DNA vaccination against human carcinoembryonic antigen; the effect of coding and non-coding sequences

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To Homma, Siavash

And Cain
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“...had life been a solely human affair, one would be issued at birth with a term, or a sentence, stating precisely the duration of one’s presence here: the way it is done in prison camps. That this doesn’t happen suggests that the affair is not entirely human; that something we’ve got no idea or control of interferes. That there is an agency which is not subject to our chronology or, for that matter, our sense of virtue. Hence all these attempts to foretell or figure out one’s future, hence one’s reliance on physicians and gypsies, which intensifies once we are ill or in trouble, and which is but an attempt at domesticating -or demonizing- the divine. The same applies to our sentiment for beauty, natural and man made alike, since the infinite can be appreciated only by the finite....”

Joseph Brodsky

“Watermarks”
Abstract

The immune system has been thought to have the potential to act against tumour cells. On this basis, a variety of strategies have been employed to stimulate anti-tumour immune responses. This thesis investigates DNA vaccination against human carcinoembryonic antigen (hCEA) as a means of stimulating responses against tumour cells that over express this antigen. DNA vaccination against hCEA in C57BL/6 mice results in weak and unreproducible responses. These responses, however, may be significantly potentiated by fusion of the antigen gene with Tetanus toxoid Fragment-c (Fr-c). Fusion of hCEA gene with a small sequence of oligonucleotides (Tag) may also augment such responses. These response-potentiating sequences may function by coding for helper epitopes or providing immunostimulatory signals (ISS) in their oligonucleotide form. An ISS of 15 base long augmented the antigen presentation ability of murine bone marrow derived dendritic cells. This sequence binds to an intracellular component of CB1 dendritic cells line. Furthermore, this ISS precipitated NF-κB p65 from the cytoplasmic extract of CB1 cells.
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## Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>EU</td>
<td>Endotoxin Units</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone Marrow derived Dendritic Cells</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CD66e</td>
<td>Cluster of Differentiation 66e; hCEA</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CFA</td>
<td>Complete Freund's Adjuvant</td>
</tr>
<tr>
<td>Con-A</td>
<td>Concanavalin-A</td>
</tr>
<tr>
<td>CS</td>
<td>Circumsporozoite</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocyte</td>
</tr>
<tr>
<td>CTLp</td>
<td>Cytotoxic T Lymphocyte precursors</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cell</td>
</tr>
<tr>
<td>ddNTPs</td>
<td>2',3'-dideoxynucleoside 5'-triphosphates</td>
</tr>
<tr>
<td>dNTPs</td>
<td>deoxynucleoside triphosphates</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbant Assay</td>
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<tr>
<td>Fr-c</td>
<td>Tetanus toxoid fragment-c</td>
</tr>
<tr>
<td>g-g.</td>
<td>gene gun</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophage-Colony Stimulating Factor</td>
</tr>
<tr>
<td>gp</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>hAAT</td>
<td>Human-α-1-antitrypsin</td>
</tr>
<tr>
<td>hCEA</td>
<td>Human Carcinoembryonic Antigen</td>
</tr>
<tr>
<td>hCEADo</td>
<td>N-terminal &amp; A1 subdomain of hCEA</td>
</tr>
<tr>
<td>hCEADoTM</td>
<td>hCEADo with a transmembrane domain</td>
</tr>
<tr>
<td>HEL</td>
<td>Hen Egg Lysozyme</td>
</tr>
<tr>
<td>hGH</td>
<td>Human Growth Hormone</td>
</tr>
<tr>
<td>hlg</td>
<td>Human Immunoglobulin</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HSA</td>
<td>Heat Stable Antigen</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes Simplex Virus</td>
</tr>
<tr>
<td>i.n.</td>
<td>Intranasal</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>ISS</td>
<td>Immunostimulatory</td>
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<tr>
<td>LCMV</td>
<td>Lymphocytic Choriomeningitis Virus</td>
</tr>
</tbody>
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Abbreviations

mAb Monoclonal Antibody
MEM Minimal Essential Medium
MHC Major Histocompatibility Complex
mIg membrane Ig
MLR Mixed Lymphocyte Reaction
MMC Mitomycin-c
NK Natural Killer Cell
NP nucleoprotein
ODN Oligodeoxynucleotide
OVA Ovalbumin
PBMCs Peripheral Blood Mononuclear Cells
RSV Rous Sarcoma Virus
rt-PCR reverse transcriptase Polymerase Chain Reaction
scFv single chain Fv
SCID Severe Combined Immunodeficiency
SLE Systemic Lupus Erythematosus
TCR T Cell Receptor
Th Helper T Cell
TcH Helper T Cell Hybridomas
TT Tetanus toxin
Chapter 1

General Introduction
Introduction

The idea that the immune system may be used as the means of combating tumour cells has been around for at least a hundred years. To achieve this, William Coley immunised animals with extracts of Mycobacterium mixed with killed tumour cells as early as 1905. Such ideas and experimentations set the foundations of the discipline of tumour immunology. In this chapter current concepts of tumour immunology will be discussed and a review of the literature on DNA immunisation and interaction of DNA with the immune system will be presented. The final section will describe human carcinoembryonic antigen that was used in our studies as a target tumour antigen.

Tumour immunology

The immune system is known to interact with tumour cells resulting in tumour regression or potentiation of growth. The aim of this section is to discuss the evidence for natural anti-tumour immune responses and the reasons for the inefficiency of such responses against tumour growth.

Immune surveillance

The concept of immune surveillance, that a function of the immune system is to seek out transformed cells and eliminate them, is now considered to apply most effectively only to limited tumour types. These are mainly virally-induced tumours, although, there is also some evidence for possible existence of surveillance for a few other tumours (see below). In 1970, Burnet proposed that the immune system developed primarily to prevent transmission of cells between members of the same species and to eliminate cells that
showed evidence of somatic mutation (i.e. cancer cells) (Burnet 1970). He proposed that the extensive polymorphism in MHC molecules evolved to allow description of a unique self (Burnet 1973). Thus, the immune system would detect any alterations in MHC molecules and consider it as non-self. A fundamental criticism of this model is that somatic mutations resulting in carcinogenesis do not have to necessarily occur in the MHC genes. Burnet also proposed that T cells were "almost solely" responsible for surveillance and antibody producing cells had a "negligible" role. There is, however, evidence showing that other cells such as NK cells are also involved in immune surveillance (Kurosawa, Harada et al. 1995). Antibodies have also been shown to play a role in immune surveillance of feline leukaemia (Essex, Sliski et al. 1975).

Despite the criticisms of Burnet's model, different lines of evidence suggest that immune surveillance does operate but mainly for virally induced tumours (Melief, Vasmel et al. 1989). Epidemiological evidence suggests that there is a higher risk of virally-induced tumours, such as Burkitt's lymphoma, Kaposi sarcoma and nasopharyngeal carcinoma, in immune suppressed patients (Penn 1988). Immune surveillance against virally-induced tumours could exist as a result of the presence of CTL against viral epitopes that are expressed by the tumour precursors. Therefore, the increased prevalence of virally-induced tumours in immunosuppressed individuals and the observed deficiency of immune surveillance may reflect defective anti-viral responses in such patients.

The risk of cancers, some of which are not virally induced, is higher in transplantation patients than normal population. In a twenty year follow-up study of 5,692 renal transplant patients in the Nordic countries, after transplantation, there was 2.5 fold increased risk of colon, larynx, lung and
bladder cancers, and in men the risk of cancers of prostate and testis, and in women cancers of cervix and vulva or vagina. These studies do not, of course, directly link immune suppression with increased risk of cancer since in renal transplant patients, apart from immune suppression, there are many other physiological anomalies compared to the general population and some of the drugs used for immunosuppression can have mutagenic effects. However, immune surveillance against non-viral cancer cells should not be completely ruled out since there are still some data that may be explained in this way. For instance, in a study of 34 human lung cancer lines, in HLA A 0201 positive cell lines the p53 mutations did not occur in the regions of p53 that could potentially bind HLA A 0201 (Wiedenfeld, Fernandez-Vina et al. 1994). In addition, in some cell lines from HLA A 0201 patients mutations did occur in HLA A 0201-restricted regions of p53, however, those tumours had lost A 0201 expression. A clinical observation that also supports the presence of immune surveillance is that some tumours undergo spontaneous regressions associated with detectable immune activity (Rosenberg 1991).

The innate immune system also takes part in immune surveillance. NK cells and macrophages have been shown to play an important role in prevention of metastasis and tumour growth. Gallo Hendrikx et al. observed that 90% of the offspring of mice that spontaneously develop primary pancreatic carcinoma developed metastatic tumours when crossed with mice carrying the beige mutation, that results in impaired activity of NK cells and macrophages, whereas crosses with mice with severe combined immunodeficiency (SCID) did not result in metastasis from their primary pancreatic tumours in any of the offspring (Gallo-Hendrikx, Copps et al. 1994). These results demonstrate that innate immunity may in some circumstances prevent metastasis. In another study, it has been shown that in the presence of IL-2, NK cells inhibit tumour growth in the absence of T or B cells (Alosco, Croy
et al. 1993). In this study, SCID and SCID-beige mice were used as syngeneic tumour recipients. In contrast to the rapid growth of the IL-2 transfected fibrosarcoma in SCID-beige mice, these cells did not grow in the mice that lack functional B and T cells (SCID mice). In addition, the inhibition of tumour growth in SCID mice was reversed by treatment with antibodies against natural killer (NK) cells demonstrating that the innate immune system may under some circumstances mount anti-tumour responses.

NK cells have been shown to express heterogeneous MHC class I receptors, NK inhibitory receptors (NKiR), that inhibit killing of target cells expressing native class I antigens (Farrell, Vally et al. 1997). This finding supports the notion of self recognition through MHC molecules that was proposed by Burnet, however, it is the NK cells that have the ability to recognise self rather than T cells of the adaptive immune system. Non-specific immunity has also been demonstrated against acute T cell leukaemias in a TCRVγ1.1Jγ4Cγ4 transgenic mouse (Penninger, Wen et al. 1995). Protection against haematopoietic tumours in this model was conferred by γδ+ T cells. The role played by the innate immune system especially in surveillance of early neoplastic cells, deserves more attention, as components of the innate immune system such as NK cells are capable of responding immediately, whereas CTL may need a lag period before being able to lyse their target cells.

**Anti-tumour immune responses**

Despite the evidence for the presence of immune surveillance, tumours do still grow. Immune surveillance is probably a function of the immune system but it may predominantly recognise neoplastic events rather than eliminate neoplastic cells by eliciting an effective anti-tumour response. In other words tumour cells are antigenic as a result of mutations but may not necessarily be immunogenic. However, tumour antigens recognised by the
Chapter 1

immune system are not all neo-antigens and may also be derived from normal
cell constituents or non-self antigens such as viral products.

Identification of Tumour antigens

The initial work on identification of tumour antigens was based on the
assumption that tumour responses were best mediated by antibodies. Some of
the approaches used will be described here.

Sera from cancer patients were analysed for antibodies against tumour
antigens using the 'autologous typing' method. Three classes of tumour
associated antigens were described by testing the sera for reactivity with
surface antigens of cultured autologous or allogeneic tumour cells and non-
malignant cells. Sera that reacted to autologous tumours exclusively were
considered to detect class 1 antigens. Class 2 antigens were identified by sera
that were reactive with autologous and allogeneic tumour cells and class 3
antigens were detected by sera with reactivity against tumour and normal cells
(Ueda, Shiku et al. 1979; Pfleudschiuh, Shiku et al. 1980).

Another approach that led to the discovery of antigens that were over-
expressed in tumour cells but had a low level of expression in normal cells, was
immunisation of animals with tumour extracts. One such antigen is human
carcinoembryonic antigen (hCEA) that was discovered by injecting rabbits with
pooled tumour extracts (Gold and Freedman 1965). The resulting antiserum were
purified by absorption with excess normal colon extract. The antiserum were
further absorbed with tumour plasma fibrin and killed gut bacteria, and then
gave a precipitin band with colonic tumour extracts in gel diffusion assays but
not with extracts of normal colon.

Another class of tumour associated antigen has been described that is
expressed in normal tissues but has an altered glycosylation pattern in tumour
cells. An epithelial tumour associated antigen, ic epithelial mucin (MUC1), was
discovered by immunisation of mice with mammary milk fat globule (Taylor
Papadimitriou, Peterson et al. 1981). A mAb (SM-3) raised against the
deglycosylated form of this antigen showed differential reactivity with the mucin produced by normal and malignant breast epithelium (Girling, Bartkova et al. 1989). SM-3 binds a region of the core protein that is not glycosylated in malignant breast carcinoma.

Currently, it is widely accepted that tumour immunity is best mediated by T cells of particularly CD8 phenotype. This is based on evidence from animal studies in which CD8 T cells, alone or in combination with CD4 cells, have been either transferred to naive animals or deleted from immunised animals resulting in effective anti-tumour immunity or loss of anti-tumour responses, respectively. In addition, in both animal and human studies it has been repeatedly observed that tumour progression is associated with down regulation of class I MHC, suggesting outgrowth of escape mutants under CD8 mediated pressure. Accordingly, in the past decade various approaches have been used to identify tumour specific T-cell epitopes.

In one approach, to identify target epitopes, the MHC class I associated peptides on tumour cells are extracted, fractionated and used for coating target cells (Cox, Skipper et al. 1994). Using this method, a peptide fragment of gp100/Mel17, a normal protein expressed by melanocytes, was identified as the epitope recognised by melanoma specific CTL derived from five different patients. Alternatively, CTL epitopes are identified by transfection of MHC matched tumour cells that do not express the antigen (i.e. are not killed by the CTL) with a cDNA library of the tumour. Sequencing of the plasmids from the cells that become susceptible to CTL lysis after transfection identifies the gene coding for CTL epitopes expressed by the original tumour cells (Brichard, Van Pel et al. 1993). This technique has been used successfully to identify epitopes recognised by melanoma specific CTL derived from differentiation antigens such as tyrosinase or melanoma specific antigens of the MAGE family (van der Bruggen, Szikora et al. 1994) that are not found on normal tissues except testis.
Chapter 1

T cells specific for tumour antigens

The T-cell repertoire that reacts against tumour antigens would be shaped by the process of thymic selection. T cells that are capable of reacting against non-self or neoantigens, regardless of their affinity, would be positively selected as these antigens are not expressed in the thymus. Virally transformed tumours may express antigens derived from viral transforming proteins (e.g. human papilloma virus E6 or E7) or structural proteins. There is good evidence that virally induced tumour cells down regulate expression of the most immunogenic viral proteins such as envelope proteins (Klein and Boon 1993). This may represent anti-tumour responses resulting in selection of tumour cells that down regulate immunogenic epitopes. Altered cellular proteins such as mutated $p53$ products, would also be recognised by T cells that have been allowed to leave the thymus as the antigen is not coded by the genome during T-cell ontogeny.

Normal cellular proteins that are recognised by tumour reactive T cells include the melanoma specific antigens coded by the MAGE family of genes that are normally expressed only in early ontogeny and tyrosinase or MART differentiation antigens that are only expressed by melanocytes. The T cells that respond to these antigens may be divided into three classes; 1) Low affinity T cells that escape negative selection in the thymus as they bind antigen in the thymus with an affinity lower than deletion threshold. 2) T cells that are not negatively selected as their target antigens are not expressed or have low expression levels in the thymus. 3) T cells against "subdominant" or "cryptic" epitopes that are also poorly presented in the thymus. This is as a result of the low affinity of these epitopes for MHC or their inefficient processing compared to "dominant" epitopes. All the classes of potentially autoreactive T cells may exist in a hyporesponsive or anergic state in the periphery, if they are not triggered to their activation threshold. This could happen as a result of low level of expression of the peptide epitopes in the periphery, lack of help or
costimulation. Alternatively, naive autoreactive T cells may remain in lymph nodes before they encounter their antigen.

The anti-tumour T cells that recognise self antigen, therefore, are probably anergic cells that are activated \textit{in vitro} under conditions that results in triggering these cells above activation threshold. These conditions include \textit{in vitro} overexpression of peptide epitopes, for instance when transfected cells are used for stimulation of T cells, or addition of cytokines such as IL-2 that are normally secreted by helper T cells or provision of costimulation by accessory cells presenting the tumour antigens.

Anergic self antigen-specific T cells may also be activated \textit{in vivo} by providing suprathreshold activation signals. An excellent animal model that demonstrates both the possibility of presence of anergic autoreactive T cells in the periphery against a model tumour antigen and also provides evidence for the possibility of turning these cells against the tumour has been reported by Speiser \textit{et al.} (Speiser, Miranda \textit{et al.} 1997). They used a mouse model that spontaneously developed pancreatic $\beta$-cell tumours that expressed transgenic lymphocytic choriomeningitis virus (LCMV) glycoprotein as a self antigen. The T cells against the viral antigen did not respond \textit{in vitro} when stimulated with LCMV glycoprotein and tumours grew progressively. However, if the mice were infected with LCMV virus the tumours regressed and the T cells against LCMV glycoprotein could be detected.
Inefficiency of anti-tumour responses

The evidence above argues that there are tumour antigens that can be recognised by CTL. The same arguments may apply to antigens recognised by other effector elements of the adaptive immune system such as B cells and helper T cells. Additionally, the presence of anergic helper T cells may contribute to B-cell and CTL anergy. An important question that arises is why tumour-specific immune cells are anergic and do not mount an effective response leading to eradication of tumours. In particular, cells specific for viral antigens or mutated self antigens would be expected to become activated as the target antigens is non-self. As mentioned before for virally induced tumours, the cells that express immunogenic antigens may be selected against but the cells that down regulate these antigens grow progressively. This might also be the case for the tumour cells expressing immunogenic mutated proteins. An effective response is also absent against the remaining tumour cells that may express antigens seen by anergic cells even when the antigen is overexpressed (e.g. MART or tyrosinase) and would be expected to efficiently trigger the responding T cells. The reasons for lack of efficient responses could be divided into factors associated with the effector phase and those associated with the induction of response.

The effector phase of the immune response may be inefficient mainly for reasons related to the tumour cells (Figure 1.1). They can be poor targets for the immune system due to loss of MHC expression or mutations in the MHC molecules or other proteins resulting in the loss of immunogenic epitopes. Other factors that make tumours poor targets include sequestration of tumour cells in a privileged environment and secretion of immunosuppressive cytokines by tumours. Tumour cells can provide an immune privileged environment by expression of Fas ligand resulting in apoptosis of Fas-positive T cells (Hahne, Rimoldi et al. 1996). Tumour cells secrete cytokines such as TGF-β, IL-10 and vascular endothelial growth factor (VEGF) that are capable of
suppression of both innate and adaptive anti-tumour responses (Chouaib, Asselin-Paturel et al. 1997). In principle, the ability of tumour cells to divide rapidly and the instability of the genome allows tumours to evolve under the pressure of the immune response and become poorer targets. The rate of tumour growth in relation to the magnitude of the immune response would also effect the outcome of the response such that rapidly growing tumours may become too great a burden for the immune response to manage.

**Figure 1.1:** Some reasons for failure of effective anti-tumour immune responses. At low levels of B7 expression CTLA4 would blocks all the binding sites for CD28. MHC molecules on tumour cells mutate and are not recognised by specific activated T cells. Tumour cells secrete immunosuppressive cytokines.

The absence of costimulatory signals on potential target cells has been shown to allow maintenance of peripheral tolerance (Guerder, Meyerhoff et al.)
1994). The mechanism of maintenance of anergic T cells and failure of induction of effective anti-tumour responses, has been proposed to be the absence of costimulatory molecules on target cells (figure 12.1). This assumes that only tumour cells, that are not professional antigen presenting cells (APCs), present tumour antigens. This assumption was made on the basis of the paradigm that class I presentation exclusively operates for endogenous antigens. However, the description of the phenomenon of cross-priming of CTL against exogenous antigens and its possible physiological significance, which probably has been underestimated (discussed elsewhere), indicates that in an anti-tumour immune response, professional APCs could potentially present tumour antigens in association with class I and II molecules.

Presentation of tumour antigen by professional APCs has been experimentally proven by induction of CD8+ CTL in mice after immunisation with GM-CSF-transfected B6 melanoma cells that lacked expression of MHC class I molecules (Huang, Golumbek et al. 1994). In another study, Huang et al. provided direct evidence that even if a tumour cell is transfected with a costimulatory molecule such as B7-1, tumour antigens are primarily presented by bone marrow derived APCs (Huang, Bruce et al. 1996). In this model H-2^b→H-2^bd bone marrow chimeras were immunised with H-2^d tumours that were transfected with a model antigen and B7-1. The CTL against the model antigen were restricted to the bone marrow haplotype (H-2^b). After repeated rounds of immunisation, however, CTL specific for the tumour haplotype also developed indicating that the tumour was also contributing to induction of the T cells but to a lesser degree.

