nucleosides and 46 thymidine (T) residues. The oligonucleotides were made up of only two different nucleosides to reduce the amount of screening required after immunisation. Sequences were 54 nucleotides in length to ensure a high molecular weight (approximately 20 000) and included five PS linkages (fig 2.9 a-c). The exact number of PS linkages required to prevent degradation of the oligonucleotide *in vivo* before an immune response occured was not known. Therefore an additional 49 nucleotide sequence consisting of O^6-MedG and T and a backbone of PS linkages, was synthesised (fig 2.9d). In addition, a self complementary oligonucleotide containing eleven nucleotides and one O^6-hydroxyEtdG was synthesised (fig 2.8e). For the purpose of the current study this sequence was to be used for screening only.

PS linkages are indicated in boldface

(a) 5' $T_5XT_5XT_5XT_5XT_5XT_5XT_5XTT_5$ 3' X=O^6MedG

(b) 5' $T_5YT_5YT_5YT_5YT_5YT_5YT_5YTT_5$ 3' Y=O^6EtdG

(c) 5' $T_5ZT_5ZT_5ZT_5ZT_5ZT_5ZT_5ZTT_5$ 3' Z-O^6n-PrdG

(d) 5' **$T_5XT_5XT_5XT_5XT_5XT_5XT_5XT$** 3'

(e) 5'CGC O^6hydroxyEtdGAA TT GCG3'

Figure 2.9. Oligonucleotides containing O^6-MedG, O^6-EtdG, O^6-n-PrdG and O^6-hydroxyEt dG for immunisation and screening.

Sequence (d) and the five 3' PS residues of sequences (a), (b) and (c) were synthesised using an adapted synthesis cycle suitable for phosphorothioates. Sequence (d) was cleaved from the solid support after synthesis. The latter 3 sequences were left attached the solid support which was then used as a starting point for the synthesis of the remaining 49 non-PS residues. These, and sequence (e) were synthesised using the standard large scale (1μmole) normal DNA coupling cycle. At the end of the synthesis the sequences were cleaved from the solid support. During oligonucleotide synthesis, after the addition of each nucleotide the

5' DMTr group was removed to allow the addition of another nucleotide. Monitoring of the colour produced by the removal of this group gave an indication of the extent of coupling of each nucleotide and the overall efficiency of synthesis (table 2.7).

Table 2.7 Average coupling of each oligonucleotide

Sequence	average coupling %
(a)	97.2
(b)	97.6
(c)	98.2
(d)	97.1
(e)	97.4

After synthesis of the sequences it was necessary to remove the phenylacetyl protecting group from the 2-amino position of guanine. Li and Swann (1989) reported the removal of this group in concentrated aqueous ammonia after 50 min at room temperature. Sequence (a) was exposed to the above conditions then tested by HPLC which showed that minimal deprotection had occurred (see appendix for raw data). The sequence was then heated to 55°C and tested by HPLC over a timecourse. Results showed that deprotection was almost complete at 24 hr (table 2.8 and appendix).

Table 2.8. Removal of the phenylacetyl protecting group

	State of Oligonucleotide
Time (temperature)	deprotected : protected (approximately)
50 min (RT)	1 : 2
4 hr (55°C)	3 : 2
12 hr (55°C)	4 : 1
24 hr (55°C)	10: 1

The remaining oligonucleotides were exposed to ammonia at 55°C for 24 hr. Purification was carried out on a column of sephadex G25. For sequences (a-d) no HPLC purification was required as failure sequences contain only T and O^6-alkyl

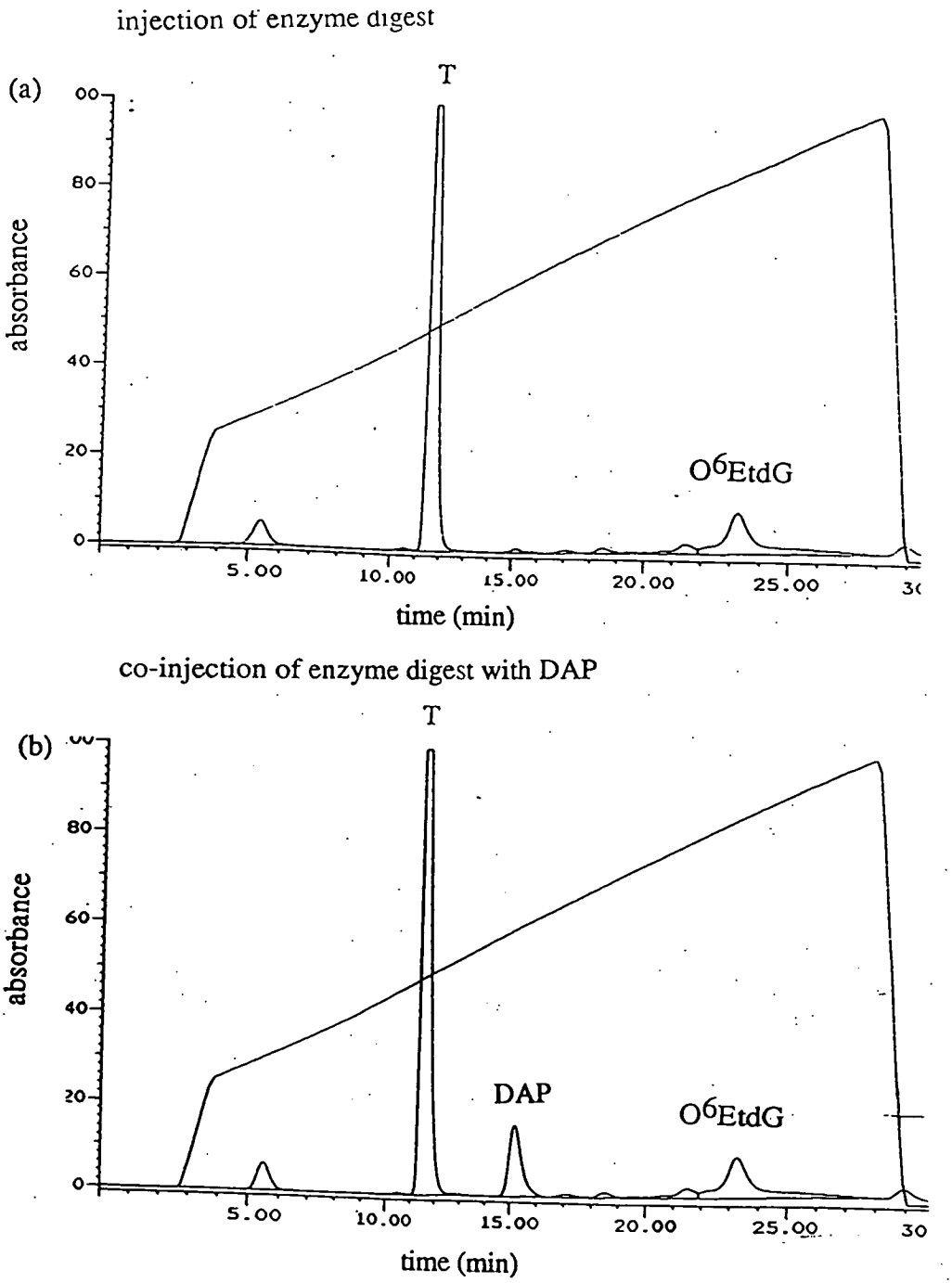
dG. If these failure sequences are sufficiently large to stimulate the immune system they will help in antibody production. If they are too small they are unlikely to cause adverse effects. Sequence (e) was purified by reversed phase HPLC.

2.2.8 Analysis of oligonucleotides

Analysis was carried out to ensure that each O^6 modified dG derivative had been successfully incorporated into oligonucleotides and had not undergone conversion to diaminopurine (DAP) during ammonia deprotection. Each oligonucleotide was enzymatically digested into nucleoside components by snake venom phosphodiesterase which cleaves the phosphodiester linkage producing 5' monophosphate residues, and alkaline phosphatase which removes the phosphate group. The digested products were analysed by HPLC and compared with standard samples of dT, O^6-alkyl dG and diaminopurine nucleoside. HPLC analysis was carried out by ultraviolet spectroscopy at 280n.m.. Injection of the digested products of sequence (b) and co-injection with dT and O^6-ethyl dG confirmed that the first peak (retention time approx. 11min) was attributable to dT residues and that the second peak (retention time approx 23min) was attributable to O^6-ethyl dG (fig. 2.10a). Co-injection with DAP (fig 2.10b) and comparison of the HPLC trace in fig 2.10a showed that minimal DAP was formed during the prolonged ammonia deprotection. The peak with a retention time of approximately 5min may have been due to the T_5 phosphorothioate sequence which may not have been readily digested by the enzymes.

Sequences (a) and (b) were also analysed by enzyme digestion and gave similar results to sequence (b). Sequence (d) was not analysed as an enzyme digest of this complete PS sequence would be unsucessful due to the presence of the enzymatically stable PS group throughout. However, the results from the first 3 sequences suggested that the O^6-MedG was intact in this sequence.

Figure 2.10. Enzyme digest analysis of O^6-alkyl dG oligonucleotides



HPLC analysis of the digested products of the sequence containing O^6-hydroxyEtdG sequence (e) showed that this derivative was no longer present (fig 2.11). It is thought that the conversion of O^6-hydroxyEt dG to dG occurred during the ammonia deprotection following oligonucleotide synthesis, as the overall structure of the ethoxyacetyl group is similar to a base labile protecting group (fig. 2.12). The incorporation of O^6-hydroxyEt dG into an oligonucleotides therefore requires alternative protection on the NH_2 position of the base, so that ammonia deprotection can be avoided.

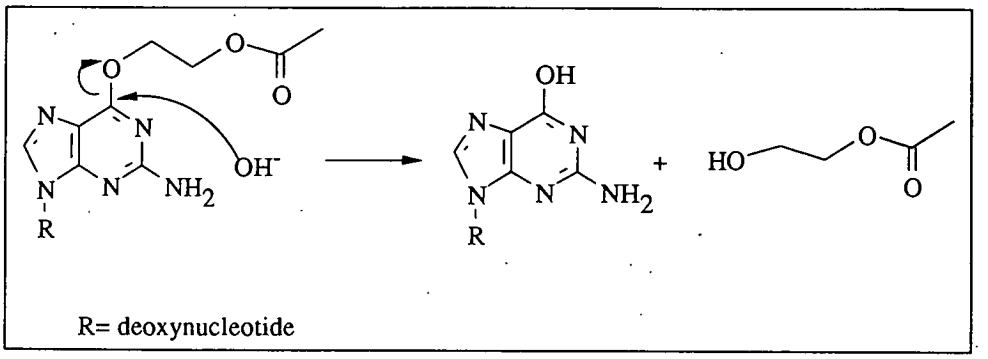
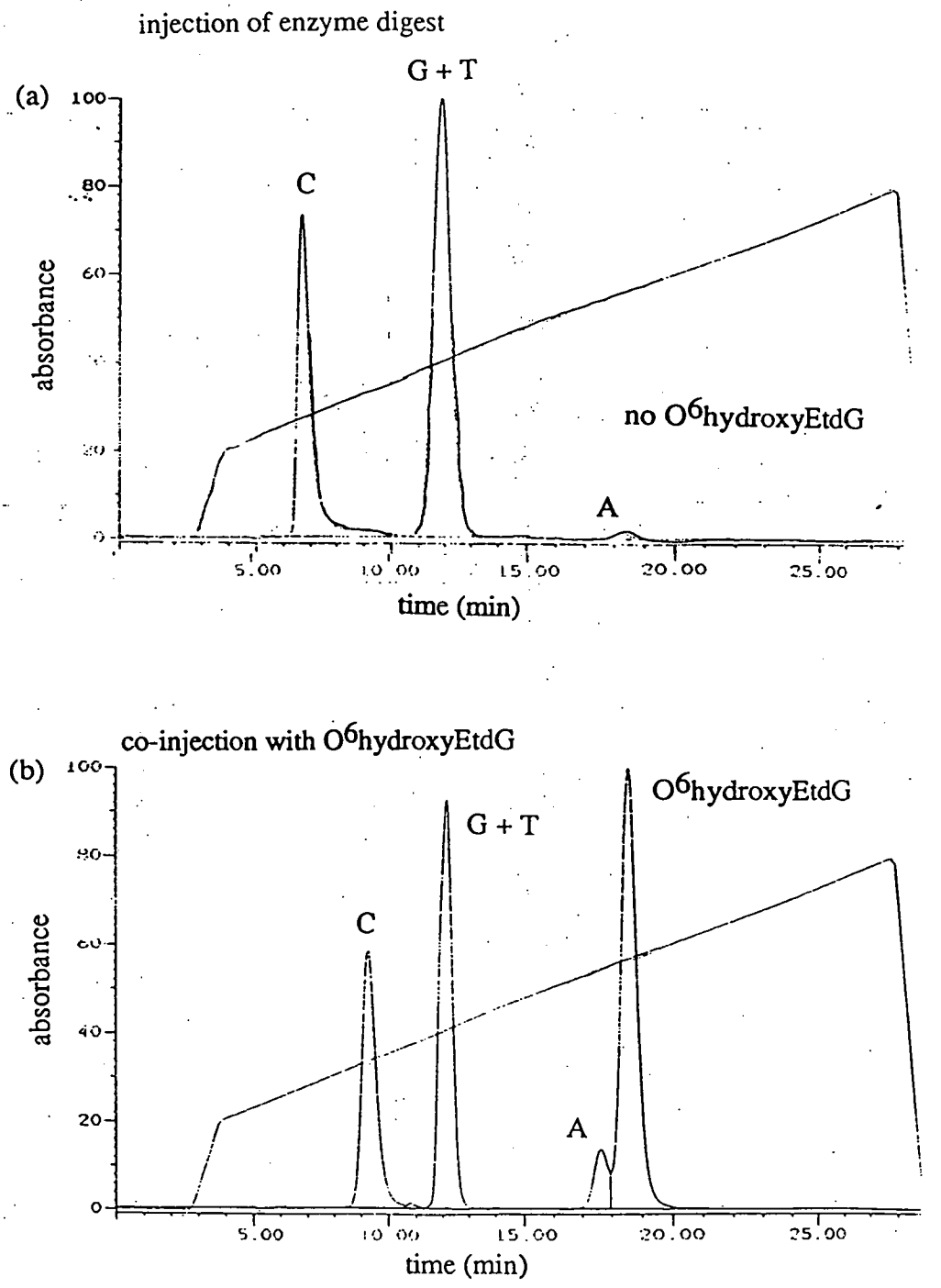


Figure 2.12. Removal of ethoxyacetyl group by ammonia

Figure 2.11. Enzyme digest analysis of O^6-hydroxyEt dG oligonucleotides



2.3. Experimental

Reagents were obtained from Fluka, unless otherwise stated. ^{1}H and ^{13}C NMR spectra were measured on a Bruker WP 200 spectrometer, mass spectra on a Kratos MS50 TC spectrometer and UV spectra on a Perkin-Elmer Lambda 15 spectrophotometer. Controlled Pore Glass Supports, pore diameter 500A, particle size 125-177μ, were obtained from Pierce. Sephadex G25 (Pharmacia NAP 10) was used for gel filtraton. Solvents were purified as follows:

DCM and TEA	distilled over CaH_2
Pyridine	distilled over KOH
MeOH	distilled over Mg/I
DMF	vacuum distilled
THF	distilled over Na benzophenone

Merck silica gel 60 was used for flash chromatography and Merck silica gel 60 F_{245} aluminium plates were used for TLC in the following solvent systems:

(1) DCM/MeOH (9:1 v/v)	(1a) =(1)+ 1%TEA
(2) DCM/MeOH (8:2 v/v)	(2a) =(2)+ 1%TEA
(3) DCM/MeOH (95:5 v/v)	(3a) =(3)+ 1%TEA
(4) nBuOH/AcOH/H_2O (3:1:1 v/v/v)	
(5) DCM/MeOH/AcOH (10:2:1 v/v/v)	(5a) =(5)+ 1%TEA
(6) CH_3CN/EtOAc (5:5 v/v)	(6a) =(6)+ 1%TEA
(7) Toluene/EtOAc (7:3 v/v)	(7a) =(7)+ 1%TEA
(8) Et_2O/EtOAc (5:5 v/v)	

The purity of all compunds synthesised was verified by one spot on TLC.

Synthesis of O^6-alkyl-2'-deoxyguanosine nucleosides for conjugation to protein carriers

(see Scheme 1)

3',5'-Bis(O-acetyl)-N^2-(acetyl)-2'-deoxyguanosine [1]

3',5'-Bis(O-acetyl)-2'-deoxyguanosine (10g, 28mmol) was dried by evaporation of anhydrous pyridine (3x30ml) then suspended in anhydrous pyridine (100ml). Acetic anhydride (17.2ml, 0.17mol, 6eqs) was added and the mixture refluxed for 3hr when TLC showed complete conversion to the triacetyl derivative. After cooling to RT the solution was poured into saturated aqueous KCl (100ml) and the product extracted with DCM (100ml). The organic layer was dried with $MgSO_4$ and evaporated to a small volume. The product was precipitated as a white powder by addition of MeOH.

(10g, 91%) R_f 0.29 (solvent 1) R_f 0.15 (solvent 3) R_f 0.74 (solvent 5) [FAB] m/z Found 394.12845 Requires 394.12845 $(M+H)^+$ δ_H[d_6DMSO] 2.06 (s, 3H, CH_3CO) 2.09 (s, 3H, CH_3CO) 2.29 (s, 3H, CH_3CO) 2.46-3.01 (m, 3H, $H_{2',2''}$) 4.31-4.65 (m, 3H, $H_{4',5',5''}$) 5.36-5.38 (m, 1H, $H_{3'}$) 6.17-6.21 (m, 1H, $H_{1'}$) 7.76 (s, 1H, H_8) 9.89 (s, 1H, $H_{1'}$)

General Method based on the method of Li and Swann (1989)

3',5'-Bis(O-acetyl)-N^2-(acetyl)-O^6-alkyl-2'-deoxyguanosine [2,3,4]

3',5'-Bis(O-acetyl)-N^2-(acetyl)-2'-deoxyguanosine (1.5g, 3mmol) was dissolved in anhydrous DCM (40ml) with gentle heating. After cooling to RT anhydrous TEA (1.7ml, 15mmol, 5eq), 2-mesitylene sulphonyl chloride (1.4g, 6mmol, 2eq) and DMAP (100mg) were added. The reaction was stirred at RT for 30min when TLC showed complete convertion to the O^6-sulphonated derivative. The solution was cooled to 0°C and N-methylpyrrolidine (3ml, 30mmol, 10eq) added. After 20min at 0°C, TLC showed conversion to the N-methylpyrrolidine quaternary salt, R_f 0-0.1. After warming to RT the solution was stirred rapidly with the appropriate alcohol (21mmol, 7eq, methanol 0.84ml, ethanol 1.2ml or n-propanol 1.56ml) and DBU (1.35ml, 9mmol, 3eq). TLC showed production of a high running compound after 40min, 2hr and 4hr for the methyl, ethyl and n-propyl derivatives respectively. Each reaction was quenched with aqueous KH_2PO_4 (100ml, 1M, pH6.5) and the organic layer extracted and dried with $MgSO_4$ then evaporated *in vacuo*. Each product was purified by flash chromatography (eluant: DCM/MeOH 95:5) and the appropriate fractions combined and evaporated *in vacuo* to a foam.

	methyl	**ethyl**	**n-propyl**
Weight/Yield	1.2g/77%	1.05g/65%	0.92g/55%
R_f:(solvent 1)	0.69	0.71	0.75
(solvent 3)	0.30	0.35	0.36
(solvent 5)	0.81	0.82	0.87
[FAB] m/z $(M+H)^+$			
Found	408	422	436
Requires	408	422	436

δ_H[d6DMSO]	methyl	ethyl	n-propyl
$\underline{CH_3}CH_2CH_2O$			0.98-1.02 (t,3)J=7.4Hz
$\underline{CH_3}CH_2O$		1.42-1.44 (t,3)J=7.1Hz	
$CH_3\underline{CH_2}CH_2O$			1.79-1.88 (m,2)
CH_3CO	1.99 (s,3)	2.02 (s,3)	2.03 (s,3)
CH_3CO	2.04 (s,3)	2.08 (s,3)	2.09 (s,3)
CH_3CO	2.44 (s,3)	2.48 (s,3)	2.46 (s,3)
H2',2"	2.50-2.67 (m,2)	2.51-2.90 (m,2)	2.52-2.90 (m,2)
CH_3O	4.12 (s,3)		
H4',5',5"	4.21-4.26 (m,3)	4.26-4.39 (m,3)	4.27-4.32 (m,3)
$CH_3CH_2\underline{CH_2}O$			4.36-4.44 (m,2)
$CH_3\underline{CH_2}O$		4.49-4.55(q,2)J=7.1Hz	
H3'	5.32-5.38 (m,1)	5.36-5.41 (m,1)	5.36-5.40 (m,1)
H1'	6.21-6.27 (t,1)J=6.6Hz	6.28-6.31 (t,1)J=6.6Hz	6.29-6.32 (t,1)J=6.6Hz
H8	7.98 (s,1)	7.92 (s,1)	7.93 (s,1)
NH	9.05 (s,1)	8.18 (s,1)	8.13 (s,1)

O^6-alkyl-2'-deoxyguanosine [6,7,8]

3',5'-Bis(O-acetyl)-N^2-(acetyl)-O^6-alkyl-2'deoxyguanosine was dissolved in pyridine then concentrated *in vacuo* to a small volume. Pyridine (6ml) and NaOH solution (2.4ml, 2.5M) were added and the solution stirred rapidly at RT. After 24hr the mixture was treated with Dowex 50-X8 pyridinium form. The Dowex was washed thoroughly with pyridine (20ml) and the resulting solution evaporated *in vacuo*. The product was purified by flash chromatography (eluant: DCM/MeOH 93:7) and the appropriate fractions combined and evaporated *in vacuo* to a foam.

	methyl	ethyl	n-propyl
Weight/Yield	0.84g/96%	0.75g/97%	0.70g/98%
Rf:(solvent 2)	0.34	0.37	0.42
(solvent 5)	0.21	0.24	0.30
(solvent 4)	0.65	0.68	0.73
[FAB] m/z $(M+H)^+$			
Found	282.11247	296.12800	310.14373
Requires	282.11240	296.12805	310.14370

δ_H[d_6DMSO]	methyl	ethyl	n-propyl
$\underline{CH_3}CH_2CH_2O$			0.92-0.97 (t,3)J=7.1Hz
$\underline{CH_3}CH_2O$		1.32-1.36 (t,3)J=7.1Hz	
$CH_3\underline{CH_2}CH_2O$			1.72-1.78(q,2)J=7.1Hz
$H_{2',2''}$	2.21-2.64 (m,2)	2.07-2.25 (m,2)	2.13-2.20 (m,2)
CH_3O	3.95 (s,3)		
$H_{5',5''}$	3.51-3.57 (m,2)	3.47-3.59 (m,2)	3.36-3.56 (m,2)
$CH_3CH_2\underline{CH_2}O$			4.33-4.36 (m,2)
$CH_3\underline{CH_2}O$		4.41-4.47(q,2)J=7.1Hz	
$H_{3'}$	3.79-3.82 (,1)	3.80-3.84 (m,1)	3.81-3.84 (m,1)
$H_{4'}$	4.36-4.41 (m,1)	4.34.4.36 (m,1)	3.36-3.56 (m,1)
$OH_{5'}$	4.98-5.03 (t,1)	4.96-5.21 (br s,1)	4.90-5.20 (br s,1)
$OH_{3'}$	5.28-5.29 (m,1)	5.32 (s, 1)	5.31 (s, 1)
H_1	6.17-6.24 (t,1)J=6.3Hz	6.19-6.23 (t,1)J=6.6Hz	6.19-6.23 (t,1)J=6.6Hz
NH_2	6.47 (s,2)	6.41 (s,2)	6.40 (s,2)
H_8	8.08 (s,1)	8.07 (s,1)	8.07 (s,1)

5',N^2-Bis-(4,4'-Dimethoxytrityl)-O^6-alkyl-2'-deoxyguanosine [10,11,12]

O^6-alkyl-2'-deoxyguanosine (2.5mmol) was stirred at RT in anhydrous pyridine (6ml) with 4,4'-dimethoxytrityl chloride (2.1g, 6.25mmol, 2.5eq), anhydrous TEA (1.5ml, 7.5mmol, 3eq) and DMAP (100mg). After 2hr the solution was evaporated *in vacuo*, redissolved in DCM (20ml) and washed with saturated KCl solution (50ml), then 1% citric acid solution (50ml). The organic layer was extracted, dried with $MgSO_4$ and concentrated *in vacuo* to give the product as an oil which was purified by flash chromatography (eluant 50:50:1 Et_2O/EtOAc/TEA) then evaporated *in vacuo* to give the *title compound.*

	methyl	ethyl	n-propyl
Weight/Yield	1.7g76/%	1.2g/53%	1.3g/57%
Rf:(solvent 3a)	0.59	0.58	0.61
(solvent 7a)	0.10	0.17	0.18
(solvent 8a)	0.29	0.32	0.35
[FAB] m/z(M+H)+			
Found	886	900	914
Requires	886	900	914

δ_H[d_6DMSO]	methyl	ethyl	n-propyl
$\underline{CH_3}CH_2CH_2O$			0.93-0.97 (t,3)J=7.1Hz
$\underline{CH_3}CH_2O$		1.22-1.26 (m,3)	
$CH_3\underline{CH_2}CH_2O$			1.72-1.78(q,2)J=7.1Hz
$H_{2',2''}$	2.08-2.45 (m,2)	2.21-2.34 (m,2)	2.15-2.61 (m,2)
OCH_3 x 4 or 5	3.36-3.72(m,15)	3.73-3.78 (s,12)	3.72-3.77 (s,12)
$H_{4',5',5''}$	3.11-3.26 (m,3)	4.09-4.21 (m,3)	3.25-3.34 (m,3)
$CH_3CH_2\underline{CH_2}O$			4.07-4.13 (m,2)
$CH_3\underline{CH_2}O$		3.73-3.78 (m,2)	
$H_{3'}$	4.17-4.26 (m,1)	4.50-4.53 (m,1)	4.71-4.73 (m,1)
$OH_{3'}$	5.26-5.28 (br s,1)	4.91-4.95 (br s,1)	5.27-5.28 (br s,1)
$H_{1'}$	6.24-6.30 (t,1)J=6.6Hz	6.17-6.22 (t,1)J=6.4Hz	6.70-6.79 (t,1)J=6.6Hz
Ar x 6	6.76-7.33 (m,26)	6.72-7.29 (m,26)	6.81-7.39 (m,26)
H_8	7.96 (s,1)	7.54 (s,1)	7.37 (s,1)
NH	10.63 (s,1)	11.72 (s,1)	7.69 (s,1)

5',N^2-(4,4'-Dimethoxytrityl)-O^6-alkyl-3'-succinyl-2'-deoxyguanosine [13,14,15]

5'N^2-(4,4'-Dimethoxytrityl)-O^6-alkyl-2'-deoxyguanosine (0.5mmol) was coevaporated with anhydrous DMF (3x5ml). Succinic anhydride (0.05g, 0.5mmol, 1eq), DMAP (5mgs, 0.05mmol, 0.1eq) and anhydrous DMF (2.5ml) were added and the solution stirred at RT. After 18hr the solution was concentrated, then coevaporated with toluene ($MgSO_4$ dried), and redissolved in DCM. This solution was washed quickly with aqueous citric acid (10ml, 10% w/v), then water (10ml). The organic layer was extracted, dried with $MgSO_4$, and evaporated *in vacuo*. The residue was purified by flash chromatography (eluant: DCM/MeOH/TEA 94:6:1) and the appropriate fractions combined and evaporated *in vacuo* to give the *title compound* as a foam.

	methyl	ethyl	n-propyl
Weight/Yield	0.29g/59%	0.24g/48%	0.27g/56%
Rf:(solvent 1a)	0.31	0.32	0.35
(solvent 2a)	0.54	0.57	0.61
(solvent 5a)	0.61	0.61	0.63
[FAB] m/z(M+H)+			
Found	986.38987	1000.40538	1014.42111
Requires	986.38981	1000.40456	1014.42111

δ_H[d_6DMSO]	methyl	ethyl	n-propyl
$\underline{CH_3}CH_2CH_2O$			0.93-0.97 (t,3)J=7.1Hz
$\underline{CH_3}CH_2O$		1.15-1.23 (t,3)J=7.1Hz	
$CH_3\underline{CH_2}CH_2O$			1.72-1.78(q,2)J=7.1Hz
$H_{2',2''}$	2.09-2.41 (m,2)	2.52-2.61 (m,2)	2.52-2.61 (m,2)
CH_2 x 2	2.51-2.58 (m,4)	2.15-2.16 (m,4)	2.15-2.16 (m,4)
OCH_3 x 4 or 5	3.73-3.74(m,15)	3.73-3.74 (s,12)	3.73-3.74 (s,6)
$H_{4',5',5''}$	3.30-3.39 (m,3)	3.31-3.33 (m,3)	3.31-3.33 (m,3)
$CH_3CH_2\underline{CH_2}O$			4.08-4.13 (m,2)
$CH_3\underline{CH_2}O$		2.86-2.92(q,2)J=7.1Hz	
$H_{3'}$	4.17-4.23 (m,1)	4.16-4.21 (m,1)	4.19-4.22 (m,1)
OH	4.71-4.75 (br s,1)	5.28-5.36 (br s,1)	5.21-5.35 (br s,1)
$H_{1'}$	6.18-6.20 (t,1)J=6.6Hz	6.16-6.20 (t,1)J=6.6Hz	6.17-6.20 (t,1)J=6.6Hz
Ar x 6	6.69-7.47(m,26)	6.70-7.28(m,26)	6.70-7.28(m,26)
H_8	7.68 (s,1)	7.37 (s,1)	7.37 (s,1)
NH	8.14 (s,1)	7.69 (s,1)	7.69 (s,1)