The above experiments show that professional APCs not only are capable of presenting tumour antigen but also stimulate effective anti-tumour responses in tumour models. The reason for inefficiency of induction of anti-tumour responses under more physiological circumstances may, therefore, lie with professional APCs. Adler et al. have demonstrated elegantly that when an antigen is expressed by parenchymal cells, bone marrow derived cells are
required for T cell tolerance to that antigen (Adler, Marsh et al. 1998). They studied T cell tolerance to an epitope of haemaglutinin (HA) in HA transgenic mice of H-2\textsuperscript{bd} haplotype. These animals were reconstituted with bone marrow from non-transgenic animals of either H-2\textsuperscript{b} or H-2\textsuperscript{d} haplotypes. Naive T cells expressing a transgenic TCR specific for a I-E\textsuperscript{d}-restricted epitope of HA from H-2\textsuperscript{bd} animals were transferred to the bone marrow reconstituted animals. In these experiments, only HA-specific T cells from mice reconstituted with H-2\textsuperscript{d} bone marrow became tolerant to HA antigen. In addition, these T cells had a phenotype consistent with antigen recognition indicating that the T cell tolerance was an active process. Apart from the fact that this transgenic TCR may have a high affinity for its target, this model is similar to the encounter of tumour specific cells with tumour cells that are not professional APCs. The interesting question here is what makes such a presentation tolerogenic.

The experiment of Adler et al. described above show that antigen presenting cells derived from bone marrow can be tolerogenic. This could occur as a result of presentation of antigen by a population of APCs different from professional APCs that are immunogenic (figure 1.1). Lymphoid derived dendritic cells have been proposed to be tolerogenic (de St Groth 1998) and might be the cells that present tumour antigen. On the other hand, the same professional APCs might present tumour antigen but have become tolerogenic as a result of either not receiving activation or maturation signals, or responding to signals that render them tolerogenic (figure 1.1). The chemokines released from the tumour site may also be different to those released from inflammatory sites, resulting in migration of either tolerogenic APCs, if they exist, or of APCs potentially capable of inducing either tolerance or activation. If the latter was the case then these APCs may become mature and capable of antigen presentation in response to other signals that may be absent at the tumour site.

There are different theories on the nature of the signals that result in maturation of APCs. On the one hand, Matzinger et al. propose that the
"danger" signals received by professional APCs result in their maturation to immunogenic APCs. In tumours, "danger" signals could be the molecules released from cells only when they undergo necrotic rather than apoptotic damage (Matzinger 1994). On the other hand, Janeway et al. propose that the innate immune system has inherited a set of primitive "pattern recognition receptors" (PRR) that recognise molecular patterns that are unique to and essential for specific groups of organisms (Medzhitov and Janeway 1997; Medzhitov, Preston-Hurlburt et al. 1997). These receptors detect the presence of foreign organisms and transduce signals that result in activation and maturation of APCs. The signals that cause maturation of APCs to immunogenic ones or result in their migration to the appropriate areas of the lymph nodes, may be absent in tumour sites or there may exist other signals that counteract them. Finally, it is possible that the APCs that take up tumour antigens do not migrate to the T cell areas of the draining lymph nodes without the presence of appropriate signals (De Smedt, Pajak et al. 1996).

A study performed by Leach et al. may provide some clues into the signals important in tolerance induction to tumour cells. In this study, it was shown that blockade of cytotoxic T lymphocyte antigen-4 (CTLA-4) result in tumour regression both in challenge and therapy experiments (Leach, Krummel et al. 1996). CTLA-4 is expressed by T cells and binds B7-1 and B7-2 with a higher affinity than CD28. This results in inhibition of T cell activation as a result of intracellular hypophosphorylation of certain T cell signalling molecules (Chambers and Allison 1997). It is thought that as CTLA-4 affinity for B7 is higher than that of CD28, in situations where B7 expression is low, for instance on immature dendritic cells, the inhibitory signal of CTLA-4 predominates, resulting in suppression of T cell activity. It may be possible that the professional APCs that present tumour antigen express limited amounts of B7 due to the lack of inflammatory signals present at the tumour site, resulting in CTLA-4 mediated T cell inhibition. Alternatively, factors secreted by tumours may result in inhibition of T cell activation by inhibition of intracellular
signalling events. It has been shown that in renal cell cancer, intracellular pathways leading to T cell activation are impaired as a result of an unknown product secreted by the tumour cells (Kolenko, Wang et al. 1997).

**Experimentally induced anti-tumour responses**

Despite the inefficiency of spontaneous immune surveillance, strong anti-tumour responses have been elicited in experimental animals. A variety of approaches to make tumours more immunogenic have been tried. These include immunisation with tumours transfected with molecules that potentiate their antigen presentation ability such as MHC class II (Ostrand-Rosenberg, Thakur et al. 1990; James, Edwards et al. 1991; Chen and Ananthaswamy 1993), or costimulatory molecules such as B7 (Baskar, Ostrand-Rosenberg et al. 1993). Cytokine transduced tumours have also been used for immunisation with some success (Dranoff, Jaffee et al. 1993). Another approach has been to increase the antigenicity of a tumour by transfection with xenogeneic antigens (Itaya, Yamagiwa et al. 1987) or allogeneic MHC class I molecules (Plautz GE 1993). All the above approaches have led to protection against the parental population as a result of spread of the immune response to otherwise weak antigenic epitopes presented by the parent population. A more direct method of stimulation of anti-tumour responses has been immunisation with recognised tumour antigens using a variety of methods to elicit mainly CTL responses. For this purpose viral vectors carrying CTL epitopes or the peptide epitopes themselves have been used. A disadvantage of these methods is that the immune responses are limited to a narrow range of T cell epitopes allowing the possibility of growth of escape mutants. In addition, single CTL epitopes are restricted to specific MHC haplotypes and may not elicit such a response in mismatched animals. Alternatively, APCs such as dendritic cells are exposed to tumour antigen, in *vitro*, and injected in to animals to elicit anti-tumour responses (Zitvogel, Mayordomo et al. 1996). In this approach, whole tumour extracts may be used that would result in broader immune responses.
A new method of eliciting CTL responses is DNA immunisation that has been used for tumour vaccination. This method has the advantage of allowing immunisation with the gene for a protein that may contain multiple CTL and B cell epitopes resulting in a broad cellular as well as humoral response in animals of different haplotypes. Moreover, there is no need to identify CTL epitopes before immunisation and the possibility of alteration in the epitopes during synthesis (for instance glycosylation patterns) is circumvented as the antigen is produced in its native form by mammalian cells, \textit{in vivo}. Another advantage of DNA immunisation is that immunomodulatory molecules such as cytokines or costimulatory molecules can be administered at the same time as the antigen. As mentioned earlier tolerant self reactive T cells may be the cells that recognise tumour antigens. It has been shown that DNA vaccination is capable of breaking self tolerance against a transgenic self antigen (Mancini, Hadchouel \textit{et al.} 1996). This may be because in DNA immunisation the antigen is synthesised under the control of a strong viral promoter, such epitopes may be presented at high concentrations if antigen presenting cells are transfected with the vector.

\textbf{DNA immunisation}

\textit{Background}

DNA immunisation is the method used for eliciting immune responses against an antigen by \textit{in vivo} administration of a eukaryotic plasmid expression vector encoding the antigen of interest. The first evidence for the possibility of raising immune responses by using the gene coding for the antigen rather than the antigen itself, came from the work on polyoma virus DNA. Atanasiu \textit{et al.} reported in 1962 that subcutaneous administration of purified polyoma virus DNA to hamsters resulted in induction of tumours and production of
antibodies against the viral proteins (Atanasiu, Orth et al. 1962). As these experiments were not entirely clear due to the possibility of contamination with viral particles, their significance was ignored until 1992 when Tang et al. reported in *Nature* that it was possible to raise antibodies against human growth hormone (hGH) and human-α-1-antitrypsin (hAAT) by introducing the gene encoding these proteins directly into mouse cells, *in vivo*. (Tang, DeVit et al. 1992). This was achieved by introduction of DNA-coated gold microprojectile particles into mouse ear skin. In 1993, Ulmer et al. demonstrated that i.m. injection of BALB/c mice with DNA encoding nucleoprotein (NP) resulted in development of antigen specific IgG antibodies and CTL responses that protected the animals against lethal challenge with the virus (Ulmer, Donnelly et al. 1993). After a short period of silence there was an explosion of reports on DNA vaccination which has continued ever since and grown by geometric progression. Most of these reports, however, demonstrate that DNA immunisation can be used to elicit antibody and/or CTL responses against antigens from a variety of organisms in different mammals. Only a small minority of reports have demonstrated novel uses of DNA immunisation or shed light on the physiology of immune response when antigen is encountered in this form.

DNA immunisation has been viewed by the biotechnology industry as a technology that could potentially generate revenue. This has led to selling of DNA immunisation as a major technological advancement. Such a technological approach, nevertheless, has yielded some useful applications for DNA immunisation. These include "genomic immunisation" for detection of antigenic epitopes of a micro-organism by immunising with a genomic library cloned into a mammalian expression vector (Barry, Lai et al. 1995). In this approach the genes encoding immunogenic products are identified by stepwise elimination of sub-libraries that do not result in an immune response.
Mechanism of the immune response

A feature of DNA immunisation that became apparent early on, was the ease with which CTL responses could be elicited, at least in the systems reported. This led to work on the feasibility of DNA immunisation as a means of eliciting CTL responses in viral infections and against tumours where they are thought to be the appropriate effectors. The conventional methods for induction of CTL responses use peptide epitopes that directly bind major histocompatibility complex (MHC) class I molecules mixed with appropriate adjuvants or use whole antigens in specific adjuvants, that direct a Th1 response. Alternatively, recombinant viruses have been used to deliver antigens to the class I processing pathway resulting in induction of CTL responses. Against this experimental background, workers initially speculated that the ease of CTL induction in DNA immunisation may be due to the fact that antigen enters the class I processing pathway as soon as it is synthesised. This led to further speculation that the cells that take up DNA and express the protein also present the antigen. Wolff et al. had shown that i.m. injection of naked plasmid DNA lead to uptake and expression of the encoded gene (Wolff, Malone et al. 1990). Therefore, for a while the debate was on whether muscle cells presented the antigen and if so what was the nature and origin of costimulatory signals. For instance, it was suggested that the inflammation due to the process of injection provided a costimulatory environment. Garlepp et al. demonstrated that muscle cells could present antigen to a T-cell hybridoma in vitro and showed that this ability was potentiated by pretreatment of muscle cells with the inflammatory cytokine interferon-γ (IFN-γ) (Garlepp, Chen et al. 1995). Demonstration of antigen presentation in DNA immunisation by bone marrow-derived rather than muscle cells (Doe, Selby et al. 1996; Fu, Ulmer et al. 1997) put an end to this debate. Other work on the mechanism of antigen presentation in DNA immunisation focussed on identification of these bone marrow-derived cells. This work is reviewed later in this chapter.
Advantages of DNA immunisation

DNA immunisation as a method of vaccination has many theoretical advantages over conventional forms of vaccination. These include the ease of preparation and the stability of DNA molecules at ambient temperatures that would lower the cost of vaccination; the absence of demonstrable responses against the vector allowing its repeated use for vaccination against other pathogens; the possibility of administration of multiple vaccines in one vector; the absence of the risk of reactivation encountered in attenuated viral vaccination and administration of the antigen in its native form. However, since the discovery of DNA immunisation very few studies have been published on the theoretical disadvantages and indeed dangers of using this method as a means of vaccinating healthy populations against pathogenic organisms. The safety issues that need to be addressed in DNA vaccination include the effect of chronic expression of the antigen; the risk of integration of DNA into the genome and the possibility of autoimmunity directed against the host DNA or proteins.

Safety of DNA immunisation

Mutations as a result of integration of plasmid DNA into host genome can potentially inactivate a cellular gene or cause transformation by inactivation of tumour suppressor genes or activation of oncogenes. This is an important issue if DNA immunisation is to be used for vaccination of healthy populations specially growing children. Integration of plasmid DNA into the genome can occur in random sites or by homologous recombination. The latter can be avoided by using plasmids that do not contain any homologous sequences to mammalian DNA. However, the real problem is random insertion that could occur at a higher rate but cannot be inhibited. In the only two reported studies on this topic, integration was not detected after plasmid DNA immunisation (Nichols, Ledwith et al. 1995) (Wolff, Ludtke et al. 1992). Nichols et al. devised a sensitive method that is capable of detecting integration of one plasmid in
150,000 nuclei and looked for such events in muscle and most other tissues (Nichols, Ledwith et al. 1995). In these experiments integration of plasmid DNA was not detected in any tissues from the twenty mice studied. On the basis of these studies, Nichols et al. estimate that the chance of plasmid DNA integration is a third of the chance for spontaneous mutations. However, to be able to make any comments about such an important issue clearly more studies need to be carried out.

The autoimmune disease systemic lupus erythematosus (SLE), is associated with the development of anti-DNA antibodies. An important issue concerning the safety of DNA vaccination, therefore, is the possibility of raising anti-DNA immunity against the host genome. Gilkeson et al. have demonstrated that immunisation with bacterial but not eukaryotic dsDNA results in development of anti-bacterial dsDNA antibodies (Gilkeson, Grudier et al. 1989). In order to induce such a response, however, E.Coli dsDNA had to be coupled to bovine serum albumin (BSA) and injected in an adjuvant. In DNA vaccination, purified naked DNA devoid of any proteins, such as histones, is used and naked DNA in this form is not immunogenic, at least in the systems studied (Xiang, Spitalnik et al. 1995; Liu, McClements et al. 1997). Moreover, anti-bacterial DNA antibodies cross react with mammalian dsDNA only when developed in SLE susceptible New Zealand Black/White (NZB/W) mice (Gilkeson, Pippen et al. 1995). Therefore, in DNA immunisation even if anti-bacterial DNA antibodies did develop, they may be cross reactive only in susceptible individuals. The factors predisposing to susceptibility, however, are not well understood but may be associated with MHC haplotypes, antigen presentation and cytokine profiles of cells responding during immunisation. Further study of such factors undoubtedly will contribute to understanding of pathogenesis in such autoimmune diseases and would also clarify the safety of DNA vaccination. For instance in one study it has been shown that the cytokine profiles of the responding cells determines the outcome of the disease. The development of anti-DNA antibodies in pre-autoimmune NZB/W mice can be
accelerated by immunisation with ds-\textit{E.Coli} DNA conjugated to BSA, however, this paradoxically protects the animals against developing the disease. Gilkeson \textit{et al.} have shown that immunisation of pre-autoimmune NZB/W mice with ds-\textit{E.Coli} DNA/BSA results in a reduction in proteinuria and prolongs survival compared to animals who spontaneously develop the disease or are induced to develop early disease by other methods (Gilkeson, Ruiz \textit{et al.} 1996). The anti-DNA antibodies induced in the protected animals have the same specificity and affinity for mammalian DNA and glomerular antigens as in control animals (Gilkeson, Ruiz \textit{et al.} 1996) however protection was associated with a shift in cytokine profiles. The splenocytes of animals immunised with bacterial DNA secreted more interleukin (IL)-12 and INF-$\gamma$ and less IL-4 than control animals (Gilkeson, Conover \textit{et al.} 1998).

There is a concern about the possibility of autoimmunity against the components of the host cells that synthesise antigen as transfection may be accompanied by an inflammatory reaction. In one study, Mor \textit{et al.} did not find any anti-myosin antibodies or evidence of myositis up to five months after intramuscular DNA immunisation (Mor, Singla \textit{et al.} 1997). The aetiology of some autoimmune disease has been attributed to viral infections and transfection of muscle cells with plasmid DNA resembles viral infection. The antigen against which the immune response is elicited, the degree of cross reactivity of that with self proteins, the cells transfected, the type of immune response and the susceptibility of the individual could influence the development of autoimmunity. Considering the complexity of this issue and other safety aspects of DNA vaccination, one cannot extrapolate and make generalised deductions from very few studies that have been performed on specific antigens in a limited number of inbred strains of mainly one species. The clinical use of DNA vaccination has to be approached with caution and may be only in selected groups in whom risk/benefit ratios are favourable. Over a long period of time such experience may clarify the degree with which these theoretical risks exist in reality.
Chapter 1

The type of immune response

An important point to consider in DNA immunisation is the type of immune response that is elicited. The effector arm of the adaptive immune response is thought to be directed by helper T-cells to develop appropriate responses so that different pathogens are counteracted according to the nature and location of their interaction with the internal environment. During the 1980's work on classification of CD4+ T cells lead to categorisation of these as T helper 1 (Th1) and 2 (Th2) cells. In 1982, MacDonald *et al.* reported clones of alloreactive T-cells that were obtained by limiting dilution which were heterogeneous in their cytokine production (MacDonald, Glasebrook *et al.* 1982). In 1986, the current terminology of Th1/Th2 responses was first defined by Mosmann *et al.* (Mosmann, Cherwinski *et al.* 1986). Thus, mouse helper T-cells have been divided into the Th1 sub-type that secretes cytokines such as IL-2, IFN-\(\gamma\) and IL-12, and the Th2 sub-type that produces cytokines such as IL-4, IL-5 and IL-10. The work of Mosmann *et al.* and subsequent studies showed that these distinct patterns of cytokine secretion are associated with provision of help for two types of immune responses. Stevens *et al.* demonstrated that Th1 and Th2 cells induce antigen specific B-cells to secrete IgG2a and IgG1, respectively (Stevens, Bossie *et al.* 1988). A similar classification has been used for human T cells, however, the divisions are not as strict. It is important to emphasis here that these classifications, that were initially based on *in vitro* observations, have become dogma leading some workers to assume that these responses are mutually exclusive. In DNA immunisation over fifty papers on the type of helper responses have been published. The initial work showed some evidence for the presence of Th1-type cytokines and IgG2a. This was followed by a series of publications that reinforced the idea that DNA immunisation results only in Th1 responses. There are, however, some very good examples that show the presence of Th2 cytokines and IgG1 antibodies or even mixed responses as a result of DNA immunisation. The type of immune response observed after DNA immunisation and studies on the potential uses
of these responses in altering disease processes are reviewed later in this chapter.

Studies on the interaction of bacterial DNA with the immune system in late 1980s lead to the discovery that specific bacterial DNA motifs stimulate splenocytes to proliferate and secrete cytokines some of which are capable of inducing Th1 responses. Therefore, these immunostimulatory (ISS) motifs are thought to be responsible for development of Th1 responses after DNA immunisation. Studies of the interaction of bacterial DNA with the immune system are reviewed in chapter 5, however, a more detailed discussion of these studies and their relevance to DNA immunisation is found later in this chapter.

Induction of immune responses after DNA immunisation

Wolff et al. demonstrated that direct injection of a plasmid encoding firefly luciferase into the skeletal muscle of mice resulted in expression of the reporter gene (Wolff, Malone et al. 1990). Although, the maximum level of reporter gene expression was at the site of injection in the skeletal muscle cells, low levels of expression were also observed in liver, spleen, skin, lungs, brain and blood cells. After intradermal injection of a plasmid, expression has been observed in the dermis and epidermis in fibroblasts, keratinocytes and cells with dendritic morphology (Raz, Carson et al. 1994). Condon et al. immunised mice using the epidermal gene gun technique and fortuitously found dendritic cells containing gold particles in the draining lymph nodes (Condon, Watkins et al. 1996).

After DNA injection, therefore, antigen can be synthesised potentially by many cells in the body. APCs could acquire the antigen synthesised by any cell by direct uptake if it is a secreted antigen or by taking up dead-cell debris or apoptotic blebs that contain the antigen. This route of antigen uptake has been termed the exogenous route and has been considered to be mainly responsible
for presentation of antigen in association with class II MHC antigens. Presentation of antigen in association with the class I MHC molecule, however, has been classically thought to happen when antigen is synthesised intracellularly. The exogenous pathway of antigen presentation was shown in 1993 to also result in presentation of antigen in association with class I MHC antigens (Kovacsovics-Bankowski and Rock 1995). This pathway of antigen presentation, however, was thought to be inefficient and not to have a significant physiologic role (Reis e Sousa and Germain 1995).

A possible explanation for induction of CTL is direct transfection of APCs and intracellular synthesis of antigen. As mentioned above, Wolff et al., demonstrated that i.m. injection of a plasmid encoding a reporter gene can result in expression of the product in many tissues distant from the site of injection (Wolff, Malone et al. 1990). Therefore, APCs could theoretically be transfected after DNA administration. However, Ulmer et al. demonstrated that transplantation of myoblasts that had been previously transfected with a plasmid was sufficient to induce both antibody and CTL responses against the encoded antigen (Ulmer, Deck et al. 1996). In these experiments, DNA could not be transferred from myoblasts to other cells because it was integrated in the genome. In addition, for induction of CTL and antibody responses the antigen had to be synthesised by viable transplanted myoblasts. Therefore, induction of CTL can occur without transfection of APCs. The question is whether muscle cells themselves present the antigen or whether professional APCs are required.