Synthesis of O^6-(4-carboxybenzyl)-2'-deoxyguanosine for conjugation to carrier proteins (see scheme 1)

3',5'-Bis(O-acetyl)-N^2-(acetyl)-O^6-(4-carbomethoxybenzyl))-2'-deoxyguanosine [5]

3',5'-Bis(O-acetyl)-N^2-(acetyl)-2'-deoxyguanosine (1.5g, 3mmol) was dissolved in anhydrous DCM (40ml) with gentle heating. After cooling to RT anhydrous TEA (1.7ml, 15mmol, 5eq), 2-mesitylene sulphonyl chloride (1.4g, 6mmol, 2eq) and DMAP (100mg) were added. The reaction was stirred at RT for 30min when TLC showed complete convertion to the O^6-sulphonated derivative. The solution was cooled to 0°C and N-methylpyrrolidine (3ml, 30mmol, 10eq) added. After 20min at

0°C, TLC showed conversion to the N-methylpyrrolidine derivative, lower R_f. After warming to RT the solution was stirred rapidly with methyl-4-(hydroxymethyl)benzoate (3.5g, 21mmol, 7eq) and DBU (1.35ml, 9mmol, 3eq). After 2hr TLC showed production of a high running compound and the reaction was quenched with aqueous KH_2PO_4 (100ml, 1M, pH6.5) and the organic layer extracted and dried with $MgSO_4$ then evaporated *in vacuo*. The product was purified by flash chromatography (eluant: DCM/MeOH 98:2) and the appropriate fractions combined and evaporated *in vacuo* to a foam. (1.7g, 90%) R_f 0.63 (solvent 1) R_f 0.31 (solvent 3) R_f 0.89 (solvent 2) [FAB] m/z $(M+H)^+$ Found 495.16752 Requires 495.16757 $\delta_H[CdCl_3]$ 2.01 (s, 3H, CH_3CO) 2.07 (s, 3H, CH_3CO) 2.38 (s, 3H, CH_3CO) 2.48-3.00 (m, 2H, $H_{2',2''}$) 3.84 (s, 3H, $COOCH_3$) 4.27-4.37 (m, 3H, $H_{4',5',5''}$) 5.35-5.38 (m,1H, $H_{3'}$) 5.54 (s, 2H, OCH_2) 6.26-6.30 (m, 1H, $H_{1'}$) 7.31-7.96 (m, 5H, H_8 & Ar) 8.31 (s, 1H, NH)

O^6-(4-carboxybenzyl)-2'-deoxyguanosine [9]

3',5'-Bis(O-acetyl)-N^2-(acetyl)-O^6-(4-carbomethoxybenzyl)-2'-deoxyguanosine (1.7g, 3.4mmol) was dissolved in pyridine then concentrated *in vacuo* to a small volume. Pyridine (20ml) and 2.5M NaOH (10ml) were added and the solution stirred rapidly at RT. TLC showed complete conversion to a low running product after 72hr. The mixture was neutralised on a column of Dowex 50-X8 H^+ form (20ml). The Dowex was washed thoroughly with pyridine/H_2O (1:1) (80ml) and the resulting solution evaporated *in vacuo*. The mixture was dissolved in 0.1M NaOH (15ml) and impurities extracted with Et_2O (20ml). The required product precipitated on addition of 0.1M AcOH (1ml) and was extracted from the solution by centrifugation, then dried *in vacuo*. (0.3g, 22%) R_f 0.21 (solvent 2) R_f 0.55 (solvent 5) R_f 0.71 (solvent 4) [FAB] m/z $(M+H)^+$ Found 402.13348. Requires 402.13353 δ_H[d6DMSO] 2.20-2.27 (m, 2H, $H_{2',2''}$) 3.45-3.62 (m, 2H, $H_{5',5''}$) 3.80-3.86 (m,1H, $H_{3'}$) 4.34-4.37 (m, 1H, $H_{4'}$) 4.75-5.52 (br s, 3H, $OH_{3'}$, $OH_{5'}$, COOH) 5.52 (s, 2H, OCH_2) 6.19-6.23 (t, J=6.1Hz, 1H, $H_{1'}$) 6.52 (s, 1H, H_2) 7.56-7.98 (m, 4H, Ar) 8.12 (s, 1H, H_8)

Synthesis of O^6-hydroxyethyl-2'-deoxyguanosine for conjugation to carrier proteins (see Scheme 2)

Removal of protecting groups (added for oligonucleotide synthesis but not required for protein conjugation)

O^6-(ethoxyacetyl)-2'-deoxyguanosine [16]

3',5'O-(1,1,3,3-tetraisopropyldisiloxyl)-N^2-(phenylacetyl)-O^6-(ethoxyacetyl)-2'-deoxyguanosine (0.7g, 1mmol) was dissolved in anhydrous THF then concentrated *in vacuo* to a small volume. THF (2.5ml) and TBAF (1.4ml, 1M in THF) were added and the solution stirred rapidly at RT. TLC showed complete conversion to a low running product after 5min. The reaction mixture was diluted with pyridine/MeOH/H_2O (3:1:1 5ml) then added to Dowex 50-X8 pyridinium form (5g in 10ml of aforementioned 3:1:1 solution) and stirred gently for 20min. The Dowex was filtered off and washed thoroughly with pyridine/H_2O (1:1) (10ml) and the filtrate evaporated *in vacuo*. The product was purified by flash chromatography (eluant 90:10 DCM/MeOH and the appropriate fractions combined and evaporated *in vacuo*. The product was dissolved in pyridine (5ml). Conc. ammonia (10ml) was added and the mixture was heated to 55^{0}C. After 72hr the solution was evaporated *in vacuo* and the product purified by flash chromatography (eluant 90:10 DCM/MeOH) to give the *title compound.* (0.16g, 83%) R_f 0.37 (solvent 1) R_f 0.61 (solvent 2) R_f 0.69 (solvent 5) [FAB] m/z $(M+H)^+$ Found 312.12301 Requires 312.12297 δ_H[d6DMSO] 2.15-2.26 (m, 2H, $H_{2',2''}$) 3.46-3.55 (m, 2H, CH_2) 3.73-3.83 (m, 3H, $H_{4',5',5''}$) 4.36-4.44 (m, 3H, $H_{3'}$ & CH_2) 4.89-4.91 (br s, 1H, OH) 5.02-5.04 (br s, 1H $OH_{3'}$) 5.29-5.31 (br s, 1H, $OH_{5'}$) 6.18-6.25 (t, J=6.1Hz, 1H, $H_{1'}$) 6.42 (s, 2H, NH_2) 8.09 (s, 1H, H_8)

5',N_2-(4,4'dimethoxytrityl)-O^6-(ethoxydimethoxytrityl)-2'-deoxyguanosine [17]

O6-hydroxyethyl-2'-deoxyguanosine (0.15g, 0.48mmol) was dried by evaporation of anhydrous pyridine (3x2ml), then dissolved in anhydrous pyridine (5ml). TEA (0.33ml, 0.23g, 2.20mmol, 5eq), 4,4'-dimethoxytrityl chloride (0.57g, 1.68mmol, 3.5eq) and DMAP (20mg) were added and the mixture stirred at RT. After 1hr the solution was evaporated *in vacuo*, redissolved in DCM (10ml), washed with KCl (10ml), dried with $MgSO_4$ and evaporated to a gum. The title compound was purified by flash chromatography (eluant DCM/TEA 99:1) (0.404g, 69%) R_f 0.63 (solvent 3a) R_f 0.19 (solvent 7a) R_f 0.31 (solvent 8a) [FAB] m/z $(M+2H)^+$ Found 1219.51498 Requires 1219.51501 δ_H[d6DMSO] 2.16-2.51 (m, 2H, $H_{2',2''}$) 2.89-2.93 (m, 4H, CH_2x2) 3.17-3.28 (m, 3H, $H_{4',5',5''}$) 3.71-3.85 (m, 18H, OCH_3x6) 4.48-4.51 (m, 3H, $H_{3'}$) 4.77-4.79 (br s, 1H $OH_{3'}$) 6.17-6.23 (t, J=6.2Hz, 1H, $H_{1'}$) 6.67-7.43 (m, 39H, Arx9) 7.66 (s, 1H, H_8) 7.99 (s, 1H, NH)

5',N^2-(4,4'-dimethoxytrityl)-3'-succinyl-O^6-(ethoxydimethoxytrityl)-2'-deoxyguanosine [18]

5',N^2-(4,4'dimethoxytrityl)-O^6-(ethoxydimethoxytrityl)-2'-deoxyguanosine (0.32g, 0.26mmol) was coevaporated with anhydrous DMF (3x5ml). TEA (0.152ml, 1.04mmol, 4eq), succinic anhydride (0.083g, 0.91mmol, 3.5eq), DMAP (7mgs, 0.06mmol, 0.22eq) and anhydrous DMF (5ml) were added and the mixture stirred at RT. After 18hr the solution was concentrated, then coevaporated with toluene ($MgSO_4$ dried), and redissolved in DCM. This solution was washed quickly with aqueous citric acid (10ml, 10% w/v), then water (10ml). The organic layer was extracted, dried with $MgSO_4$, and evaporated *in vacuo*. The residue was purified by flash chromatography (eluant: DCM/MeOH/TEA 94:4:1) and the appropriate fractions combined and evaporated *in vacuo* to give the *title compound* as a foam.(eluant DCM/TEA 99:1) (0.235g, %) R_f 0.33 (solvent 1a) R_f 0.73 (solvent 2a) R_f 0.79 (solvent 5a) [FAB] m/z $(M+H)^+$ Found 1318.53110 Requires 1318.53105 δ_H[d6DMSO] 2.49-2.57 (m, 2H, $H_{2',2''}$) 2.78-2.88 (m, 4H, CH_2x2) 3.10-3.58 (m, 3H, $H_{4',5',5''}$) 3.62-3.74 (m, 18H, OCH_3x6) 4.22-4.24 (m, 3H, $H_{3'}$) 4.78-4.80 (br s, 1H OH) 6.21-6.23 (m, 1H, $H_{1'}$) 6.64-7.41 (m, 40H, Arx9 & H_8) 7.74 (s, 1H, NH)

General Method

Conjugation of 3'-tritylated, 5'-succinylated, O^6-deoxyguanosine derivatives to Protein (see scheme 3)

Protein (BSA or CγG) (5mg) was dissolved in H_2O/pyridine 4:1 (0.5ml). The modified nucleoside (10mg) was dissolved in DMSO/H_2O 4:1 (0.5ml) and 1-ethyl-3(dimethylaminopropyl)carbodiimide (10mg) was added. The nucleoside solution was added quickly by syringe to the protein solution. After 3hr at 37°C the mixture was purified by Sephadex G-25 (equilibrated with EtOH/H_2O 50:50). The extent of conjugation was measured by change in UV absorbance over the range 220n.m.to 300n.m.

Detritylation of Conjugates

AcOH (5ml, 80% in H_2O) was added to each conjugate. After 6hr the mixture was evaporated *in vacuo* to approximately 1ml, then purified by Sephadex G-25.

General Method

Preparation of modified deoxynucleotide monomers for DNA synthesis

O^6alkyl dGs (see scheme 4)

3',5'-Bis(O-acetyl)-2'-deoxyguanosine [24]

2'-deoxyguanosine monohydrate (10g, 35mmol) was dried by evaporation of anhydrous pyridine (3x30ml) then dissolved in a mixture of anhydrous pyridine (113ml) and anhydrous DMF (180ml). Acetic anhydride (43ml, 0.42mol, 12eqs) was added and the reaction stirred at RT in darkness with the exclusion of moisture. After 48hr ethanol (20ml) was added and stirring continued for a further 20min., after which the solution was evaporated *in vacuo* and the product recrystallised from ethanol/water (9:1) to give the *title compound* (12 g, 97%) R_f 0.13 (solvent 1) R_f 0.63 (solvent 4) R_f 0.50 (solvent 5) [FAB] m/z $(M+H)^+$ Found 352. Requires 352 δ_H[d_6DMSO] 2.02 (s, 3H, CH_3CO) 2.07 (s, 3H, CH_3CO) 2.39-2.50 (m, 2H, $H_{2',2''}$) 4.19-4.23 (m, 3H, $H_{4',5',5''}$) 5.32 (br d, J=6.0 Hz,1H, $H_{3'}$) 6.12 (dd, J=5.9Hz, 1H, $H_{1'}$) 6.55 (s, 2H, H_2) 7.94 (s, 1H, H_8) 10.4 (s, 1H, H_1)

3',5'-Bis(O-acetyl)-N^2-(phenylacetyl)-2'-deoxyguanosine [25]

3',5'-Bis(O-acetyl)-2'-deoxyguanosine (10g, 0.028mol) was coevaporated with anhydrous pyridine (3x30ml). Phenylacetic anhydride (40g, 0.16mol) and anhydrous pyridine (140ml) were added and the solution stirred under reflux, with the exclusion of moisture, for 30min. After cooling to RT the solution was poured into saturated aqueuos $NaHCO_3$ (400ml). The bicarbonate solution was extracted with DCM (300ml) and the organic layer dried with $MgSO_4$ and evaporated *in vacuo* to a gum. The residue was purified by flash chromatography (eluant: DCM/MeOH 96:4) and the appropriate fractions combined and evaporated *in vacuo* to a foam, redissolved in DCM (5ml) and precipitated into diethyl ether to give the *title compound* (10g, 75%) R_f 0.43 (solvent 1), R_f 0.19 (solvent 3) R_f 0.84 (solvent 5) [FAB] m/z $(M+H)^+$ Found 473.18331 Requires473.18322 δ_H[d_6DMSO] 2.03 (s, 3H, CH_3CO) 2.09 (s, 3H, CH_3CO) 2.59-2.94 (m, 3H, $H_{2',2''}$) 3.81 (s, 2H, $\underline{CH_2}Ph$) 4.17-4.28 (m, 3H, $H_{4',5',5''}$) 5.30 (br d, J=6.2 Hz, 1H, $H_{3'}$) 6.24 (dd, J=5.9Hz, 1H, $H_{1'}$) 7.29-7.36 (m, 5H, $CH_2\underline{Ph}$) 8.26 (s, 1H, H_8) 11.9 (s, 1H, $H_{1'}$)

Phenylacetic anhydride

Phenylacetic acid (20g, 0.14mol) and acetic anhydride (34.7ml, 0.36mol, 2.5eq) were refluxed and after 2hr the solution was evaporated *in vacuo* and recrystallised from Et_2O (100ml) to give the *title compound* (10g, 86%).

R_f 0.56 (solvent 8) M.p 72 [EI] m/z (M) Found 136. Requires 136 $\delta_H[d_6DMSO]$ 7.23-7.32 (m, 10H, Arx2) 3.6 (s, 4H, CH_2x2) IR anhydride carbonyl peaks at 1875 and 1760cm^{-1}

3',5'-Bis(O-acetyl)-N^2-(phenylacetyl)-O^6-alkyl-2'-deoxyguanosine [26,27,28]

3',5'-Bis(O-acetyl)-N^2-(phenylacetyl)-2'-deoxyguanosine (10g, 0.02mol), 2-mesitylene sulphonyl chloride (9.4g, 0.04mol, 2eq) and DMAP (120mg, 1.0mmol) were stirred together at RT in anhydrous TEA (12ml, 0.1mol, 5eq) and anhydrous DCM (100ml). The reaction was monitored by TLC until the starting material was completely converted to the O^6-sulphonated derivative, with higher R_f (60min). The solution was cooled to 0°C and N-methylpyrrolidine (22ml, 0.2mol, 10eq) added. After 20min at 0°C, TLC showed conversion to the N-methylpyrrolidine derivative, lower R_f. After warming to RT the solution was stirred rapidly with the appropriate alcohol (0.14mol, 7eq, methanol 5.6ml, ethanol 8.2ml or n-propanol 10.4ml) and DBU (9.0ml, 0.06mol, 3eq). TLC showed production of a high running compound after 60min, 90min and 200min for the methyl, ethyl and n-propyl derivatives respectively. Each reaction was quenched with aqueous KH_2PO_4 (100ml, 1M, pH6.5) and the organic layer extracted and dried with $MgSO_4$ then evaporated *in vacuo*. Each product was purified by flash chromatography (eluant: DCM/MeOH 95:5) and the appropriate fractions combined and evaporated *in vacuo* to a foam.

	methyl	**ethyl**	**n-propyl**
Weight/Yield	7.8g/64%	4.6g/43%	3.2g/32%
R_f:(solvent 1)	0.73	0.79	0.81
(solvent 3)	0.36	0.40	0.42
(solvent 5)	0.87	0.87	0.89
[FAB] m/z(M+H)+			
Found	483	497	511
Requires	483	497	511

δ_H[d_6DMSO]	methyl	ethyl	n-propyl
$\underline{CH_3}CH_2CH_2O$			0.95-1.02 (t,3)J=7.4Hz
$\underline{CH_3}CH_2O$		1.39-1.42 (t,3)J=7.1Hz	
$CH_3\underline{CH_2}CH_2O$			1.77-1.91(q,2)J=7.4Hz
CH_3CO	1.98 (s,3)	2.01 (s,3)	2.04 (s,3)
CH_3CO	2.08 (s,3)	2.06 (s,3)	2.09 (s,3)
H2',2"	2.47-2.68 (m,2)	2.29-2.51 (m,2)	2.48-2.62 (m,2)
$\underline{CH_2}Ph$	3.84 (s,2)	3.92 (s,1)	4.05 (s,2)
CH_3O	4.07 (s,3)		
H4',5',5"	4.21-4.26 (m,3)	4.19-4.28 (m,3)	4.27-4.47 (m,3)
$CH_3CH_2\underline{CH_2}O$			4.27-4.47 (m,2)
$CH_3\underline{CH_2}O$		4.29-4.36 (m,2)	
H3'	approx. 5.41 (m,1)	5.37-5.41 (m,1)	5.34-5.42 (m,1)
H1'	approx. 6.40 (br t,1)	6.31-6.39 (t,1)J=6.4Hz	6.27-6.37 (m,1)
$CH_2\underline{Ph}$	7.21-7.30 (m,5)	7.19-7.31 (m,5)	7.22-7.33 (m,5)
H8	8.40 (s,1)	8.01 (s,1)	7.93 (s,1)
NH	10.71 (s,1)	9.97 (s,1)	9.98 (s,1)

N^2-(phenylacetyl)-O^6-alkyl-2'-deoxyguanosine [29,30,31]

3',5'-Bis(O-acetyl)-N^2-(phenylacetyl)-O^6-alkyl-2'-deoxyguanosine (6mmol) was dissolved in pyridine (8ml) and concentrated *in vacuo* to a small volume. Pyridine (20ml) and NaOH solution (8ml, 2.5M) were added and the solution stirred rapidly at RT. After 5min the mixture was treated with Dowex 50-X8 pyridinium form. The Dowex was washed thoroughly with pyridine (20ml) and the resulting solution evaporated *in vacuo*. The product was purified by flash chromatography (eluant: DCM/MeOH 93:7) and the appropriate fractions combined and evaporated *in vacuo* to a foam.

	methyl	ethyl	n-propyl
Weight/Yield	1.9g/76%	2.0g/79%	2.0g/80%
R_f:(solvent 1)	0.30	0.32	0.33
(solvent 2)	0.48	0.49	0.57
(solvent 5)	0.54	0.56	0.57
[FAB] m/z(M+H)+			
Found	400	414	428
Requires	400	414	428

δ_H[d_6DMSO]	methyl	ethyl	n-propyl
$\underline{CH_3}CH_2CH_2O$			0.99-1.01 (t,3)J=7.1Hz
$\underline{CH_3}CH_2O$		1.30-1.40 (t,3)J=7.2Hz	
$CH_3\underline{CH_2}CH_2O$			1.81-1.94(q,2)J=7.1Hz
H2',2"	2.62-2.69 (m,2)	2.34-2.56 (m,2)	2.41-2.53 (m,2)
$\underline{CH_2}Ph$	3.96 (s,2)	4.05 (s,1)	3.99 (s,2)
CH_3O	4.07 (s,3)		
H4',5',5"	3.50-3.83 (m,3)	4.17-4.22 (m,3)	4.01-4.14 (m,3)
$CH_3CH_2\underline{CH_2}O$			4.17-4.38 (m,2)
$CH_3\underline{CH_2}O$		4.27-4.38(q,2)J=7.2Hz	
H3'	4.13-4.18 (m,1)	5.04-5.16 (m,1)	4.82-4.95 (m,1)
$OH_{5'}$	4.92-4.97 (br s,1)	4.89-4.92 (t,1)J=5.2Hz	4.98-5.03 (br s,1)
$OH_{3'}$	5.32-5.41 (br s,1)	5.29-5.32(d,1)J=3.5Hz	5.28-5.34 (br s,1)
H1'	6.32-6.39 (t,1)J=7.0Hz	6.09-6.21 (m,1)	6.19-6.27 (m,1)
CH_2Ph	7.21-7.32 (m,5)	7.19-7.31 (m,5)	7.24-7.35 (m,5)
H8	8.44 (s,1)	8.12 (s,1)	8.09 (s,1)
NH	10.73 (s,1)	10.34 (s,1)	10.07 (s,1)

5'-(4,4'-Dimethoxytrityloxy)-N^2-(phenylacetyl)-O^6-(alkyl/ethoxyacetyl)-2'-deoxyguanosine [32,33,34,42]

N^2-(phenylacetyl)-O^6-(alkyl/ethoxyacetyl)-2'-deoxyguanosine (2.5mmol) was stirred at RT in anhydrous pyridine with 4,4'-dimethoxytrityl chloride (1g, 3mmol, 1.2eq), anhydrous TEA (0.73ml, 5mmol, 2eq) and DMAP (100mg). After 60 min the solution was evaporated *in vacuo*, redissolved in DCM (20ml) and washed with saturated KCl solution (50ml). The organic layer was extracted, dried with $MgSO_4$ and concentrated *in vacuo* to give the product as an oil which was purified by flash chromatography (eluant: DCM/MeOH/TEA 99:0.5:1) then evaporated *in vacuo*.

	methyl	ethyl	n-propyl	ethoxy-acetyl
Weight/Yield	1.3g/75%	1.2g/71%	1.2g/69%	1.1g/57%
Rf:(solvent 1a)	0.76	0.79	0.84	0.85
(solvent 3a)	0.35	0.30	0.30	0.34
(solvent 6a)	0.28	0.29	0.31	0.31
[FAB]m/z(M+H)+				
Found	702	716	730	774.30615
Requires	702	716	730	774.30608

δ_H[d_6DMSO]	methyl	ethyl	n-propyl	ethoxyacetyl
$\underline{CH_3}CH_2CH_2O$			0.92-1.09 (t,3)J=7.1Hz	
$\underline{CH_3}CH_2O$		1.39-1.44 (t,3) J=7.4Hz		
$CH_3\underline{CH_2}CH_2O$			1.77-2.05 (q,2) J=7.1Hz	
H2',2"	2.51-2.78 (m,2)	2.39-2.50 (m,2)	2.52-2.59 (m,2)	2.03-2.16 (m,2)
CH_3CO				2.03-2.16 (m,3)
OCH_3 x 2	3.70 (s,6)	3.73 (s,6)	3.71 (s,6)	3.63-3.68 (m,6)
$\underline{CH_2}Ph$	3.82 (s,2)	3.97 (s,2)	3.99 (s,2)	3.99 (s,2)
CH_3O	4.06 (s,3)			
H4',5',5"	4.58-4.64 (m,3)	3.24-3.51 (m,3)	3.29-3.34 (m,3)	3.24-3.31 (m,3)
$CH_3CH_2\underline{CH_2}O$			4.37-4.44 (m,2)	
$CH_3\underline{CH_2}O$		4.47-4.58 (q,2) J=7.4Hz		
CH_2x2				2.45-2.68 (m,4)
H3'	3.96-4.00 (m,1)	4.04-4.19 (m,1)	4.61-4.64 (m,1)	4.55-4.59 (m,1)
OH3'	5.33-5.39 (br s,1)	4.64-4.67 (br s,1)	4.16-4.20 (br s,1)	4.78-4.81 (br s,1)
H1'	approx. 6.31-6.39 (t,1)	6.50-6.44 (t,1) J=6.7Hz	6.41-6.49 (m,1)	6.10-6.16 (m,1)
Ar x 4	6.72-7.30(m,18)	6.73-7.39(m,18)	6.71-7.40(m,18)	6.65-7.34 (m,18)
H8	8.34 (s,1)	7.96 (s,1)	7.97 (s,1)	7.79 (s,1)
NH	10.61 (s,1)	10.65 (s,1)	8.13 (s,1)	8.12 (s,1)

5'-(4,4'-Dimethoxytrityloxy)-N^2-(phenylacetyl)-O^6-(alkyl/ethoxyacetyl)-2'-deoxyguanosine-3'-(2-cyanoethyl-N,N-diisopropyl)-phosphoramidite [35,36,37,43]

5'-(Dimethoxytrityl)-N^2-(phenylacetyl)-O^6-(alkyl/ethoxyacetyl)-2'-deoxyguanosine (1.5mmol) was coevaporated with anhydrous DCM. Diisopropylethylamine (0.98ml, 0.73g, 6mmol, 4eq) and 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (0.45ml, 0.45g, 2.2mmol, 1.5eq) were added and the solution stirred at RT with the exclusion of air. After 2-3hr when TLC showed each reaction was complete, diethyl ether (30ml) was added and each solution was washed with $NaHCO_3$ (30ml), then brine (3x30ml). The organic layer was extracted, dried with $MgSO_4$, evaporated *in vacuo* to an oil then flushed with nitrogen. The product was purified by flash chromatography, (eluant: DCM/ethyl acetate/TEA 20:80:2) and the appropriate fractions combined, evaporared *in vacuo*, redissolved in DCM and precipitated into hexane (500ml, Na dried) avoiding prolonged exposure to air, to give the *title compound* as a white powder.

	methyl	**ethyl**	**n-propyl**	**ethoxyacetyl**
Weight/Yield	0.69g/54%	0.72g/57%	0.51g/40%	0.54g/41%
Rf:(solvent 1a)	0.34	0.40	0.42	0.39
(solvent 2a)	0.45	0.51	0.52	0.48
(solvent 3a)	0.61	0.63	0.67	0.66
[FAB] m/z(M+H)+				
Found	902	916	930	974
Requires	902	916	930	974
31P[$CDCl_3$]	149.16 (s, 1P)	no data	no data	no data

O^6hydroxyethyl dG (see scheme 5)

N^2-(phenylacetyl)-2'-deoxyguanosine [38]

3',5'-Bis(O-acetyl)-N^2-(phenylacetyl)-2'-deoxyguanosine (11.5g, 24mmol) was stirred vigorously at RT with pyridine (50ml) and 2.5M NaOH (17ml). After exactly 5min TLC showed complete conversion to a low running product and the solution was neutralised by Dowex 50-X8 pyridinium form (100ml). The mixture was evaporated *in vacuo* and the required product was precipitated by addition of MeOH. (7.4g, 79%) R_f 0.23 (solvent 1) R_f 0.68 (solvent 2) R_f 0.75 (solvent 5) [FAB] m/z (M+H)+Found 386.13870 Requires 386.13861 δ_H[d_6DMSO] 2.23-2.35 (m, 2H, $H_{2',2''}$) 3.46-3.88 (m, 3H, $H_{4',5',5''}$) 3.80 (s, 1H, $\underline{CH_2}Ph$) 4.36-4.39 (m, 1H, $H_{3'}$) 4.95-

5.01 (t, J=5.4Hz, 1H $OH_{5'}$) 5.33-5.35 (d, J=3.7Hz, 1H, $OH_{3'}$) 6.18-6.26 (m, 1H, $H_{1'}$) 7.24-7.35 (m, 5H, Ar) 8.25 (s, 1H, H_8) 11.95 (m, 2H, H_1 & NH)

3', 5',O-(1,1,3,3-tetra-isopropyldisiloxyl)-N2-(phenylacetyl)-2'-deoxyguanosine [39]

N^2-(phenylacetyl)-2'-deoxyguanosine (2g, 5.6mmol) was dried by evaporation of anhydrous pyridine (3x5ml) then dissolved in anhydrous pyridine (15ml). 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (2.2ml, 2,2g, 6.8mmol, 1.2eq) was added and the solution stirred at RT. After 1hr the reaction was quenched with MeOH (2ml), evaporated *in vacuo*, redissolved in EtOAc, washed with saturated aqueous $NaHCO_3$, then evaporated to a gum. The residue was purified by flash chromatography (eluant: DCM/MeOH 95:5) and the appropriate fractions combined and evaporated *in vacuo* to give the *title compound.* (2.5g, 78%) R_f 0.79 (solvent 1) R_f 0.51 (solvent 3) R_f 0.24 (solvent 6) [FAB] m/z $(M+H)^+$ Found 628.29091 Requires 628.29084 $\delta_H[CdCl_3]$ 0.72-1.60 (m, 24H, CH_3x8) 2.41-2.52 (m, 2H, $H_{2',2''}$) 3.75-3.83 (m, 3H, $H_{4',5',5''}$) 3.86 (s, 1H, $\underline{CH_2}$Ph) 3.96-3.99 (m, 4H, CH_3x4) 4.55-4.67 (m, 1H, $H_{3'}$) 6.02-6.07 (m, 1H, $H_{1'}$) 7.28-7.35 (m, 5H, Ar) 7.89 (s, 1H, H_8) 12.08 (m, 2H, H_1 & NH)

Monoacetyl ethylene glycol

Ethylene glycol (24g, 21ml, 0.4mol) was stirred with anhydrous pyridine (20ml) and acetic acid (10g, 9.1ml, 0.1mol, 0.25eq). After 10min TLC showed presence of a higher running product which was purified by fractional distillation. (4.9g, 48%) R_f 0.55 (solvent 1) R_f 0.74 (solvent 2) R_f 0.21 (solvent 3) [EI] m/z (M) Found 103. Requires 103 $\delta_H[CdCl_3]$ 1.96 (s, 3H, CH_3) 3.63-3.68 (m, 2H, CH_2) 4.02-4.07 (m, 2H, CH_2) 4.67 (s, 1H OH)

3',5'O-(1,1,3,3-tetra-isopropyldisiloxyl)-N2-(phenylacetyl)-O6-(ethoxyacetyl)-2'-deoxyguanosine [40]

3',5'O-(1,1,3,3-tetraisopropyldisiloxyl)-N^2-(phenylacetyl)-2'-deoxyguanosine (2g, 3mmol) was dried by evaporation of anhydrous DCM (3x5ml) then dissolved in anhydrous DCM (20ml). TEA (1.7ml, 15mmol, 5eq), 2-mesitylene sulphonyl chloride (1.4g, 6mmol, 2eq) and DMAP (100mg, 0.6mmol, 0.2eq) were added and the reaction stirred at RT for 30min when TLC showed complete conversion to the O^6-sulphonated derivative. The solution was cooled to 0°C and N-methylpyrrolidine (3ml, 30mmol, 10eq) added. After 20min at 0°C, TLC showed conversion to the N-methylpyrrolidine quaternary salt, lower R_f. After warming to RT the solution was stirred rapidly with monoacetyl ethylene glycol (2.2g, 2.4ml 21mmol, 7eq) and DBU (1.35ml, 9mmol, 3eq). TLC showed production of a high running compound after

2hr and the reaction was quenched with aqueous KH_2PO_4 (100ml, 1M, pH6.5) the organic layer extracted, dried with $MgSO_4$ then evaporated *in vacuo*. The product was purified by flash chromatography (eluant: DCM/MeOH 96:4) and the appropriate fractions combined and evaporated *in vacuo* to a foam. (0.7g, 34%) R_f 0.84 (solvent 1) R_f 0.57 (solvent 3) R_f 0.29 (solvent 6) [FAB] m/z $(M+H)^+$ Found 714.32755 Requires 714.32762 $\delta_H[CdCl_3]$ 1.01-1.08 (m, 24H, CH_3x8) 2.02 (s 3H, CH_3CO) 2.53-2.63 (m, 2H, H2',2") 3.78-3.86 (m, 2H, CHx2) 4.11 (s, 2H, $\underline{CH_2}$Ph) 4.16-4.20 (m, 2H, CHx2) 4.40-4.71 (m, 3H, H4',5',5") 5.21-5.34 (m,1H, H3') 6.25-6.28 (m, 1H, H1') 7.25-7.35 (m, 5H, Ar) 7.97 (s, 1H, NH) 8.08 (s 1H H_8)

N^2-phenylacetyl-O^6-(ethoxyacetyl)-2'-deoxyguanosine [41]

3',5'O-(1,1,3,3-tetraisopropyldisiloxyl)-N^2-(phenylacetyl)-O^6-(ethoxyacetyl)-2'-deoxyguanosine (0.7g, 1mmol) was dissolved in anhydrous THF then concentrated *in vacuo* to a small volume. THF (2.5ml) and TBAF (1.4ml, 1M in THF) were added and the solution stirred rapidly at RT. TLC showed complete conversion to a low running product after 5min. The reaction mixture was diluted with pyridine/MeOH/H_2O (3:1:1 5ml) then added to Dowex 50-X8 pyridinium form (5g in 10ml of aforementioned 3:1:1 solution) and stirred gently for 20min. The Dowex was filtered off and washed thoroughly with pyridine/H_2O (1:1) (10ml) and the filtrate evaporated *in vacuo*. The product was purified by flash chromatography (eluant 90:10 DCM/MeOH and the appropriate fractions combined and evaporated *in vacuo* to give the *title compound.* (0.3g, 64%) R_f 0.42 (solvent 1) R_f 0.69 (solvent 2) R_f 0.21 (solvent 3) [FAB] m/z $(M+H)^+$ Found 484. Requires 484 δ_H[d6DMSO] 2.23-2.34 (m, 2H, H2',2") 3.38-3.41 (m, 3H, CH_3) 3.46-3.64 (m, 5H, CH_2 & H4',5',5") 3.81 (s, 2H, $\underline{CH_2}$Ph) 3.84-3.88 (m, 2H, CH_2) 4.38-4.40 (m, 1H, H3') 4.95-5.00 (t, J=5.2Hz, 1H $OH_{3'}$) 5.32-5.34 (d, J=3.5Hz, 1H, $OH_{5'}$) 6.19-6.25 (m, 1H, H1') 7.25-7.36 (m, 5H, Ar) 7.97 (s, 1H, NH) 8.08 (s, 1H, H_8)

Synthesis, deprotection and analysis of oligonucleotides containing O^6-alkyl dG

Oligonucleotide synthesis was carried out on an Applied Biosystems 380B DNA synthesiser on a 1.0 micromole scale following standard procedures, unless otherwise indicated. A total of 4 oligonucleotides were synthesised. Initially 5mer phosphorothioate poly T sequences were synthesised. The phosphorothioate linkages were introduced by replacing the oxidising agent (iodine) with tetraethyl thiuram disulphide on the DNA synthesiser. 49mer non-phosphorothioate sequences incorporating 3 of the modified nucleotides were then synthesised using the phosphorothioate 5mers on the solid support, producing 3 related 54mers as shown (where boldface indicates presence of phosphorothioate linkages).

5' 3'

TTTTTXTTTTTXTTTTTXTTTTTXTTTTTXTTTTTXTTTTTXTTTTTXT*TTTTT*

1. X=O6-MedG
2. X=O6-EtdG
3. X=O6-n-PrdG

Additionally 1 49mer, where every nucleotide possessed a phosphorothioate linkage, was synthesised, as shown.

5' 3'

TTTTTXTTTTTXTTTTTXTTTTTXTTTTTXTTTTTXTTTTTXTTTTTXT

X=O6-MedG

Following synthesis, sequences were deprotected by incubation with ammonia at 55°C for 24 hr, then analysed by HPLC, on a C_{18}. revesed phase column, using 0.1M NH_4OAc (buffer A) and 0.1M NH_4OAc in 20% acetonitrile (buffer B), with the following gradient:

Time (min)	%B
0	0
3	0
4	15
26	80
28	80
29	0
30	0

No HPLC purification was necessary. All oligonucleotides were desalted by sephadex G-25, then freeze dried.

Analysis by enzyme digest

Snake venom phosphdiesterase (50µl of 1mg/100µl in H_2O) and alkaline phosphatase (10µl of 1µl/19µl in H_2O) were added to 10 OD units of each 54mer in 50mM Tris.HCl/1M NaCl (pH8, 0.5ml). The mixtures were incubated at 37°C overnight, then analysed by reversed phase HPLC, using the above conditions. Standard solutions of dA,dG,dC,dT,O^6-MedG and diaminopurine (DAP) were subjected to HPLC under the same conditions to allow identification of the digested components of the 54mers.

Chapter 3. Production and Selection of Monoclonal Antibodies to O^6-alkyl dG

3.1. Introduction

3.1.1. The immune response

The immune response protects animals from foreign materials such as viruses, bacteria and toxins which may enter the body. The underlying molecular mechanisms of the system involve cell to cell communication and secretion of regulatory and signalling molecules. There are 2 types of immune response, i) innate and ii) acquired. The former removes foreign particles by phagocytosis and macrophage action, but fails to recognise the same particles on repeated exposure. The latter response which forms the basis of many immunochemical techniques leads to i) the activation of lymphocytes which produce cell surface receptors and ii) the production of circulating antibodies. Foreign particles which initiate the production of antibodies are known as antigens. The first exposure of an animal to an antigen results in a slow immune response, and after further exposure the antibodies are produced more rapidly and in vast quantities.

A vast repertoire of antibodies, with different antigen binding sites, can be synthesised by the immune system. There are millions of pre-determined receptor sites for antigens. The activation of these leads to the secretion of a specific antibody and stimulation of cell division of the antibody producing cells.

The production of an antibody to a particular antigen involves macrophages, T lymphocytes and B lymphocytes. Immediately after entering the body, foreign particles are engulfed non-specifically by macrophages or similar cells collectively known as antigen presenting cells (APCs) (Unanue, 1978,1984). The APCs then fuse with lysosomes and the antigen is partially degraded. The resulting antigen fragments are displayed on the surface of the APC (fig 3.1). A cell surface glycoprotein complexes with the antigen fragments; the complex is known as the major histocompatibility complex (MHC) (Forman, 1984). The MHC binds specifically with helper T lymphocytes. The receptors on the T cells are specific for a particular antigen-glycoprotein complex, therefore only a small number of the total T cell population in an animal will respond to any one complex. Binding to the MHC leads to proliferation and differentiation of T cells. This growth is helped by the production of interleulin 1; a growth factor produced by the APC (Dower and Urdal, 1987). A second growth factor, interleukin 2 (Oppenheim *et al*, 1986)

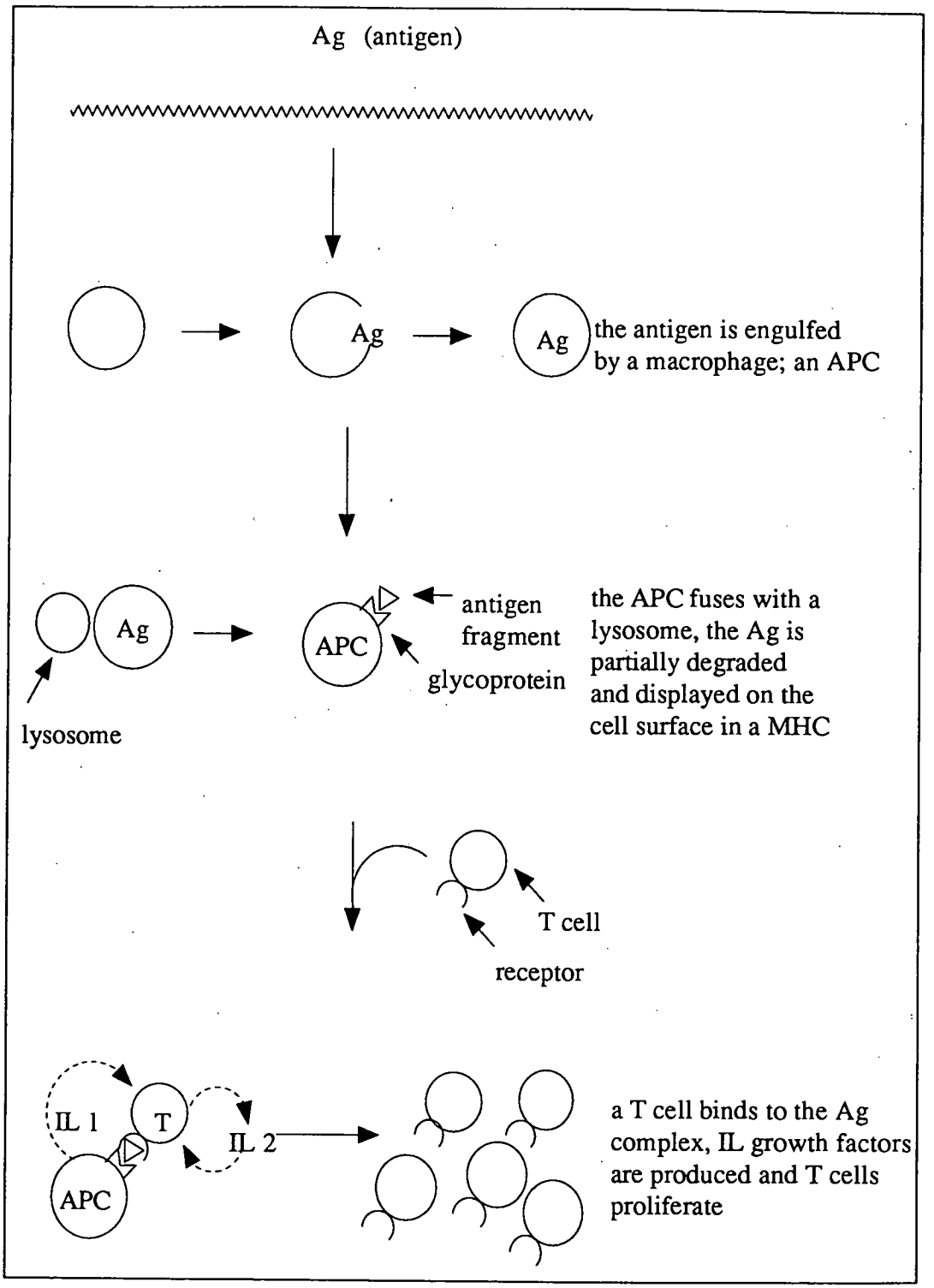


Figure 3.1. The proliferation of T cells

induces the T cells to reproduce exponentially. The growth factors are secreted as long as T cells bind to the MHC. Reduction in amounts of antigen, resulting in a decrease in T cell binding to the MHC arrests the production of interleukin 1 and 2 and hence the T cell proliferation diminishes.

During the proliferation of T cells, B lymphocytes are also being activated by the antigen (fig 3.2). The B cells have a specific antibody type receptor on their cell surface (Cambier *et al*, 1993). Following antigen binding the receptor and antigen are internalised, and the antigen is partially degraded then presented as a MHC on the cell surface. T cells which are specific for this complex (already abundant in the circulation) bind to the MHC on the B lymphocytes stimulating them to synthesise growth factor receptors (Coutinho *et al*, 1984; Parker, 1993). The T cells then produce interleukin 4 (Howard *et al*, 1982) which initiates the proliferation and differentiation of B lymphocytes. After binding interleukin 4 the B cells grow exponentially and begin to respond to interleukin 5 (Swain *et al*, 1983) which results in the differentiation of B lymphocytes into plasma and memory cells. These cells travel through the blood and lymphatic systems and accumulate in lymphoid tissues (lymph nodes and spleen in mammals). Antibody is secreted in vast quantities by the plasma cells, which are devoted to this purpose but are short lived. In contrast the memory cells do not secrete antibody and remain in circulation.

During differentiation and maturation of the B cells, mutations occur which lead to the production of antibodies with differing affinities (Kishimoto, 1985). The cells producing high affinity antibodies proliferate more readily as the B cell surface receptor binds more efficiently to the antigen. Class shifting also occurs during differentiation (Harreman, *et al*, 1993). Large IgM antibodies which are produced initially are limited in their movements across membranes and are therefore often replaced by smaller IgG antibodies. These IgGs often have higher affinity due to the maturation process. When the antigen is eliminated by the action of antibodies, the plasma cells and hence antibody production decline. A second exposure to the same antigen results in activation of the memory cells and a secondary response occurs immediately. The presence of many helper T cells and the memory B cells ensure that the first antibodies to be produced are of high affinity and are IgGs.

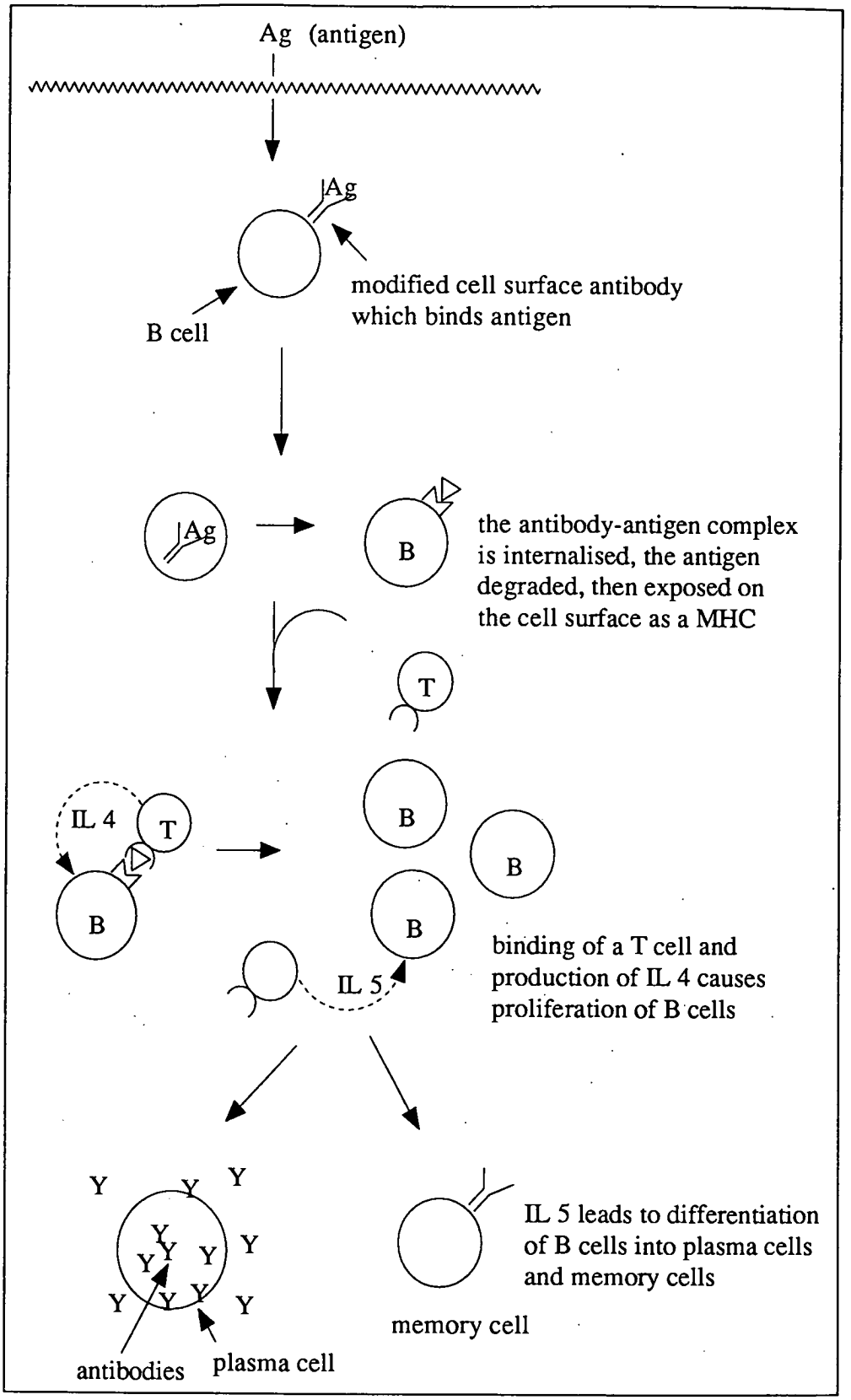


Figure 3.2. The production of antibodies by B-cells

3.1.2. Polyclonal and monoclonal antibodies

A polyclonal antibody response occurs following the introduction of an antigen to an animal (i.e. many different antibodies are produced, each from a single stimulated lymphocyte and its clonal progeny). The antibodies may be of different classes (i.e. IgG, IgM, IgE, IgA, IgD) have different affinities, or recognise different epitopes on the antigen. Despite this, immunisation procedures can be controlled to favour the predominant production of an antibody of a particular class, specificity and affinity. Polyclonal antibodies can be useful in recognising multiple determinants and in immunoprecipitation experiments (Dunbar and Skinner, 1990). However the type, specificity and affinity of these antibodies can alter over time due to the *in vivo* maturation response, previously discussed. Consequently, polyclonal antibodies are disadvantageous for immunoassay and immunoaffinity techniques, which require the constant supply of an antibody with a pre-defined specificity and affinity.

The development of hybridoma technology, by Kohler and Milstein (1975) allows the continuous production of an antibody with a pre-defined specificity. This is achieved by fusing an antibody producing B lymphocyte (which cannot survive in tissue culture), with a non-antibody producing myeloma cell (a B tumour cell specially adapted to efficient growth in culture). The hybrid possesses the antibody secreting capacity of the B lymphocyte, and the longevity of the myeloma. Hybridomas can be grown in culture, or be reintroduced to an animal and grow as an antibody producing tumour. Antibodies produced by this technique are referred to as monoclonal, since the total antibody present comes from a single lymphocyte and its identical progeny. The antibodies produced are therefore identical in structure and function. The production of monoclonal antibodies (MAbs) is described in many texts (Kennett *et al*, 1980; Kohler, 1980; Goding, 1986; Brown and Ling, 1988)

MAbs are advantageous for immunoassay and immunoaffinity experiments for 3 major reasons: i) a single antibody recognises one determinant, therefore there is minimal cross reactivity in assays, ii) large quantities of an antibody can be obtained and easily purified and iii) an antibody with specific characteristics can be selected during screening procedures.

3.1.3. The production of monolconal antibodies

The production of monoclonal antibodies can be divided into 4 separate techniques, i) immunisation, ii) screening, iii) hybridoma production and iv) cloning. After immunisation and boosts, test bleeds can be taken to allow a screening technique to

be developed (fig 3.3). On confirmation (by screening techniques) that the antibody is present in the sera, the appropriate lymphoid tissue (i.e. the spleen) is removed from the animal. The animal is usually re-exposed to the antigen to obtain maximum antibody production prior to this operation. The splenocytes are extracted and fused with myeloma cells, and after a period of 1-2 weeks the resulting hybridomas can be screened for production of a specific antibody. Hybridomas producing the desired antibody are cloned by limiting dilution to obtain a single clone secreting the antibody of interest.

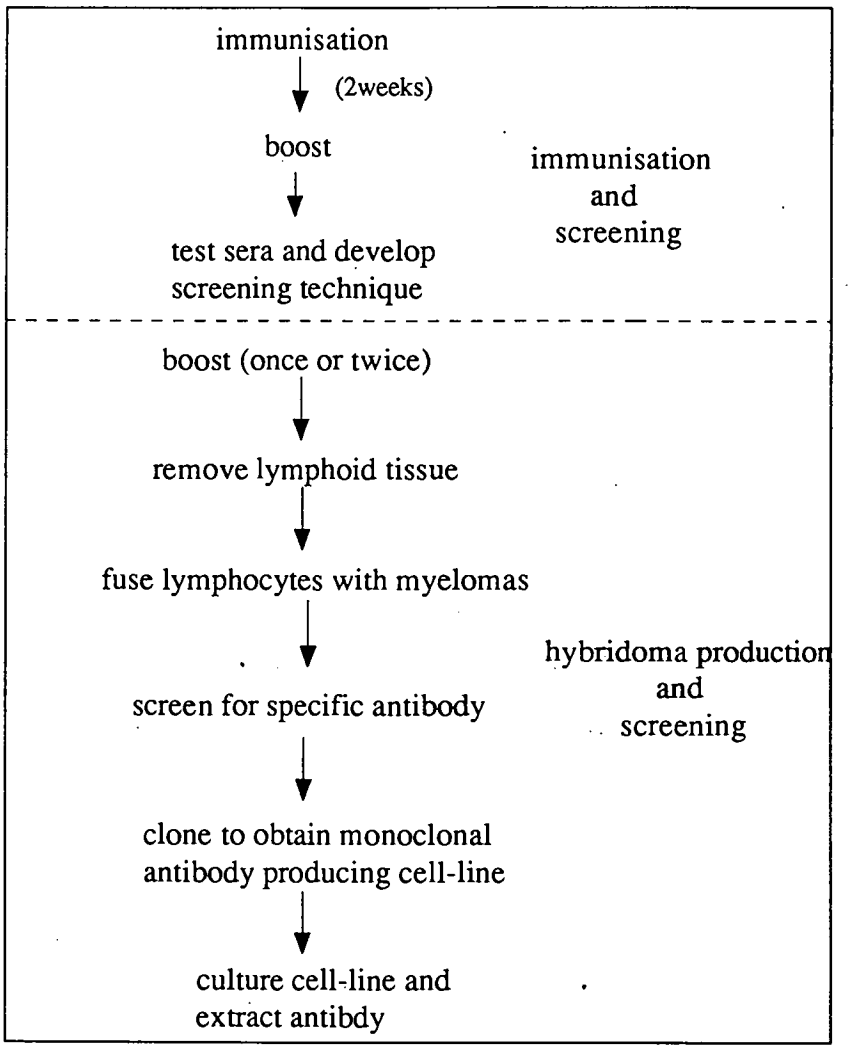


Figure 3.3 An outline of monoclonal antibody production

3.1.3.1. Immunisation and bleeds

There appears to be no universal immunisation procedure, as different antigens may require completely different protocols to ensure optimum antibody production. It is important that the splenocytes and myelomas, to be fused, come from the same strain

of animal. The myeloma cell lines commonly available are balb/c in origin, therefore immunisations for monoclonal antibody production are generally carried out in these mice.

Soluble antigens are mixed with an aqueous adjuvant mixture prior to immunisation. Freunds adjuvant, 'complete' or 'incomplete', is normally used for small molecules (Freund, 1947). The initial immunisation is carried out in 'complete' which contains mineral oil, and heat killed *Myobacterium tuberculosis*; further boosts are conducted in 'incomplete' which does not contain bacteria. The oil is believed to form an emulsion protecting the antigen from rapid degradation *in vivo*. The bacteria contain a muramyl dipeptide toxin which leads to non-specific stimulation of the immune response (Ellouz *et al*, 1974). The induction of MAbs does not require immunisation with a pure antigen, unlike immunisations to produce polyclonal antibodies. The route of injection may influence the type of antibody response. Adjuvant-antigen mixtures are injected subcutaneously (200µl maximum) or intramuscularly (50µl maximum), to favour slow antigen release to the local lymph nodes.

It is essential to ensure the presence of specific antibody before the final boosts and removal of the spleen. This is determined by taking a preliminary bleed, normally from the tail vein of the mouse, and screening the sera for antigen binding activity.

3.1.3.2. Screening of antibodies

The screening assay is used at 3 stages in the production of monoclonal antibodies: i) subsequent to immunisation to test for the presence of specific antibody, ii) following fusion of the splenocytes and myelomas, to select appropriate hybridomas and iii) after cloning to select a monoclonal cell line producing the antibody of interest. Many cell lines are generated during a fusion and screening experiments may be repeated many times. The ideal assay is therefore simple, easily reproduced and rapid so that immediate attention can be given to selected cell lines.

Most screening assays involve 2 stages. Firstly, antibodies are tested for activity against the antigen and, secondly, those giving positive results are retested against a range of antigens to ensure that the antibody binds selectively to the antigen of interest. These tests are referred to respectively as non-competitive and competitive assays. Several specific screening assays have previously been discussed in chapter 1. ELISA (Wisdom, 1976; Voller, 1979) is generally regarded as the most

convenient assay as it does not require the use of radiolabeled substrates and has been shown to be more sensitive than other assays (Poirier *et al,* 1980; Santella *et al,* 1988).

Non-competitive ELISA can be carried out in 96 well plastic microtitre plates, or on nitrocellulose or nylon membranes. A reagent is added, incubated, then washed from the plate or membrane (fig. 3.4).

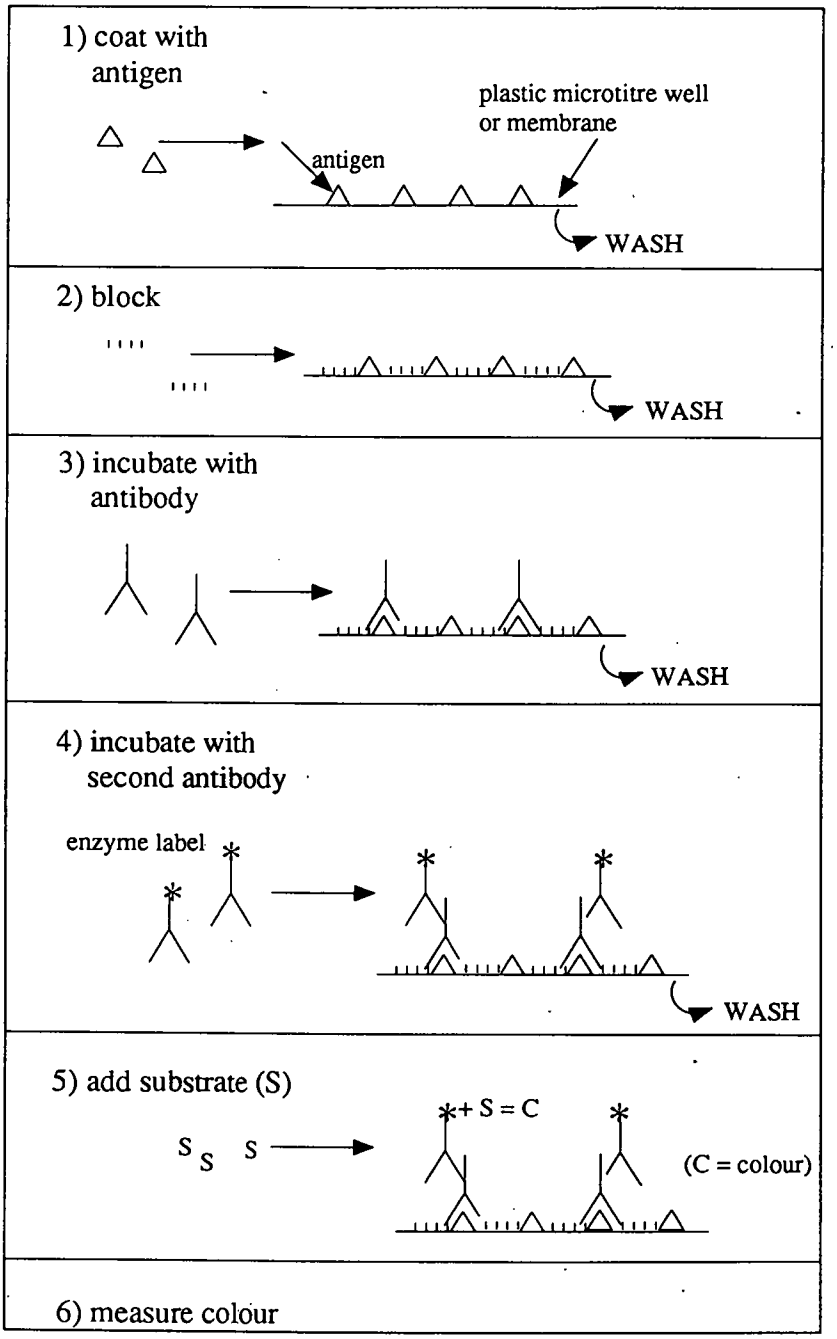


Figure 3.4. Non-competitive ELISA

Firstly the antigen is coated on the wells of the plate, or dotted onto a membrane. After incubation and washing the unoccupied sites on the plate/membrane are blocked (usually with a protein) to prevent non-specific binding of the antibody, to uncoated areas (Trivers *et al,* 1983). The antibody of interest is then incubated with the coated plate or membrane. After allowing antibody-antigen binding to reach equilibrium excess antibody is removed by washing. An enzyme conjugated second antibody, which binds to the first antibody, is then added. The conjugated enzyme, commonly alkaline phosphatase or horseradish peroxidase, is capable of converting a substrate to give a coloured product. The final step of the assay involves the addition of the appropriate substrate and the measurement of the resulting colour. The amount of colour is proportionate to the quantity of antibody bound to the antigen on the microtitre well or membrane. Normally an antibody is diluted across a plate or membrane allowing a characteristic dilution curve to be plotted. The antibody titre (the dilution at which the antibody half maximally binds to the plate) can be determined from this curve. An estimate of titre is necessary for carrying out a competitive assay.