Corr. et al. demonstrated that in i.m. DNA immunisation CTL priming is by bone marrow-derived cells (Corr, Lee et al. 1996). They reconstituted lethally irradiated mice of H-2^b,d haplotype with bone marrow of either H-2^b or H-2^d haplotype. Intramuscular immunisation of these mice with a plasmid encoding
NP antigen of influenza virus resulted in priming CTL that were restricted only to the haplotype of the donor bone marrow.

It follows that, in the myoblast transplantation experiments (Ulmer, Deck et al. 1996), for CTL induction, antigen synthesised by the myoblasts would have to be transferred to bone marrow derived APCs. This would mean that the process of "cross priming" is responsible for CTL induction after DNA immunisation, unless there is another yet unknown process by which antigen is transferred from muscle to the class I pathway. Recent observations show that cross priming may be a major physiologic mechanism of CTL induction. Sigal et al. have constructed a transgenic mouse model in which only non-haemopoietic cells can be infected by a virus and demonstrated that efficient CTL priming against the viral epitopes does occur (Sigal, Crotty et al. 1999). They argue that this could be an important mechanism for CTL induction against mutant viruses that avoid infection of professional APCs.

Professional bone marrow-derived APCs responsible for antigen presentation after DNA immunisation could be macrophages, dendritic cells or B cells. In an in vitro model, Rouse et al. demonstrated that macrophages stimulated a CTL response by taking up naked DNA whereas dendritic cells had to be transfected to be able to do so (Rouse, Nair et al. 1994). This finding may be a result of using mature dendritic cells that were incapable of DNA uptake. In these experiments, splenic dendritic cells were used. The experience in our laboratory with splenic dendritic cells is that within a few hours after isolation they acquire a mature phenotype and are no longer capable of endocytosis.

In an in vivo model, dendritic cells and not B cells have been shown to present antigen after DNA immunisation (Casares, Inaba et al. 1997). Casares et al. used a plasmid encoding a polypeptide that contained a helper T cell and a B
cell epitope (VH-Tb). Three, 7 or 11 days after i.m. injection of mice with this plasmid, B cells and dendritic cells were sorted using antibodies against B220 and CD11c, respectively. The sorted cells were used to stimulate specific helper T cell hybridomas (TcH). In this study only the dendritic cells could stimulate the TcH. Furthermore, in the presence of the VH-Tb peptide both sorted populations could stimulate the TcH, demonstrating that the B-cell fraction was also able to present the peptide, however, only the dendritic cells had acquired that ability in vivo.

In DNA immunisation bone marrow-derived antigen presenting cells are responsible for induction of the immune response. Dendritic cells are the most potent known APCs but a role for macrophages cannot be ruled out. Antigen can be synthesised in APCs or in other cells and then be transferred to APCs for presentation. In addition, skin-derived dendritic cells could directly be transfected using the particle bombardment technique.

**DNA immunisation results in both Th1 and Th2 responses**

The immune system has evolved to have at its disposal different effector mechanisms for combating invading pathogens. A combination of the most appropriate effector mechanisms ought to develop for an optimum immune response. The choice of effectors would, therefore, be influenced by the invading pathogen/antigen; where in the body it is encountered and other molecules from the external environment that are introduced with the pathogen/antigen. In response to these stimuli a variety of signals are initiated by the immune system and other cells in the body. In turn the combination of these signals results in selection of the most appropriate effector response. The pathogens have also evolved ways of evading the immune response by for instance mimicking inappropriate immune signals. In addition, the immune
system of an individual is based on an inherited genetic framework and is conditioned throughout life by unique environmental factors. Therefore, in response to the same pathogen, although in two individuals similar combination of effectors may develop, they would not be identical and may vary for instance in intensity of different components. As mentioned earlier the mouse immune response has been observed to polarise to Th1 and Th2 types, however, such polarisation is not absolute. It is important to bear these arguments in mind when discussing immune responses to DNA immunisation on the basis of detection of individual cytokines. Considering the complexity of this system and the multifactorial nature of the outcome, caution should be used in extrapolating from one experimental system to another. The rest of this section will use examples in the literature to illustrate the points mentioned above.

Raz et al. directly compared the type of responses elicited by DNA and protein immunisation. They demonstrated that intradermal (i.d.) immunisation of mice with β-galactosidase (β-gal) in saline or alum induced IgG1 and IgE in association with IL-4 and 5 whereas IgG2a in association with IFN-γ were induced when plasmid DNA encoding β-gal was injected i.d. (Raz, Tighe et al. 1996). There are other reports which also conclude that DNA immunisation results in Th1-type of immunity. Shiver et al. immunised mice against human immunodeficiency virus (HIV)-1 glycoproteins (gp) 120 or 160 and detected 100-fold more IFN-γ than IL-4 in the supernatants of splenocytes restimulated with the recombinant protein (Shiver, Perry et al. 1995). On this basis they concluded that this was a Th1 response. Manickan et al. detected IgG2a and type-1 cytokines (IL-2 and IFN-γ and not IL-4) after immunisation of mice with herpes simplex virus (HSV) gp-B (Manickan, Rouse et al. 1995). There are many other reports of detection of Th1-type cytokines and IgG2a antibody after DNA
immunisation, however, there are also some reports that demonstrate the opposite and many others that show a mixture of the two.

**Route or method of immunisation**

One of the factors that has been thought to result in development of Th2 responses after DNA immunisation is the use of intradermal or gene gun (g.g.) routes for delivery of immunogen (figure 1.2). Boyle et al. compared the immune responses when different routes were used to immunise mice. They used vectors encoding ovalbumin (OVA), hen egg lysozyme (HEL) and human immunoglobulin (hIg) (Boyle, Silva et al. 1997). In this study i.m. immunisation resulted in marginally higher levels of IgG2a against OVA and hIg in comparison to i.d. immunisation. This experiment, however, was not reported for HEL. In the case of OVA, i.d. immunisation resulted in secretion of both IL-4 and IFN-\(\gamma\) by restimulated splenocytes whereas splenocytes from i.m. immunised animals only secreted IFN-\(\gamma\) after restimulation. Moreover, both IgG1 and IgG2a were produced in response to all three antigens regardless of the route of immunisation. This paper has, rather misleadingly, been quoted by many subsequent publications to support the statement that i.m. immunisation induces Th1 whereas i.d. immunisation results in Th2 responses. Other reports also demonstrate a tendency for i.d. or g.g. immunisations to skew the response to a Th2-type. For instance, Pertmer et al. report that immunisation of the nucleoprotein of the influenza virus by g.g. or i.d. resulted in production of IgG1 but IgG2a antibodies were produced when the same vector was injected i.m. (Pertmer, Roberts et al. 1996). In this study, only the g.g. route resulted in IL-4 synthesis, whereas, both g.g. and i.m. routes of immunisation induced production of comparable levels of IFN-\(\gamma\). However, the cytokines secreted by cells from i.d immunised animals were not studied and since in g.g. immunisation considerably lower levels of DNA are used (in this study 1/100), the difference in the results cannot be solely attributed to the route of immunisation.
The amount of vector has been equalised in another experiment where the intranasal route of immunisation was used. Intranasal (i.n.) administration of HSV gp-B has been reported to induce IL-5 synthesis and more IgG1 than 2a whereas i.m. administration of the same vector results in no IL-5 and more IgG2a than 1 (Kuklin, Daheshia et al. 1997). Both methods also resulted in secretion of IFN-γ which supports a skewing role for i.n. immunisation rather than an all or none switching function.

**Figure 1.2:** DNA immunisation can result in both Th1 and Th2 responses. Intradermal or gene gun immunisation may result in direct transfection of Langerhan’s cells. In intramuscular immunisation a larger amount of DNA is used that potentially could reach lymph nodes and influence the immune response by stimulating cytokine secretion.
Different ports of entry for antigen, therefore, seems to result in different effector responses. However, the route of immunisation as the reason for differences in outcome has been disputed by Felquate et al. (Felquate, Heaney et al. 1997). They claim that instead of routes of immunisation different methods are responsible for the differences such that g.g. immunisation into muscle or dermis results in Th2-type whereas needle injection i.m. or i.d. results in Th1-type responses. Felquate et al. found that the IgG1/IgG2a ratios were higher when DNA encoding influenza haemagglutinin was administered by g.g., whereas IFN-γ/IL-4 ratios were higher after needle injection regardless of the location of DNA administration. In these studies the DNA dose was taken into account by using as little as 1 μg of DNA in i.m. immunisation. These results, however, are in contrast to other work that shows Th2-type responses after i.d. immunisation with a needle (Raz, Tighe et al. 1996; Boyle, Silva et al. 1997; Li, Sambhara et al. 1998). This could be due to the fact that different antigens have been used in different studies.

An important signal for development of Th1 responses is IL-12 (Manetti, Parronchi et al. 1993). Hexameric motif sequences in DNA have been shown to induce IL-12 secretion and Th1 responses (Chu, Targoni et al. 1997). These motifs will be discussed later in this chapter, however, they are mentioned here to point out that the interaction of DNA molecule with the immune system may be altered by the method or route of immunisation. In the g.g. method very little DNA may be found extracellularly and the only cells stimulated by the DNA molecule would be the transfected cells, whereas in the other methods a large quantity of DNA is normally injected in to the extracellular space that could potentially become available to many different cells (figure 1.2).
**Antigen presentation and structure**

The differences in the type of immune response, whether as a result of the route or method of immunisation, could be due to differences in presentation of antigen. In the skin, Langerhans cells (LC) may be transfected with the vector resulting in intracellular synthesis, processing and the presentation of the antigen. In the muscle, the antigen source might be mainly transfected muscle cells. Antigen produced by muscle cells would be taken up and presented through the extracellular antigen processing pathway by APCs other than LCs. Therefore, in the skin and the muscle, antigen would be expected to be presented mainly in association with class I and II MHC, respectively. Processing and presentation of antigen in association with MHC class-I or II antigens would result in stimulation of different effector cells (CD8+ T vs B and CD4+ T cells), however, it cannot be responsible for determining the type of effector responses. for two reasons; firstly, the phenomenon of cross priming shows that antigen that is captured through the extracellular route is presented in association with both class I and II MHC molecules, and secondly, the division of immune responses starts at the level of helper T cells that are CD4+ and can only interact with class II MHC molecules.

The influence of antigen itself on the effector response after DNA immunisation has not been systematically studied. However, there is enough evidence to assume that the antigen structure and/or composition does influence the outcome. Sixt *et al.* have reported that mice immunised with the haemagglutinin of canine distemper virus (CDV) using the g.g. method developed exclusively IgG1 whereas in the same study, mice immunised with the fusion protein of the same virus produce both IgG1 and 2a antibodies (Sixt, Cardoso *et al.* 1998). In another study, Boyle *et al.* have shown that DNA immunisation with different forms of OVA (secretory, membrane bound or cytoplasmic) results in CTL responses of different magnitude (Boyle, Koniaras *et al.* 1997). Since CTL are stimulated as part of Th1 responses, it is reasonable to assume that different magnitudes of CTL responses represent the intensity of a
Th1 response. The structure of an antigen could influence the immune response as a possible result of intracellular or extracellular location of the antigen. e.g. cytoplasmic vs endoplasmic or localisation to different parts of lymph nodes or spleen. The location of antigen may in turn result in presentation of antigen by different APCs capable of inducing different responses.

**Conditioning of the immune system**

An interesting example of the influence of conditioning of the immune system on the type of an effector response is when an antigen alters the type of immune response elicited to another antigen. This phenomenon has been reported to occur in the experimental autoimmune encephalomyelitis (EAE) model after immunisation of mice with DNA encoding for Vβ8.2 (Waisman, Ruiz et al. 1996). The susceptible PL/J mice develop pathology after immunisation with myelin basic protein (MBP). Pathogenesis is mediated by T cells that express a T-cell receptor (TCR) from the Vβ8.2 family and secrete IFN-γ and IL-2 in response to antigen stimulation. Waisman et al. showed that immunisation of PL/J mice with DNA encoding Vβ8.2 inhibited development of pathology. This was not due to depletion or anergy of the Vβ8.2 T cells as these cells were present at a normal frequency and proliferated in response to MBP, in vitro. As a result of DNA immunisation, however, these T cells acquired a Th2 phenotype such that they only secreted IL-4.

**Changes of T helper phenotypes with time**

On the basis of the available experimental data it is still difficult to predict the type of immune response to an antigen after DNA immunisation. This may simply reflect the complexity of the immune system, however, it may also be due to the fact that there are many uncontrolled variables between studies. Two of the most important of these variables are the time of sampling and the effect of boosting. There is evidence for a change in the effector response, in studies that the isotype of the antibodies or cytokine profiles before
and after boosting. These studies, however, do not clarify whether the changes were due to boosting or time alone. For instance, Mor et al. demonstrated that i.m. immunisation of BALB/c or NZB/W mice with DNA encoding circumsporozoite antigen of Plasmodium yoelii resulted in high IL-4/IFN-γ and IgG1/IgG2a ratios whereas boosting of the mice with the same vector reversed these ratios (Mor, Klinman et al. 1995). In another study, Pertmer et al. showed that BALB/c mice immunised i.m or epidermally with a plasmid encoding an influenza nucleoprotein developed IgG2a or IgG1 antibodies, respectively (Pertmer, Roberts et al. 1996). The splenocytes from all the mice secreted a high level of IFN-γ in response to the antigen but only the splenocytes from epidermally immunised mice produced IL-4, in vitro. In keeping with the IFN-γ synthesis all the mice also developed strong CTL responses regardless of the method of immunisation. In this study, the ratio of antibody isotypes and the strength of CTL responses remained the same after boosting, however, IFN-γ levels declined in all the mice whereas IL-4 levels increased in the animals immunised in the epidermis. This study demonstrates many important issues concerning effector responses. These include; a) the time of sampling is important as the cytokine levels can change during the course of a response, b) at least after a boost, the cytokine ratios or levels may not correspond with the strength of the expected effector responses, c) detected antibody isotypes or cytokines alone are not sufficient to categorise a response as Th1 or Th2 types.

One consistent feature of reports on DNA vaccination is that, whenever sought, IFN-γ has been found to be produced. As mentioned earlier, this is thought to be initiated by ISS. The peculiarity of immune response to DNA vaccination is probably to a large degree determined by ISS. The interaction of ISS sequences with the immune system and their role in DNA immunisation will be discussed below.
DNA was advertised and sold to the French public as a potion with general "immunostimulatory" properties during the 1960's. This particular property of DNA may only represent the affinity of a population towards medication and at best may have a placebo effect, however, in the light of new findings that will be discussed later one cannot help but wonder about the origins of some folklore. Immunologists traditionally considered DNA as an immunologically inert molecule until as late as ten years ago. A line of evidence contributing to the underestimation of the immune properties of DNA came from early studies of DNA binding properties of sera from SLE patients and normal human subjects (NHS). Because DNA from only a few sources was tested, these studies had led to the belief that anti-DNA antibodies only occurred in SLE sera. A wide panel of DNA was never considered necessary as it was thought that anti-DNA antibodies recognised either ssDNA or dsDNA in a conformational dependent rather than a sequence specific manner. In the late 1980's, however, using a wide panel of DNA from different sources, it was demonstrated that there were anti-DNA antibodies in both SLE and NHS sera that could specifically recognise DNA from *Micrococcus lysodeikticus* and *Staphylococcus epidermis* (Karounos, Grudier et al. 1988). Subsequently, Pisetsky et al. demonstrated that both ss and ds bacterial-DNA were more potent immunogens than mammalian DNA when conjugated to BSA (Gilkeson, Grudier et al. 1989). In these experiments, bacterial DNA induced some antibodies that bound to target sites unique to bacterial DNA and other antibodies that could also recognise binding sites on mammalian DNA. These studies revived the investigation of a possible role for bacterial DNA in the pathogenesis of SLE.

Another line of work that changed the traditional view on immunogenicity of DNA came from studies of the anti-tumour properties of *Mycobacterium bovis* extract. Tokunaga et al. isolated the active fraction (MY-1) of this extract and showed that it was entirely made of RNA and DNA
(Tokunaga, Yamamoto et al. 1984). The anti-tumour activity was sensitive to DNAase but not RNAase. It was later shown that the DNA component present in MY-1 was able to activate natural killer (NK) cell activity of peripheral blood cells from cancer patients (Mashiba, Matsunaga et al. 1988). Yamamoto et al. continued this work by synthesising 45-mer sequences of DNA which were found in MY-1 and tested their ability to augment NK cell activity of mouse splenocytes, in vitro. They noticed that these 45-mers that were able to activate NK cells all contained palindromic sequences (Tokunaga, Yano et al. 1992). On this basis Yamamoto et al. carried out a study of a group of synthetic palindromic sequences and found a group that could activate NK cells (Yamamoto, Yamamoto et al. 1992). However, it was later shown that it was not the palindromes but their central dinucleotide sequence that conferred the immunostimulatory properties to these oligonucleotides.

Krieg et al. showed that synthetic oligonucleotides containing unmethylated CG sequences caused proliferation of mouse splenocytes and activation of B cells (Krieg, Yi et al. 1995). Later, the same group showed that the presence of palindromic sequences, as inferred from the work of Yamamoto et al., was not essential but a hexameric motif with a central unmethylated CG flanked by two 5'purines and two 3'pyrimidines was responsible for proliferation of lymphocytes and secretion of a variety of cytokines (Klinman, Yi et al. 1996). Krieg et al., in 1995, proposed an evolutionary significance for immune stimulation by bacterial DNA motifs that contained unmethylated CG sequences (Krieg, Yi et al. 1995). This was based on the phenomenon of "CG suppression". This is the term given to the observation that CG dinucleotides occur in mammalian genome with a lower frequency than expected. Krieg et al. proposed that "CG suppression" was selected as it conferred an advantage in recognition of bacteria and viruses by the mammalian immune system. In the past few years, these immunostimulatory sequences (ISS) have been shown to have an extensive and complex relationship with the mammalian immune
system. The importance of these sequences in DNA vaccination and their mechanism of interaction with the immune system will be discussed below.

**ISS are adjuvants that induce cytokines synthesis**

Sato *et al.* demonstrated that elimination of CG containing ISS from DNA vaccines resulted in a significant loss in both antibody and CTL responses that could be restored by coinjection of plasmids containing these sequences (Sato, Roman *et al.* 1996). Klinman *et al.* demonstrated that methylation of plasmid vectors used for DNA immunisation significantly reduced the antibody responses (Klinman, Yamshchikov *et al.* 1997). They also showed that although methylation resulted in a reduction in protein expression, the inactivation of ISS was mainly responsible for the decline in antibody responses and not reduced protein expression. Therefore, unmethylated ISS appear to act as essential adjuvants in DNA immunisation.

It was mentioned before that DNA immunisation has been shown to stimulate both Th1 and Th2-type effector responses and it was argued that the outcome could depend on a variety of factors. The hexameric motifs found in ISS that have been subject of many studies appear to be the factors that contribute to development of Th1 responses. Roman *et al.* showed the Th1 response obtained by DNA immunisation against β-gal could also be achieved by coadministration of the ISS found in the vector with the protein (Roman, Martin Orozco *et al.* 1997). Chu *et al.* demonstrated the same phenomenon for HEL in both Th2-biased BALB/c and Th1-biased B10-D2 mice (Chu, Targoni *et al.* 1997). The ability of ISS to stimulate Th1 responses has been investigated for their potential in treatment of Th2-type pathologies such as allergic diseases. Broide *et al.* have shown that inhaled or i.p. administered ISS oligonucleotides inhibited allergen induced airway hypersensitivity and local or systemic eosinophilia (Broide, Schwarze *et al.* 1998). This effect was associated with an increase in IFN-γ and a reduction in IL-5 production by allergen specific splenocytes, *ex vivo*. This effect of ISS could be inhibited, *in vivo*, by antibodies.
to IFN-\( \gamma \), demonstrating that the underlying mechanism is development of a Th1 response.

As DNA immunisation can also result in development of Th2 responses, there have to be other factors that drive the response towards a Th2-type. These factors as mentioned in earlier sections may include the route of immunisation and the antigen itself, however, in the same way that some sequences induce Th1 responses there may exist other sequences that could drive the response to a Th2-type. There have not yet been any reports on Th2-inducing sequences, however, it has been shown that changes to an oligonucleotide can result in production of different cytokines. Lipford et al. have discovered two oligonucleotides that stimulate IL-12 synthesis but only one of them also stimulates TNF-\( \alpha \) production (Lipford, Sparwasser et al. 1997). These oligonucleotides are 18 and 20 bases long and have an identical central sequence of 10 bases containing a hexameric ISS motif but differ in their flanking regions. Therefore, it is not inconceivable that DNA may contain, in a non-classical way, encoded information on synthesis of specific groups of cytokines that can be read and interpreted by the mammalian immune system. In other words, the ability of the immune system to relate a specific DNA sequence unique to a group of organisms and mount an appropriate immune response against those organisms may have been selected during mammalian evolution. It is interesting that the known Th1- inducing sequences may be recognised by the more ancient part of the immune system; the innate machinery. This may indicate the importance of these sequences in decision making by the immune system.