Competitive ELISA (fig. 3.5) which must be carried out in microtitre wells is essentially similar to the non-competitive assay. A pre-determined concentration of antibody (from non-competitive ELISA) is mixed with a known amount of free antigen before being added to the plate. If the antibody is specific for the added antigen, the amount of antibody binding to the antigen on the plate decreases. This decrease is determined by direct comparison with a sample with no antigen added and the percentage inhibition by the free antigen can be calculated. A dose response effect can be obtained by mixing decreasing amounts of antigen with the antibody. If a small amount of free antigen prevents antibody binding to the coated plate the antibody has high specificity and affinity for that antigen. These assays allow the selection of the appropriate cell lines throughout the procedure of monoclonal antibody production.

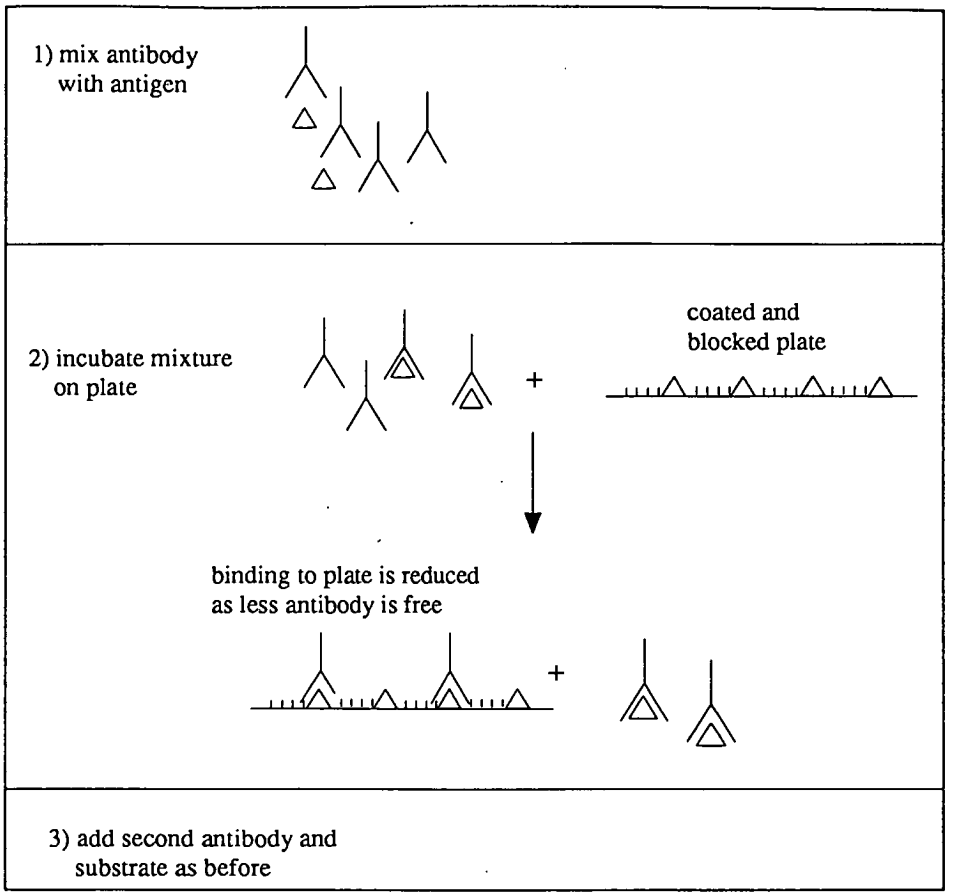


Figure 3.5 Competitive ELISA

3.1.3.3. Hybridoma production

The fusion of splenocytes with myelomas is not a spontaneous process. Fusion is commonly induced by the presence of polyethylene glycol (PEG), by a mechanism which is poorly understood (Pontecorvo, 1975). It has been suggested that a contaminant in the PEG plays a role in the fusion process (Wojciezsyn *et al*, 1983). After the fusing of the cell membranes heterokaryons (multinucleate cells) are formed. The next cell division produces daughter cells with a fused nuclei containing a share of the genetic information from a myeloma and a splenocyte. In addition to the desired splenocyte-myeloma hybrids there are undesirable products including splenocyte-splenocyte and myeloma-myeloma hybrids, and also multiple cell products. Splenocyte-splenocyte hybrids and unfused splenocytes cannot survive in culture and die within 7-10 days.

Myeloma-myeloma hybrids and unfused myelomas do not die naturally. Their elimination is achieved by a selection procedure devised by Littlefield in 1964. Aminopterin (a folic acid antagonist) is added to the cell culture medium to block the

main cellular DNA biosynthetic pathway. The desired hybridomas can continue to synthesise DNA *via* an alternative salvage pathway, in which the enzymes hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and thymidine kinase play a role. The former converts hypoxanthine and guanine to guanosine monophosphate, and the latter thymine to deoxythymidine monophosphate (fig 3.6).

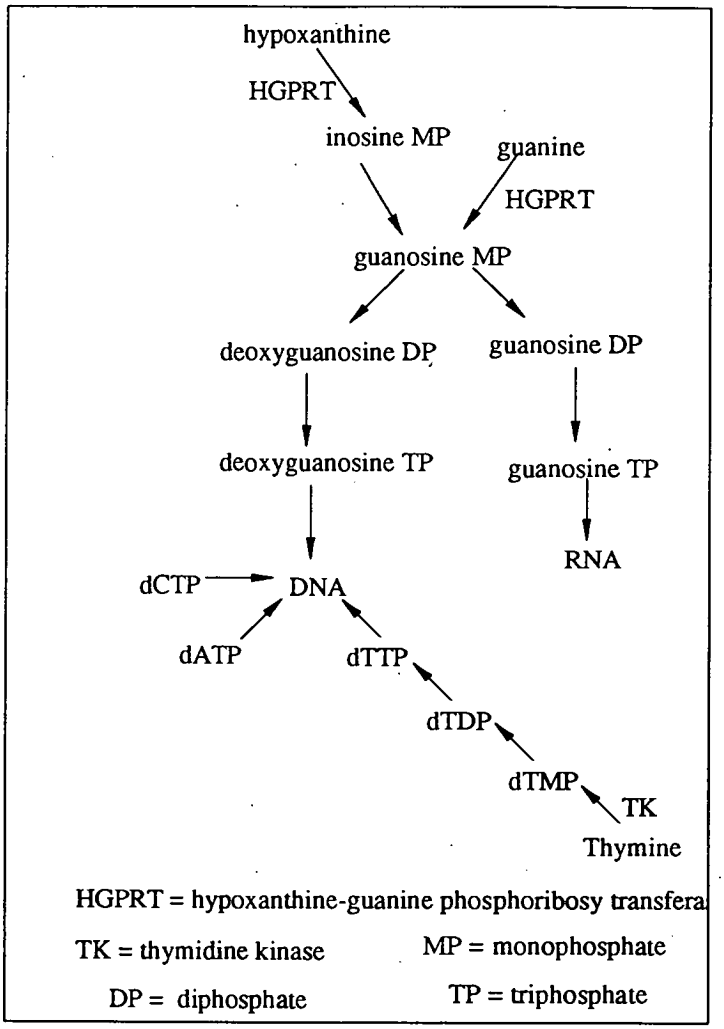


Figure 3.6. The role of HGPRT and TK in cellular DNA synthesis

Hypoxanthine and thymidine are added to the culture medium (in addition to the aminopterin) to ensure the survival of the hybridomas by this pathway. The myeloma cells routinely used in fusions are previously cultured to be deficient in the HGPRT enzyme (the most commonly used myeloma cell lines, sp2/0Ag14 and X63-Ag8.653 are commercially available). The salvage pathway is non functional in these cells, so that after the addition of aminopterin the myelomas die. However a

myeloma fused with an HGPRT positive splenocyte will synthesise DNA by the salvage pathway and hence survive.

There is some variation in fusion techniques, but most methods are based on the 50% PEG stirring procedure of Galfre *et al* (1977), or the 30% PEG spinning procedure of Geftre *et al* (1977), which was adapted by Kennett in 1978. Generally 10^8 lymphocytes (derived from 1 mouse spleen) are mixed with approximately 10^7 myeloma cells which must be in the exponential phase of growth. The cells are centrifuged to form a pellet, then diluted by the addition of PEG (30-50%). Temperature, pH, myeloma:splenocyte ratio, the presence of contaminants such as erythrocytes and the molecular weight of the PEG, are thought to influence the success of the fusion (Zola and Brooks, 1982). The actual fusion of the cells is normally carried out in serum free medium, then the fused products are immediately introduced to medium containing growth supplements (i.e. foetal calf serum) and antibiotics to prevent infections, then the hypoxanthine, aminopterin and thymidine (HAT) medium is added. The serum is believed to provide minerals and hormones essential to cell growth, but the exact components which aid cell growth are not known (Barnes and Sato, 1980).

The hybrid products are distributed into microtitre wells, to increase the chances of obtaining only one clonal product per well. The number and size of wells used for this purpose varies. Galfre and Milstein (1981) used 48 x 1ml wells. The cells are normally supplemented every 2-3 days by the removal of half the medium and addition of fresh material, containing HAT. This removes waste material in addition to diluting out antibody that is being released by the dying unfused splenocytes. Myeloma cells die within one week in HAT solution, therefore its addition can be discontinued at this point. Aminopterin is metabolised slowly by cells, whereas hypoxanthine and thymidine are metabolised more quickly. For this reason, hypoxanthine and thymidine (HT) medium is added to the cells for several days after discontinuing HAT addition, as the salvage pathway remains to be the main biosynthetic route for DNA synthesis while traces of aminopterin remain.

3.1.3.4. Cloning

The wells containing cells which are producing the antibody of interest are identified by screening procedures. The contents of these wells are cloned by limiting dilution to prevent overgrowth of the desired cells by non-secreting hybrids. The cloning

procedure aims to dilute the cells on a microtitre plate and achieve wells which contain only one clone (visible by light microscope). After these single cells have proliferated screening techniques may be employed to identify those producing the desired antibody. Normally it is necessary to clone several times before obtaining a single healthy clone producing the desired antibody. The growth of hybridoma cells at high dilutions is normally unsuccessful, but growth can be aided by the addition of mouse thymocyte feeder cells (Lernhardt *et al*, 1978).

Cells producing monoclonal antibodies can be grown continually in culture and expanded to larger wells and flasks. A culture medium change is required every 2-3 days. Larger quantities of antibody can be produced by growing the hybridomas as tumours in animals. In addition cells can be frozen and stored in liquid nitrogen for several years with a good recovery rate (Goding, 1986). Purification of the antibody is normally required before use. The traditional method involves precipitation of the antibody in high salt concentrations, but this can lead to contamination bythe precipitation of other serum proteins. Gel filtration, affinity chromatography and ion-exchange chromatography are also employed in antibody purification schemes (Ey *et al,* 1978; Bruck *et al,* 1982). It is important to determine the class and subclass of an antibody as this influences the biological and physical properties, in addition to determining the purification technique employed. These can be determined by simple immunochemical assays.

3.2. Results and Discussion

3.2.1. Immunisation

The O^6-alkyl dG derivatives which were conjugated to a protein or incorporated into an oligonucleotide were suitable for both the immunisation and screening procedures required for the production of a class specific monoclonal antibody. The ability to synthesise such compounds created the potential for a vast array of immunisation, screening and fusion protocols. There may have been numerous ways of producing and obtaining the desired O^6-alkyl dG monoclonal antibody. However the most successful route to this end was impossible to determine prior to experimentation. Most of the O^6-alkyl dG compounds (table 3.1) were therefore used for immunisations to allow the identification of the best antigens. Each antigen was administered to at least 2 mice. The mice with the highest antibody response were employed in fusion experiments.

Table 3.1. Immunisations and fusions

Antigen	Number of mice immunised
O^6-MedG-BSA	5
O^6-EtdG-BSA	2
O^6-n-PrdG-BSA	2
O^6-hydroxyEtdG-BSA	2
O^6-CBdG-BSA	2
oligo Me	2
oligo Et	2
oligo n-Pr	2
oligo MeCP	2

oligo Me = 3' phosphorothioate (PS) 54mer incorporating O^6-MedG
oligo Et = 3' PS 54mer incorporating O^6-EtdG
oligo nPr = 3' PS 54mer incorporating O^6-n-PrdG
oligo MeCP = complete PS incorporating O^6-MedG

3.2.2. Initial screening and immunoassay development

In the time available for this study it was not possible to test each immunised mouse for antibody production, neither was it possible or necessary to use all mice for

fusion experiments. Bleeds from oligonucleotide immunised mice were tested to estimate the success of antibody production by this novel method. Additionally, three of the O^6-MedG-BSA, one of the O^6-hydroxyEt dG-BSA and one of the O^6-CBdG-BSA immunised animals were bled. The sera were screened by competitive ELISA to determine the antibody titre, and non competitive ELISA to determine the antibody specificity.

ELISA requires a coating of antigen on each microtitre well of a plastic plate as previously described. Free nucleosides do not normally bind to this surface and protein conjugates, which bind non-covalently by an unknown mechanism, are often used. The O^6-alkyl dG-protein conjugates were considered suitable for screening antibodies raised to the oligonucleotide antigens. Antibodies specific for the thymidine residues in the oligonucleotides went unrecognised in this assay, whereas O^6-alkyl dG antibodies cross-reacted with the O^6-alkyl dG derivative of the protein conjugate on the plate. CγG conjugates were used to screen the serum of the O^6-alkyl dG-BSA conjugate immunisations.

At this stage the competitive ELISA was not designed to test for class specificity, so, for example serum from a mouse subjected to an O^6-MedG antigen was also screened and tested for inhibition with this derivative. A 10µl/ml solution of each free O^6-alkyl dG nucleoside was used to calculate the percentage inhibition of antibody binding to the plate. The actual amount of inhibition was therefore not of great importance. However <10% to 0% inhibition generally indicated that minimal amounts of specific antibody were being produced, and higher amounts of inhibition suggested that a significant amount of specific antibody was present. The concentration of antibody used for this purpose (i.e. the antibody titre) was determined from the dilution curves which were plotted from the results of the non-competitive ELISA (see fig. 3.7 for examples).

Comparison of the antibody titres indicated a variation in antibody response between mice (table 3.2). For example, the immunisation of two mice with an oligonucleotide containing O^6-EtdG resulted in a high response in mouse 'a', titre 1:1200, and a low response in mouse 'b', titre 1:500. This variation made it difficult to determine 'good' and 'bad' antigens. However, on balance, there appeared to be a significantly higher titre and percentage inhibition in the mice immunised with oligonucleotide antigens, particularly the complete phosphorothioate oligonucleotide. The high response to the CP oligonucleotide may have been

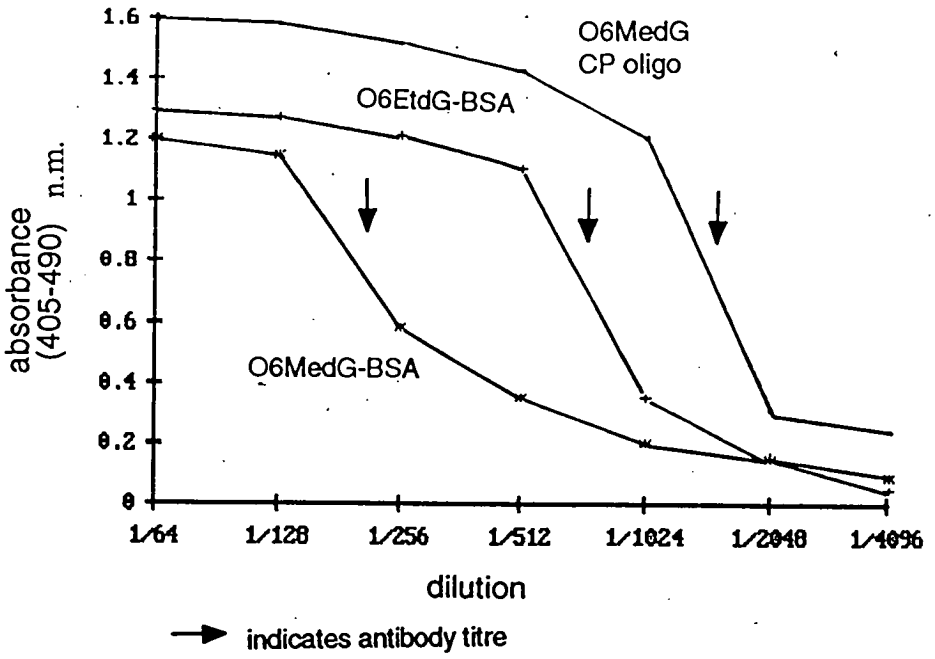


Figure 3.7. Variation in antibody response and titre

attributable to, i) resistance to enzymatic degradation, resulting in persistence of the oligonucleotide in the mouse after immunisation and ii) the presence of the sulphur atom which makes the phosphorothioate oligonucleotide less like a 'self molecule'. However the sample size was too small to draw unambiguous conclusions.

Table 3.2. Initial screening results from non-competitive and competitive ELISA, showing compounds used, immunisation and plate coating, for the initial screening of serum.

		ELISA non-comp.	ELISA comp.	
Antigen/ mouse	**Plate coating (1μg/ml)**	**Titre**	**Inhibitor (10μg/ml)**	**Inhibition (%)**
oligo Me/a	O^6-MedG-BSA	1:200	O^6-MedG	0
/b	"	1:500	"	6
oligo Et/a	O^6-EtdG-BSA	1:1200	O^6-EtdG	32
/b	"	1:500	"	19
oligo n-Pr/a	O^6-n-PrdG-BSA	1:800	O^6-n-PrdG	6
/b	"	1:1100	"	25
oligo MeCP/a	O^6MedG-BSA	1:1100	O^6-MedG	43
/b	"	1:1300	"	24
O^6-MedG-BSA/a	O^6-MedG-CγG	1:800	O^6-MedG	20
/b	"	1:700	"	34
/c	"	1:500	"	19
O^6-hydroxyEtdG-BSA /a	O^6-hydroxyEtdG-CγG	1:800	O^6-hydroxyEtdG	38
O^6-CBdG-BSA /a	O^6-MedG-CγG	1:1000	O^6-MedG	19

The 3' PS oligonucleotide containing O^6-MedG elicited the weakest immune response (1:200 mouse 'a', and 1:500 mouse 'b') (table 3.2). It is unlikely that the O^6-methyl group created a less antigenic compound than higher alkyl groups, as the O^6-MedG-BSA antigen showed a high titre. The surprisingly low titre may have been due to inter-mouse variation, or degradation of the antigen.

The results shown in table 3.2 indicated that in all cases, except for the 3'PS Me oligo, a specific antibody response occurred. Mice demonstrating a high antibody

response (high titre), and the presence of a specific antibody (high inhibition) were used in fusion experiments.

3.2.3. Fusions and cloning

A total of eight fusions were carried out. In all cases approximately 10^8 splenocytes were mixed with 10^7 myelomas and 40% PEG was added to initiate the fusion reaction. The resulting splenocyte/myeloma/hybridoma mixture was divided into the inner 60 wells of six, 96-well sterile microtitre plates. The cells were cultured for 14 days to favour the proliferation of the hybridomas. Fusions were generally considered successful if 90% of the wells contained hybridomas. The hybridomas were then screened to ascertain the presence of the desired antibody (table 3.3). Initially, non-competitive ELISA was employed to detect specificity towards the O^6-alkyl dG used for immunisation. Negative wells were immediately eliminated.

Table 3.3 Success of fusions, showing hybridoma production and initial screening results

Antigen/ mouse	Hybridoma Production(%)	Non comp. ELISA results	Plate coating (1μg/ml)
oligo Et/a	failed	-	-
oligo n-Pr/a	10	2 +ve wells	O^6-n-PrdG-BSA
oligo MeCP/a	90	23 +ve wells	O^6-MedG-BSA
O^6-MedG-BSA/a	90	31 +ve wells	O^6-MedG-CγG
/b	90	35 +ve wells	"
/c	90	11 +ve wells	"
O^6-hydroxyEtdG-BSA /a	90	59 +ve wells	O^6-hydroxyEtdG-CγG
O^6-CB-BSA /a	90	34 +ve wells	O^6-n-PrdG-CγG

Positive wells were retested by competitive ELISA with the initial O^6-alkyl dG and a second or third O^6-alkyl dG to identify wells with hybridomas producing a class specific antibody. During this procedure cells were also tested for cross reactions with dG. Hybridomas producing antibodies which cross-reacted with dG were discarded. Cells producing group specific antibodies (i.e. an antibody which recognised only one O^6-alkyl dG derivative) were also identified, cultured, then stored at -70°C, for future studies.

The 2 positive wells from the oligo n-Pr fusion and the 34 positive wells from the O^6-CB-BSA fusion showed no inhibition in a competitive assay with O^6-MedG, O^6-EtdG or O^6-n-PrdG. One of the three O^6-MedG-BSA fusions became infected and was discarded. A second showed inhibition with O^6-MedG and O^6-EtdG, but also showed the same extent of inhibition with dG, and was also discarded. A small number of the positive hybridomas from the three remaining fusions demonstrated inhibition with more than one O^6-alkyl dG derivative. Only wells which showed >30% inhibition were considered, and are described below.

a) oligo MedG CP fusion: three wells demonstrated inhibition with O^6-MedG, two of which also showed inhibition with O^6-EtdG, **wells 5D8** and **6E5**
b) O^6-MedG-BSA fusion: four wells demonstrated inhibition with O^6-MedG, one of which also showed inhibition with O^6-EtdG and O^6-n-PrdG, **well 2C8**
c) O^6-hydroxyEt dG-BSA fusion: eleven wells demonstrated inhibition with O^6-hydroxyEt dG, ten of which also showed inhibition with O^6-EtdG. Nine of these wells demonstrated inhibition with O^6-n-PrdG, and two of these showed inhibition with O^6-MedG, **wells 2G2** and **2E3**.

Immediate attention was given to cells from wells **2C8**, **2G2** and **2E3**, as results suggested that cells from these wells may be secreting class specific antibodies. They were expanded into 2ml wells, and aliquots of approximately 200 cells from each well were mixed with feeder thymocytes and cloned by limiting dilution. Monoclonal wells were identified under a microscope then tested by ELISA. One positive monoclonal well from each clone plate was chosen, and recloned. Monoclonality of the chosen cell lines was confirmed by ELISA on the third or fourth generation of clone plates, when the presence of any number of clones in a well correlated with the amount of colour produced on screening. An absence of cells resulted in no colour. The detailed cloning procedures and results are described for **2C8**, **2G2** and **2E3**, in figs. 3.8, 3.9 and 3.10 respectively.

The cells from wells **5D8** and **6E5** which recognised only O^6-MedG and O^6-EtdG were expanded into 2ml wells, then frozen and stored for future reference.

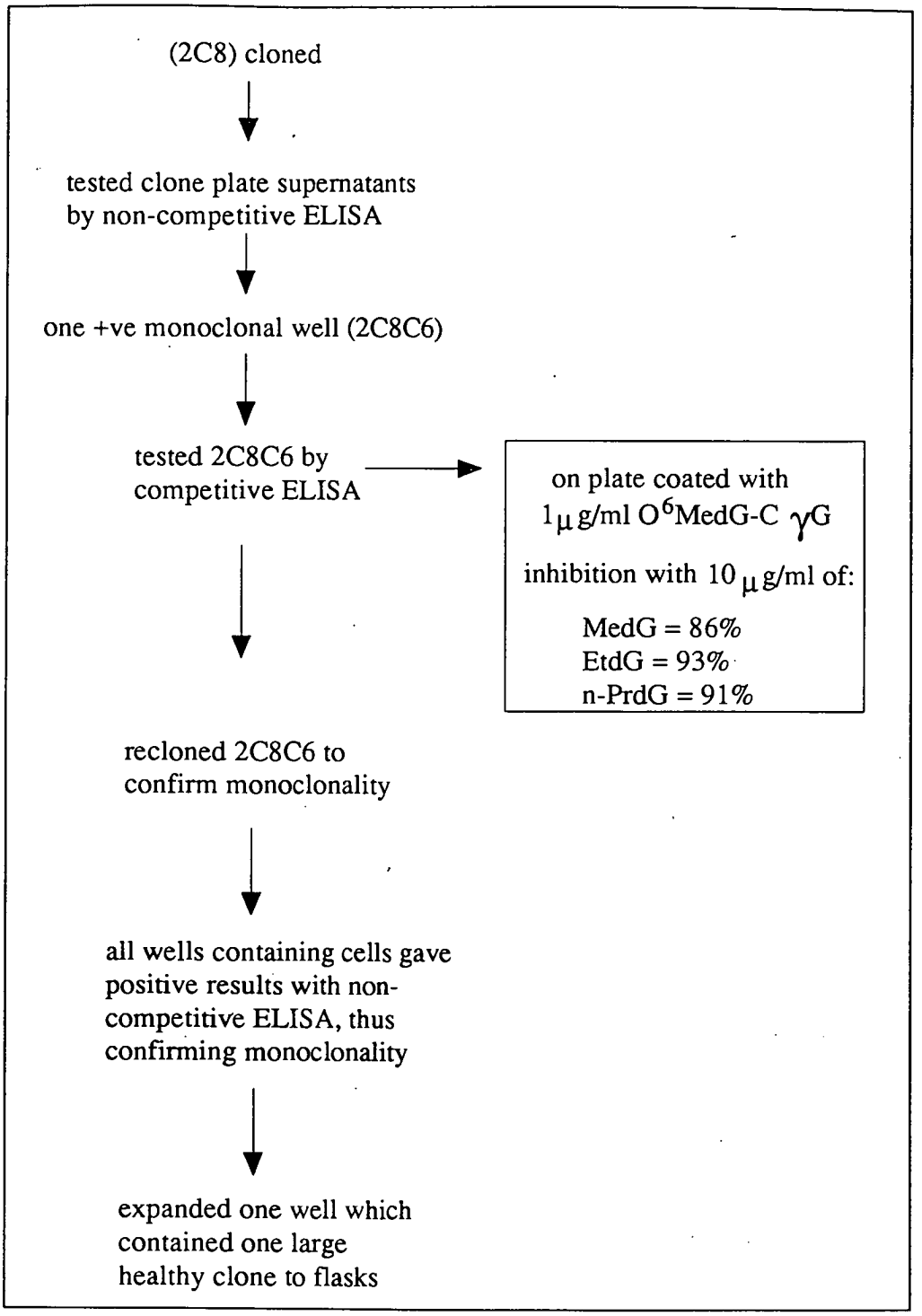


Figure 3.8. Cloning of 2C8

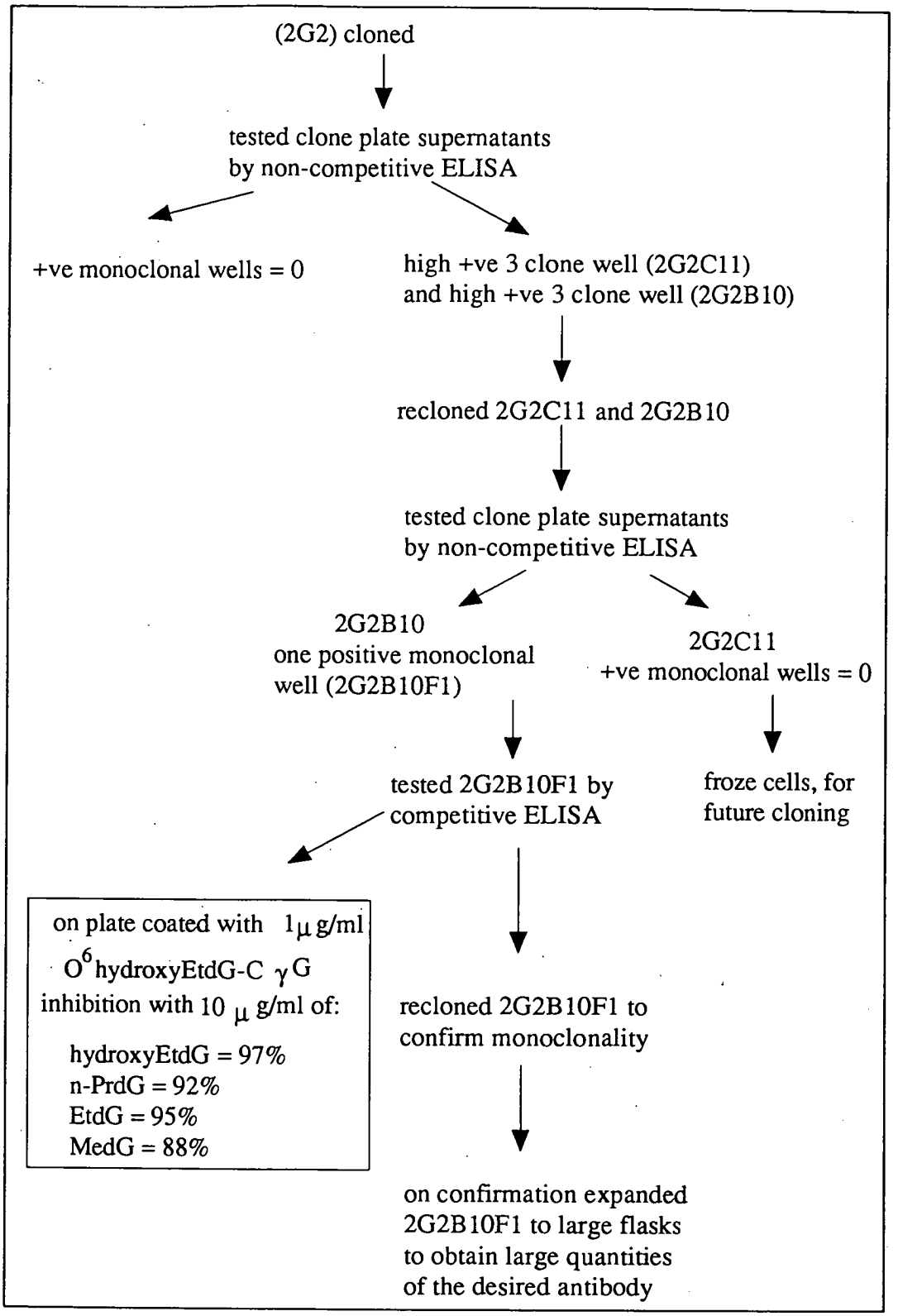


Figure 3.9. Cloning of 2G2

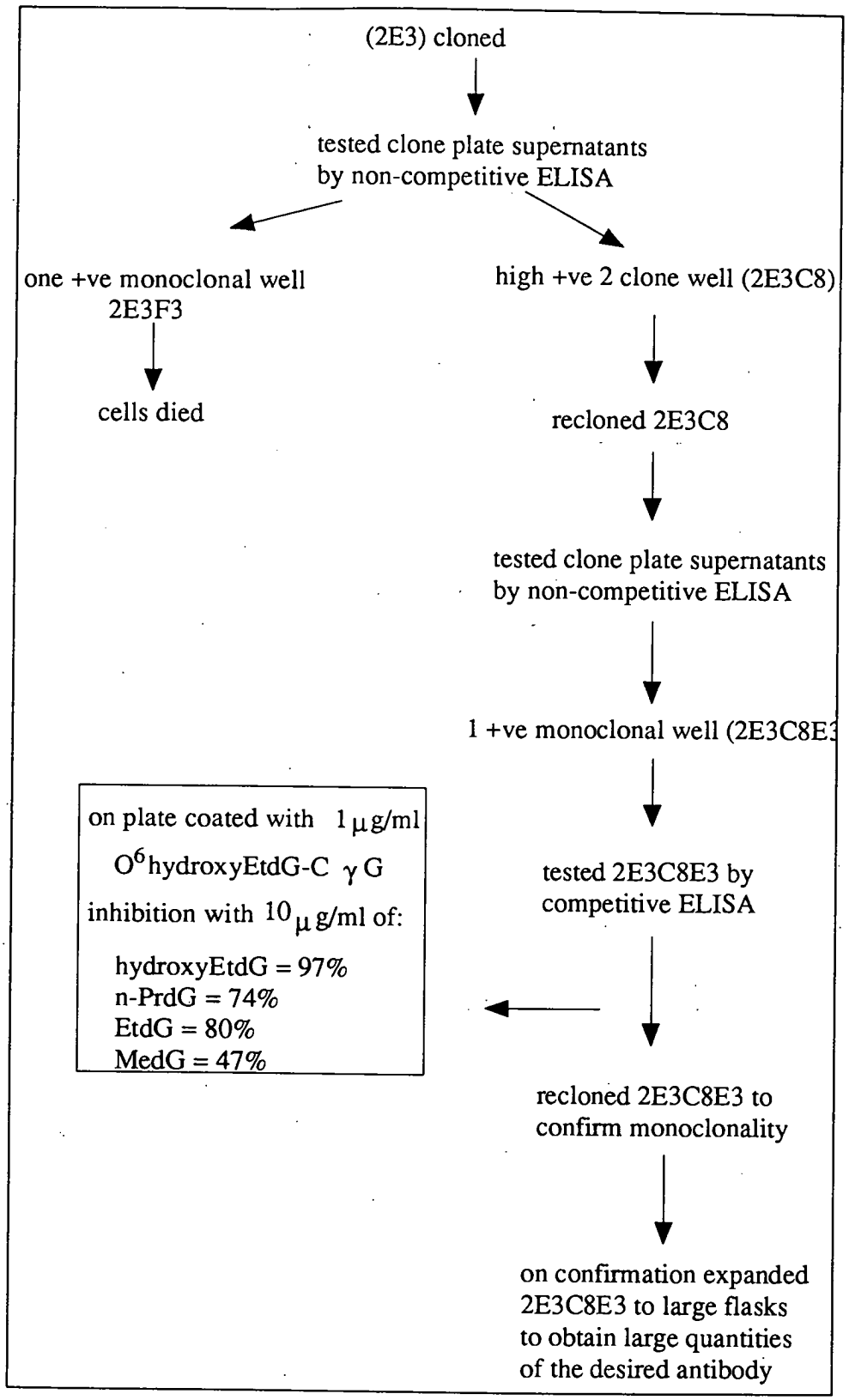


Figure 3.10. Cloning of 2E3

3.2.4. Characterisation of 2C8C6H5, 2G2B10F1 and 2E3C8E3

The three monoclonal cell lines were grown in large cell culture flasks for 4-6 weeks. The number of cells in each flask was maintained at a fairly constant level by the removal of some cells every 2-3 days. The supernatant was removed, accumulated and stored at 4^{o}C,and fresh medium was added to the cells.

3.2.4.1. ELISA to determine comparative antibody affinity for each O^{6}-alkyl dG derivative

These experiments compared the affinity of each of the three monoclonal antibodies, for each O^{6}-alkyl dG by non-competitive and competitive assays. There were many possible assay designs, in terms of, i) the O^{6}-alkyl dG protein conjugate immobilised on the plate, and ii) the free O^{6}-alkyl dG derivative competing for the antibody. Both factors strongly influence the results. For example, an antibody with high affinity for O^{6}-EtdG would require a large amount of O^{6}-MedG or O^{6}-n-PrdG to obtain inhibition on an O^{6}-EtdG coated plate. Furthermore, O^{6}-EtdG would then exhibit more inhibition on an O^{6}-MedG or O^{6}-n-PrdG coated plate, than on an O^{6}-EtdG coated plate. To avoid over-estimating results in this way, competitive inhibition assay were carried out on plates coated with the O^{6}-alkyl dG for which the antibody exhibited highest affinity.

Results obtained from the competitive ELISAs during cloning, indicated that 2C8C6H5 had marginally higher affinity for O^{6}EtdG, and 2G2B10F1 and 2E3C8E3 had marginally higher affinity for O^{6}-hydroxyEt dG. 2C8C6H5 was therefore tested on an O^{6}-EtdG-CγG plate and 2G2B10F1 and 2E3C8E3 on an O^{6}-hydroxyEtdG-CγG coated plate.

2C8C6H5

This antibody resulted from an O^{6}-MedG-BSA immunisation. Competitive ELISA showed that 2C8C6H5 was inhibited by O^{6}-MedG, O^{6}-EtdG and O^{6}-n-PrdG, and exhibited no inhibition with dG (table 3.4).

Compared to dG, the O^{6}-alkyl dG derivatives have a different structural arrangement around the N^{1} of the purine ring. It was possible that the antibody was specific for this area of O^{6}-alkyl dG. Differing affinity of the antibody towards each O^{6}-alkyl derivative suggested that specificity towards the alkyl group was also involved.

Table 3.4 Inhibition of 2C8C6H5 by O^6-alkyl dG derivatives at 3 concentrations, showing concentration giving 50% inhibition (IC_{50})

	% inhibition by:				
Conc. Molar	**O^6-MedG**	**O^6-EtdG**	**O^6-n-PrdG**	**O^6-hydroxyEtdG**	**dG**
40×10^{-6}	52	93	61	0	0
4×10^{-6}	29	76	43	0	0
0.4×10^{-6}	15	20	16	0	0
IC_{50} Molar	37.8×10^{-6}	1.94×10^{-6}	18.5×10^{-6}	-	-

It was surprising to find that 2C8C6H5 exhibited higher affinity for O^6-EtdG than for the original antigen, O^6-MedG. It was possible that, i) the kinetics of antibody-antigen binding for O^6-MedG were slow, or ii) O^6-EtdG was easily accommodated in the antibody binding site, whereas the methyl derivative may have been slightly small. Following the same principle the n-propyl derivative may have been slightly large, and the hydroxyEt derivative too large to allow antibody binding. In the case of the latter derivative the polar electronegative hydroxyl group may also have prevented antibody binding.

An additional experiment investigated the amount of inhibition demonstrated by O^6-MedG competing for 2C8C6H5 antibody on an O^6-MedG-CγG coated plate. Under these conditions 24.8×10^{-6}M gave 50% inhibition, compared to 37.8×10^{-6}M required to give inhibition on an O^6-EtdG-CγG coated plate. Again the ethyl and n-propyl derivatives exhibited the highest inhibition.

2G2B10F1 and 2E3C8E3

These antibodies resulted from an O^6-hydroxyEtdG immunisation. 2G2B10F1 exhibited inhibition with O^6-MedG, O^6-EtdG, O^6-n-PrdG and O^6-hydroxyEtdG and showed no specificity for dG (table3.5). 2E3C8E3 was also inhibited by each of these four O^6-alkyl dG derivatives but showed some cross reactivity with dG (table 3.6). The inhibition of 2E3C8E3 by dG did not demonstrate a characteristic dose response curve (fig 3.12), as maximal inhibition was 28% at 40×10^{-6}M, and zero inhibition was not observed within the limits of the assay. It is therefore possible

that 2E3C8E3 adhered non-specifically to dG. It may also be possible that antigen binding site of 2E3C8E3 was extremely flexible. The screening procedures to obtain an antibody which recognises more than one antigen may have favoured the selection of an antibody with low specificity for one molecule. It may be that this antibody recognised part of the dG molecule, which resembled part of an O^6-alkyl dG, and bound loosely with this area.

2E3C8E3 demonstrated 100% inhibition with some O^6-alkyl dGs which initially suggested that this was the highest affinity antibody. However, comparison of the dose response curves (figs 3.11 and 3.12) and the IC_{50} values indicated that both 2E3C8E3 and 2G2B10F1 exhibited similar affinities for each O^6-alkyl dG. The greatest variation between the antibodies was shown with O^6-MedG, as 2G2B10F1 gave IC_{50} at 8.31×10^{-6}M , while 2E3C8E3 gave IC50 at 7.92×10^{-6}M with this derivative. This variation may have been due to experimental error. The concentration of O^6-EtdG giving 50% inhibition was $<0.16 \times 10^{-6}$M for both antibodies, but exact values were not determined as the experiment could not be repeated under exactly the same conditions, due to the limited amounts of available plate coating material.

Table 3.5 Inhibition of 2G2B10F1 by O^6-alkyl dG derivatives at 9 concentrations, showing concentration giving 50% inhibition

	% inhibition by:				
Conc. Molar	O^6-MedG	O^6-EtdG	O^6-n-PrdG	O^6-hydroxyEtdG	dG
40×10^{-6}	95	95	95	93	0
20×10^{-6}	85	95	95	92	0
10×10^{-6}	60	95	95	90	0
5×10^{-6}	37	95	95	83	0
2.5×10^{-6}	14	95	95	70	0
1.25×10^{-6}	0	95	85	50	0
0.62×10^{-6}	0	95	78	15	0
0.31×10^{-6}	0	90	65	10	0
0.16×10^{-6}	0	83	43	4	0
IC_{50} Molar	8.31×10^{-6}	$<0.16 \times 10^{-6}$	0.21×10^{-6}	1.25×10^{-6}	-

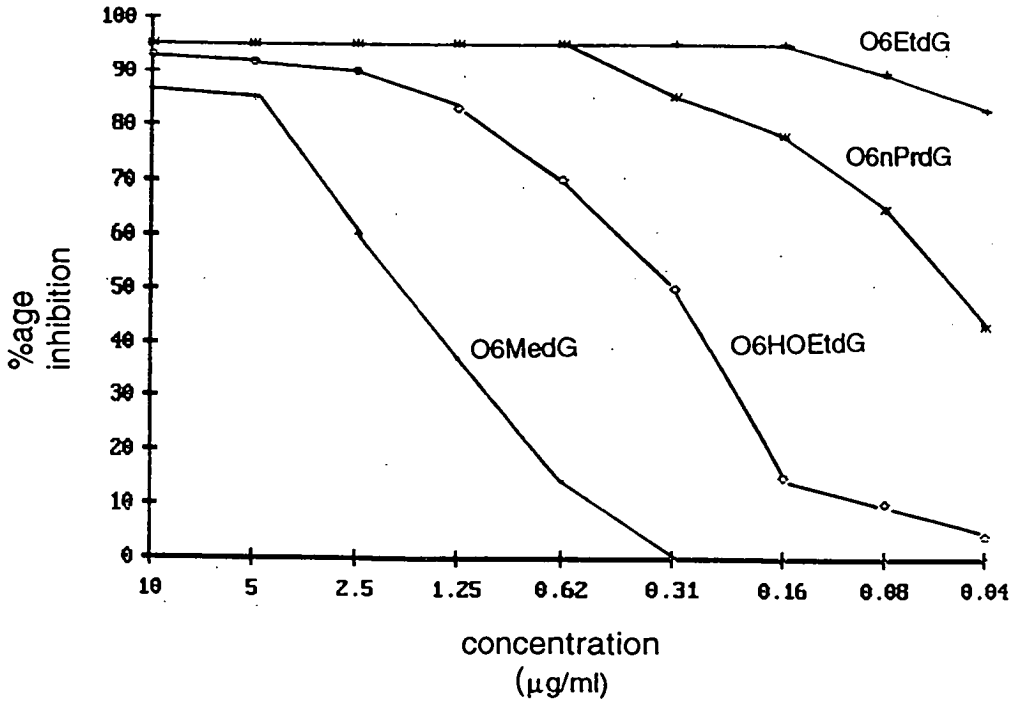


Figure 3.11. Dose response curves for 2G2B10F1

Table 3.5 Inhibition of 2E3C8E3 by O^6-alkyl dG derivatives at 9 concentrations, showing concentration giving 50% inhibition

	% inhibition by:				
Conc. Molar	O^6-MedG	O^6-EtdG	O^6-nPrdG	O^6-hydroxyEtdG	dG
40×10^{-6}	84	100	100	100	28
20×10^{-6}	83	100	100	100	28
10×10^{-6}	65	100	100	100	25
5×10^{-6}	38	100	100	100	25
2.5×10^{-6}	14	100	100	97	23
1.25×10^{-6}	0	100	100	48	10
0.62×10^{-6}	0	100	85	25	10
0.31×10^{-6}	0	100	74	10	10
0.16×10^{-6}	0	97	50	0	10
IC_{50} Molar	7.92×10^{-6}	$<0.16 \times 10^{-6}$	0.16×10^{-6}	1.28×10^{-6}	-

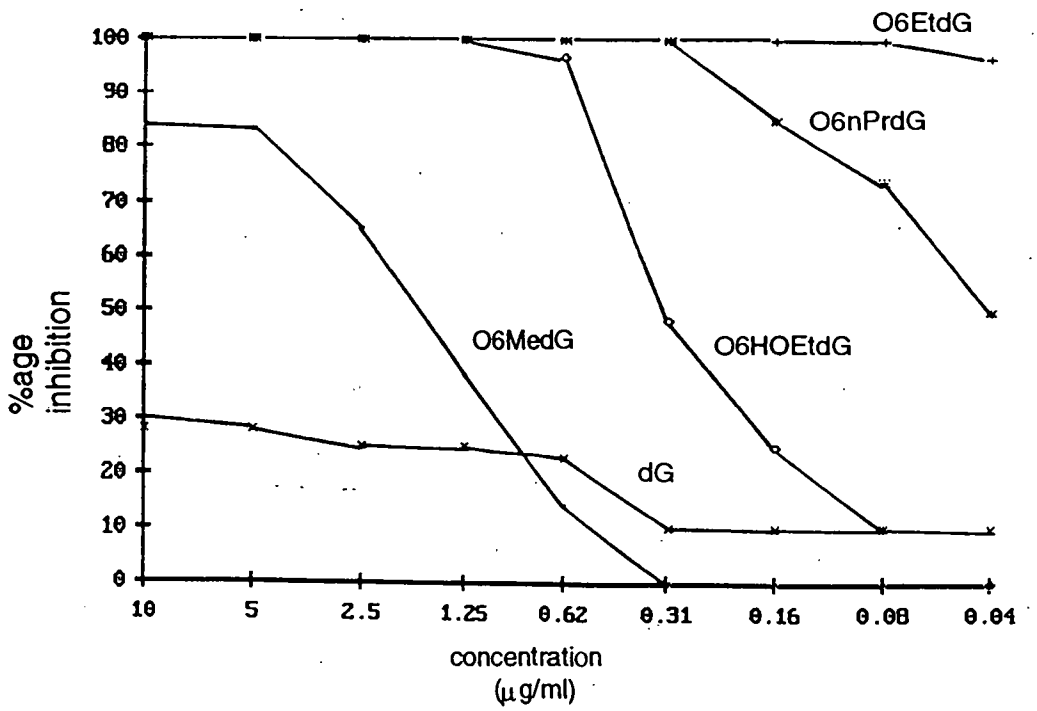


Figure 3.12. Dose response curves for 2E3C8E3

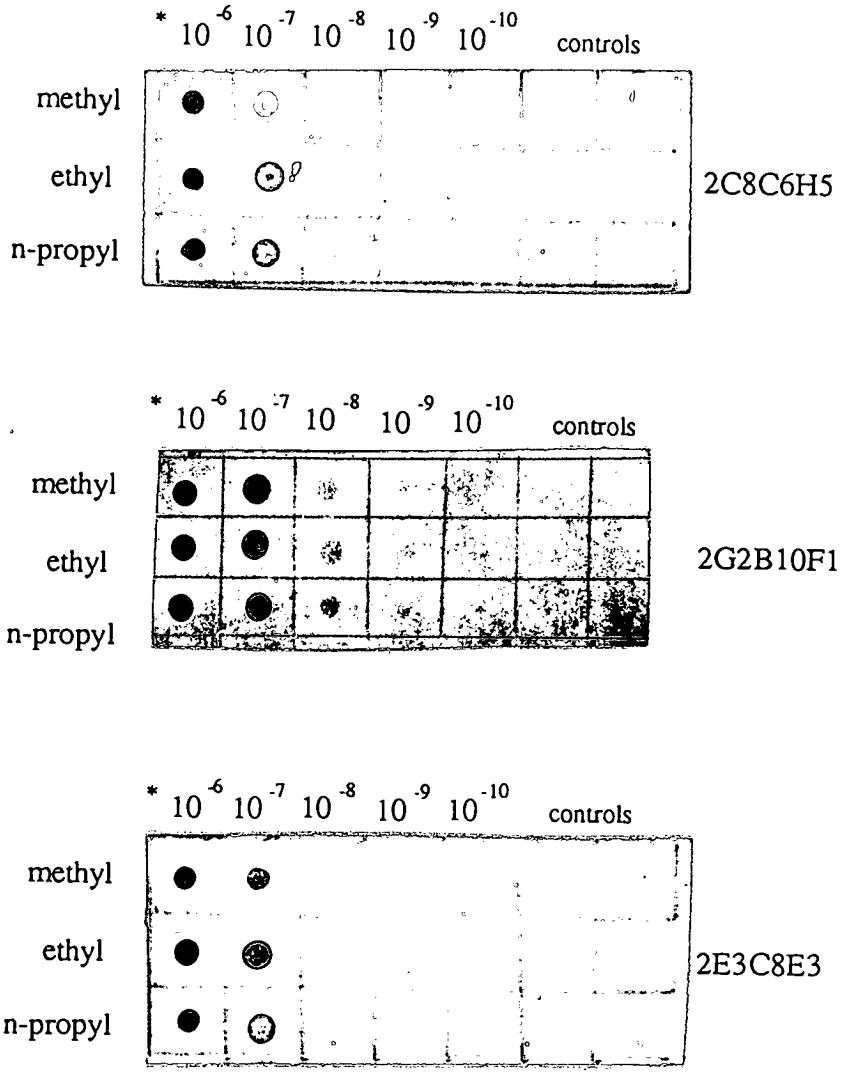
2G2B10F1 and 2E3C8E3 showed an overall higher affinity for the O^6-alkyl dG derivatives than 2C8C6H5. IC_{50}s indicated a 5-10 fold lower affinity of the latter antibody (tables 3.4, 3.5 and 3.6). It was surprising to find that antibodies 2G2B10F1 and 2E3C8E3, produced by an O^6-hydroxyEt dG immunisation, had higher affinity for O^6-EtdG and O^6-n-PrdG, than for O^6-hydroxyEt dG. Again, least inhibition was demonstrated with O^6-MedG. These antibodies therefore followed the pattern set by 2C8C6H5, except for recognition of O^6-hydroxyEt dG. This implies that for an antibody to cross react with hydroxy alkylated dG, the hydroxyl group may have to be present at the time of immunisation.

To summarize:
i) 2E3C8E3 recognised four O^6-alkyl dG derivatives, in the order O^6-EtdG> O^6-n-PrdG> O^6-hydroxyEtdG> O^6-MedG, but also cross-reacted with dG.
ii) 2G2B10F1 also recognised these four O^6-alkyl dG derivatives in the same order, had similar affinity and had the advantage of exhibiting no cross reactivity with dG.
iii) 2C8C6H5 was the lowest affinity antibody, recognising only three O^6-alkyl dG derivatives, in the order O^6-EtdG> O^6-n-PrdG> O^6-MedG.

3.2.4.2. Dot Blots to determine comparative antibody affinity for each O^6-alkyl dG derivative

The antibodies were tested by dot blot assays to give some indication of the relative affinties towards each antigen in an alternative assay. Dot blots provided a quick and simple result as, i) different O^6-alkyl dG derivatives were not competing for the antibody and ii) the oligonucleotide antigens were immobilised for screening, so the exact number of O^6-alkyl dG derivatives per oligonucleotide was known. The affinity of antibodies 2G2B10F1, 2E3C8E3 and 2C8C6H5 for O^6-MedG, O^6-EthdG and O^6-n-PrdG were tested at concentrations of 10^{-10} to 10^{-6}M. O^6-hydroxyEt dG binding was not investigated as an oligonucleotide incorporating this derivative was not available.

For all three antibodies the point at which the antibody no longer recognised the antigen was at a concentration between 10^{-8} and 10^{-7} M (fig. 3.13). The results showed that in this assay system the variation in antibody affinity for each O^6-alkyl dG derivative was no greater than a factor of 10. In addition, the results suggested that the three different antibodies had overall affinities within a similar range.



*concentration of O^6alkyl dG derivative (Molar)

Figure 3.11 Dot blots showing the reaction of 2C8C6H5, 2G2B10F1 and 2E3C8E3 with O^6alkyl dG oligonucleotides

3.2.4.3. The effect of antibody class on affinity

The affinities of monoclonal antibodies rely heavily on the properties of the amino acids involved in the antigen binding site. In addition, the structure of the entire antibody molecule, i.e. the class or sub-class of the heavy or light chain, also affects the binding to antigens. Antibody class-typing experiments determined that 2G2B10F1 and 2E3C8E3 were bivalent IgG molecules, and 2C8C6H5 was a decavalent IgM molecule (i.e. has 10 antigen binding sites). IgGs are the most commonly produced antibody in an animal following repeated exposure to an antigen, and normally exhibit higher affinity than IgMs. IgMs are normally only produced following the initial exposure to an antigen, although it has previously been observed that an IgM response is elicited continually when an antigen contains a high hapten substitution (Erlanger, 1980).

IgMs are rejected by some researchers, as they commonly produce excellent results with one assay technique but fail to recognise the antigen with another. The variation in results between the non-competitive ELISAs and the dot blot assay for 2C8C6H5 were probably due to the multivalency of the antibody. Low inhibition in the non-competitive ELISA was perhaps due to the large amount of inhibitor required to block the ten binding sites to prevent this antibody binding to the plate.

3.3 Experimental

Phosphate buffered saline (PBS) tablets were obtained from Oxoid (Unipath); peroxidase conjugated rabbit immunoglobulins to mouse immunoglobulins (second antibody) from DAKOPATTS (Denmark), Hibond nylon membrane from Amersham and immunoplates, maxisorb f96 cert and tissue culture plates from Nunc, Gibco. All other cell culture plasticware was obtained from Costar. Cell culture media was obtained from ICN Flow (except Gibco myoclone plus foetal calf serum). Polyethylene glycol (PEG 1500) was obtained from Sera-Lab and all other reagents from Sigma. Plates were read on a Molecular Devices kinetic microplate reader using a Softmax computer program.

Buffers and Cell Culture Media

PBS (pH 7.4)	from tablets (Oxoid) 1 per 100ml dH_2O.
PBS/Tween (PBS/Tw)	0.05% w/v Tween 20 in PBS pH 7.4.
Coating buffer (pH 9.8)	sodium carbonate (0.015M) and sodium bicarbonate (0.03M)
ABTS	substrate, 0.63mg/ml in citrate phosphate buffer (0.1M, pH 4.3) containing 0.0036% hydrogen peroxide.
Serum free medium (1):	RPMI 1640, penicillin (100Iu/ml),streptomycin (100ug/ml).
Complete medium	serum free with L-glutamine (2mM), sodium pyruvate(1mM), 10% v/v myoclone plus foetal calf serum, fungizone (2.5ug/ml).
Serum free medium (2):	complete without myoclone foetal calf serum.
HAT medium	complete with 1:50 dilution of HAT stock (=5mM hypoxanthine, 20uM aminopterin, 800uM thymidine) to give final conc of 100uM hypoxanthine, 0.4uM aminopterin, 16uM thymidine.
HT medium	as HAT medium without aminopterin.
Freezing medium	90% foetal calf serum, 10% DMSO v/v.

ELISA

Non-competitive assay

96-well microtitre plates were coated with 100µl/well of the required O^6-alkyl dG conjugate (1µg/ml in bicarbonate buffer [1.59g Na_2CO_3 + 2.93g $NaHCO_3$/l dH_2O]) overnight at 4°C. The coating solution was then removed by washing with PBS/Tw (3x). Serum or culture supernatant (100µl/well) was added and after 2 hr incubation at RT the plates were washed with PBS/Tw (3x). Peroxidase-conjugated rabbit anti-mouse IgG immunoglobulin (1/1000 dilution, 100µl/well) was added and the plates incubated for a further 1 hr then washed again with PBS/Tw (5x). The presence of specific antibody was indicated by the development of a green colour (intensity of which is directly related to the amount of antibody present) on the addition of ABTS (enzyme substrate solution 0.6mg/ml, 100µl/well). This colour was quantified spectophotometrically by A_{405} minus A_{490} on a microtitre plate reader.

Competitive inhibition assay

A limiting amount of serum or culture supernatant (the dilution which half maximally bound the antibody, determined by non-competitive ELISA) was incubated with decreasing concentrations of each free O^6-alkyl dG derivative. After 10min each mixture (100µl/ml) was applied to a coated plate and incubated for 2hr at RT. Washing, addition of 2nd antibody and substrate were carried out as previously described. Wells with free O^6-alkyl dG derivative were compared with control wells without free O^6-alkyl dG and the %age decrease in colour measured.

Dot blot assay

Wash solutions

PEE= PBS containing 1mM EDTA and 1mM EGTA.

PEET= PEE containing 0.5% Tween 20.

Each 54mer (2mg) was dissolved in dH_2O (50µl) to give 40mg/ml solutions. 10 fold dilutions were made and 0.5µl of each solution spotted onto a nylon membrane giving coating concentrations of 1 x 10^{-6}M to 1 x 10^{-10}M of each oligonucleotide. The membrane was exposed to UV irradiation for 5min to allow attachment of the oligonucleotides (via thymine residues) followed by incubation for 30min at RT with 10% w/v fat free skimmed milk in PEE (10ml), to block the uncoated areas of the membrane. After washing with PEET (20ml) then PEE (20ml) the membrane was incubated at RT with antibody solution (1:1000 in PBS, 10ml). After 30min the membrane was washed as before, then incubated at RT with 2nd antibody (1:1000 in

PBS, 10ml) for 30min, followed by washing. 3,3'diaminobenzidine tetrachloride (10ml of 0.5mg/ml in 20mM Tris.HCl with 0.06% hydrogen peroxide) was incubated with the membrane for 10-30min, to allow the colour reaction to proceed. The reaction was stopped by washing the membrane as before.

Typing of antibody

Each antibody was tested by non-competitive ELISA with, i) IgG second antibody and ii) IgM second antibody (1:1000 100µl/well). On the addition of ABTS test i) gives a stronger colour with an IgG and test ii) gives a stronger colour with an IgM.

Immunisation Procedure

250µl of a 1mg/ml solution of each antigen was emulsified with 350µl of complete Freunds adjuvant. For each antigen 2 or 3 BALB/c mice were immunised subcutaneously with 200µl the above mixture (=100µg antigen/mouse). After 21 days booster injections were given (100mg antigen/mouse in incomplete Freunds adjuvant). Serum was screened by non-competitive ELISA for specific antibody. 2 pre-fusion boosts, consisting of 100µg antigen made up to 200µl total volume with PBS were carried out on the fourth and third days prior to fusion, by intraperitoneal injection.