*Innate immunity and Th1 cytokine synthesis*

Evidence suggests that recognition of ISS by the innate immune system sets off cascades of proinflammatory cytokine production (figure 1.3) that eventually result in Th1 responses. ISS stimulate synthesis of TNF-\( \alpha \) by macrophages (Stacey, Sweet et al. 1996). Macrophages have also been shown to
secrete IL-12 in response to bacterial DNA (Chase and Prochaska 1976). In the same study, Chase et al. also showed that IFN-γ is secreted by NK cells in response to IL-12 that is induced by bacterial DNA. Ballas et al. demonstrated that ISS augment NK cell activity indirectly by inducing the secretion of IL-12, TNF-α and IFN-I. Moreover, T and B cells were not required for this effect (Ballas, Rasmussen et al. 1996).

**Figure 1.3:** Immunostimulatory sequences (ISS) may interact with a variety of cells and induce cytokine cascades resulting in inflammatory and Th1 responses.

ISS, therefore, stimulate the secretion of proinflammatory cytokines that result in the acute phase response that is in turn integrated in Th1 responses to increase the efficiency of cellular immunity. In a Th1 response, acute phase cytokines such as IFN-γ as well as TNF-α and IFN-I increase the microbicidal
activity of macrophages and increase resistance to viral infection. Microorganisms are more efficiently killed by the complement system as a result of the presence of Th1-associated complement fixing antibodies. CTL activity is augmented by IFN-γ. This and induction of complement fixing antibodies depend on differentiation of naive helper T cells to the Th1 phenotype. This is driven by IL-12 through its ability to increase IFN-γ synthesis and reduce IL-4 production of naive helper T cells (Fearon and Locksley 1996).

Detection of ISS by the innate immune system, therefore, may not only drive a Th1 response but also may augment such a response. This, however, does not exclude the possibility of interaction of ISS with some component of the adaptive immune system such as the B cells as mentioned in earlier sections. The augmentation of a Th1 response should be distinguished from the adjuvant effect of ISS. The latter may involve interaction of ISS with the adaptive immune system.

*ISS my act as adjuvants by augmenting costimulation*

The adjuvant effect of ISS has been demonstrated both in experiments where they were added to protein antigen (Roman, Martin Orozco et al. 1997) and where they were used as an integral part of a DNA vaccine vector (Sato, Roman et al. 1996). They appear to have this effect by influencing the events occurring during antigen presentation. Krieg et al. demonstrated that the mouse B-cell line CH12.LX could be induced to secrete immunoglobulins when it was incubated with ISS and its specific target antigen (Krieg, Yi et al. 1995). This is not a demonstration of "costimulation", however, it suggests that ISS may be able to give a second signal for activation of antigen specific B cells. The ability of ISS to upregulate costimulatory signals was first demonstrated in highly purified T-cell depleted human peripheral blood cells. Liang et al. showed that incubation of these B cells with ISS resulted in upregulation of B7-2 and IL-2 (Liang, Nishioka et al. 1996). Activated B cells have been considered to be capable of antigen presentation, however, in DNA immunisation Casares et al.
showed that DCs present the antigen and not the B cell (Casares, Inaba et al. 1997). This does not, however, exclude a role for B cells in antigen presentation. Sprent et al. have demonstrated that activated B cells are capable of providing bystander costimulation for mouse CD8+ T-cells (Sun, Cai et al. 1996). In their system the CD8+-T cells were responding to antigen presented by Drosophila APC that only express transfected mouse MHC class-I antigens but no mammalian costimulatory molecules. B cells were activated by Drosophila DNA that contained ISS and also by synthetic ISS oligonucleotides to upregulate B7-2 and ICAM-1. These B cells were essential to activation of the CD8+ T-cells responding to the engineered APCs.

It is possible that ISS also interact with professional APCs to augment antigen presentation. Our experiments described in chapter 5 provide evidence for such interactions.

Carcinoembryonic antigen

Background

Human carcinoembryonic antigen (hCEA) is a member of the immunoglobulin super family. It was first detected by studies concerned with antigenic analysis of human colonic adenocarcinoma (Gold and Freedman 1965). Pooled tumour extracts were injected into rabbits and the resulting antisera were purified by absorption with excess normal colon extract. The antisera were further absorbed with tumour plasma fibrin and killed gut bacteria, and then gave a precipitin band with colonic tumour extracts in gel diffusion assays but not with extracts of normal colon. Immunoprecipitation studies using these antisera showed that the antigen was present in endodermally derived gastrointestinal and pancreatic tumours and fetal tissues, but not in normal adult tissue (Gold and Freedman 1965). The authors
concluded that they had demonstrated a specific cancer antigen of embryonic origin and they named it carcinoembryonic antigen.

In 1969, hCEA was detected by radioimmunoassay in the serum of patients with colorectal cancer but not in patients with carcinomas outside the gastrointestinal tract or in normal control subjects (Thomson, Krupey et al. 1969). However, later studies also revealed elevated serum hCEA in patients with cancer of the breast, bronchus, prostate and bladder and in patients with non-malignant diseases such as hepatic cirrhosis, pulmonary emphysema, chronic pancreatitis and ulcerative colitis (Chu, Reynoso et al. 1972; Kupchik and Zamcheck 1972; Egan, Pritchard et al. 1977). hCEA levels were also elevated in some healthy subjects (Chu, Reynoso et al. 1972).

The first immunostaining studies for hCEA were carried out in 1968 on colorectal tumours with anti-hCEA antisera (Gold, Gold et al. 1968). This and the majority of subsequent investigations using light microscopy have shown a linear pattern of staining for hCEA along apical borders of the epithelial cells lining glandular spaces within colorectal adenocarcinomas and staining of intra-glandular debris (Gold, Gold et al. 1968). Staining for hCEA has been reported in 60% of colonic adenocarcinoma and 14% of normal colonic mucosa in formalin fixed wax embedded tissue (Goldenberg, Sharkey et al. 1976) while 100% of normal and cancerous colon tissues have been found to stain when frozen samples were used (Huitric, Laumonier et al. 1976).

In normal colonic mucosa hCEA is localised predominantly on the apical surface of epithelial cells lining the upper crypts and mucosal surface (Primus, Clark et al. 1981; Ahnen, Nakane et al. 1982), and ultrastructural localisation of hCEA with anti-hCEA antibodies has shown hCEA to be intimately associated with the glycocalyx at the apical border of colonic carcinoma cells (Gold, Krupey et al. 1970). Later ultrastructural localization of hCEA in the normal colonic epithelium with polyclonal hCEA antibodies demonstrated uniformly staining along the microvilli, and in the sub nuclear spaces, endoplasmic reticulum, golgi complex and cytoplasmic vesicles of many but not all columnar
cells. The strongest staining was seen in mature absorptive cells. A similar pattern of staining has been observed in colonic tumours (Ahnen, Nakane et al. 1982). hCEA has also been detected in most epithelial tumours, including breast carcinoma (Shousha and Lyssiotis 1978; Shousha, Lyssiotis et al. 1979), and bronchogenic carcinoma (Corson and Pinkus 1982). It is also expressed in normal cervix (Sanders, Stocks et al. 1992).

**Description and molecular biology**

Clones for human CEA cDNA have been isolated from cDNA libraries derived from a colonic tumour (Paxton, Mooser et al. 1987), normal colon (Oikawa, Imajo et al. 1987; Oikawa, Nakazato et al. 1987) and colonic tumour cell lines (Zimmermann, Ortlieb et al. 1987). The amino acid sequence as deduced from the nucleotide sequence of cDNA shows that hCEA is synthesized as a precursor of 702 amino acids; this consists of a leader peptide of 34 amino acids an N-terminal domain of 108 amino acids, three repeat AB domains of 178 amino acids and a carboxyl domain of 26 amino acids (Oikawa, Nakazato et al. 1987)(figure 1.4).

The molecular weight (minus leader) is approximately 80,000. The final 180,000 molecular weight for hCEA is achieved by glycosylation. There are twenty eight N-glycosylation sites on the hCEA peptide and the average carbohydrate chain length is approximately 20 residues (Oikawa, Imajo et al. 1987; Oikawa, Nakazato et al. 1987; Paxton, Mooser et al. 1987). Glycosylation inhibition studies have shown the protein moiety to consist of a single polypeptide chain with an apparent molecular weight of approximately 80,000 (Kuroki, Moore et al. 1988). Such heavy glycosylation may hinder the binding of antibodies to protein epitopes or be antigenic in itself (Hammarstrom, Shively et al. 1989). hCEA isolated from different sources shows different nature of glycosylation and variability in the major sugar chains have been observed in
hCEA isolated from the seminal plasma, meconium and colon adenocarcinoma (Nagata, Miura et al. 1991). This is probably due to the difference in the fetal and adult glycosylation.

The 3' untranslated region of the processed hCEA mRNA contains a truncated Alu type repetitive sequence flanked by a pair of direct repeats, this region is apparently unique to hCEA in colorectal tissue and has no homology with corresponding regions in other members of hCEA family (Zimmermann, Weber et al. 1988). Two hCEA mRNA transcripts have been identified with molecular weight of 3 and 3.5 kb from colorectal tissue by northern hybridisation, these appear to be encoded by one gene with two polyadenylation sites (Oikawa, Nakazato et al. 1987).

The amino acid sequence indicates that hCEA is a member of the immunoglobulin super family (Oikawa, Imajo et al. 1987). This conclusion is based on the observation that there is a repeated domain structure each of which contains a disulphide bridge (figure 1.4). Moreover, comparison of the deduced amino acid sequence has shown conservation of critical amino acids compared with other immunoglobulin super family members (Paxton, Mooser et al. 1987). The hCEA domains also reveal a high degree of sequence homology amongst themselves, having between 67% and 73% of their amino acids in common, and each contain four cystine residues at precisely the same positions (Zimmermann, Weber et al. 1988).

Analysis of the gene organisation indicates a strong correlation between the exon and domain structure. As shown below in figure 1.4, the first exon of the hCEA gene contains the 5' untranslated region and part of the leader domain, the second exon encodes the rest of leader and the complete N-terminal domain. The following six exons encode repeated A and B domains. The ninth exon encodes the C-terminal hydrophobic membrane domain and
part of the 3' untranslated region. The tenth contains the rest of the hCEA 3' untranslated region (Schrewe, Thompson et al. 1990).

**Figure 1.4:** Diagramatic representation of hCEA gene and protein structure.

Since identification of the hCEA cDNA in the 1987, numerous CEA-related genes have been cloned in different laboratories. This has resulted in the human CEA gene family being divided into two subgroups, the CEA subgroup and the pregnancy specific glycoprotein (PSG) subgroup. Recently the hCEA gene family has been designated as CD66. In this system the CEA subgroup is known as CD66e, and the PSG subgroup as CD66f.

hCEA is post-translationally modified by replacement of the hydrophobic membrane domain with the glycosyl phosphatidylinositol (GPI) moiety, which is released from the membrane by digestion with phosphatidylinositol-specific phospholipases. It is possible that release of hCEA into the serum is controlled in vivo by these phospholipases. Such mechanisms may be responsible for the rapid turnover of CEA in the normal mucosa (Kuroki, Moore et al. 1988).

**Clinical significance of hCEA**

The role of the hCEA as a tumour marker has been the subject of numerous studies since its original description. There are quantitative differences between CEA levels in serum of patients with tumours and normal control sera, but these are perhaps better considered as prognostic rather than diagnostic factors. For example, serum hCEA concentration is a useful
parameter in post-operative surveillance of cancer patients, and failure of plasma CEA to return to normal levels following surgery is suggestive of residual disease (Thomson, Krupay et al. 1969; Thompson, Mossinger et al. 1993).

**hCEA Functions**

hCEA has been considered to function as a cell adhesion molecule on the basis of the studies of hCEA mediated homophillic aggregation in cultured human colon carcinoma cells (Benchimol, Fuks et al. 1989), and of heterophillic interaction between hCEA and the nonspecific cross reactive antigen (NCA) in CHO cells transfected with CEA cDNA and NCA cDNA (Oikawa, Inuzuka et al. 1989).

It has been shown in vitro that CEA has affinity to bind the strains of E-coli bacteria found in the human gut flora (Leusch, Hefta et al. 1990). On this basis it has been suggested that one function of hCEA may be to help maintain the steady state level of these bacteria in the gut. This interaction is achieved by a binding of bacterial lectin to hCEA carbohydrate (Leusch, Hefta et al. 1990). It has also been shown that a hCEA transfected cell line binds to collagen type I (Pignatelli, Durbin et al. 1990). This observation has given rise to suggestions that it may have a role in mediating interaction of metastasising tumour cells with the basement membrane and thereby functioning as an adhesion molecule. The function of hCEA in normal or malignant cells remains uncertain.

**Carcinoembryonic antigen as a target tumour antigen**

CEA has been used as a target tumour antigen in experimental animals and in human clinical trials. Three Macaca virus monkeys developed antibodies against hCEA that did not cross react with NCA after immunisation with hCEA (Ruoslathi, Engvall et al. 1976). Immunisation of rats with rat CEA in complete Freund's adjuvant (CFA) was shown to result in protection against syngeneic
CEA positive tumours (Diakun, Wilhelm et al. 1986). Anti-hCEA antibodies have been used in human clinical trials with little success. In one study, it was shown that anti-idiotypic antibodies against hCEA binding site developed in 5 out of 10 patients who had received mouse anti-hCEA antibodies (Traub, Dejager et al. 1988). Anti-idiotypic antibodies against hCEA antibodies have also been developed for use as tumour vaccines. Immunisation of rabbits with an anti-idiotypic antibody with the image of an epitope of hCEA resulted in development of antibodies with reactivity to hCEA (Gaida, Fenger et al. 1992). Antibody and T cell proliferative responses against hCEA have also been demonstrated after immunisation with another anti-idiotypic antibody with a hCEA image (Durrant, Denton et al. 1992).

The change of emphasis on T-cell role in anti-tumour immunity and cloning of hCEA gene led to the generation of a vaccinia vector encoding hCEA. Kantour et al. demonstrated that vaccination of mice with this vector resulted in anti-hCEA antibody responses as well as cell mediated delayed type hypersensitivity, lymphoproliferative and CTL responses (Kantor, Irvine et al. 1992; Kantor, Irvine et al. 1992). Furthermore, challenge experiments in vaccinia-hCEA immunised mice with a mouse colorectal carcinoma resulted in growth retardation or complete eradication of tumours.

This thesis investigates the hypothesis that DNA vaccination may be used to stimulate tumour responses against tumour antigens and explores some mechanism that may potentiate the response to DNA immunisation. On the basis of these results and the evidence showing that DNA immunisation can result in CTL induction (reviewed earlier in this chapter), we designed a set of experiments to elicit anti-hCEA responses in mice with a view to study the anti-tumour effect. These experiments are described in chapters 3. In chapter 4, the effects of addition of Lipopolysaccharides (LPS) to a DNA vector coding for hCEA, and fusion of the nucleotide sequence of tetanus toxoid fragment-c (Fr-c) with hCEA gene, on anti-hCEA responses were studied. These experiments were based on the hypothesis that LPS and Fr-c augment costimulatory signals
of activation. As discussed earlier in this chapter, certain sequences that may be present in DNA vectors have immunostimulatory properties. Studies on bone marrow derived dendritic cells as targets for such sequences are presented in chapter 5. In the experiments of chapter 6, the mechanism of action of these sequences in dendritic cells is investigated using an immortalised dendritic cell and a known immunostimulatory oligonucleotide.
Chapter 2

Material and Methods
In this chapter general materials and methods will be described. More specific points on methodology relevant to each chapter can be found at the beginning of each of the result chapters.

**Plastics**

Standard disposable plasticware manufactured by Falcon, UK, was used that were supplied by ICRF. Plastics for tissue culture were radiation sterilised by the manufacturers.

**Mice**

C57BL/6 (H-2<sup>b</sup>), BALB/c (H-2<sup>d</sup>) and CBA (H-2<sup>k</sup>) female mice were obtained from Biological Services, Imperial Cancer Research Fund (ICRF) Clare Hall Laboratories. They were kept in the animal facilities at the Biological Services Unit, University College London, and used in experiments at 6-12 weeks of age.

**Reagents**

Unless otherwise indicated, all reagents were obtained from Sigma or BDH and were of the highest grade available. Storage procedures for certain reagents are shown in Table 2.1.

**Radioactive Isotopes**

Tritiated thymidine ([<sup>3</sup>H]TdR, 76.0 Ci/mmol), Na<sup>51</sup>CrO<sub>4</sub> (5mCi/ml), γ-<sup>[32P]</sup>-ATP (>1000Ci/m mole), α-[<sup>35S</sup>]dATP (>1000Ci/m mole) were all
purchased from Amersham. Na\textsuperscript{51}CrO\textsubscript{4} was used for up to two weeks after the delivery date, and then allowed to decay.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Procedure used to prepare and store stock solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokines</td>
<td>lyophilised protein was dissolved in water to 100ng/μl stored in 25μl aliquots at -80°C</td>
</tr>
<tr>
<td>β-mercaptoethanol (2-ME)</td>
<td>5 \times 10^{-2} \text{M} in IMDM medium. Filtered through 0.2 μm. Stored in aliquots at -20°C.</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>1 mg/ml in PBS. Filtered through 0.2 μm. Stored protected from light at 4°C for 1-2 weeks maximum.</td>
</tr>
</tbody>
</table>

**Table 2.1**: Storage procedures for certain reagents.

**Peptides and Proteins**

SV-9 peptide shown in Table 2.1 was synthesised by the Peptide Synthesis Laboratory at ICRF, Lincoln’s Inn Fields and received in powder form. The quality of the peptide was checked by mass spectrometry and HPLC (by the Peptide Synthesis Laboratory). Tetanus toxoid Fragment-c-7 (Fr-c-7) peptide (King, Spellerberg et al. 1998) was a kind gift from Dr. C.A. King of the Molecular Immunology Group, Southampton University Hospital, UK. To coat target cells for CTL assays, these peptides were dissolved at 20mg/ml (~20mM) in DMSO and stored in 20μl aliquots in eppendorf tubes at -80°C. Peptides were freshly dissolved in PBSA at 10x concentration and then diluted in medium for each experiment.
Human carcinoembryonic antigen (hCEA) was initially extracted from a human colorectal metastasis using a perchloric acid extraction technique followed by a fast performance liquid chromatography step. This procedure proved to yield hCEA that was contaminated with unacceptable levels of LPS for our purposes. Subsequently, a commercial hCEA (Calbiochem-Novabiochem (UK) Ltd., Cat. No. 219369) was used that was purified from a established human cell line and was delivered in a lyophilised solid form.

<table>
<thead>
<tr>
<th>Derivation of peptide</th>
<th>MHC which presents peptide</th>
<th>Sequence of peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetanus Toxoid Fr-c-7</td>
<td>H-2K(^b)</td>
<td>SNWYFNHL</td>
</tr>
<tr>
<td>Sindai Virus 9 nucleoprotein 324-332</td>
<td>H-2K(^b)&amp;Db</td>
<td>FAPGNYPAL</td>
</tr>
</tbody>
</table>

**Table 2.2:** Details of peptides used in these studies.

**Media**

**Media used for mammalian cell cultures**

RPMI: RPMI 1640 supplemented with 2g/litre sodium bicarbonate and 2mM L-glutamine (ICRF media production).

IMDM: Iscove’s modified Dulbecco’s medium (Gibco) supplemented with 2mM L-glutamine, 3.02g/l sodium bicarbonate, 100 units/ml penicillin and streptomycin and 5 μg/ml transferrin.
Chapter 2

DMEM: Dulbecco's modified Eagles's medium (Gibco) supplemented with 4mM L-glutamine, 1.5 g/l sodium bicarbonate, 100 units/ml penicillin and streptomycin.

MEM: Minimal essential medium (ICRF media production).

*Media used for bacterial cell cultures*

**Luria-Bertani (LB)**

- Bacto-tryptone 10g
- Bacto-yeast extract 5g
- NaCl 10g

Dissolved in 1000ml dH₂O and pH adjusted to 7.5 with 5M NaOH. The medium was then autoclaved.

**2TY**

- Bacto-tryptane 16g
- Yeast Extract 10g
- Sodium Chloride 5g

dissolved in 1000ml dH₂O and autoclaved.

**LB Agar**

Bacto-agar 15g added to 1000ml of LB medium and re-autoclaved. For plates containing ampicillin, 50µg/ml Ampicillin was added prior to pouring.