Cell Fusion

Spleen Cell Preparation

Pre-fusion boosts were carried out as previously described. The mouse was then killed by ether anaesthesia and a sterile dissecting kit used to remove the spleen which was then placed in cold serum free (SF) medium. The following operations were carried out in a laminar flow cabinet. The spleen was transferred to a sterile petri dish and the fatty tissue removed. A superficial longitudinal cut was made along the length of the spleen with a scalpel. The splenocytes were dispersed by injecting SF medium (37°C) through 2 10ml syringes with 25 gauge needles, along the length of the spleen. The medium containing the cells (typically 10^8) was centrifuged at 400g for 5min and the resulting pellet resuspended in SF medium (5ml).

Myeloma cell preparation

Sp2 or 653 myeloma cell lines were grown and maintained at approximately 0.5-1 x 10^7 cells/ml in complete medium (20ml) in 250ml rectangular flasks.
Approximately 5 x 10^7 cells were used for each fusion. Immediately before each

fusion the cells were scraped from the flask surface, divided into 3 20ml universal containers and centrifuged at 400g for 5 min to produce 3 pellets which were each resuspended in SF medium (5ml/pellet).

PEG fusion

Before commencing the fusion all culture medium required was allowed to warm to 37°C in appropriate syringes as follows: 6x10ml of SF medium, 6x6ml of complete medium and 6x0.3ml of PEG.

The myeloma and splenocyte cell suspensions were combined (=20ml) then divided equally into 6 20ml universal containers, each of which was centrifuged at 400g for 5 min to produce 6 pellets. After complete removal of the supernatant each pellet was agitated with a 25 gauge needle, followed by the addition of PEG over a period of 30 sec. Without delay each mixture was diluted over a period of 60 sec. with SF medium (10ml) followed by centrifugation at 400g for 5min. Each pellet was resuspended in complete medium (6ml) then applied to the inner 60 wells (100µl/well) of a 96 well microtitre plate. The plates were placed in a 37°C CO_2-incubator, then after 3-4 hr. HAT medium was added (100µl/well). Every 2-3 days 100µl was carefully removed from each well and replaced with 100µl of fresh HAT medium, ensuring immediate return of the plates to the incubator. After 10 days each well was tested by non-competitive ELISA to identify wells producing antibody of interest. Cells in wells giving positive results were expanded to 2ml wells and competitive ELISA was carried out. Wells showing high inhibition were cloned.

Thymocyte cell preparation

A 4-6 week old BALB/c mouse was killed by ether anaesthesia. Using sterile dissecting instruments an incision was made along the mid-line of the thorax to the neck region. The thoracic cavity was opened and the 2 thymus lobes, lying anterior to the heart, were carefully removed and placed in cold SF medium in a 20ml sterile container. After transfer to a petri dish containing SF medium (10ml) the thymus was teased with forceps allowing the thymocytes to be released. The cell containing medium was centrifuged at 400g for 5min and the pellet resuspended in complete medium (20ml).

Cloning by limiting dilution

This procedure aims to dilute cells in a 96-well microtitre plate to achieve 2-3 rows with 1 cell/well. 4ml of thymocyte suspension was added to approximately 200 hybridomas. 2.4ml of this mixture was distributed evenly over rows A and B of a

96-well microtitre plate (theoretically 5 cells/well). Another 2.4ml of thymocyte suspension was added to the remaining 1.6ml hybridoma mixture and after gentle mixing 2.4ml was distributed evenly over rows C and D (2 cells/well). The remaining 1.6ml of mixture was mixed with a further 1.6ml of thymocyte suspension and again 2.4ml was distributed over rows E and F (1 cell/well). Finally the remaining 0.8ml of hybridoma mixture was mixed with the remaining 1.6ml of thymocyte suspension and the resulting 2.4ml distributed over rows G and H (1 cell/2 wells). 150µl of complete medium was added to each well before culturing the plate at 37°C in a CO_2-incubator. After 10-14 days the number of clones/well, visible microscopically, were recorded and the culture supernatants screened by non-competitive ELISA. Positive wells of monoclonal origin were expanded to 2ml wells. Cloning was repeated twice for each cell line to confirm monoclonality, which was indicated by antibody production in 100% of cell containing wells. Monoclonal cell lines were expanded into flasks and maintained at 0.5-1 x 10^7 cells/ml changing medium every 2-3 days. At each stage of cloning cells were frozen for safety.

Freezing cells

Freezing medium (90% foetal calf serum + 10% DMSO) was prepared and cooled on ice. For each cryotube 10^6 cells were centrifuged at 400g for 5min. The supernatant was discarded and the pellet resuspended in 0.5ml ice cold freezing medium then transferred to a cold cryotube. Cryotubes were placed in a polystyrene box and stored at -70°C overnight, then transferred to liquid nitrogen for long term storage.

Transferring cells to serum free culture conditions

Complete medium (10% serum) was removed from hybridomas growing in 200ml culture flasks and medium containing 7.5% serum was added (20ml). After 2 days the cells were examined microscopically and the cell culture supernatant was tested by competitive ELISA. Healthy hybridomas which continued to secrete antibody were introduced to media containing decreasing amounts of serum i.e. 5%, 2.5%, 1% then 0, over a period of at least 4 wks. At each stage cells were tested for antibody production.

Chapter 4. Preparation and Application of Immunoaffinity Columns for the Purification of O^6-alkyl-2'-deoxyguanosine

4.1 Introduction

Immunoaffinity chromatography (IAC) is considered to be one of the most powerful techniques for the purification of chemical and biological material to which antibodies can be induced. A single step experiment can result in purification factors of 2 000-20 000-fold (Goding, 1986). Prior to the advent of monoclonal antibodies, polyclonal antibodies were employed as immunoaffinity reagents, enabling the purification of many enzymes and proteins implicated in medical disorders (Livingston, 1974; Robb *et al*, 1976). Problems with quality of antibody, low binding capacity and specificity, discouraged the widespread application of the technique. The development of monoclonal antibodies with precise characteristics eliminated these shortcomings. Monoclonal antibodies are now widely applied in affinity chromatography, and have been used to purify cell surface antigens (Parham, 1979), vaccines (van Wesel and van der Marel, 1982) and many biologically important proteins (Secher and Burke, 1980; Mescher *et al*; 1983; Bigio *et al*, 1989).

4.1.1. Principles of immunoaffinity purification

IAC is a separation technique and relies on specific antibody-antigen binding and the dissociation of the antibody-antigen complex (Wilchek, *et al*, 1984). It can be divided into 3 steps: i) immobilisation of the antibody on a solid support (matrix), ii) binding of the antigen to the antibody-matrix, and iii) elution of the antigen. Generally, the antibody-matrix is packed into a column and a crude mixture containing the desired 'antigen' is applied. As the antigen comes into contact with the antibody, binding results. The remaining material in the crude extract elutes from the column, aided by washing. The purified antigen is then dissociated from the antibody-matrix by introducing a change (e.g. pH, polarity, denaturants) to the column environment. An overview of the procedure is described in figure 4.1. The antibody-matrix can also be stirred with the crude mixture to extract the desired antigen (the stirred tank method, Chase, 1984), although the column method is more common for laboratory scale experiments (< 100mgs), (Yarmush *et al*, 1992).

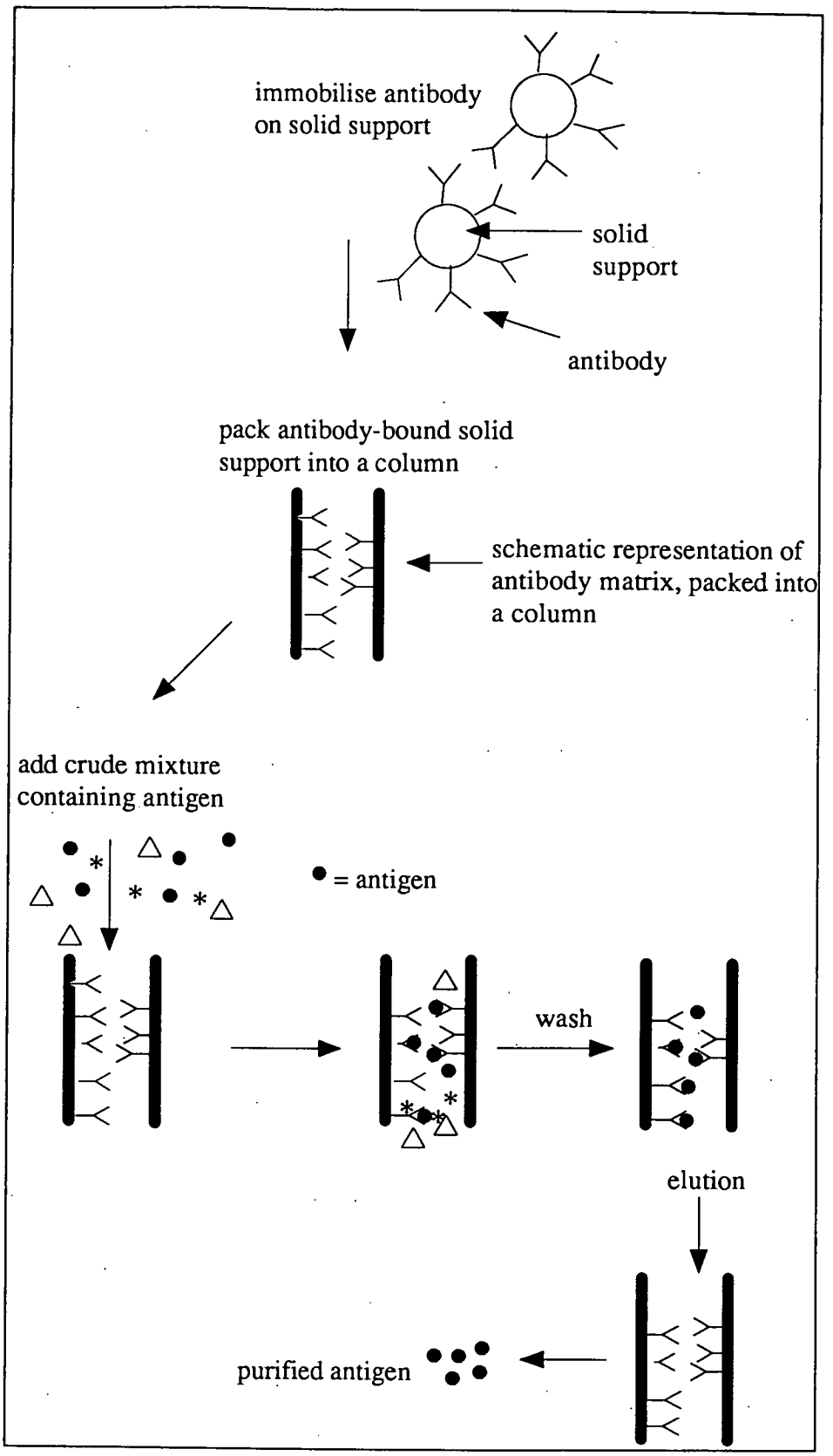


Figure 4.1. Overview of immunoaffinity purification

4.1.1.1. Preparing immobilised antibodies

Many solid supports and coupling methods have been investigated for the immobilisation of antibodies (reviewed by Chase, 1984; Yarmush, 1985; Yarmush *et al*, 1992). The most popular method for covalently attaching antibodies to a solid support is *via* an activated bead (Scouten, 1987). Alternatively, the antibody can be activated. The former method offers a wider range of possibilities as the beads can withstand harsher activation conditions than the antibodies. Agarose, (figure 4.2) in the form of a bead, is the most commonly employed matrix. It possesses ideal qualities for a matrix such as, minimal non-specific adsorption, absence of charged groups and high porosity, which provide a high surface area (Yarmush *et al*, 1992). In addition, it is chemically stable and contains functional groups which can be activated. Most commercially available agarose has been chemically and mechanically stabilised by the addition of a cross-linking agent. The cross-linked product is often referred to as Sepharose.

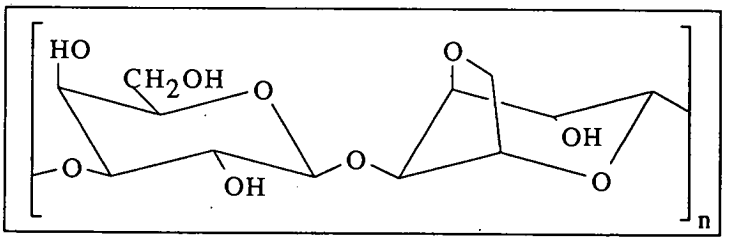


Figure 4.2. Agarose

There are many methods for the activation of agarose. Cyanogen bromide (CNBr) activation (fig 4.3a), which was the most popular for a long period of time, is simple, can be carried out in an aqueous environment, and results in high loading on the agarose (Axen *et al*, 1967; March *et al*, 1974). However, following the immobilisation of the antibody on the matrix an unstable isourea bond is formed (Wilchek, 1975; Lasch and Koelsch,1978). An extra charge is introduced by the presence of this bond, subsequently causing the matrix to act as an ion exchanger at low salt concentrations (Goding, 1986). Additionally, over a period of time the antibody begins to leak from the support (Lowe, 1979). Leakage is accelerated on exposure to high temperatures, extremes of pH and exposure to nucleophiles (Wilchek, 1975). Triazine activation of agarose (fig 4.3.b) which yields a stable and neutral antibody-matrix linkage (Lang *et al*, 1977), is a modern alternative to CNBr activation. It has the added advantage of exhibiting high coupling, immobilising at least 90% of the antibody in most cases. This is due to the ability of the activated

matrix to react with amine, thiol and hydroxyl groups of the antibody, producing triazine ether bonds (Lily *et al*, 1976). The only obvious disadvantage of this method is the toxicity of triazine.

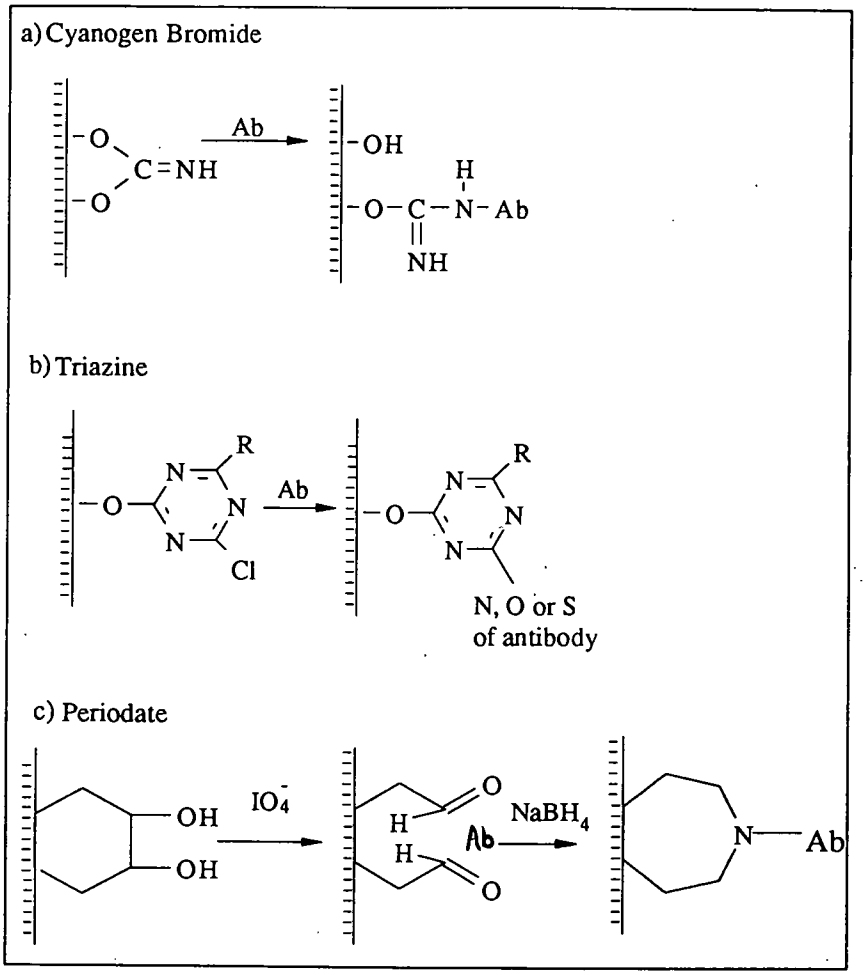


Figure 4.3. Activation of agarose

CNBr and triazine activation methods involve the addition of an active group to the agarose. In contrast, periodate activation (fig 4.3c) involves the alteration of the agarose backbone to produce an activated group (Fischer, 1985). Periodate converts an agarose diol to a dialdehyde, which reacts with the amine groups of the antibody. Following the addition of the antibody an unstable imine is produced and reduction by borohydride is necessary to produce a stable antibody-matrix-complex. Of the 3 activated supports described, only periodate activated sepharose is not commercially available, as the dialdehydes may be reduced on storage. There are many more ways in which agarose can be activated, some are described in table 4.1.

Table 4.1. Methods for the activation of agarose for antibody coupling

Activating reagent	Reference	Disadvantage
Carbonyl-diimidazole	Bethal *et al* 1979	none known
p-Toluene sulphonyl chloride	Nilson & Mossbach, 1980 & 1984	charged at neutral pH
Hydroxysuccinimide	Cuatrecasas & Parik, 1972	non-specific binding
Epichlorohydrin	Porath & Axen 1976	long activation time

It is important that the monoclonal antibody to be coupled to the activated support is pure. It is generally accepted that ammonium sulphate precipitation yields antibody of sufficient purity. However, extensive dialysis is required to extract ammonium sulphate, as the presence of ammonia ions inhibits the coupling reaction (Goding, 1986). The predominant reaction of antibodies with the activated support is nucleophilic attack by the lysine amino groups of the antibody. This reaction is rapid at slightly alkaline pH, as the amino groups are not protonated. Over-coupling of the antibody can lead to inactivation of the antibody binding sites (Cuatrecasas and Anfinsen, 1971), presumably due to steric hindrance. It is important to inactivate any uncoupled active groups and to wash the antibody-matrix complex extensively prior to loading of the antigen. The amount of antibody immobilised on the activated support can be estimated by recording the A_{280} of the antibody solution before and after the coupling reaction, providing there are no by-products of the reaction which absorb at this wavelength.

4.1.1.2. Loading of antigen and washing

Immobilisation of the antibody, results in an alteration of the kinetics of antibody-antigen binding, therefore it takes longer for equilibrium to be reached. The time taken for the antigen solution to travel through the column (flow rate) is normally sufficient for high affinity antibodies to capture the antigen. However, low affinity antibodies have been shown to bind minimally in this time, making it necessary to use an excess of antibody-matrix for equilibrium to be reached (Harlow and Lane, 1988). It is important for the column capacity to be greater than the amount of antigen to be bound, as under these conditions flow rate is no longer important (Yarmush *et al*, 1992). In many cases where the column capacity is less than the

amount of antigen loaded, a break-through of antigen occurs at the column outlet (Chase, 1984). For large scale immunoaffinity purification, i.e. in industrial use it is essential that loading of the column is maximised, to avoid wasting antibody or antigen (product). In these cases column capacity and antibody-antigen equilibrium are calculated theoretically (described by Yarmush *et al*, 1992).

It is generally desirable to remove contaminants from the crude antigen mixture before loading the column. Aggregated materials and debris may block the column, and impurities such as enzymes and proteins may degrade or non-specifically bind to the support.

After the loading solution has travelled through the column the unbound material is removed by extensive washing. This procedure also removes some of the material which has bound non-specifically to the support by ionic and hydrophobic interactions. Modified wash buffers have been developed for the removal of impurities, e.g. detergent containing buffers remove impurities bound by hydrophobic interactions (Smith *et al*, 1982) and non-specifically bound proteins are removed by alterations in pH (Schneider *et al*, 1982). However, deleterious effects on the anibody-antigen binding have been observed during the use of modified buffers (Stallcup *et al*, 1981).

4.1.1.3. Elution of the antigen

The elution step involves the dissociation of the antibody-antigen complex to obtain the pure antigen. It has been shown that the ease of dissociation does not depend on the affinity of the antibody (Parham, 1983). The antibody-antigen interactions can be disrupted by, i) treating with harsh conditions or, ii) adding an excess of a compound that mimics the antigen. The latter is not commonly used as displacement of the antigen may take several hours resulting in a broad elution profile. In addition, there is often contamination by the displacing reagent (Harlow and Lane, 1988) rendering the product too impure for further use. Conditions for technique 'i)' must be carefully chosen to avoid degradation or denaturation of the antigen. Most elution methods rely on the reversible denaturation of antibodies. Most proteins, including antibodies, have been shown to alter in tertiary structure in the presence of many chemical reagents, and renature after the removal of the reagents (Hager and Burgess, 1980). Due to the differing properties of antibodies it is necessary to carry out a range of tests to identify suitable elution conditions.

The most popular method of dissociation is to change pH. Low pH (i.e. 100mM glycine, pH 1.8) is generally preferred, as high pH (i.e. 100mM phosphoric acid, pH 12.5) can potentially cause irreversible denaturation of antibodies (Tanford, 1968). Chaotropic ions disrupt water structure, break hydrogen bonds and weaken hydrophobic interactions. These ions disrupt antibody-antigen binding in the following order (Goding, 1986):

$$SCN^- > I^- > ClO_4^- > NO_3^- > Br^- > Cl^- > CH_3COO^- > SO_4^{2-} > PO_4^{3-}$$

$$Ba^{2+} > Ca^{2+} > Mg^{2+} > Li^+ > Na^+ > K^+ > Cs^+ > NH_4^+$$

Thiocyanate is the most effective chaotropic ion, but it is difficult to remove this ion completely and the small traces can render the column unusable (Harlow and Lane, 1988). Organic solvents, which alter the polarity of the elution solvent (e.g. pyridine, acetonitrile, DMSO, dioxane ethylene glycol and aqueous acetone) can be used, and columns can normally be reused if they are washed extensively immediately after the elution of the antigen. The protein denaturants commonly used are 5 to 8M urea and 4 to 6M guanidine-HCl. These reagents are normally employed when all other methods of antibody-antigen dissociation fail, because irreversible antibody denaturation commonly occurs (Goding, 1986).

4.1.2. Application of immunoaffinity columns

During the last several years IAC has been applied to allow the detection and quantification of modified DNA derivatives . It has generally been used as a purification step in combination with ^{32}P-postlabelling, HPLC, flourescence techniques, or electrochemical methods for the quantification of O^6 and 7-alkyl dG, methyladenine and benz[a]pyrene diol epoxide DNA derivatives. The characteristics of the columns used in some of the studies are described in table 4.3. The column capacity varies between studies and is largely dependent on the orientation of the antibody on the sepharose and the affinity of the antibody.

Table 4.3. Recent applications of immunoaffinity chromatography

DNA derivative	Matrix (sepharose)	Column capacity#	Antigen recovery	Reference
O^6-butylguanine	CNBr	1.53nmol/ 1.25mg Ab	62 +/- 5%	Bonfanti *et al*, 1990
benzo[a]pyrene diol epoxide derivatives	CNBr	no information given	85-100%	Manchester *et al*, 1990
O^6-methyl guanine	CNBr	1.4nmol/mg Ab	>95%	Cooper *et al*, 1992
	hydrazide	1.5nmol/mg Ab		
	periodate	6.2nmol/mg of Ab		
7-methyl deoxyguanosine	protein A	606pmol/ml sepharose*	>90%	Bianchini *et al*, 1993

the amount of DNA derivative which binds to the immobilised Ab
*Ab loading not known

It was anticipated that similar results to those shown in table 4.3 could be achieved by immobilising the O^6-alkyl dG class specific antibodies (produced in chapter 3), in order to allow the purification of O^6-alkyl dG from native DNA.

4.2. Results and Discussion

Antibodies 2C8C6H5 and 2G2B10F1 were chosen to be employed in IAC experiments. In general, low affinity antibodies, such as IgMs (i.e. 2C8C6H5) are considered unsuitable for IAC. However, 2C8C6H5 was the first class specific antibody to be generated during the project, therefore many initial IAC experiments were conducted with this product. In addition, ELISA and dot blot assays indicated that the affinities for the O^6-alkyl dGs by this antibody were only 10-fold lower than affinities exhibited by 2G2B10F1 antibody. Due to the similarities between 2G2B10F1 and 2E3C8E3 only the former was investigated for IAC experiments

The chosen antibodies were purified from the cell culture supernatant by ammonium sulphate precipitation. The purity of 2C8C6H5 was enhanced by growing the cell line in serum free media, prior to precipitation. The change in cell culture conditions was introduced gradually (over a course of 4-6 weeks) and the supernatant was tested at each stage to ensure that the antibody maintained specificity and affinity towards O^6-alkyl dG, despite the change in cell culture environment. Following precipitation, minimal contaminant serum proteins were present, which allowed a more accurate calculation of the amount of antibody. Cell line 2C8C6H5 gave an average antibody production of 40-50µg/ml of supernatant, whereas 2G2B10F1 (which was not introduced to a serum free environment) gave an average overall protein product of 50-60µg/ml of supernatant. These amounts varied depending upon the number of healthy cells present at the time of collecting the culture supernatant.

4.2.1. Immobilisation of 2C8C6H5 on three types of sepharose and purification of O^6-alkyl dG

The performance of immobilised antibodies has been shown to be dependent upon the nature of the solid support (Cooper *et al*, 1992). Therefore 3 different types of activated sepharose: CNBr, triazine and periodate, were examined. This allowed comparison of the amount of antibody immobilised and the relative amount of antigen captured by the immobilised antibody. Firstly, 2C8C6H5 was immobilised on each activated sepharose following the manufacturers' instructions. The extent of immobilisation varied greatly between the activated supports (table 4.4).

Table 4.4. Comparison of sepharose binding characteristics of 2C8C6H5 antibody

Sepharose	Amount	Antibody immobilised	Coupling efficiency
CNBr	1g	9.8mgs	75%
TrAS	1g	29mgs	90%
PIAS	1g	2mgs	35%

The coupling efficiency gave an indication of the amount of antibody immobilised as a percentage of the total amount of antibody exposed to the solid support. Triazine activated sepharose (TrAS) appears to have bound significantly larger amounts of antibody than cyanogen bromide activated sepharose (CNBrAS) and periodate activated sepharose (PIAS). TrAS was exposed to higher levels of antibody which may have resulted in more antibody coupling. All 3 types of sepharose couple to amine groups of antibodies, but TrAS also couples with antibody hydroxyl and thiol groups, which may explain the high antibody loading on this support. Similar coupling efficiencies were expected for CNBrAS and PIAS, as both bind antibodies *via* amine groups. However the latter exhibited approximately 50% less binding. PIAS, which is not commercially available, was synthesised prior to the experiment and it is possible that this support possessed less activated groups than the commercially available CNBrAS (which will be synthesised routinely at optimal conditions).

The column capacity for each antibody matrix was estimated by loading 10μg of an O^6-alkyl dG derivative, washing the column, determining the amount of O^6-alkyl dG in the wash, eluting the bound material from the column, and determining the amount of O^6-alkyl dG in the eluted sample. O^6-EtdG was used for these experiments as the antibody has highest affinity for this derivative. Initial experiments were conducted on CNBrAS-antibody and PIAS-antibody complexes, and the presence of O^6-EtdG in the wash and elution samples was determined by competitive ELISA. The samples were tested for ability to inhibit 2C8C6H5 antibody binding to the plate. The samples that show inhibition contain O^6-EtdG. The wash and elution samples were collected in fractions and the inhibition demonstrated by each is shown in table 4.5.

Table 4.5. % Inhibition shown by wash and elution fractions from CNBr-antibody and PIAS-antibody IACs.

Support	Fraction	% Inhibition of 2C8C6H5
CNBrAs	wash 1	50
	wash 2	38
	wash 3	21
	wash 4-10	0
	elution 1-2	0
	elution 3	45
	elution 4	15
	elution 5-10	0
PIAS	wash 1-10	0
	elution 1-6	0
	elution 7	68
	elution 8	50
	elution 9	30
	elution 10	0

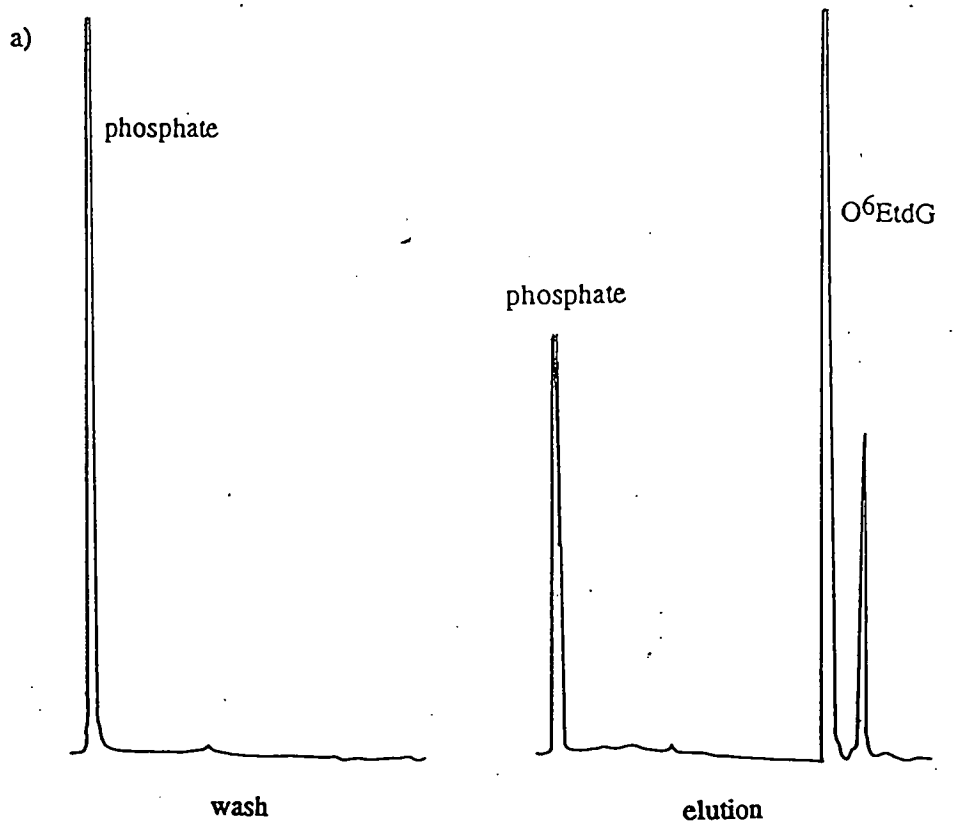
CNBrAS-Ab bound O^6-EtdG (shown by the inhibition in elution fractions 3 and 4, table 4.5). However higher inhibition shown by the wash samples 1, 2 and 3 indicated that not all of the O^6-EtdG derivative has bound. Further experiments with CNBrAS involved tumbling O^6-EtdG with the support over a range of times (i.e. 10-120min), but identical results were obtained. PIAS demonstrated no inhibition in the wash samples and high inhibition in the elution samples (table 4.5). 2C8C6H5 immobilised on PIAS was therefore more effective at extracting O^6-EtdG, than 2C8C6H5 immobilised on CNBrAS. In addition, the PIAS column was reusable, whereas the antibody bound to the CNBrAS became less active during subsequent usage. CNBrAS was therefore omitted from further investigation.

The O^6-EtdG in each fraction could not be quantified from these inhibition results. In addition, it was possible that small amounts of O^6-EtdG were present in samples, but at levels too low to result in inhibition. The calculated amount of O^6-alkyl dG that 2mgs of antibody (the amount immobilised on the PIAS) can theoretically bind, is less than 10μg[1]. Consequently, not all of the 10μg applied to the column was binding to the column, and a small amount of O^6-EtdG was unaccounted for. The wash and elution samples from subsequent IAC experiments with PIAS and TrAS were therefore examined by HPLC analysis to estimate the ratio of bound to unbound O^6-alkyl dG. HPLC allowed smaller amounts of O^6-alkyl dG to be detected.

4 μg of O^6-EtdG were applied to a PIAS-antibody and a TrAS-antibody column. The presence of O^6-EtdG in only the elution sample from the PIAS (fig 4.4a) indicated that this support was capable of binding 4μg of this derivative. This is almost maximal binding for the 2 mgs of antibody immobilised on this support (maximum calculated to be 6.6μg). In comparison, the majority of the O^6-EtdG was present in the wash sample after being applied to a TrAS-Ab column (fig 4.4b). A small amount of O^6-EtdG (< 1μg) was present in the elution sample. As 29mgs of antibody were immobilsed on this support higher binding of O^6-EtdG was expected. Extensive use of TrAS would result in rapid consumption of antibody, and the subsequent purification of small amounts of O^6-alkyl dG.

The finding that 2mgs of antibody, immobilised on PIAS, bound more O^6-EtdG than 29mgs of antibody immobilised on TrAS may be attributable to the orientation of the antibody on the support. TrAS, which binds the antibody via amine, hydroxyl and thiol groups, is likely to bind a higher amount of antibody than PIAS which binds antibody *via* only the amino groups. This may result in i) an excess of binding *via* antibody binding sites, rendering them inactive, or ii) binding at many areas of the antibody causing a distortion in the overall shape, ultimately affecting the 3 dimensional structure of the antibody binding sites. The immobilisation in this study was further complicated by the use of an IgM, which probably bound to the sepharose *via* many sites and became distorted.

(Mw of an IgM = 900 000, therefore 2mgs = 2.22×10^{-9}mol
Assuming that the 10 binding sites of the IgM are available to capture antigen, then 2.22×10^{-8}mol of antigen should be bound.
2.22×10^{-8}mol of O^6alkyldG (Mw = 300) = 6.6μg.



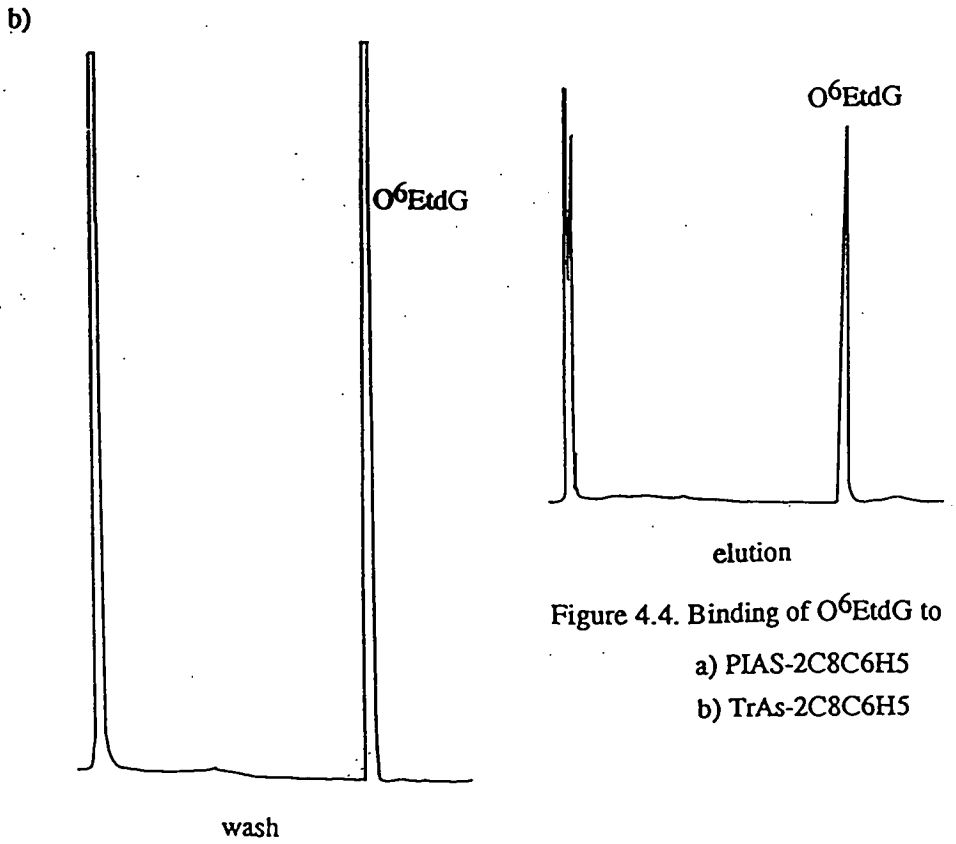


Figure 4.4. Binding of O^6EtdG to
a) PIAS-2C8C6H5
b) TrAs-2C8C6H5

The results of antibody immobilisation and subsequent O^6-EtdG capture are summarised in table 4.6.

Table 4.6. Comparison of 3 types of activated support (summary)

Support	Efficiency of Ab immobilisation	Column capacity	Comments
PIAS	poor	4µg/ 2mg Ab	almost 1:1 Ab to O^6-EtdG binding, column reusable
TrAS	excellent	<1µg/ 29mg Ab	poor O^6-EtdG binding, high Ab consumption
CNBrAS	average	1-5µg/ 10mg Ab	reasonable O^6-EtdG binding, column not reusable

4.2.2. Recognition of O^6-MedG, O^6-n-PrdG and dG by PIAS-2C8C6H5

The capture of O^6-MedG and O^6-n-PrdG by PIAS-2C8C6H5 was tested by applying 4µg of each to the column. Approximately 75% of O^6-MedG was observed in the wash fraction, and the remaining 25% in the elution fraction (fig. 4.5a), therefore 1µg bound to the support. Similarly, O^6-nPrdG was present in both the wash and elution fractions at a ratio of 1 : 1 (fig. 4.5b), indicating that 2µg of O^6-n-PrdG bound to the support. These figures and the O^6-EtdG figures, showed that immobilised 2C8C6H5 had lowest affinity for O^6-MedG, a 2-fold higher affinity for O^6-n-PrdG, and a 4-fold higher affinity for O^6-EtdG (table 4.7). The comparative affinities of 2C8C6H5 for O^6-alkyl dG followed a similar pattern as that shown by competitive ELISA in chapter 3.

Table 4.7. Amount of each derivative binding to PIAS-2C8C6H5

	Amount in:	
Derivative	Wash	Elution
O^6-MedG	1µg	3µg
O^6-EtdG	0	4µg
O^6-n-PrdG	2µg	2µg

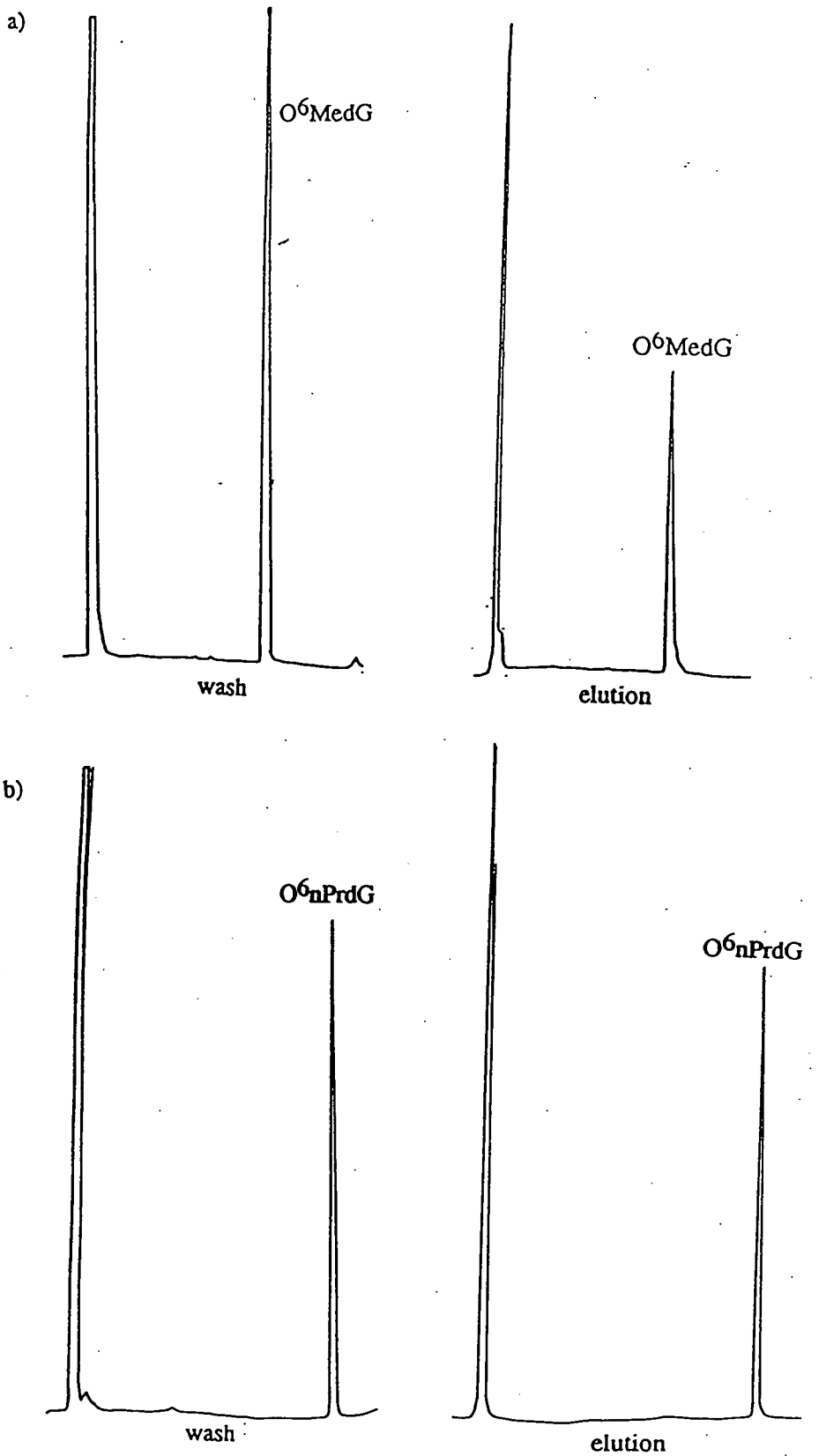


Figure 4.5. Binding of a) O^6MedG and b) O^6nPrdG to PIAS-2C8C6H5

The columns were tested for non-specific binding to dG, following the procedure for binding of O^6-alkyl dG derivatives. The majority of the dG sample is present in the wash fraction (fig 4.6), therefore minimal non-specific binding was occurring.

4.2.3. Extraction of O^6-alkyl dG from of an excess of dG

The ultimate purpose of the immunoaffinity columns was to extract O^6-alkyl dG from native DNA samples. It was firstly important to test the efficiency of the columns in distinguishing O^6-alkyl dG derivatives from other DNA components, in particular dG.

4.2.3.1. Three-fold excess of dG

A mixture containing O^6-MedG, O^6-EtdG and O^6-n-PrdG (1μg of each) and a 3-fold excess of unmodified dG (9μg) was applied to the PIAS-2C8C6H5 antibody column. After washing and elution O^6-EtdG and O^6-n-PrdG were detected in the elution sample, indicating that these derivatives bound efficiently to the column in the presence of an excess of dG (fig. 4.7). In contrast, O^6-MedG showed minimal binding to the column, as most of this derivative was present in the wash sample (fig. 4.7). Previous experiments showed that 1μg of O^6-MedG could be bound to this column in the absence of other O^6-alkyl dGs or dG. In the present experiment the immobilised 2C8C6H5 preferentially extracted the larger O^6-alkyl dGs. This may be advantageous as O^6-MedG is believed to be present at higher frequencies than O^6-EtdG and O^6-n-PrdG in DNA (Chambers, 1991). Therefore, an antibody with lower affinity for O^6-MedG and greater affinity for the higher alkyl derivatives may recognise large amounts of O^6-MedG at similar efficiencies to smaller amounts of O^6-EtdG and O^6-n-PrdG.

4.2.3.2. 10^8-fold excess of DNA

O^6-alkyl dG has been shown to occur in human DNA samples in amounts varying from 0.01 to 0.7μmol per mole of dG (Foiles *et al*, 1988; Hall *et al*, 1991). Exact levels of O^6-alkyl dG in human samples are not clear, but techniques which allow a minimum detection limit of 1 O^6-alkyl dG in 10^8 normal DNA residues are generally considered sufficiently sensitive (Cooper *et al*, 1992).

The amount of O^6-alkyl dG previously employed in IAC experiments in this study (i.e.1-4μg) would have required the presence of 100-400g of normal DNA to mimic a 'real' situation with a 10^8 excess of DNA. It was therefore necessary to dramatically decrease the amount of O^6-alkyl dG applied to further evalidate the

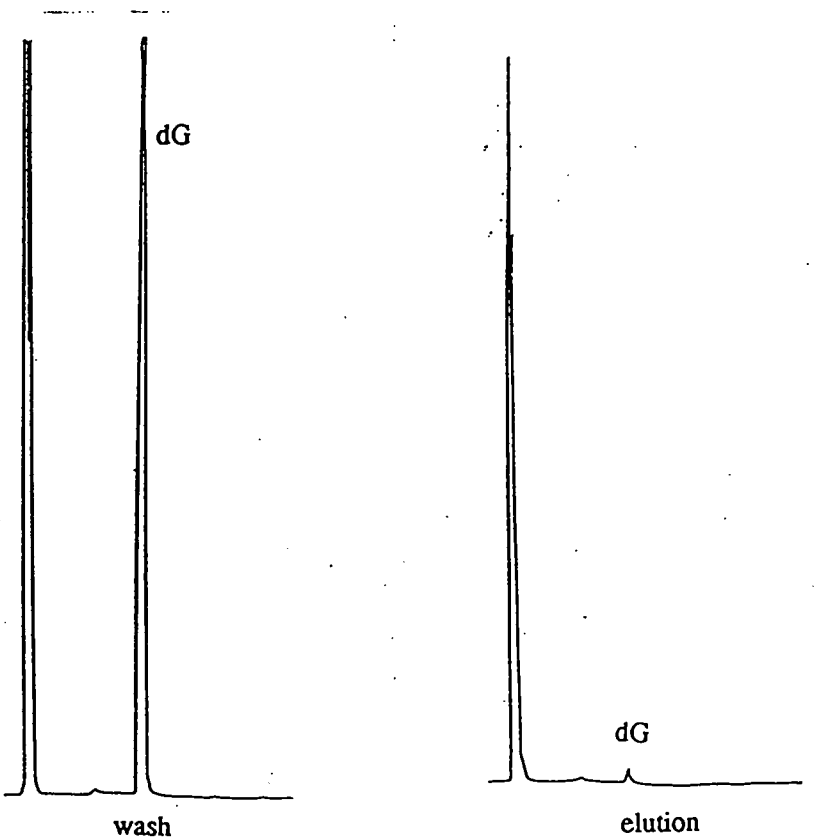


Figure 4.6. Binding of dG to PIAS-2C8C6H5

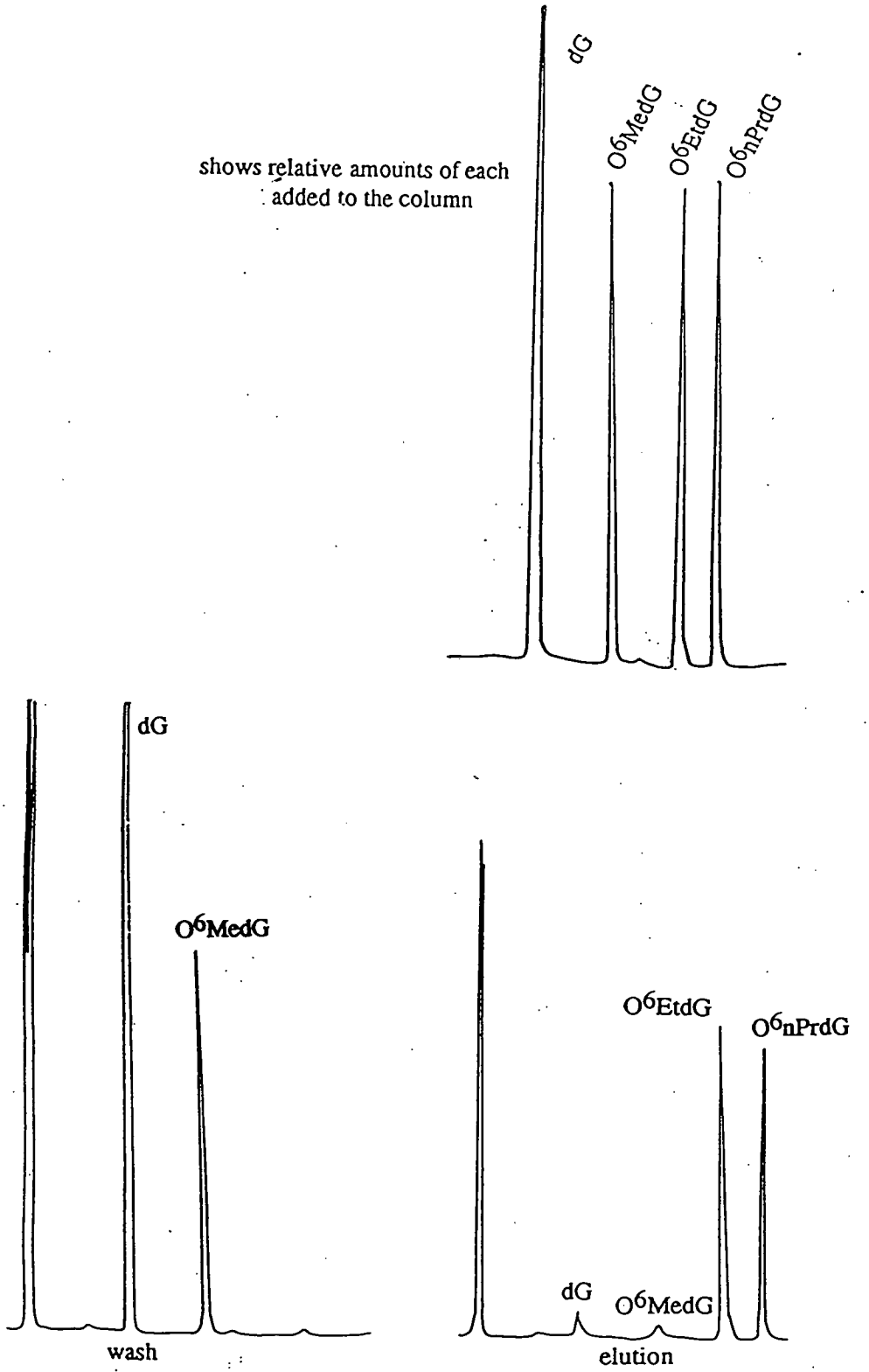


Figure 4.7. Binding of O^6MedG, O^6EtdG and O^6nPrdG to PIAS-2C8C6H5 in the presence of a 3-fold excess of dG

immunoaffinity column. This meant using an amount of O^6-alkyl dG which could not be efficiently detected by HPLC. Tritium labelled O^6-alkyl dG was therefore synthesised, as small amounts of radioactivity can be easily detected. O^6-MedG was chosen to be labelled, as it was assumed that any binding of this derivative also implied binding of O^6-EtdG and O^6-n-PrdG, since the antibody had higher affinity for the latter 2 derivatives.

Approximately 1000dpm of 1',2'-^{3}H-O^6-MedG (1×10^{-14}mol), in an excess of 10^8 of normal DNA (1×10^{-6}mol), was loaded onto and eluted from the column. HPLC analysis combined with scintillation counting determined the composition of the wash and elution fractions. The normal DNA residues did not bind non-specifically to the column, shown by their presence in only the wash sample (fig. 4.8). The distribution of the radioactivity between the wash and elution samples, indicated that approximately 10% of ^{3}H-O^6-MedG bound to the column. A maximum of 10% binding may be due to, i) the presence of the excess of normal DNA which may partially obscure O^6-MedG from the antibody, or ii) the affinity of the immobilised antibody which may be too low to recognise very small amounts of O^6-MedG.

The ability of immobilised 2C8C6H5 to recognise small amounts of O^6-MedG in the absence of normal DNA, was investigated. Immobilised 2G2B10F1 was also investigated in this experiment, to allow a comparison of the extent of binding by each immobilised antibody. It was important to consider the amount of antibody immobilised and the theoretical binding of each column. Column capacities of 20% of the theoretical binding are generally reported (Campbell *et al*, 1981). The following calculations, therefore assume that only 20% of the antibody binding sites are available to bind antigen.

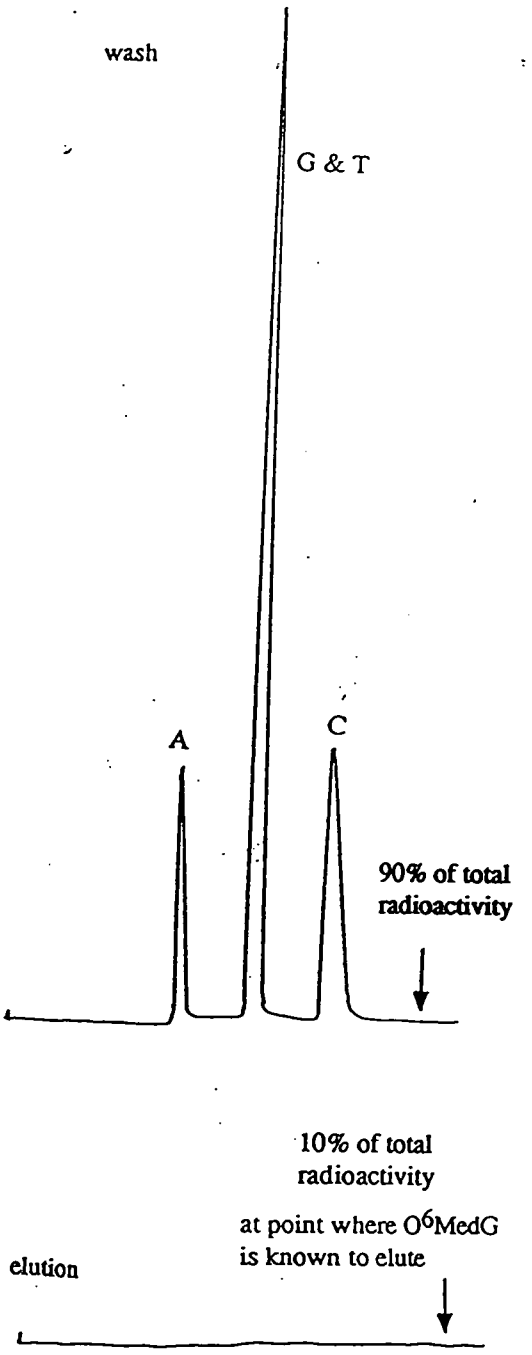


Figure 4.8. HPLC of the wash and elution fractions when adding $^{3}HO^{6}MedG$ and deoxynucleosides to 2C8C6H5-PIAS, in a ratio of $1 : 10^{8}$

	2C8C6H5	2G2B10F1
Ab immobilised per g (3.5ml) of sepharose	2mg	4mg
Experiment used 0.25 ml of support =	0.14mg (1.5×10^{-10}mol) IgM	0.28mg (1.9×10^{-9}mol) IgG
Ab : Ag binding	1 :10 = 1.5×10^{-10}:1.5×10^{-9}mol	1 : 2 = 1.9×10^{-9} : 3.7×10^{-9}mol
Assuming 20% max. total theoretical antigen binding =	**3×10^{-10}mol**	**7.4×10^{-10}mol**

From these calculations, a column containing 0.25ml of 2C8C6H5-sepharose or 2G2B10F1-sepharose, should theoretically bind 3×10^{-10}mol and 7.4×10^{-10}mol of O^6-MedG respectively. To cover this range of concentrations ^{3}H-O^6-MedG was applied to the columns in 10-fold dilutions from 1×10^{-14}mol to 1×10^{-6}mol. For both 2C8C6H5 and 2G2B10F1 the maximum amount of binding of O^6-MedG was no greater than 8% of the total amount of O^6-MedG added (table 4.8).

Table 4.8. Binding of ^{3}H-O^6MedG to PIAS-2C8C6H5 and PIAS-2G2B10F1

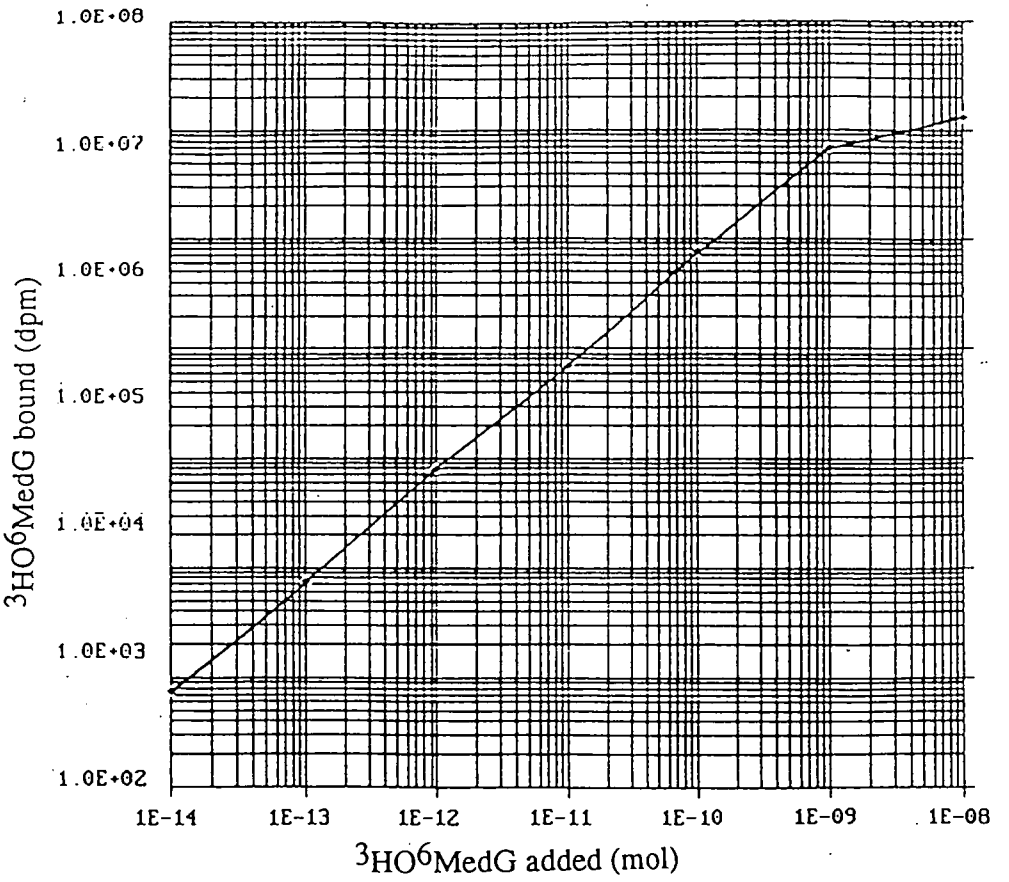
	2C8C6H5		2G2B10F1	
Amount of ^{3}H-O^6-MedG added	Amount of ^{3}H-O^6-MedG bound	% bound	Amount of ^{3}H-O^6-MedG bound	% bound
1×10^{-14}	4×10^{-16}	4.0	ND	ND
1×10^{-13}	5.5×10^{-15}	5.5	ND	ND
1×10^{-12}	3.9×10^{-14}	3.9	6×10^{-15}	0.6
1×10^{-11}	3.8×10^{-13}	5.4	8×10^{-14}	0.8
1×10^{-10}	4×10^{-12}	4.0	4.6×10^{-12}	4.6
1×10^{-9}	7×10^{-11}	7.9	4.5×10^{-11}	4.5
1×10^{-8}	2.1×10^{-10}	2.1	4.6×10^{-10}	4.6
1×10^{-7}	ND	ND	5×10^{-10}	0.5
1×10^{-6}	ND	ND	2×10^{-9}	0.2

ND = not determined

An increase in the amount of O^6-MedG added (to 1 x 10^{-8}) resulted in an overall increase in the amount bound. As expected, with lower concentrations of O^6-MedG less binding occurred. The binding also decreased as the concentration of O^6-MedG exceeded the theoretical binding capacity of the column (fig. 4.9 and 4.10). It was assumed that saturation was occurring at the point where the percentage binding began to fall.