**Sera**

Fetal calf serum (FCS, Gibco) was routinely heat inactivated at 56°C for one hour before freezing in aliquots.
Buffers

Phosphate buffered saline Dulbecco's A (PBSA, 137 mM NaCl, 3.3 mM KOH, 1.7 mM anhydrous KH₂PO₄, 10mM anhydrous Na₂HPO₄ adjusted to pH7.4 with HCl) was obtained from ICRF Clare Hall. The recipes for all the other buffers are given where they are mentioned in the text.
### Antibodies

<table>
<thead>
<tr>
<th>Designation</th>
<th>Target molecule</th>
<th>Isotype</th>
<th>Source</th>
<th>Conjugate (if any)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1H12</td>
<td>hCEA</td>
<td>Mouse IgG&lt;sub&gt;1&lt;/sub&gt;</td>
<td>ATCC</td>
<td>none</td>
</tr>
<tr>
<td>A5B7</td>
<td>hCEA</td>
<td>Mouse IgG&lt;sub&gt;1&lt;/sub&gt;</td>
<td>ATCC</td>
<td>none</td>
</tr>
<tr>
<td>EA77</td>
<td>hCEA</td>
<td>Mouse IgG&lt;sub&gt;2a&lt;/sub&gt;</td>
<td>ATCC</td>
<td>none</td>
</tr>
<tr>
<td>C6G9</td>
<td>hCEA</td>
<td>Mouse IgG&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Sigma</td>
<td>none</td>
</tr>
<tr>
<td>L3T4</td>
<td>Mouse CD4</td>
<td>Rat IgG&lt;sub&gt;2a,κ&lt;/sub&gt;</td>
<td>Pharmingen</td>
<td>Biotin</td>
</tr>
<tr>
<td>Ly-2</td>
<td>Mouse CD8α</td>
<td>Rat IgG&lt;sub&gt;2a,κ&lt;/sub&gt;</td>
<td>Pharmingen</td>
<td>Biotin</td>
</tr>
<tr>
<td>L3T4</td>
<td>Mouse CD4</td>
<td>Rat IgG&lt;sub&gt;2a,κ&lt;/sub&gt;</td>
<td>Pharmingen</td>
<td>FITC</td>
</tr>
<tr>
<td>Ly-2</td>
<td>Mouse CD8α</td>
<td>Rat IgG&lt;sub&gt;2a,κ&lt;/sub&gt;</td>
<td>Pharmingen</td>
<td>FITC</td>
</tr>
<tr>
<td>Ly-2</td>
<td>Mouse CD8α</td>
<td>Rat IgG&lt;sub&gt;2a,κ&lt;/sub&gt;</td>
<td>Pharmingen</td>
<td>PE</td>
</tr>
<tr>
<td>A85-1</td>
<td>Mouse IgG&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Rat IgG&lt;sub&gt;1,κ&lt;/sub&gt;</td>
<td>Pharmingen</td>
<td>HRP</td>
</tr>
<tr>
<td>R12-3</td>
<td>Mouse IgG&lt;sub&gt;2b&lt;/sub&gt;</td>
<td>Rat IgG&lt;sub&gt;2a,κ&lt;/sub&gt;</td>
<td>Pharmingen</td>
<td>Biotin</td>
</tr>
<tr>
<td>R19-15</td>
<td>Mouse IgG&lt;sub&gt;2a&lt;/sub&gt; and IgG&lt;sub&gt;2c&lt;/sub&gt;</td>
<td>Rat IgG&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Pharmingen</td>
<td>HRP</td>
</tr>
<tr>
<td>Red-T/ G297-289</td>
<td>Mouse IL-12 p35 subunit of p70</td>
<td>Hamster IgG /Rat IgG&lt;sub&gt;2a&lt;/sub&gt;</td>
<td>Pharmingen</td>
<td>none</td>
</tr>
<tr>
<td>C17.8</td>
<td>Mouse IL-12 p40 subunit of p70</td>
<td>Rat IgG&lt;sub&gt;2a&lt;/sub&gt;</td>
<td>Pharmingen</td>
<td>Biotin</td>
</tr>
<tr>
<td>Y3</td>
<td>Kb</td>
<td>Mouse IgG&lt;sub&gt;2b&lt;/sub&gt;, k</td>
<td>ATCC</td>
<td>none</td>
</tr>
<tr>
<td>Tib 93</td>
<td>I-A κ</td>
<td>IgG&lt;sub&gt;2b&lt;/sub&gt;</td>
<td>ATCC</td>
<td>none</td>
</tr>
<tr>
<td>Tib 120</td>
<td>I-A (b, d, q)</td>
<td>Rat IgG&lt;sub&gt;2b&lt;/sub&gt;, k</td>
<td>ATCC</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>I-E (d, k)</td>
<td>Rat IgG&lt;sub&gt;2b&lt;/sub&gt;, k</td>
<td>ATCC</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>Antibody Description</td>
<td>Species</td>
<td>Company</td>
<td>Biotin</td>
</tr>
<tr>
<td>-----</td>
<td>--------------------------------------------------------------------------------------</td>
<td>------------------</td>
<td>-------------------</td>
<td>--------</td>
</tr>
<tr>
<td>M1/70.15</td>
<td><strong>CD11b (Mac1): macrophages, monocytes, granulocytes and NK cells</strong></td>
<td>Rat IgG2b</td>
<td>Sera-lab</td>
<td>none</td>
</tr>
<tr>
<td>N418</td>
<td><strong>a-chain of a 150,90 kDa integrin (CD11c) on dendritic cells. Not on lymphocytes</strong></td>
<td>Hamster mAb</td>
<td>Alexandra Livingstone, Imperial College.</td>
<td>biotin</td>
</tr>
<tr>
<td>RA3-6B2</td>
<td><strong>CD45-B220</strong></td>
<td>Rat IgG2a</td>
<td>Pharmingen</td>
<td>biotin</td>
</tr>
<tr>
<td>KT3.1.1</td>
<td><strong>CD3</strong></td>
<td>Rat IgG2a</td>
<td>ATCC</td>
<td>none</td>
</tr>
<tr>
<td>16-10A1</td>
<td><strong>CD80 (B7-1)</strong></td>
<td>Hamster mAb</td>
<td>Pharmingen</td>
<td>biotin</td>
</tr>
<tr>
<td>GL1</td>
<td><strong>CD86 (B7-2)</strong></td>
<td>Hamster mAb</td>
<td>Pharmingen</td>
<td>biotin</td>
</tr>
<tr>
<td>NLS-G</td>
<td><strong>Mouse NF-κB p50</strong></td>
<td>Goat polyclonal IgG</td>
<td>Santa Cruz</td>
<td>none</td>
</tr>
<tr>
<td>K-27</td>
<td><strong>Mouse NF-κB p52</strong></td>
<td>Rabbit polyclonal IgG</td>
<td>Santa Cruz</td>
<td>none</td>
</tr>
<tr>
<td>C-20-G</td>
<td><strong>Mouse NF-κB p65</strong></td>
<td>Rabbit polyclonal IgG</td>
<td>Santa Cruz</td>
<td>none</td>
</tr>
</tbody>
</table>

**Table 2.3:** Primary (1st layer) antibodies used in cell staining and western blot analysis. Target molecules are all murine.
<table>
<thead>
<tr>
<th>Target Ig</th>
<th>Host</th>
<th>Conjugation</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat IgG2b</td>
<td>Goat</td>
<td>FITC</td>
<td>Nordic</td>
</tr>
<tr>
<td>Rat IgG</td>
<td>Rabbit</td>
<td>FITC</td>
<td>Sigma</td>
</tr>
<tr>
<td>Rat Ig</td>
<td>Goat</td>
<td>FITC</td>
<td>Nordic</td>
</tr>
<tr>
<td>Mouse Ig</td>
<td>Rabbit</td>
<td>HRP</td>
<td>Dako</td>
</tr>
<tr>
<td>(IgA, IgG, IgM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hamster Ig</td>
<td>Goat</td>
<td>FITC</td>
<td>Nordic</td>
</tr>
<tr>
<td>Biotin</td>
<td>N/A (streptavidin)</td>
<td>HRP</td>
<td>Zymed</td>
</tr>
<tr>
<td>Biotin</td>
<td>N/A (streptavidin)</td>
<td>Mini-Macs beads</td>
<td>Miltenyi Biotech</td>
</tr>
<tr>
<td>Biotin</td>
<td>N/A (streptavidin)</td>
<td>FITC</td>
<td>Pharmingen</td>
</tr>
<tr>
<td>Biotin</td>
<td>N/A (streptavidin)</td>
<td>Cy 5</td>
<td>Pharmingen</td>
</tr>
</tbody>
</table>

**Table 2.4:** Secondary antibodies and Streptavidin-Cychrome C used in ELISA assays, in cell separation experiments or to visualise staining of cells with unconjugated or biotinylated first layer antibodies.
Cell lines

The cell lines used in this project are summarised below (Table 2.5).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>MHC expression</th>
<th>Growth medium</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>EL4</td>
<td>Murine thymoma</td>
<td>Kb, Db</td>
<td>RPMI/5% FCS</td>
<td>ATCC</td>
</tr>
<tr>
<td>RMA</td>
<td>Murine T cell lymphoma</td>
<td>Kb, Db</td>
<td>RPMI/5% FCS</td>
<td>K. Kärre, Stockholm</td>
</tr>
<tr>
<td>P815</td>
<td>Murine mastocytoma</td>
<td>Kd, Dd, Ld</td>
<td>RPMI/5% FCS</td>
<td>ECACC</td>
</tr>
<tr>
<td>CMT-93</td>
<td>Murine colorectal carcinoma</td>
<td>H-2b</td>
<td>RPMI/5%FCS</td>
<td>ICRF</td>
</tr>
<tr>
<td>COS-7</td>
<td>African green monkey renal carcinoma</td>
<td>-</td>
<td>DMEM/5%FCS</td>
<td>ATCC</td>
</tr>
<tr>
<td>CB1</td>
<td>Immortalised murine dendritic cell line</td>
<td>H-2d I-A &amp; I-Ed</td>
<td>RPMI/5%FCS</td>
<td>P. Paglia, Italy</td>
</tr>
</tbody>
</table>

Table 2.5: Cell lines used for targets in CTL assays and for transfection of hCEA.

Cell counting

10μl of cell suspension was diluted 1:1 with trypan blue solution (0.2% w/v in PBSA with 3mM NaN3). The cells were then viewed under phase and counted on an improved Neubauer counting chamber using a light microscope. Viable cells that excluded trypan blue were counted.
Cryopreservation and retrieval of cells

Cells for cryopreservation were counted, centrifuged and resuspended in FCS with 10% v/v of DMSO. 5x10^6 cells/ml were slowly frozen in 1ml cryotubes (Nunc) in an expanded polystyrene block at -80°C for 24-48 hours and then transferred to the vapour phase of liquid nitrogen.

Cells were retrieved from cryopreservation by quick warming in a 37°C water bath. As soon as the mixture had thawed it was transferred to a T25 flask and fresh medium at 37°C was added dropwise, centrifuged and resuspended in fresh medium. After overnight incubation cells were split 1:5 into fresh medium and culture was continued at 37°C/5%CO2 in a humidified incubator for all cell types.

Transfection of Mammalian cells

Cells in suspension were transfected by electroporation and adherent cells by lipofection.

Electroporation

The cells used for electroporation were split 1:2 at mid-log phase growth and >90% viability for 2 days prior to electroporation. For each electroporation 5x10^6 cells were used. After two washes in cold PBS the cells were resuspended in 600μl of IMDM to which 25μg of plasmid DNA or dH2O as control and 50μl of Salmon sperm DNA (at 10mg/ml and freshly boiled) were added. The mixture was then transferred to cold 4 mm electroporation cuvettes (BioRad, UK) and placed on ice for 5 minutes and then electroporated in a BioRad electroporation apparatus as follows;
RMA  200V  960μF
P815 800V 25μF
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EL4  450V  125μF

After electroporation the cells were immediately placed on ice for a further 5 minutes and then the volume was made up to 10 ml with IMDM/10% FCS. Electroporated cells were transferred to 5 wells of a 24 well tissue culture plates (Falcon, UK) and incubated at 37°C/5% CO₂ in a humidified incubator for 48 hours. The culture medium was then changed to RPMI/7.5% FCS and the appropriate selection antibiotic was added. The concentration of antibiotic was determined for each cell line prior to electroporation by determining the minimum antibiotic required to kill all the cells that were seeded at 4x10⁵ cells/ml, in 3-4 days. Using the limited dilution method single cell colonies were grown in U-bottom 96 well tissue culture plates (Falcon, UK).

**Lipofection**

Adherent cells were split in T-25 tissue culture flasks (Falcon, UK) and transferred to six well tissue culture plates (Falco, UK) for a few days before lipofection to ensure that they were between 40-60% confluent on the day of transfection. The medium was taken off and the cells were washed twice with serum free medium (SFM) and replaced by 3 ml of . Plasmid DNA at 30μg in 300μl of SFM or SFM alone and 60μl of lipofectamine reagent (GIBCO BRL, UK) in 300μl of SFM were added together and left at room temperature for 15 minutes. The volumes of the transfection mixture or control were made up to 3 ml with SFM and to each well 1 ml was added. The flasks were placed in a humidified incubator at 37°C/5% CO₂ overnight. The medium was replaced the next day with the medium supplemented with FCS. The appropriate selection medium was used as soon as there was a sign of cell growth. A few days after all the controls were dead, single cell colonies could be detected that were removed using sterile forceps and filter paper and placed along with the
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filter paper in 24 well tissue culture plates (Falcon, UK) initially in 250μl of medium that was raised gradually to 1 ml as the cells started growing.

**Immunofluorescence staining of cell surface markers**

Cells suspended in PBSA were aliquotted into a 96 well flexible plate (Dynatech microtitre plate) at about 2x10^5/well. They were pelleted by centrifugation at 300 x g for 2 minutes. The supernatant was discarded by inverting the plate and the cells resuspended by agitating on a whirlimixer. The cells were incubated on ice with the appropriate first layer antibody (Table 2.3) at a predetermined optimum dilution for twenty minutes then washed three times with ice cold PBSA. Both directly conjugated and unconjugated antibodies were used in different experiments. For unconjugated or biotinylated antibody a fluorescence conjugated second layer of the appropriate anti-species Ig or streptavidin FITC was used (Table 2.4). When two layer staining was performed some cells were stained only with the second layer as a control for non-specific staining. When staining cells for two markers experiments were designed so as to avoid using second layer antibodies which could bind both first layer antibodies. Stained cells were examined and quantified using a FACScan (Becton Dickinson) with FACScan software.

**Intracellular staining and confocal microscopy**

All staining steps were carried out on permeabilised cells in the dark at room temperature. Cells were fixed and permeabilised by adding to each well 50μl of PermeaFix (Ortho) diluted 1:2 in PBS. Plates were then agitated in the dark for 40 min at room temperature. Cells were then washed in RPMI supplemented with 10% FCS, twice. 1st layer biotinylated oligonucleotides
were added, and cells were agitated for 30min, washed twice, and then 2nd layer antibody was added and left for 30 min and washed as above. Cells were then washed twice, fixed in PBSA/1% paraformaldehyde, washed twice more and then resuspended in 50μl PBSA. Controls of second layers without first layers, and of staining one target molecule only were also included.

**Mounting cells onto slides**

50μl cell suspensions were pipetted onto poly-L-lysine coated 6mm diameter wells of 8-well glass slides (Roboz Surgical Instruments) and left at 4°C for 30min to allow the cells to adhere. Excess PBSA was then removed from the wells, and a drop of mounting medium (20% glycerol plus anti-fade reagent) was added before a cover slip was lowered and secured with Jet Set Red nail varnish (Rimmel). Slides were stored at 4°C.

**Confocal microscopy**

Mounted cells were viewed through an inverted Zeiss Axiovert 100 microscope in conjunction with a Bio-Rad MRC 1024 Laser Scanning Confocal Imaging System. This uses a krypton-argon laser run by LaserSharp software in an OS/2 Warp Connect operating system which acquires digital images. Representative images were then processed using Adobe Photoshop and Microsoft Powerpoint software, and printed on Epson photographic quality paper by an Epson Stylus colour printer.

**Enzyme Linked Immunosorbent Assay (ELISA)**

Immunoglobulins directed against hCEA from mice immunised with hCEA vectors or hCEA protein were detected by ELISA. At a desired time point tail bleeds were performed under anaesthesia. Blood was allowed to coagulate
in eppendorf tubes at room temperature for 4 hours, then tubes were spun at 13,000rpm for 4 minutes. Sera was removed into fresh eppendorfs and stored at -20°C before use in ELISA.

ELISA plates (DynaTech) were coated with 50μl/well of 2μg/ml hCEA (Calbiochem-Novabiochem Corp., UK) in coating buffer (8.4 g/l sodium bicarbonate at pH 8.2 in millipore dH2O) at 4°C overnight. Next day all wells were washed (using washing buffer, PBS-A with 0.05% Tween 20) three times and then blocked with 150μl of PBS-A/1% Bovine serum albumin (BSA) at room temperature for 1 hour. Wells were washed as above and 50μl of sera or standard antibodies against hCEA were added in serial dilutions and incubated at room temperature for 2 hr. Wells were washed as above and then 50μl of 1:400 Horseradish peroxidase (HRP)-conjugated rabbit anti mouse Ig (Dako) or 1:500 HRP-conjugated anti-mouse isotypes (IgG1 or IgG2c from Pharmingen) added for 1hr at room temperature. After washing, 50μl of substrate solution (Dako TMB One-Step substrate solution) was added to each well and plates were incubated in the dark at room temperature until the colour developed. 25μl stop solution (10% sulphuric acid) was then added to each well and the OD450 of each well was read using a Dynatech MR700 microplate reader.

**Sandwich ELISA**

Sandwich ELISA was used to detect cytokine levels in supernatant of bone marrow derived dendritic cells treated with oligonucleotides. The method used for detection of interleukin-12 (IL-12) ELISA will be described below as only the results of this assay are reported in this thesis. This assay had a minimum IL-12 detection level of 16 ng/ml.

ELISA plates (DynaTech) were coated with 100μl/well of 10μg/ml IL-12 capture antibody cocktail at 4°C overnight. The capture antibody cocktail was a
commercially prepared mixture of two antibodies, Red T and G297-289 (Pharmingen, UK) that bind the mouse IL-12 p35 subunit of the IL-12 p70 heterodimer and the coating buffer used was 0.1M Na₂HPO₄ at pH9. Next day all wells were washed (using washing buffer, PBS-A with 0.05% Tween 20) four times and then blocked with 200µl of PBS-A/1%BSA at room temperature for 1 hour. Wells were washed three times and 100µl of the samples or standard IL-12 (recombinant mouse IL-12, Pharmingen, UK, a range of 2.3 to 1667ng/ml) were added serially diluted in blocking buffer and incubated at 4°C overnight. Wells were washed four times and then 100µl of 1:500 of C17.8 in blocking buffer (biotin-conjugated rat anti mouse IL-12 monoclonal antibody that binds the p40 subunit of IL-12 p70, Pharmingen, UK) was added for 1hr at room temperature. The detection antibody was then washed five times and 100µl of HRP-conjugated streptavidin (ZYMED, USA) diluted 1:4000 in blocking buffer was added for 30 minutes at room temperature. After six washes, 100µl of substrate solution (Dako TMB One-Step substrate solution) was added to each well and plates were incubated in the dark at room temperature until the colour developed. 50µl stop solution (10% sulphuric acid) was then added to each well and the OD₄₅₀ of each well was read using a Dynatech MR700 microplate reader.

**Enriching dendritic cells from spleen**

Dendritic cells were enriched from mouse spleens by taking advantage of two properties of these cells: transient adherence and low buoyant density. Strongly adherent cells (e.g. macrophages) remain stuck to plastic after an overnight incubation whereas dendritic cells do adhere initially, but detach after 2 hours and are non-adherent after an overnight incubation. These non-adherent, low density dendritic cells can subsequently be separated from other
non-adherent, high density cells such as lymphocytes by centrifugation on a layer of metrizamide.

Spleens from young adult mice were teased through a sterile plastic mesh to make a single cell suspension which was then washed once and resuspended at 5x10^6 cells/ml in RPMI/10% FCS. 10^8 spleen cells were seeded in a T75 flask and incubated overnight. The following day non-adherent cells were harvested, centrifuged and resuspended at 10^7 cells/ml in RPMI/10% FCS. 8 ml of cells were layered on top of 2 ml 14.5% (w/v) metrizamide in RPMI/10% FCS in a universal tube. The cells were centrifuged for 10 minutes at 600 x g (1800 rpm). The low density cells at the interface were collected and washed. These cells were routinely >40% N418+ve dendritic cells. hCEA or control antigens were incubated at 1mg/ml with the splenic DC's for eight hours and washed with RPMI/10% FCS. These cells were used to restimulated splenocytes from mice that had been immunised with hCEA protein or DNA vectors.

**Enriching lymphocytes from spleen**

CD4^+ , CD8^+ T cells or B cells were positively selected from non-adherent splenocytes using Mini-Macs magnetic beads (Miltenyi Biotech). The purity of both positively and negatively selected fractions can be accurately assessed by staining when using directly conjugated beads. Following a 40 min plastic adherence step, non-adherent cells were resuspended in cold Mini-Macs buffer (PBSA, 0.5% BSA, 5 mM EDTA) 10^6 cells/ml and incubated on ice for 20 minutes with 50µl of 1:500 Biotin-conjugated B220 antibody (Pharmingen) per 2x10^5 cells. The unbound antibodies were then washed three times. The pellet was resuspended in buffer at 10^8 cells/ml with 10µl of streptavidin-conjugated Mini-Macs beads per 10^7 cells. Suspensions were incubated in universal tubes
in the fridge for exactly 15 mins, then each applied in 500μl buffer to a Mini-Macs column (on a magnet) which had been primed by flushing through 500μl of Mini-Macs buffer to clear the hydrophilic coating. The column was used without a resistor, in order to speed up the flow and thus optimise the purity of the positive fractions. The negative fraction, consisting of CD4 and CD8 enriched T cells were collected for positive selection as above but by using Biotin-conjugated L3T4 and Ly-2 CD4 and CD8 cells were positively selected, respectively. Columns were washed by running through 2 aliquots of 500μl of buffer, and the positive fractions were then eluted in 1 ml of buffer off the magnet using the syringe plungers provided. The purity of positively selected fractions was checked by appropriate staining and flow cytometric analysis, and was routinely greater than 95%, with less than 5% contaminating cells.

Proliferation assay

Splenocytes or lymphocytes were induced to proliferate by a variety of methods that are mentioned in the specific method sections of the result chapters. After a desired interval, to measure the level of proliferation each well was pulsed for 14-16 hours with 10μl PBSA containing 1μCi [3H]TdR before being harvested onto glass fibre filters using a cell harvester (Skatron). Incorporation of [3H]TdR was assessed by liquid scintillation using the LKB betaplate system.

CTL assay

Cytotoxic T cell activity was determined in a 51Cr release assay. The assay uses the release of chromium from pre-labelled target cells to measure lysis of target cells after contact with effector T cells. All assays were carried out
in 96 well U-bottom non-tissue culture treated assay plates. The assay medium used was RPMI/5% FCS.

**Target cells**

All target cells were seeded at mid-log phase growth and >90% viability the night before the assay. On the day of the assay target cells to be peptide labelled were cultured at 10^6 cells in 50μl for 1 hr at 37°C with 100μM peptide. All target cells were radioactively labelled with 50 mCi Na^{51}CrO_4 by adding 10 ml of 5 mCi Na^{51}CrO_4 for a further hour. Cells were then washed three times, counted and resuspended at 5x10^4/ml.

**Effector cells**

After the indicated days of culture of stimulators with responders, viable cells were counted, washed and resuspended at 5x10^5/ml in assay medium. Serial 2-fold dilutions in triplicate were then set up in assay plates before addition of target cells.

Replicate wells with 100 μl medium or 100 μl 0.5% (w/v) SDS representing spontaneous and maximum release respectively were prepared on separate plates. Target cells were then added at 5x10^3/well to all wells generating cultures with various effector cell:target cell ratios. Assay plates were then spun briefly once to pellet cells and cultured at 37°C for 4 hr. After this time 100 μl supernatant was harvested from each well and γ-radioactivity was counted using a LKB Wallac 1272 Clinigamma γ-counter linked to an Olivetti PCS 286 computer. The "% specific release" was calculated as:

\[
\frac{(\text{experimental release}-\text{spontaneous release})}{(\text{maximum release - spontaneous release})} \times 100\%.
\]
Restriction enzyme digests

Specific cutting restriction enzymes from Pharmacia or Promega were employed to digest double stranded DNA. The concentrations of enzymes were chosen to give 1 unit per μg of DNA with a glycerol concentration of not more than 5%. One Phor All buffer (Pharmacia) was used at 1x or 2x as appropriate for each enzyme. Incubations were carried out at 37°C for 1 to 2 hours followed by enzyme inactivation at 65°C for 15 mins, and small amounts of the digests were run on an agarose gel and visualised with ethidium bromide and if required isolated from NuSieve low melting point gels (see below).

Purification of DNA from agarose gels

DNA was run on a low melting point NuSieve gel (see below), the band of interest was excised under long wave ultraviolet light, made up to 10mM EDTA, 100mM NaCl, melted in a water bath at 65-70°C for 10 minutes. The DNA was then extracted using the Wizard clean up system (Promega, UK) or with phenol/chloroform. After two phenol/chloroform extractions the DNA was precipitated by addition of 1/10 volume 3M sodium acetate pH5.2 and 2.5 volumes absolute ethanol. DNA was then recovered by centrifugation at 13,000 rpm for 10 minutes and washed once in 70% ethanol before resuspension in water or TE pH8.0.

Estimation of nucleic acid concentration

Purified DNA or RNA solutions were diluted in distilled water by a factor of 50-100. The optical density (OD) of the solutions was measured in a
quartz cuvette at a wavelength of 260 nm on a spectrophotometer (LKB Ultrospec II), using distilled water as the zero reference. The nucleic acid concentration (µg /ml) was calculated by multiplying the absorbance reading at 260 nm by a factor of 50 for double-stranded DNA, 40 for single-stranded DNA and RNA and 20 for single-stranded oligonucleotides, taking into consideration the dilution factor. The OD\textsubscript{260}/OD\textsubscript{280} ratio was examined as a measure of purity, a value greater than 1.7 indicating low protein contamination.

**Ligation of restriction fragments in to plasmids**

Purified fragments of DNA were subcloned by ligation in to plasmids that had been digested with appropriate enzymes to create compatible ends. Vector and insert DNA were mixed at molar ratios of 1:1, 1:3, and 1:10. Two control tubes with vector alone, with and without ligase, were always included. Ligations were performed in 10 µl, containing 10-20 ng vector DNA in 1 µl, 1-7 µl insert DNA, 1 µl 10x ligase buffer and 100U of T4 DNA ligase (promega). 10x ligase buffer consisted of 0.5M Tri-HCl, pH7.6, 0.1M MgCl\textsubscript{2}, 100 mM dithiothreitol (DTT), 10mM ATP, 0.5 mg/ml(BSA).

**Transformation of E.coli with plasmid DNA**

Electrocompetent TG1 E. coli were prepared by growing the bacteria in 2TY medium until an OD\textsubscript{550} of 0.48 was reached. They were then rapidly chilled to 4°C by addition of presterilised plastic ice (used for cooling white wine, Tesco UK) and shaking the flask in an ice container. The bacteria were then pelleted by centrifugation at 2500 rpm for 5 minutes, at 4°C. They were then gently resuspended in 4 ml of ice cold buffer (10 mM sodium-MOPS, 10
mM KCl, 15 mM CaCl₂ and 15% (v/v) glycerol). Aliquots of 100 µl were snap frozen on liquid nitrogen and stored at -80°C.

One vial of electrocompetent bacteria was thawed at room temperature and then placed on ice. The electroporator was pre-set at 2.5kV, 25 µF and 200 ohms. 40µl of TG1s together with 2µl of plasmid DNA were placed in a microcentrifuge and incubated on ice for 1 minute. The contents was then transferred to a pre-chilled 1mm cuvette (BioRad, UK) and placed in a cold electroporation chamber and pulsed. The time constant was documented (should be approximately 4.5s). 1ml of LB was added immediately to resuspend the bacteria and incubated at 37°C for 30 minutes at 250rpm. The solution was then centrifuged at 13,000rpm for 4 minutes, the supernatant removed and the cells resuspended in 100µl of LB. Serial dilutions of the culture were made and plated out on LB agar plates and incubated at 37°C overnight.

Plasmid preparation

Small and medium scale plasmid preparations were performed using the 'miniprep' and 'maxiprep' systems (Promega, UK), respectively. Large scale plasmid preparations (6-8 mg/preparation) were performed for use in DNA immunisations using the Qiagen EndoFree plasmid Giga Kit that had a endotoxin removal step. The manufactureres claimed that this method resulted in endotoxin levels of less than 1 EU/preparation. All the plasmids were prepared exactly as directed by the manufacturer except that the final products were dissolved in non-pyogenic sterile saline if they were to be used for injections.
DNA amplification

Oligonucleotide primers

Oligonucleotide primers were designed to flank the first two domains of hCEA and to create a mutation which introduced two new restriction endonuclease sites at these regions. The two endonuclease target sites were a NOT I site (the palindromic 5’GCAGCGAG3’t sequence) at the 3’ flanking region, and a HIND III site (the palindromic 5’ACCATGGAGT3’ sequence) at the 5’ flanking region. The full sequence of the two primers were as follows;

hCEA reverse;

5’GCAGACAAAGCTTACCATGGAGTCTCCCTCGG3’

hCEA forward;

5’GGGGAAATGGTGGGGCAGATGCATGCAGGACATTAGGATG3’

These primers were synthesised and cartridge purified by Genosys (Cambridge, U.K.).

To join the first two domains of hCEA with tetanus toxoid fragment-c the hCEA forward primer was used in conjunction with a linker primer along with a fragment-c reverse primer (methods, chapter 4). The latter two oligonucleotides were a kind gift from Dr. Delin Zu of Southampton University Hospital, the sequences of which are as follows;

Linker; 5’GGACCCCGAGCTAAAAACCTTTGATTGTTGGGTCGAC3’

Fragment-c reverse; 5’TATGCCCGCTTTAGTCGTTGGGTCCAAC3’

Polymerase Chain Reaction (PCR)

The Gene Amp PCR Reagent Kit from Perkin Elmer was used to carry out PCR amplification of the genes of interest or cDNA. The reaction buffer
used was 10 mM Tris-HCL, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.001%w/v gelatine. The four deoxynucleoside triphosphates (dNTPs) were diluted from their individual original stock concentrations of 10 mM to 1.25 mM in sterile distilled water as a mixture, and used at a working concentration of 0.2 mM. Oligonucleotide primers were used at a final concentration of 0.5 mM each to target h/m C E A cDNA. The enzyme AmpliTaq DNA polymerase (Perkin Elmer, UK) was used at a final concentration of 0.05 U/µl. Plasmid vectors containing the genes of interest were used as targets. Each reaction was carried out in a final volume 100 µl and was protected from evaporation by overlaying three drops of light mineral oil.

A Biometra Trio-thermoblockTM (Biometra Ltd, UK) was used to perform the PCR in 30 cycles; each consisting of 1 minute denaturation at 95°C, 1 minute for primer annealing at 65°C followed by 1 minute for elongation at 74°C. Resulting products of amplification were visualised in agarose gel.

**cDNA synthesis**

cDNA of hCEA was synthesised by PCR for cloning purposes or as a method of confirming the presence of hCEA mRNA in hCEA transfected mouse cell lines. The cDNA was reverse transcribed from total cell RNA that was extracted from cells using RNAzolTM B (TEL-TEST INC., USA).

**RNA extraction**

To minimise RNA degradation clean gloves were used throughout the procedure; all the reactions were performed on ice; all solutions used were treated with 0.1% diethyl pyrocarbonate (DEPC, a strong but not absolute inhibitor of RNase) and then autoclaved; all the tubes, pipette tips and benches were treated with DEPC water. According to the manufacturer’s instructions, RNAzolTM B promotes formation of complexes of RNA with guanidium and
water molecules, and abolishes hyrophilic interactions of DNA and proteins. 1ml of RNAzolTM B was used to resuspend a pellet of 5x10^6 cells which were washed once with cold PBS. The resuspension results in the lysis of the cells and interaction of RNA with guanidium and water. To the lysed cells 0.1 volume of chloroform was added. The mixture was vortexed for 5 seconds then placed on ice for 5 minutes. The aqueous phase was recovered by centrifugation for 15 min. at 12,000g. The RNA was precipitated by incubation with an equal volume of isopropanol on ice for 15 min. and then recovered by centrifugation for 15 min, at 12,000g at 4°C. The RNA pellet was washed once with 75% ethanol to reduce the salt content and resuspended in 25 µl of DEPC treated water and stored at -70°C.

**Reverse Transcription Reaction**

The RNA was reverse transcribed using avian myeloblastosis virus reverse transcriptase (AMV RT) (GIBCO BRL, UK). The optimum activity of AMV RT is at 42°C and hence problems associated with RNA secondary structures are reduced. The reaction is performed in reverse transcriptase (RT) buffer (500mM Tris-HCl pH8.3, 50mM MgCl2, 50 mM DTT, GIBCO BRL, UK). Thus, 10µl of RT buffer, 10µl of DEPC dH2O, 10µl of 250mM KCl (GIBCO BRL, UK), 1µl of 40U/µl rRNasin (Promega, UK), 1µl of 10mM d-NTPs (Bohringer, UK), 1µl of 0.01U/µl pd(N)6 and 1µl of 2.5U/µl AMV RT were added together and then 10 µl of total cell RNA extracted from 5x10^6 cells was added and the mixture was incubated at 42°C for 2 hours.
Gel electrophoresis

Agarose Gel Electrophoresis

Horizontal slab gel electrophoresis was used analytically to resolve nucleic acids in preparative procedures such as PCR, in manipulative procedures such as endonuclease digestion or ligation, to test for transcription of a gene of interest by amplification of the cDNA, and for purification of nucleic acids of interest from a mixture of nucleic acids. For instance after double digestion of a vector in order to isolate the insert. The analytical gels were prepared in 1X TBE (0.089M-Tris base, 0.089M-boric acid, 0.002M-EDTA), and the purification gels were prepared in 1XTAE (0.04M-Tris, 0.04M-acetate, 0.001M-EDTA pH 7.7 with glacial acetic acid). For analytical purposes, 0.7% agarose slurry of 50 ml volume was melted in a microwave oven, allowed to cool to 60°C, then 5μl of ethidium bromide was added. Finally the slurry was poured into a gel mould and was allowed to set. For purification purposes exactly the same method was followed except for the gel concentration which was a 1% NuSieve low melting temperature agarose (GIBCO BRL, U.K.).

The gels were bathed in their appropriate electrophoresis buffer and DNA samples were loaded into wells with 0.1 volume gel loading buffer (30% (w/v) glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF in distilled water). Electrophoresis of samples was carried out at a constant voltage of 50 volts for 0.7% agarose gels and of 38volts for 1% NuSieve gels for a period of 30 minutes and 1 hour, respectively.
**Polyacrylamide Gel Electrophoresis (PAGE)**

PAGE was used to resolve DNA from sequencing reactions, protein and oligonucleotides in gel shift assays (chapter 6) or protein that was extracted from the cytoplasmic extract of a dendritic cell lines (chapter 6).

Sequencing reactions were resolved in 6% (w/v) polyacrylamide gels using a Biorad gel electrophoresis apparatus. The glass plates were thoroughly cleaned with detergent and rinsed with tap water. After drying the plates with soft tissue paper, one side of the smaller of the two plates was coated with siliconising agent (Repel). Plates were assembled with the appropriate spacers to form a 0.4 mm thick rectangular mould, then sealed with waterproof gel sealing tape.

Sever Biotech Ltd. (Worcs., UK) predisolved polyacrylamide gel mix (acrylamide : Bisacrylamide ratio 19:1 in 7M Urea 1XTBE) was used which carries a lower risk of neurotoxicity than the dried form. Polymerisation was initiated by adding 600µl of freshly made 10% (w/v) ammonium persulphate and 50µl of TEMED (N,N,N',N'-tetramethylehylene-diamine) to 100µl of the gel mix and mixing thoroughly. The gel was poured with the mould at a 45° angle, then it was laid flat and rectangular combs were placed at the top between the glass plates to ensure a straight edge at the free side of the gel. The gel was left to polymerise over night.

**DNA Sequencing**

**Single stranded DNA preparation**

Selected colonies grown on 2TY agar plates were picked out with a plastic loop and grown up in 3ml of 2TY at 37°C for 1 hour in an orbital shaker. To the bacterial culture 3µl of VCsM13 helper phage (Stratagene) was added
Chapter 2

(titre > 1x10^{11}) and incubation was continued for a further 1 hour then 6μl of kanamycin (final concentration of 25mg/ml) was added and incubation continued for a further 5 hours shaking at 300 rpm.

The solution was then centrifuged at 12,000 rpm for 5 minutes and 1ml of the supernatant transferred to an eppendorf tube. To the supernatant 100μl of 20% PEG/2.5M NaCl was added and kept at room temperature for 30 minutes. The suspension was centrifuged for 15 minutes and the supernatant removed, a further centrifugation was performed to remove any remaining supernatant. The tube was then air dried. The dried pellet was resuspended in 100μl of TE buffer (pH 8.0) and 100μl Phenol added. The solution was vortexed for one minute to ensure thorough mixing and centrifuged at 13,000 rpm for 5 minutes. 90μl of the top aqueous phase (containing the DNA) was removed to a fresh tube. To this was added 10μl 3M Na Acetate pH 6.0 and 300μl absolute alcohol and incubated at -70°C for 2 hours. The solution was centrifuged at 13,000 rpm for 15 minutes and the supernatant discarded, the pellet was washed with 1ml of 95% ethanol (prechilled at -20°C) and a further centrifugation performed for 2 minutes. 950μl was removed and the tube vacuum dried (speed vac). Once completely dry the pellet was resuspended in 20μl of TE buffer, pH8.0 (10mM Tris-HCl, 1mM EDTA pH8.0). 2μl of the preparation was analysed on a 1% agarose gel.

**Sequencing reaction**

A single-stranded DNA template was used for sequencing using a modified version of the dideoxy chain-termination DNA sequencing method (Sanger, Nicklen et al. 1992). The DNA template is synthesised in vitro from the single-stranded template using a DNA polymerase. This is initiated at a site where an oligonucleotide primer anneals to the template and terminated by the
incorporation of a nucleotide analog that will not support continued chain elongation. The chain-termination nucleotide analogues are 2',3'-dideoxynucleoside 5'-triphosphates (ddNTPs) that lack the 3'-OH group necessary for DNA chain elongation. When a mixture of dNTPs and one of the four ddNTPs are used, polymerisation is terminated in a fraction of the population of chains where ddNTP is incorporated generating DNA of various lengths that are all terminated with a known base. Using all four dNTPs and resolving the four populations of DNA side by side on a gel reveals the sequence of the original template. A radioactively labelled nucleotide $[^{35}\text{S}]\text{dATP}$ is incorporated so that labeled chains of various lengths can be visualised by autoradiography after separation by high-resolution electrophoresis. Sequencing was carried out using the Sequenase$^\text{TM}$ Version 2.0 DNA Sequencing Kit (Amersham, UK) exactly as directed by the manufacturer. All materials used except $[^{35}\text{S}]\text{dATP}$ were supplied with this kit. After establishing the method the sequencing was carried out by a commercial laboratory (ABC centre, Charring Cross hospital).
Chapter 3

DNA immunisation against hCEA
Introduction

In this century there has been some encouraging experimental and clinical evidence in support of the efficacy of therapy for cancer based on vaccination against tumour antigens. In the past two decades, a better understanding of the mechanisms by which various immune cells interact, the development of techniques to manipulate these interactions and the ability to identify tumour antigens have revived the field of tumour immunology. Tumour immunologists have tried various methods of vaccination such as the use of peptide epitopes, viruses as carriers of tumour antigens and DNA immunisation in order to stimulate both cellular and humoral immune responses to tumours. Against such a background we decided to investigate the feasibility of vaccination against the human carcinoembryonic antigen (hCEA) using the naked DNA technique. hCEA was chosen primarily as a 'model' antigen as its over production and mode of expression in a variety of carcinomas make it a relevant target antigen. The over expression of hCEA in carcinomas could potentially result in a higher avidity of interaction between anti-hCEA T cells and carcinoma cells because of the presence of more hCEA peptides bound to MHC. This could result in a better chance of T cells reaching their activation threshold. Additionally, the pattern of expression of hCEA is deregulated in tumours, especially in poorly differentiated colorectal carcinomas, such that it can be found on both apical and basolateral cell membranes (Shirota, Minassian et al. 1988). However, in normal epithelial tissues, hCEA is expressed only on the apical surfaces which may account for the inaccessibility of anti-hCEA antibodies to these cells (Blumenthal, Sharkey et al. 1992). Hypothetically, this could confer protection from antibodies to normal hCEA expressing epithelial cells, while tumours are vulnerable. This
argument, however, does not apply to anti-hCEA T cell reactivity against normal epithelial cells.

The aim of the experiments described in this chapter was to use DNA vaccination as the means of stimulating immune responses against hCEA in mice and to investigate the claimed advantages of this new method (Ulmer, Donnelly et al. 1993). The advantages relevant to an anti-tumour response include raising an immune response with a cytotoxic T cell component and breaking tolerance to self antigens (see the introductory chapter). We therefore planned to study the nature of immune responses to hCEA as a foreign antigen in C57BL/6 mice with a view to studying the breaking of self tolerance in a hCEA transgenic C57BL/6 model.