The percentage binding for 2G2B10F1 gave a characteristic plateau shaped curve, showing the point at which the antibody, i) first recognised the antigen and, ii) became saturated (fig. 4.10). For 2C8C6H5 the saturation point was shown but the point at which the antibody first recognised the antigen was not detected (fig. 4. 9). Binding with less than 1 x 10^{-14}mol of ^{3}H-O^6-MedG could not be investigated as the radioactivity was undetectable below this quantity.

The results indicate that, i) the columns were capable of binding low levels of O^6-MedG, and ii) the column capacities were similar to theoretical values. However reasons for a maximum binding of only 8% of ^{3}H-O^6-MedG, irrespective of the amount added, were uncertain. The purity of ^{3}H-O^6-MedG was ascertained by HPLC to ensure that the radioactivity was an integral part of the derivative. In addition the immobilised antibodies were tumbled with ^{3}H-O^6-MedG to increase the time available for antibody-antigen binding, but a maximum of 8% binding was continually observed. It was possible that the incorporation of the radiolabel altered the specificity of the antibodies towards O^6-MedG. However a comparison of the immunoreactivity of O^6-MedG and ^{3}H-O^6-MedG by competitive ELISA indicated that the antibodies had similar specificity towards both labelled and non-labelled O^6-MedG. The results therefore implied that these columns could not be applied for the identification of O^6-alkyl dG in native DNA samples, as the immobilised antibodies exhibited low affinity for small amounts of ^{3}H-O^6-MedG.



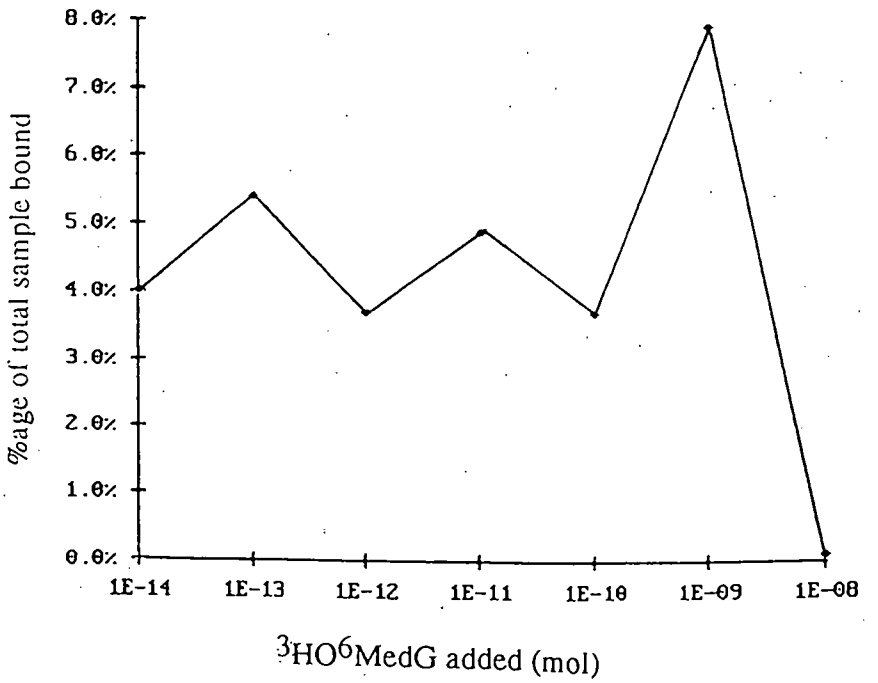
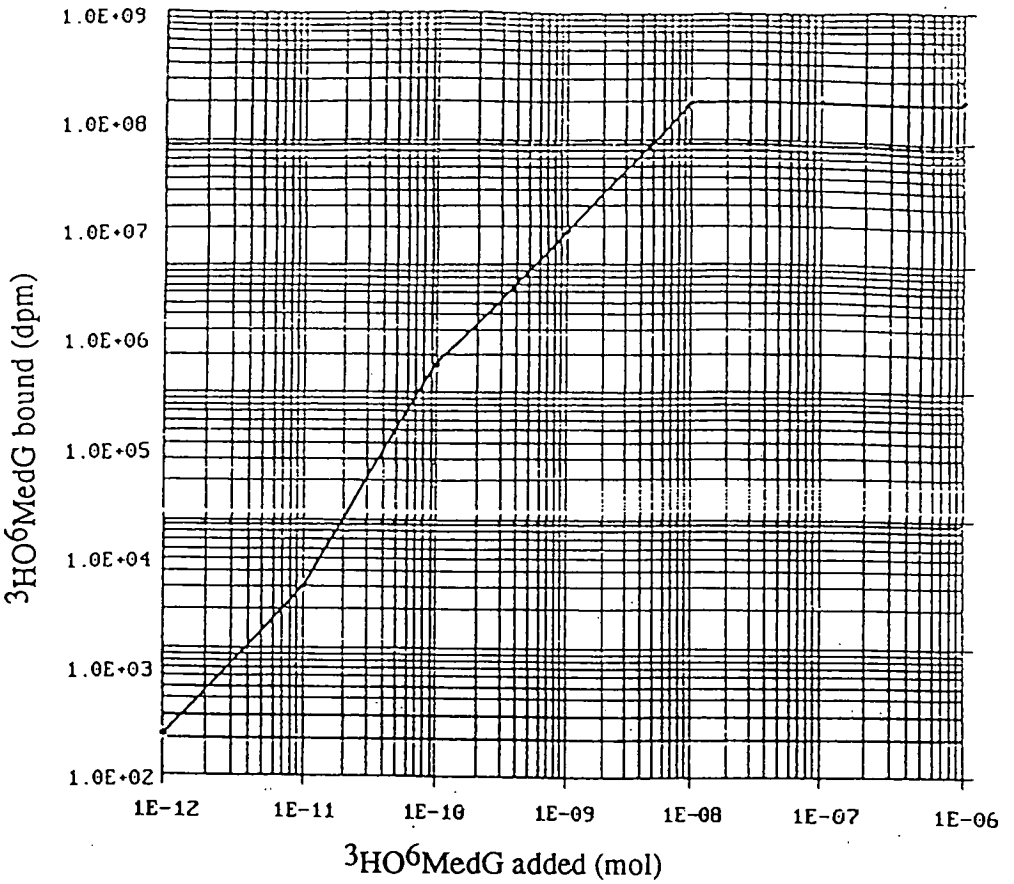


Figure 4.9. Binding of $^3HO^6MedG$ to 2C8C6H5



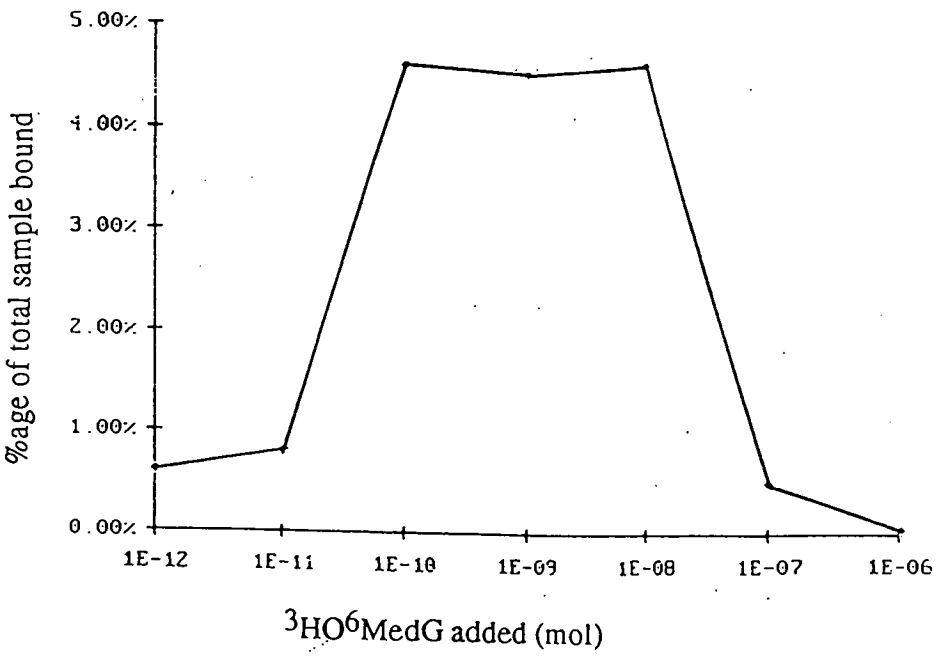


Figure 4.10. Binding of $^{3}HO^{6}MedG$ to 2G2B10F1

4.3.Experimental

IgG precipitation

Saturated ammonium sulphate (410ml, 4°C) was added slowly to hybridoma culture supernatant (500ml, 4°C). As the solution reached 45% ammonium sulphate a white precipitate appeared and the mixture was stirred gently for 30min, allowed to settle for 15min then centrifuged at 7000rpm for 15 min. The supernatant was discarded and the pellet redissolved in a minimum volume of coupling buffer (see below).

Preparation of immunocolumns

Coupling buffers

1. 0.1M sodium bicarbonate, 0.5M sodium chloride, pH8.3.
2. 50mM sodium acetate, pH 5
3. 0.01M phosphate (pH 7.4), 0.1M sodium carbonate, 0.067M sodium chloride, pH 9.5.

Cyanogen bromide activated sepharose

1g of sepharose was suspended in aqueous HCl (1mM, 20mls) to allow swelling of the beads, which were then washed extensively on a glass sintered funnel with aqueous HCl (1mM, 500ml), then coupling buffer 1 (50ml). The sepharose was transferred to a 20ml universal container and tumbled with purified antibody (5-10mls of a 5-10mg/ml solution) in coupling buffer 1, at RT. After 2hr the sepharose was collected by filtration and washed with coupling buffer 1 (20ml) [the filtrate and wash solution were kept to allow calculation of the amount of antibody immobilised on the sepharose]. The unreacted sites of the sepharose were blocked by tumbling with glycine (0.2M, 10ml). After 2hr the sepharose was washed with acetate buffer (0.1M, with 0.5M NaCl, pH4, 50ml) and was ready for use. The amount of antibody bound to the sepharose was estimated by measuring the A_{280} of the total antibody used and subtracting the A_{280} value of the filtrate and wash solution.

Triazine activated sepharose

1g of sepharose was suspended in distilled H_2O (dH_2O)(10ml) for 15min to allow swelling of the beads, which were then washed with dH_2O (500ml), then coupling buffer 2 (50ml). After the addition of antibody (5-10ml of a 5-10mg/ml solution) in coupling buffer 2, the sepharose was tumbled at RT for 2hr, washed with dH_2O

(20ml) then blocked with ethanolamine (2M, 10ml). After washing with dH_2O (25ml) then ethanol/dH_2O (8:2, 25ml) the sepharose was ready for use.

Periodate activated sepharose
CL6B sepharose (35g) was washed with dH_2O (250ml) on a glass sintered funnel then transferred to a 200ml plastic bottle. Sodium periodate (70ml,0.07M in dH_2O) was added and the mixture tumbled end over end at RT. After 45min ethylene glycol (35ml, 2M in dH_2O) was added and tumbling continued for a further 30min, followed by filtering and washing of the sepharose with dH_2O (200ml) then 0.01M $NaCO_3$ (500ml, pH 9.5). Purified antibody (10ml of 5-10mg/ml solution in coupling buffer 3) was added to 10g of activated sepharose and the mixture tumbled at 4°C. After 72 hr the sepharose was collected by filtration and washed with ice cold PBS (50ml). The sepharose was placed in a 100ml plastic bottle and tumbled at RT with sodium borohydride (20ml of 1mg/ml in PBS, made immediately before use). After 1 hr the sepharose was filtered and washed with ice cold PBS (200ml), then acetone/H_2O 9:1 (100ml), then ice cold PBS (500ml) and was ready for use.

Use of immunocolumns
HPLC conditions for all immunocolumn experiments = [C18, 5ODS column, buffer A= 75mM KH_2PO_4, buffer B= 80:20 MeOH/H_2O, gradient 0-70% B over 15min, 280nm, 0.1AUFS]

Cyanogen bromide activated sepharose
1g of sepharose was washed with PBS (50ml), then tumbled at RT with O^6-EtdG (1ml of a 10µg/ml solution in PBS). After 1hr the mixture was poured into a small plastic filter column and 1ml of supernatant was collected (fraction 1). Unbound O^6-EtdG was removed by passing PBS/Tw (10ml) through the column and 10x1ml fractions were collected (fractions 2-11). O^6EtdG bound to the immobilised antibody was eluted with 9:1 acetone/H_2O (10ml) and 10x1ml fractions were collected (fractions12-21). All fractions were evaporated, then used in a competitive ELISA where any fractions containing substantial amounts of O^6-EtdG inhibit the binding of antibody to the plate.

Triazine and Periodate activated sepharose
Immunoaffinity columns were constructed by pouring sepharose (0.5-1ml) into shortened pasteur pipettes with a glass wool filter. Columns were washed with PBS (10 volumes) prior to use. O^6-alkyl dG samples (1-10µg/ml) were passed through

the columns in PBS under gravity. After loading a sample columns were washed with 10 volumes of PBS to remove unbound material, then any O^6-alkyl dG bound to the sepharose was eluted with acetone/H_2O (5-10 volumes). Wash and elution fractions were evaporated then analysed by ELISA or HPLC.

Synthesis of ^3H-O^6-MedG

Dephosphorylation of ^{3}H-dG-triphosphate

Alkaline phosphatase (Sigma Type III p4242, 10µl, 2 units) was incubated at RT with deoxy(1',2'-^{3}H)guanosine-5'-triphosphate (250µCi, 7.5 x 10^{-9}mol) in dH_2O (250µl). After 2hr an analytical HPLC analysis of the mixture combined with scintillation counting showed that 90% of the radioactive material was ^{3}H-dG. This was purified using a C18 Bond elute column (J. T. Baker) (^{3}H-dG-triphosphate eluted immediately with 1ml dH_2O, ^{3}H-dG eluted later with 1ml MeOH). The product was dried by blowing with N_2.

Preparation of ^{3}H-O^6-MedG

N-methyl-N-nitroso-p-toluenesulphonamide (2g) was dissolved in Et_2O (30ml) in a specially adapted reaction tube with clear glass joints and a side arm to collect the product. 5ml of an ethanolic potassium hydroxide solution (4g KOH in 100ml EtOH) was added slowly and the mixture heated to 60°C in a water bath. An ethereal solution of diazomethane (3ml) distilled off and was kept cool on ice. ^{3}H-dG was dissolved in MeOH (100µl) and ethereal diazomethane (200µl) was added. The mixture was stirred at 0°C for 2hr then dried under N_2 and redissolved in dH_2O (100µl). Analytical HPLC and scintillation counting confirmed the presence of ^{3}H-O^6-MedG, which was purified by prep HPLC. The fractions containing the product were combined to give 16.5µCi, 3.66 x 10^7dpm, 5 x 10^{-10}mol, yield 6.6%.

Using ^3H-O^6-MedG on immunoaffinity columns

Antibody (1mg) immobilised on sepharose (0.25ml) was poured into a shortened pasteur pipette containing a glass wool filter, and the resulting column washed with 10 volumes of PBS. ^{3}H-O^6-MedG (35000 DBMS 1 x 10^{-12}mol) was diluted with cold O^6-MedG increasing the total amount present to give four samples containing 1 x 10^{-11}, 1 x 10^{-10}, 1 x 10^{-9} and 1 x 10^{-8} mol of O^6-MedG. Each sample was added to the top of an immunoaffinity column in PBS (200µl). The column was then washed with 10 volumes of PBS and the wash solution collected. Any bound material was eluted with acetone/H_2O and the elution solution collected. Both the

wash and elution samples were evaporated to dryness, redissolved in dH_2O (200µl) and mixed with scintillation counting fluid (4ml). The radioactivity in each sample was measured to determine the amount of capture of O^6-MedG on the column.

5. Conclusions and future perspectives

5.1. Conclusions

i) O^6-alkyl dG protein conjugates and O^6-alkyl dG oligonucleotides were capable of eliciting a specific immune response in mice, to produce antibodies towards O^6-alkyl dG

ii) Immunisation with a specific O^6-alkyl dG produced an antibody which cross reacted with other O^6-alkyl dGs. Often the affinity towards the immunising O^6-alkyl dG was less than the affinity for related derivatives.

iii) The antibody raised to O^6-MedG did not cross react with O^6-hydroxyEtdG, but the antibody raised to O^6-hydroxyEt dG cross reacted with O^6-MedG.

iv) The immobilised antibody specific for O^6-alkyl dG bound high amounts of O^6-alkyl dGs on the addition of an excess, but bound only up to 8% of the sample when non saturating amounts were added.

v) The monoclonal antibodies generated in this study could not be applied in an immunoaffinity mode to extract small quantities of O^6-alkyl dG from native DNA samples.

5.2. Future perspectives

There are many alternatives for future work, but they broadly fall into 3 categories:

i) employ 2C8C6H5 and 2G2B10F1 for the extraction of O^6-alkyl dG by altering or eliminating the immunoaffinity procedure,

ii) defrost cell lines created in the study and select antibodies specific for each O^6-alkyl dG,

iii) carry out more immunisations and fusions altering the screening protocol to favour the selection of an IgG with highest affinity for O^6-MedG.

i) The immobilisation of 2C8C6H5 and 2G2B10F1 resulted in low affinity immobilised products. This problem may be overcome by the immobilisation of antibody fragments. The Fab fragments of antibodies (fig. 4.11) are produced by enzymatic digestion creating active fragments with molecular weight approximately 50 000. Most reported antibody digestion protocols refer to IgGs (Lamoyi, 1986; Prisyazhnoy *et al*, 1988; Yarmush *et al*, 1992). However IgMs have also been successfully fragmented, to produce immunoreagents with superior properties (Gorini *et al*, 1972; Matthew and Reichardt, 1982). Generally, more Fab fragments can be immobilised per volume of sepharose than intact antibody. In addition, there are techniques for determining the orientation of the fragment, to allow the maximum number of binding sites to become available (Yarmush *et al*, 1992). A single

monovalent interaction between the antibody and antigen is believed to be the key parameter in IAC (Mason and Williams, 1980), making Fab fragments ideal reagents for this purpose.

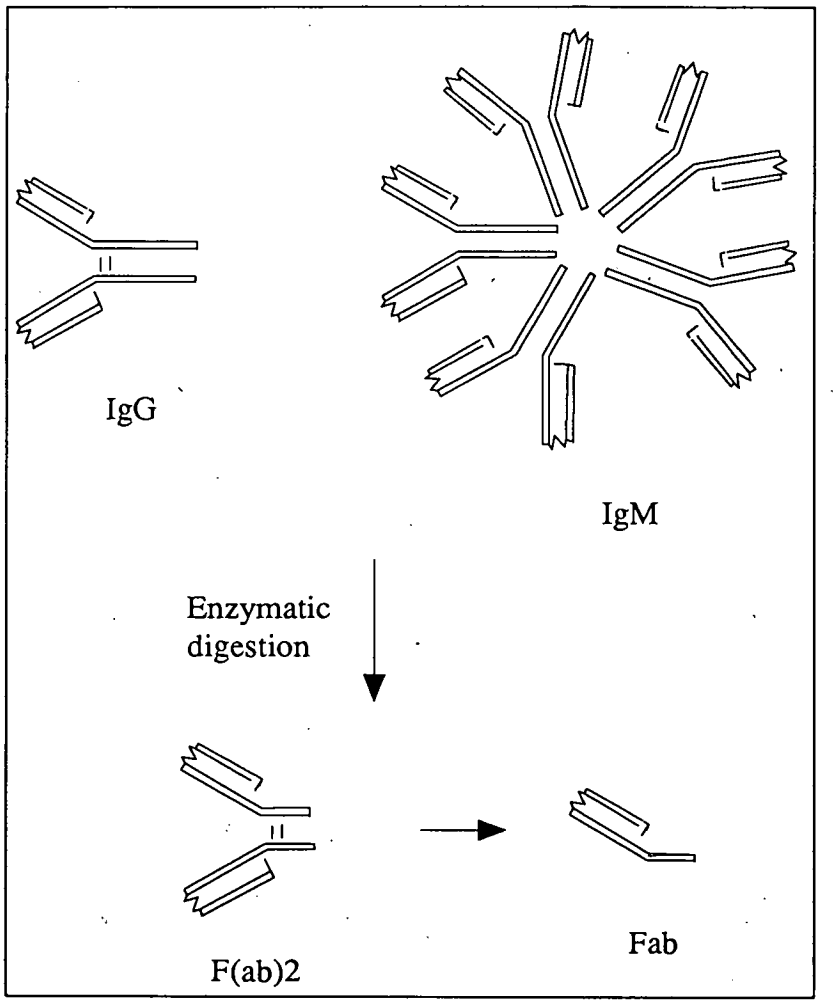


Figure 4.11. Antibody fragmentation

Assuming that 2C8C6H5 and 2G2B10F1 can be easily digested to produce Fab fragments, these antibodies may be useful in the identification of O^6-alkyl dG in an immunoaffinity mode.

Identification of O^6-alkyl dG with 2C8C6H5 and 2G2B10F1 may also be possible if immobilisation of the antibodies was eliminated. One possible technique involves the use of biotinylated antibodies and a streptavadin-agarose column (Hofmann *et al*, 1978; Updyke and Nicholson, 1984; 1986). The biotinylated antibodies are bound to the antigen in solution, and the antibody-antigen complexes are extracted on an agarose-streptavidin column, followed by elution of the antigen. This technique takes advantage of the high affinity of streptavidin for biotin. In addition antibodies are

easily biotinylated in aqueous conditions and streptavidin can be attached to agarose following antibody immobilisation methods. Generally this method increases the level and speed of antibody-antigen binding, as both are in free solution.

The employment of size exclusion membrane filters ('Microconcentrators', Amicon) may provide another possible method for the identification of O^6-alkyl dG by 2C8C6H5 and 2G2B10F1 without the requirement for immobilised antibodies. The antibody and antigen could be mixed in solution, allowing maximum binding activity of the antibody. Subsequent purification on a size exclusion filter would remove any small molecules not bound to the antibody. It would then be possible to remove the bound antigen from the antibody, using conditions similar to IAC elution. Further purification on a size exclusion filter would yield the pure antigen for quantification.

It is therefore possible to manipulate the immunoaffinity column procedure and perhaps increase the binding efficiency of the available class specific antibodies. However the affinity of these antibodies may be too low to extract O^6-alkyl dG from a pool of normal DNA, irrespective of the technique employed.

ii) During antibody production many immunisations were carried out and antibody producing cell lines were cryopreserved at each stage of cloning. It is therefore possible to return to these immunised mice or cell lines and select alternative antibodies. From these reserves it may be possible to obtain antibodies which are not class specific but recognise only one or two of the O^6-alkyl dGs. A mixture of these antibodies in appropriate amounts may produce a combination which recognises the O^6-alkyl dGs.

iii) The screening protocols employed in this study favoured the selection of an O^6-alkyl dG antibody with higher affinity towards the higher alkyl derivatives. More immunisations and fusions concentrating on the production and selection of antibodies which primarily recognise O^6-MedG should be carried out. The implementation of screening procedures which identify high affinity IgG molecules with specificity towards O^6-MedG may provide a more appropriate starting ground. These antibodies can then be tested for cross reactivity with other O^6-alkyl dG derivative in the search for a class specific antibody.

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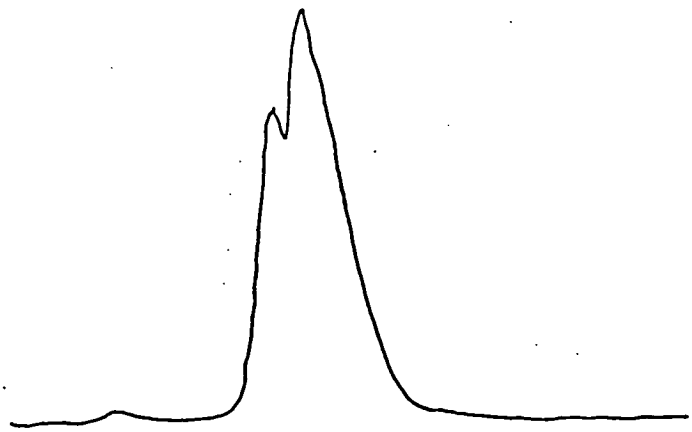
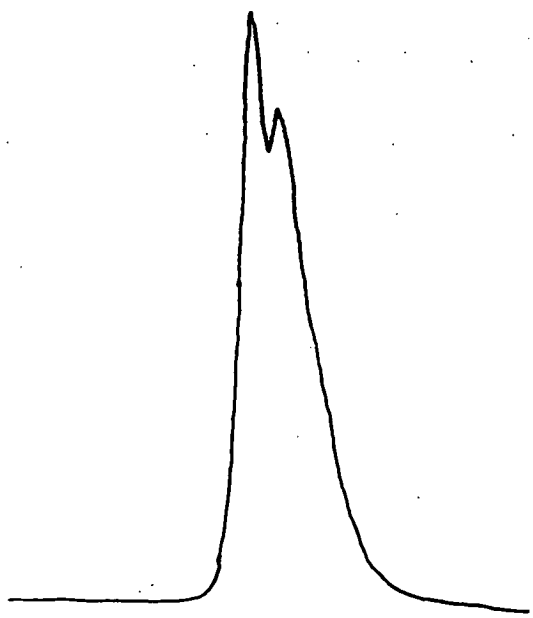
Appendix

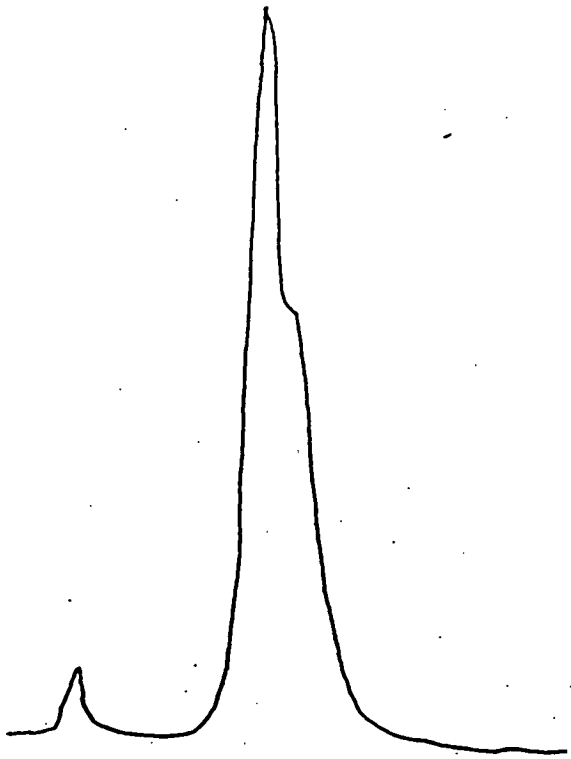
Ammonia deprotection of an O^6-alkyl dG oligonucleotide, over 24 hours



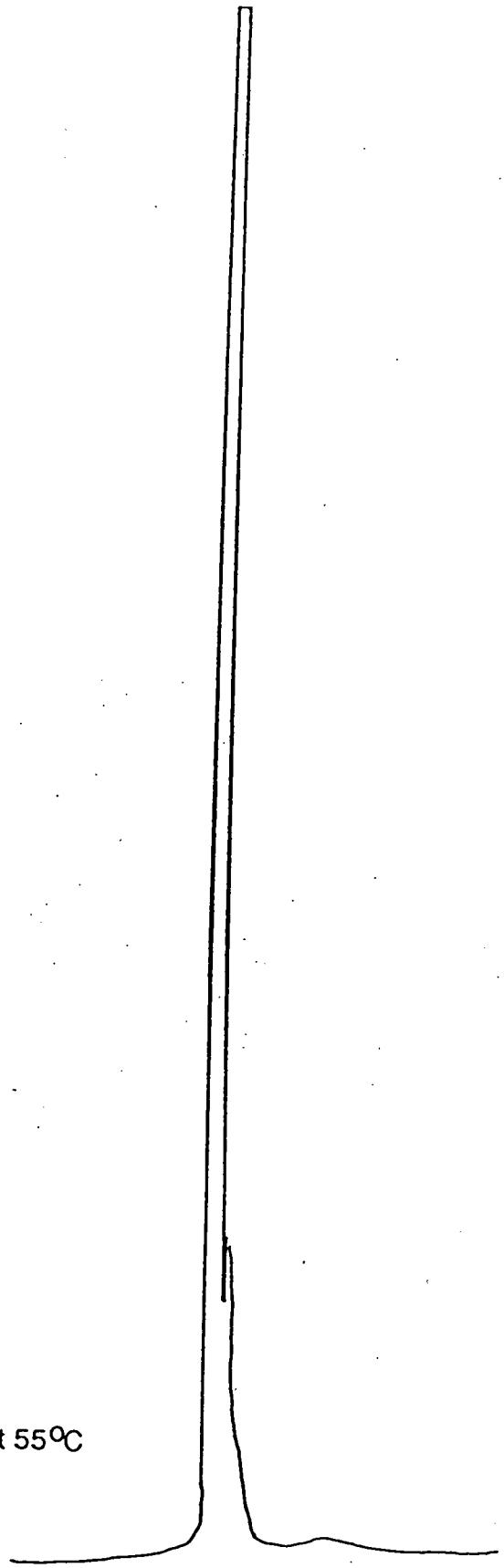
50 minutes at room temperature



4 hours at 55° C



12 hours at 55°C



24 hours at 55°C