The humoral and cellular responses generated against hCEA are examined in this chapter. An important subsidiary issue was to make sure that the constructs did express hCEA. This is dealt with at the beginning of the results section of this chapter. Our initial experiments with small groups of mice showed that some mice did not generate any detectable responses so that a second set of experiments were carried out with larger groups. Nevertheless the preliminary experiments did show that DNA vaccination could stimulate responses. Hence, some of these data have been included for illustrative purposes.

Finally, a protein challenge experiment is described that was performed to investigate whether the problem of weak and unreproducible antibody responses could be overcome by availability of antigen in large concentrations.
Methods

**Vaccination constructs**

Initially, two different DNA constructs were used; one containing the whole of the hCEA gene and another containing only the first two N-terminal domains. The first construct contains a sequence starting 70 nucleotides upstream of the hCEA gene and ends 274 nucleotides downstream of the gene. This sequence was a kind gift of Dr. J. Thompson from the group which generated the hCEA transgenic C57BL/6 mouse (Eades-perner et al., 1994). The inclusion of the 70 upstream nucleotides could increase the chances of including regulatory elements, such as ribosomal binding sequences, in the construct (Kozak, 1991) especially as the 3’ non-coding sequence of hCEA may play a role in the control of transcription (Oikawa, Nakazato et al. 1987).

The truncated construct contains the leader, N-terminal and the A1 subdomain sequences of hCEA, hereafter, called hCEADo (fig.3.1). The presence of the leader sequence and the absence of a transmembrane domain should result in a secretory product. This construct was designed for two reasons. Firstly, hCEA family members express a variable number of immunoglobulin-like domains. Minimising the number of these in the construct might reduce crossreactivity. Secondly, the hCEADo construct was designed to evaluate if the immune responses to the secreted antigen differ in strength from those raised against a membrane bound form. Therefore, a control construct was also designed which coded for hCEADo and a transmembrane region (hCEADoTM) (see methods chapter). Finally, a construct was also made that coded for hCEADo followed by a streptavidin binding tag to allow detection or purification of hCEADo (hCEADoTag).
The first criterion for the vaccination vectors to be functional is their ability to express the protein antigen. It is possible to detect reporter gene expression as a result of direct intramuscular plasmid injection (Wolff, Williams et al. 1991), however, detection of other antigens is more difficult since very low levels of the protein are expressed. For instance, in one study, a maximum of 15 ng/ml of human-α-1-antitrypsin (hAAT) was detected in the serum at the peak of expression of the plasmid after injection (Levy, Barron et al. 1996).
Figure 3.1: Diagramatic representation of the vectors used. hCEA and hCEADoTM were cloned in the Hind III and XbaI sites. hCEADo was cloned in the HindIII and NotI sites and was the rtPCR product of RNA from human colorectal samples obtained using two primers that span the N terminal and A1 domains of hCEA gene (see methods chapter).
Considering such low levels of antigen and that hAAT is a secreted protein, it is likely that detection of membrane bound hCEA would require a very sensitive sandwich ELISA assay or radioimmunoassay. An alternative way of showing that vaccine vectors are capable of expression, is to transfect them into mammalian cell lines and look for mRNA and protein synthesis. The latter was the method we used to assess the functionality of the vectors.

**Immunisation schedule**

The mice were immunised in the quadriceps muscles under anaesthesia either with a single dose consisting of 100 μg of DNA at 1 mg/ml in saline or were first immunised with 100 μg and then boosted with 50 μg of DNA. For protein immunisations the animals were immunised, subcutaneously, with 25 μg of hCEA protein in Complete Freund's Adjuvant (CFA), and boosted later, intraperitoneally, with 50 μg of the protein in saline.

**Measurement of anti-hCEA antibody levels**

An Enzyme Linked Immunosorbant Assay (ELISA) was used to measure anti-hCEA antibody levels. The ELISA plates were coated with hCEA protein in PBS. The protein used for coating was initially prepared from a human colorectal tumour metastasis by affinity column and fast performance liquid chromatography. However, a high background was obtained when this source of antigen was used. Using a commercial source of protein (Calbiochem-Novabiochem (UK) Ltd) the background was reduced to zero.

Initially the ELISA was performed on a set of dilutions of the pooled sera and a curve was constructed for each pool. To obtain the relative value of anti-hCEA responses, OD$_{450}$ at the mid-point of the linear part of each curve was divided by the corresponding point on the curve obtained for 1H12 mAb
supernatant in that assay. In later experiments, mAb units were used to enable comparison of the response levels between different experiments. These units were based on the A5B7 mAb. OD\textsubscript{450} curves for different dilutions of the standard and the samples were constructed. The A5B7 standard curves were constructed such that the optical density obtained with 1\mu g/ml of A5B7 in each ELISA plate was taken to be equivalent to 1000 mAb units. An OD\textsubscript{450} reading of a sample dilution that was in the linear part of its curve was compared to the mAb units for that OD\textsubscript{450} in the standard curve and then multiplied by the sample dilution to obtain the mAb units of anti-hCEA antibody in the serum.

**Lymphoproliferative assay**

Six to fourteen days after the final injection, spleens of the immunised mice were removed and after preparation of a single cell suspension T cells were enriched from splenocytes by B cell depletion, using B220 mAb and a magnetic bead system. To stimulate the anti-hCEA Th cells, 10^5 T cell enriched splenocytes from the immunised mice were coincubated with 4\times10^5 naive irradiated (30 Gy) feeder splenocytes and a range (12.5 to 100\mu g/ml) of hCEA protein or BSA as a control. The background proliferation levels were measured by incubating the T cell enriched splenocytes in medium alone or with irradiated feeder cells. After 48, 72 and 96 hours the cells were pulsed with ^3\text{H}-Thymidine for 14 to 16 hours and proliferation was measured as the level of ^3\text{H}-Thymidine incorporation into the DNA of the cells.

**Cytotoxic T Lymphocyte assay**

In order to detect anti-hCEA CTL responses of C57BL/6 mice, target cells were prepared by transfecting mouse tumour cells expressing H-2^b MHC class I antigens with hCEA. The mouse T cell lymphomas EL4 and RMA were
electroporated with the pRCCMV/hCEA vector that contained the whole of the hCEA gene. The transfectants were selected with neomycin and then the cells with the highest levels of expression of hCEA were FACS sorted using the A5B7 anti-hCEA mAb. Single cell colonies were then grown from the sorted population using the limiting dilution method. The expression of hCEA was tested in these cells by rtPCR and FACS staining using both A5B7 and 1H12 anti-hCEA mAbs. Figure 3.2 shows a typical FACS profile of these single cell colonies. Despite repeated rounds of sorting the target cells, a high level of expression of hCEA was never achieved. This could have been due to a variety of reasons such as the choice of promoter in the transfection vector. Moreover, the processes of transcription, translation or post-translational modification of the protein, in the native hCEA producing cells, may differ from those in the transfected cell lines.

To detect anti-hCEA CTL, ten days after the final injection of the vectors the responder splenocytes were cultured for six days with mitomycin-c (MMC) treated RMA/hCEA stimulator cells at a 10:1 ratio. A standard 51Cr release assay was used to assess the level of target cell lysis.

**Figure 3.2:** Staining of a RMA/hCEA single cell colony with A5B7 mAb. The curves for isotype control + FITC anti-mouse Ig or FITC anti-mouse Ig alone are superimposed (———). A5B7 + FITC anti-mouse Ig (········).
Results

*Transfection of mammalian cell lines with vaccination vectors*

The vectors used in this part of the study were called pVAC vectors (fig.3.1) and were engineered by a different group (Hawkins, R.E., MRC, Cambridge) not to contain the neomycin resistance gene. The neomycin resistance gene had been removed with the possibility in mind of using these vectors in human clinical trials. The lack of a gene conferring resistance to antibiotics used against mammalian cells, made the selection of transfectants with these vectors difficult. Since only a low percentage of mammalian cells can be transfected with even the most efficient techniques and because transfected cells grow slower than the untransfected ones in the population, detection of the transfected protein can be a difficult task. However, it is relatively easy to detect synthesis of the relevant mRNA. Therefore, the pVAC vectors were transfected, initially for their ease of transfection, into the African green monkey COS-7 cells, and later into the mouse CMT-93 colorectal carcinoma.

Using primers which span the N-terminal and the A1 subdomain of hCEA, mRNA expression in the transfected cells was analysed by RT-PCR. In order to rule out the possibility of amplification of CEA sequence from the genomic or transfected plasmid DNA the mRNA preparations were also used as PCR templates at the same concentrations as they were for RT-PCR. PCR of the cDNA made from COS-7 or CMT-93 cell lines transfected with pVAC vectors containing the genes for hCEA, hCEADo, hCEADoTag or hCEADoTM (fig. 3.1), amplified a band of approximately 700 b.p. long (fig. 3.3). This band corresponds to the 747 b.p. long region spanned by the primers. However, PCR of the mRNA preparations or of the cDNA from the cells transfected with pVAC/empty (using hCEA primers) failed to amplify any products (fig. 3.3).
Moreover, the absence of hCEA band in the control sample was not due to cDNA degradation or failure of reverse transcription, since bands corresponding to the housekeeping gene HPRT were obtained for all the samples when HPRT primers were used. Therefore, hCEA mRNA was being transcribed in those cell lines transfected with pVAC/hCEA, hCEADo, hCEADoTag and hCEADoTM.

Figure 3.3: Verification of expression of hCEA. PCR amplification of N-terminal and A1 subdomain of hCEA (747 b.p.) from RNA or cDNA extracted from CMT-93 cell line transfected with different hCEA expression vectors (Top panel). PCR of cDNA from all the transfected cell lines using HPRT primers shows that the RNA was not degraded during the process of cDNA synthesis (bottom panel).

There still remained the question of whether the hCEA mRNA was translated after transfection of pVAC vectors. This question was indirectly
answered by subcloning the inserts with a transmembrane domain (hCEA and hCEADoTM) into vectors containing the neomycin resistance gene and transfecting them into mouse cell lines. The CMT line and the mouse lymphoma EL4 and RMA were transfected and stained positive with various anti-hCEA antibodies (fig. 3.4). Although these protein products were coded by inserts in different vectors, this shows that the mRNA coded by these inserts, which was shown to be transcribed from pVAC vectors, is translatable.

**Figure 3.4:** Staining of cells, transfected with pCDNA3 vectors coding for hCEA and hCEATM, with A5B7 mAB. Isotype control + FITC anti-mouse Ig (-----), A5B7 + FITC anti-mouse Ig (---------).
Initial immunisations with pVAC/hCEA and pVAC/hCEADo

Humoral responses

In order to gain a sense of the steps involved in DNA immunisation, initially small groups of mice were immunised. Hence, three groups of four mice were immunised with a single dose of DNA. One group received pVAC/empty and the others either pVAC/hCEADoTag or pVAC/hCEA vectors. The mice were bled 2 and 4 weeks post immunisation and their sera were tested for anti-hCEA antibodies.

![Comparison of pooled anti-hCEA responses to 2-domain or whole hCEA genes](image)

**Figure 3.5:** The relative levels of anti-hCEA responses of pooled sera from four mice immunised with pVAC/empty, hCEADoTag or hCEA, determined by ELISA.

At 2 weeks post immunisation no antibodies to hCEA could be detected in any mice. However, the pooled sera of mice immunised with pVAC/hCEA and pVAC/hCEADoTag reacted more strongly with hCEA antigen compared to those immunised with pVAC/empty at 4 weeks post immunisation (fig. 3.5).
In this experiment, there appears to be a higher anti-hCEA activity in the sera of mice immunised with pVAC/hCEADoTag compared to those immunised with pVAC/hCEA. There is also a high background level of non-specific binding to hCEA; the activity of the control sera (pVAC/empty) reaches 40% of that of the highest responder. Therefore, in this experiment, due to the high background, the presence of a response to hCEA as a result of immunisation with these vectors is doubtful. The background obtained with the sera of pVAC/empty immunised mice was reduced to near zero when a commercial source of hCEA protein was used in the ELISA assays. Moreover, titration of individual sera of the responding mice showed that they have higher anti-hCEA activity compared to a control serum up to a dilution of 1 in 729 (figure 3.6). On the other hand, only one out of four mice had responded at the time when the peak response is expected in conventional immunisations (2-4 weeks). In retrospect, later time points should have been included in the experiment since later experiments showed that the peak antibody response was at 6-8 weeks post immunisation (fig.3.11).

The above experiment was repeated with the same number of mice, however, two mice from each group were boosted twice at two week intervals. Figure 3.7 shows the anti-hCEA responses of the sera from individual mice at 6 weeks post immunisation. Two mice in each experimental group developed responses. One of the two responders in each group gave significantly higher responses, however, unlike the previous experiment the responses to pVAC/hCEADoTag were not stronger. Moreover, the mice which had been boosted did not always produce the stronger responses.
**Figure 3.6:** Titration of the anti-hCEA responses of the sera of the responder mice compared to the 1H12 anti-hCEA mAb (In house hCEA was used). Each bar represents a single animal or a mAb.

**Figure 3.7:** Individual anti-hCEA responses of groups of four mice immunised with pVAC/empty, hCEADoTag or hCEA. Two mice in each group were boosted twice. Each bar represents a single animal. (Commercial hCEA was used in this and all the subsequent ELISA assays).
**Cellular responses**

The presence of both Th and CTL responses were assessed in DNA immunised mice which had produced humoral responses. As a positive control for lymphoproliferative responses three mice were immunised with hCEA protein in Complete Freund’s Adjuvant (CFA), and boosted 12 weeks later with protein in saline. The DNA immunised mice that were assessed for lymphoproliferative responses were injected with pVAC/hCEADoTag or pVAC/hCEA and then boosted twice with DNA at two week intervals. Six to fourteen days after the last injection, T cells were enriched from splenocytes by B cell depletion. To stimulate the anti-hCEA Th cells, T cell enriched splenocytes from the immunised mice were coincubated with naive irradiated feeder splenocytes and a concentration range of hCEA protein or BSA as a control. The background proliferation levels were measured by incubating the T cell enriched splenocytes in medium alone or with irradiated feeder cells.

Figure 3.8 shows that the splenocytes from two mice immunised against hCEA using either of the immunisation methods incorporated approximately 7-8 times more $^3$H-Thymidine in response to 50 $\mu$g/ml of hCEA protein than in response to the same amount of BSA. Without antigen the T cell enriched splenocytes and feeder cells together incorporated less than a 1000 cpm of $^3$H-Thymidine at 120 hours.

The peak levels of $^3$H-Thymidine incorporation in response to Con-A were reached at 48 hours and were different for protein and DNA immunised mice; 18,420 and 54,000 cpm respectively. A weak response to mitogen may be due to the presence of in vivo activated effector cells in the culture that are not capable of responding to mitogen. On the other hand, the maximum responses to hCEA are only approximately 4000 cpm. In the literature on DNA immunisation against hCEA the proliferation responses reported are also not
remarkable. In three papers, proliferation of spleen cells of a total of 15 mice immunised with hCEA vectors has been reported (Conry, LoBuglio et al. 1994; Conry, LoBuglio et al. 1995; Conry, Widera et al. 1996). In response to hCEA protein, the splenocytes of six of these mice incorporated 1000 cpm or less $^3H$-Thymidine. Another six showed incorporation levels of between 3000 and 6,900 cpm and the last three had incorporated 9000, 11,000 and 26,000 cpm of $^3H$-Thymidine each. These data indicate that, firstly, the lymphoproliferative responses to hCEA immunisation with plasmid DNA are variable, and secondly, even the best responders do not generate very strong lymphoproliferative responses.

**Figure 3.8:** In vitro proliferation of T cell enriched splenocytes of individual mice immunised with hCEA protein in Complete Freund’s Adjuvant (CFA) or hCEA encoded in pVAC/hCEA plasmid. The splenocytes were restimulated *in vitro* with 50 μg/ml of hCEA or BSA proteins and their proliferation was measured as $^3H$-Thymidine incorporation into cellular DNA. The background proliferation of splenocytes and feeder cells together was <1000 cpm for both methods of immunisation. Each point is the mean of a triplicate.
In this experiment, the splenocytes from another 3 mice immunised with the same plasmid did not respond (data not shown). Moreover, control splenocytes from mice immunised with the empty vector were not used in the assay. Therefore, it is not possible to conclude that a specific anti-hCEA response was demonstrated. This assay was repeated in subsequent experiments where larger groups of mice were used, however, a specific proliferative response to hCEA could not be demonstrated. Moreover, proliferation at later time points and with alterations in other parameters such as T cell enrichment by B cell adhesion to plastic, varying the numbers and types of feeders (e.g. splenic DC's) were also studied with no success in demonstration of specific anti-hCEA Th responses.

In order to raise CTL responses against hCEA, mice were injected intramuscularly and boosted twice at two weeks intervals with pVAC/hCEA. Ten days after the final injection responder splenocytes were cultured for six days with mitomycin-c (MMC) treated RMA/hCEA stimulator cells.

Figure 3.9 shows that at different effector to target ratios (E:T) more RMA/hCEA cells were lysed than RMA cells which do not express hCEA. However, at each E:T ratio between 100:1 and 25:1, 2/3 of RMA cells compared to RMA/hCEA were also lysed. The level of this 'nonspecific' lysis was proportional to the E:T ratio. On the other hand, between E:T ratios of 25:1 and 3.125:1 a plateau of 20% lysis of RMA/hCEA has been reached. Therefore, the 20% may be the maximum specific lysis achievable by the CTLs in this splenocyte population within the 4 hour assay time and the further increase in lysis, between E:T ratios of 25:1 to 100:1, may be nonspecific. However, it is possible that since RMA/hCEA were used *in vitro* for restimulation of these
splenocytes, the lysis of RMA/hCEA is only an artefact caused by *in vitro* priming. In order to rule out this artefact, control splenocytes that were incubated *in vitro* with RMA/hCEA had to be tested in parallel with cells from pVAC/hCEA immunised mice.

**Mice immunised with pVAC/hCEA, restimulated in vitro with RMA/hCEA cells**

![Graph showing percentage cytotoxicity of splenocytes](image)

**Figure 3.9:** Percentage cytotoxicity of splenocytes from a mouse immunised with pVAC/hCEA and restimulated *in vitro* with RMA/hCEA against RMA and RMA/hCEA cell lines.

Figure 3.10 shows the % cytotoxicity of naive and pVAC/hCEA immunised splenocytes against RMA/hCEA and RMA target cells after six days of coincubation with MMC treated RMA/hCEA. The splenocytes from a DNA immunised animal lysed more target cells than the ones from a naive mouse at every E:T ratio. The level of lysis of RMA cells by both populations of splenocytes was zero at all E:T ratios (not shown).
Figure 3.10: Percentage cytotoxicity of splenocytes from a naive or a pVAC/hCEA immunised animal against RMA and RMA/hCEA cell lines. Both populations were coincubated in vitro with RMA/hCEA for six days before the cytotoxicity assay.

There is a very high background of lysis of RMA/hCEA in this experiment. This could be attributed to in vitro priming against epitopes presented by RMA/hCEA cells. Nevertheless, the higher levels of cytotoxicity by pVAC/hCEA splenocytes could not be attributed to anti-hCEA CTL without cloning the CTL line. This is because there is a possibility that other cell types such as non specific NK cells were responsible for the cytotoxicity since DNA immunisation may boost NK cell numbers and activity (Ballas, Rasmussen et al. 1996). Appropriate controls for this could be pVAC/empty immunised mice. Moreover, even if CTL were responsible for the cytotoxicity, they could have been raised against other epitopes transcribed by both pVAC/hCEA and the pRCCMV/hCEA which was used for transfection of RMA cells. Indeed, recently it has been shown that in DNA vaccination strong CTL responses
against the product of a transcript from the ampicillin gene sequence can arise (van Hall, van de Rhee et al. 1998). This ampicillin sequence is transcribed by the upstream promoter activity of the viral promoter in the vaccine vector.

In a repeat experiment when pVAC/empty immunised mice were used as controls, pVAC/hCEA immunised splenocytes did not show specific cytotoxicity. Therefore, in these experiments a clear and reproducible anti-hCEA CTL response was not demonstrated. All the experiments described so far were performed in small numbers of mice. Even in such small numbers there was evidence of variation of responses. This raised the possibility that due to variation in responses and pooling of splenocytes, the responding cells were diluted and hence demonstration of cellular responses was hindered. Therefore, it was decided that immunisations should be repeated in groups with larger mice in order to obtain a clearer picture of the frequency of responders. Moreover, the cellular responses in individual mice ought to be investigated.

**Variability of response in larger groups of mice**

In order to investigate cellular and humoral responses in individual mice, during an immune response that appeared to be variable, larger numbers of mice were immunised. Eight mice were injected in the quadriceps muscles with pVAC/hCEA and boosted 2 weeks later. Three control mice were immunised in the same way with pVAC/empty. In the previous experiments, humoral responses were not detectable until four weeks post immunisation and hence the kinetics of the response was slower than that of conventional immunisations. Hence, in this experiment the mice were bled 2, 4 and 6 weeks after the initial immunisation in an attempt to cover later stages of the response.

Figure 3.11 shows that there is a considerable variation in the anti-hCEA antibody responses between mice immunised with pVAC/hCEA. Moreover,
only three out of eight mice produced detectable levels of anti-hCEA responses. The mean of anti-hCEA response increased with time (fig. 3.11b), however, this increase was not statistically significant. This could have been due to the low numbers of responders in the group. The sera from the responding mice showed very low titres. Anti-hCEA antibody activity diluted rapidly even in those sera with the highest titres (fig. 3.11, mouse #5). This may have been due to the presence of high affinity antibodies in low concentrations. Such a situation would require isotype switched plasma cells in low numbers.

The splenocytes from all the mice in this experiment were evaluated for recall responses in both lymphoproliferative and CTL assays. However, no positive responses was demonstrated.

In this experiment, it became clear that the humoral responses were weak and varied between different mice. The variation may be because in some mice the activation signals to Th cells had not reached the required threshold levels. On the other hand the signals could have reached the activation threshold levels but were not enough to result in large detectable expansions of the responding cells. The current understanding of T cell activation is based on the paradigm that for T cells to be activated a threshold level has to be reached as a result of cumulative antigen specific signalling through the T cell receptor and a second signal provided by costimulatory molecules. In this experiment, lack of the antigen specific signal could have had several explanations. The immunisation procedure may fail to transf ect enough muscle cells or any other cell types that would function as antigen synthesis depots, resulting in antigen not being available. The rate of antigen synthesis that is dependent on the promoters used, could have resulted in low antigen levels. The lack of availability of antigen to APCs could have resulted in a failure to reach the
Chapter 3

threshold. On the other hand, lack of costimulation due to the absence of adjuvants could have been a reason for the low levels and variation in responses. This was addressed in later experiments and will be discussed in the next chapter.

**Figure 3.11:** a) The relative levels of anti-hCEA antibody responses in a group of eight mice immunised with pVAC/hCEA (1-8) compared with three mice immunised with pVAC/empty ((1)-(3)). Each bar represents a single animal. b) Mean of anti-hCEA antibody levels of all the eight mice immunised with pVAC/hCEA.
Other groups have addressed the first possibility, that the immunisation procedure may not effectively transflect muscle cells. It was found that longitudinal instead of perpendicular i.m. injection (Levy, Barron et al. 1996), injection of DNA in saline rather than hypertonic fluids (Wolff, Williams et al. 1991) and injection of mice between 4-6 weeks of age (Wells and Goldspink 1992) gave better levels of antigen expression by muscle cells. Hence, the above findings were taken into account in our subsequent experiments. The promoters used in our plasmids were Rous sarcoma virus (RSV) early promoter. This promoter was used in many DNA vaccination studies. It was also compared with other promoters for the level of protein expression in muscle (Levy, Barron et al. 1996) and was found to be as effective as the other commonly used promoters.

The form of antigen could influence the availability of antigen to APCs. If cells other than APC produce the antigen, a secreted antigen would be more readily available. On the other hand, for the T cell response, epitopes generated within the APC from a given antigen are critical for class I-restricted CTL responses. These points were considered in the next experiment.

Immunisation with hCEA genes coding for different forms of hCEA

In the previous experiments it became clear that the responses to hCEA were weak and not reproducible after DNA immunisation. In this experiment we set out to compare the immunogenicity of hCEA with that of hCEADo in larger groups of mice. Since hCEADo is a secreted protein but hCEA is membrane bound, for comparison of the immunogenicity of epitopes of hCEA with those of hCEADo, a construct was made which coded for a membrane bound form of hCEADo (hCEADoTM). To explore the effect of antigen shedding a group was also included that were injected with the construct
coding for the secreted form of hCEADo. Moreover, to construct the pVAC/hCEADoTM a short sequence of DNA that coded for a 12 a.a. long tag sequence was removed from the end of hCEADo sequence and replaced by a sequence coding for a transmembrane domain (see fig. 3.1). Therefore for the purpose of comparison, the same tag sequence was replaced with a stop codon to construct the pVAC vector that coded for the secreted form of hCEADo.

Groups of ten mice were injected in the quadriceps muscles with each one of the plasmids and boosted 2 weeks later. The mice were then bled 4, 6 and 8 weeks after the initial injection. Figure 3.12 shows the anti-hCEA antibody responses of these mice. The units were based on the A5B7 mAb such that the optical density obtained with 1μg/ml of A5B7 in each ELISA plate was taken to be equivalent to 1000 mAb units.

As was observed in previous experiments, in this experiment there was also a high degree of variation and a low level of response. The only group of mice that developed consistently any anti-hCEA responses was the one immunised with pVAC/hCEA. This made interpretation of the data in terms of the immunogenicity of secreted versus non secreted antigen impossible. However, figure 3.12 shows that in this experiment pVAC/hCEA is more immunogenic than pVAC/hCEADo or pVAC/hCEADoTM. Comparison of the responses to pVAC/hCEADo in this experiment with earlier experiments in smaller groups of mice shows that there is a much lower number of responders than expected in this experiment. This could be due to the high degree of variability that has been observed all along, however, this comparison is not valid since the vector expressing hCEADo used earlier was slightly different from the one used in this experiment. The vector used for delivering hCEADo in the previous experiments had an extra 36 base pairs coding for a tag
sequence (see fig. 3.1). In order to assess the role of tag in immunogenicity the next experiment was performed.

**Figure 3.12:** Anti-hCEA responses of four groups of 10 mice immunised with pVAC vectors coding for hCEA, hCEADo or hCEADoTM. As a control pVAC/empty was used. Each bar represents a single animal. (1000 mAb units=OD$_{450}$ obtained by 1µg/ml of A5B7 mAb)
Anti-hCEA responses after immunisation with hCEADoTag

In the previous experiment, mice immunised with pVAC/hCEADo without a 36 b.p. long sequence of DNA coding for a tag peptide, did not develop any convincing anti-hCEA responses. This was in contrast to the earlier experiments in which the tag sequence was included immediately downstream of the hCEADo insert (see figs. 3.5, 3.6, 3.7). The tag sequence codes for a peptide that binds streptavidin (Schmidt and Skerra 1993). The sequence of tag is as follows:

GCC GCA AGC GCT TGG CGT CAC CCG CAG TTC GGT GGT
ala ala ser ala trp arg his pro gln phe gly gly

The nucleotide sequence of tag contains two CpG immunostimulatory motifs (in bold letters) and the amino acid sequence is made of mainly hydrophobic residues (in bold letters). In all the previous experiments the plasmids that resulted in development of any immune response coded for proteins with a hydrophobic tail. For instance pVAC/hCEA or pVAC/hCEADoTag or pVAC/hCEADoTM have hydrophobic C-terminal residues. This could allow the anchoring of these proteins into membrane structures. Therefore, in this experiment four groups of 10 mice were immunised with pVAC/empty, hCEADo, hCEADoTag, hCEADoTM to address two questions. First, the ability of tag to increase the number of responders and second, whether anchoring of the antigen has any role in the increased number of responders.

Four groups of ten mice were injected in the quadriceps muscles with each one of the plasmids and boosted 2 weeks later. The mice were bled 4 and 6 weeks after the first injection. At 4 weeks post immunisation there were not any detectable anti-hCEA antibody responses. However, at 6 weeks post immunisation half of the mice in the group that were immunised with
pVAC/hCEADoTag had developed significant anti-hCEA antibody responses (fig. 3.13). These results clearly demonstrate that the presence of the tag sequence immediately down stream of hCEADo gene result in an increase in the number of mice that develop an anti-hCEA antibody response. Moreover, the hypothesis stating that the increased number of responders, when tag is present, is due to the anchoring ability of tag is not supported in this experiment, since pVAC/hCEADoTM immunisation did not stimulate any responses.

The effect of tag in this experiment could have two explanations; first, the nucleotide sequence of tag could have an immunostimulatory effect and second the amino acid sequence coded by it could contribute to the increased immunogenicity. The next experiment addresses these questions.

![Antibody response 6 weeks after immunisation](image)

**Figure 3.13:** Anti-hCEA responses of four groups of 10 mice immunised with pVAC vectors coding for hCEADo, hCEADoTag or hCEADoTM. As a control pVAC/empty was used. Each bar represents a single animal. (1000 mAb units=OD450 obtained by 1μg/ml of A5B7 mAb)
Does the nucleotide or protein sequence of tag increase hCEADo immunogenicity?

The increased immunogenicity observed in the previous experiment could be due to the nucleotide or the amino acid sequence of tag or both. If the nucleotide sequence is responsible for the effect then the non transcribed form of the tag sequence should also have the same effect. On the other hand if the protein sequence is responsible then only the transcribed form can have an effect. Moreover, if the tag peptide had caused increased immunogenicity, it could have worked as part of the antigen structure or independent of the antigen molecule. However, this experiment was designed only to question what form of tag was responsible for the observed effect.

Five groups of eight mice were immunised as described in table 3.1 and boosted two weeks later with the same amounts of DNA. The mice were bled 4, 6 and 8 weeks after the initial immunisation.

<table>
<thead>
<tr>
<th>Group</th>
<th>Plasmid</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pVAC/empty</td>
<td>80µg in 80µl of saline</td>
</tr>
<tr>
<td>2</td>
<td>pVAC/hCEADo</td>
<td>80µg in 80µl of saline</td>
</tr>
<tr>
<td>3</td>
<td>pVAC/hCEADoTag</td>
<td>80µg in 80µl of saline</td>
</tr>
<tr>
<td>4</td>
<td>pVAC/hCEADo+pVAC/emptyTag</td>
<td>80µg+80µg in 160µl of saline</td>
</tr>
<tr>
<td>5</td>
<td>pVAC/hCEADoTag+pVAC/empty</td>
<td>80µg+80µg in 160µl of saline</td>
</tr>
</tbody>
</table>

Table 3.1: Dose and combinations of plasmid used for immunisation of 5 groups of 8 mice.

The data obtained from this experiment (fig. 3.14) did not shed any light on the question of the way tag had increased immunogenicity in the previous experiment (fig. 3.13). This could have been due to the high degree of variability of responses, as observed throughout our experience with DNA vaccination,
resulting in undetectable responses. The data may suggest that in this experiment there is only one responder and the attempt to immunise the other animals had failed. However, when the same animals were boosted with hCEA protein, it became clear that many had been primed against hCEA by the DNA immunisation (see below).

**Antibody responses after protein boost**

To investigate whether following DNA immunisation the problems of weak antibody responses and low numbers of responding mice could be overcome by boosting with soluble protein antigen, groups of mice were immunised with the pVAC/hCEADo (human carcinoembryonic antigen domains) vector that was shown in this chapter to give weak responses in a few mice. A group of sixteen mice were immunised with 100 µg DNA and then boosted two weeks later with 50 µg of DNA. Five mice were immunised in the same way with pVAC/empty as controls. Over the ten week period three of the mice in the experimental group produced low levels of anti-hCEA antibodies (fig. 3.15). Therefore, as expected weak antibody responses were detected in a low proportion of the animals.

After the last bleed, at 10 weeks, all the mice were immunised i.p. with a single dose of 25 µg of hCEA protein in PBS. Figure 3.16 shows that during the three weeks following protein challenge none of the mice initially immunised with pVAC/empty produced anti-hCEA responses. One week after the protein challenge, however, anti-hCEA antibodies were detected in five mice, two of which had not produced detectable responses previously. A total of 5 new responders were detected in the group initially immunised with pVAC/hCEADo during the 3 weeks period after antigen challenge (fig. 3.16).
Figure 3.14: Anti-hCEA antibody responses of five groups of mice 4, 6 and 8 weeks after immunisation with vectors that either expressed Tag or only contained Tag nucleotide sequence. Each bar represents a single animal. (1000 mAb units=OD450 obtained by 1μg/ml of A5B7 mAb; (1-8): mouse numbers).
Figure 3.15: Anti-hCEA antibody response of mice immunised with pVAC/hCEADo or pVAC/empty. The animals were boosted two weeks after initial immunisation with the respective vectors.

The level of anti-hCEA antibody in the mice that had produced detectable responses as a result of DNA vaccination increased 10-100 fold during the 3 week period after antigen challenge. The kinetics of antibody responses in these mice were varied; two of the mice showed 10 fold elevation of anti-hCEA at one week and reached peak levels by 2 weeks after challenge whereas in another mouse the peak levels were reached 3 weeks post protein challenge. The two mice that showed faster kinetics had also produced the strongest responses after DNA vaccination alone.
Figure 3.16: Anti-hCEA responses of mice after i.p. challenge with hCEA protein in PBS. These mice were immunised 10 weeks and boosted 8 weeks prior to protein challenge with pVAC/empty or pVAC/hCEADo.
Discussion

In this chapter, a set of experiments were described which were designed to assess the immune responses to hCEA DNA constructs in C57BL/6 mice. The evaluation of these responses proved to be difficult since, firstly, only a low number of mice gave detectable humoral responses, and secondly, these responses were very weak. Therefore, the mice used for evaluation of cellular responses had either not responded or their responses were so weak that they could not be detected. The variability, low number of responders and the inability to demonstrate T Helper (Th) responses despite immunoglobulin (Ig) class switching, seems to be a common experience of DNA vaccination workers [F. Stevenson and others, personal communications].

Although the literature on DNA vaccination concentrates on the relative successes of this technique, the variability of antibody levels and the absence of reports on Th responses are noticeable (Peet, Delves et al. 1995). The strong cytotoxic T lymphocyte (CTL) and antibody responses reported in some systems could be dependent on the particular antigens used (Ulmer, Donnelly et al. 1993; Martins, Lau et al. 1995; Iwasaki, Stiernholm et al. 1997). For instance, most of the reported CTL responses are against viral antigens while only poor humoral responses to hCFA have been reported (Conry, LoBuglio et al. 1994). In a report on immunisation against the major outer membrane protein (MOMP) of *Chlamydia trachomatis* antibodies could not be detected in groups of 10 mice immunised using a variety of immunisation regimens, whilst the same workers demonstrated immune responses to other antigens successfully (Strugnell, Drew et al. 1997). The experiments of Iwasaki A. et al. clearly demonstrate the antigen dependency of the strength of immune responses in DNA vaccination (Iwasaki, Stiernholm et al. 1997). They showed that the CTL responses to the
Kd-restricted NP 147-155 epitope from influenza A/PR/8/34 nucleoprotein were reduced almost to zero when three amino acids of the protein were altered. These amino acids were in a region remote from the CTL epitope. The mutant protein was still processed and presented since transfection of the vaccination vectors into P815 mastocytoma (H-2Kd) made them susceptible to lysis by influenza-specific CTL. Moreover, coinjection of mice with the mutant vector and an IL-12 expressing vector restored the CTL activity against this epitope. The authors have proposed the possibility of reduced levels of processing of the epitope causing the effect. A reduced level of peptide bound to MHC could indeed result in a reduction in the level of antigen specific signals delivered to T cells. On the other hand, the mutations could have resulted in reducing the costimulatory signals of T cell activation. This could have been caused by eliminating Th epitopes that may have been present in the protein, or by altering CpG immunostimulatory sequences that could have been present in the antigen gene. It is interesting that a Th stimulating cytokine such as IL-12 restored the CTL activity. Considering the low levels of antigen in DNA immunisation, it can be assumed that factors such as the presence of Th epitopes in the antigen can become very important. Thus, due to the low antigen concentration the T cells may be suboptimally stimulated. This could result in T cell tolerance or development of memory rather than effector T cells (see below).

Comparison of the immunogenicity of hCEA with hCEADo did not produce an unequivocal answer. However, in one experiment hCEA seemed to be more immunogenic (fig. 3.10). This may be explained by the possible presence of more Th epitopes in hCEA. For activation of Th cells the antigen specific signal is delivered by the binding of TCR to MHC class II and peptides originating mainly from proteolytic cleavage of the antigen in the
phagolysosomes. The MHC molecules are restricted in the choice of peptides that they can bind. This restriction depends on the structure of the MHC peptide binding groove. Thus, in an antigen such as hCEA only a limited number of peptide fragments can bind a specific MHC haplotype (H-2^b in the case of C57BL/6 mice). Therefore, it is possible that the whole of hCEA contains more peptides that can bind H-2^b than the two domains of hCEA. However, the availability of digested peptides to bind H-2^b is subject to very complex factors and mechanisms such that not all the potential H-2^b binding peptides may be processed. The selection of the peptides that are processed could depend on the structure and the folding of the antigen (Sercarz, Lehmann et al. 1993). Our current knowledge of antigen processing is not sufficient to predict accurately whether for instance the two domains or whole hCEA can provide more antigenic epitopes and the amino acid motifs of class II binding peptide are not easily predictable. This is because anchor residues and other interaction sites of class II binding peptides are degenerate in their specificity (Rammensee, Friede et al. 1995). Therefore, the empirical method remains the only completely reliable way of determining whether immune responses are stronger against hCEA or hCEADo.

Our data on the effect of antigen shedding on the outcome of DNA immunisation were uninterpretable. DNA vaccination with the secreted or the membrane bound forms of the rabies virus glycoprotein (Xiang, Spitalnik et al. 1995) or *Mycobacterium tuberculosis* antigen 85 (Ulmer, Liu et al. 1997) did not resulted in any detectable differences in the immune response.

It is reasonable to expect low antigen concentration to result in an appropriately weak antibody response. Considering the low antigen levels of DNA immunisation, in the experiments discussed in this chapter some of the mice may have had primed anti-hCEA T and B cells but at very low frequencies
resulting in low or undetectable antibody titres. Another feature of DNA immunisation is the longer time taken for seroconversion (Cox, Zamb et al. 1993; Hoffman, Sedegah et al. 1994; Peet, Delves et al. 1995). In all the experiments described in this chapter antibody responses could not be detected earlier than four weeks after the initial injections. Moreover, the kinetics of T cell proliferation in figure 3.8 also appears to be different. The proliferation of splenocytes from the protein immunised mouse is detected earlier than proliferation of DNA immunised splenocytes. Although a conclusion can not be made from a single experiment, it is interesting to consider that the faster kinetics may be due to a higher frequency of responder cells. The higher frequency occurring where a considerably larger amount of antigen is available for the priming of the same numbers of naive antigen specific T cells. In DNA immunisation the antigen concentration is very low and as a result a low frequency of antigen specific effector or memory cells may be present in the splenocyte population. The protein boost experiments provide support for this hypothesis.

After antigen challenge, three groups of mice could be identified. The first group were the mice that showed detectable antibody responses to DNA vaccination alone and produced strong antibody responses with fast kinetics after protein challenge. The second group did not show detectable responses to DNA vaccination but after protein challenge developed weak responses with slow kinetics. And the third group were the animals that did not develop any detectable responses to either DNA or protein immunisation. In the second group anti-hCEA responses had been primed by DNA immunisation but were not detectable. In this group the kinetics of response were slower than the first group. Slower kinetics might be attributed to a lower frequency of antigen
specific cells in these mice. In these animals, antigen alone was sufficient to
induce expansion of the responding clones to a detectable level.

In DNA immunisation, therefore, low antigen dose may contribute to
irreproducibility by not supporting large detectable expansions of the
responding cells. In the third group, antigen specific cells could not have
expanded sufficiently for detection after injection of antigen in PBS. This may
be either due to the failure of DNA immunisation in priming antigen specific
cells or the failure of protein immunisation to cause sufficient expansion. The
former could be because the antigen dose is below a threshold required for
induction of naive cells or within a range that could induce low zone tolerance.
The latter might have been as a result of such a low frequency of antigen
specific cells that the transient presence of antigen in PBS was not sufficient to
cause their expansion to detectable levels.

Challenge of the mice in the first two groups with protein in PBS
resulted in 10-100 fold boosts in antibody levels within two weeks. These levels
of anti-hCEA response had not been seen previously when pVAC vector was
used. In the absence of adjuvant, therefore, antigen dose appeared to be the
limiting factor in the magnitude of the antibody responses.

It is difficult to categorise the cells that responded to the protein
challenge as memory or effector cells. It is possible that ten weeks after DNA
immunisation, as a result of the presence of small amounts of antigen
continuously synthesised by the transfected cells, clones of effector cells with a
slow turnover were still present. As a result of antigen challenge these clones
may have expanded to produce the observed antibody response. If this was the
case, these cells could not be functionally distinguished from memory cells and
might represent immunological memory in situations such as persistent viral
infection or DNA immunisation. Alternatively, clones of T and B cells
responding to the protein challenge might have originated from memory cells that developed in response to DNA immunisation.

Despite the demonstration of increased antibody responses, helper T-cell responses could not be demonstrated after administration of protein antigen. In general, it appears that demonstration of lymphoproliferation after immunisation with DNA vectors is difficult (Conry, LoBuglio et al. 1994; Conry, LoBuglio et al. 1995; Conry, Widera et al. 1996; Boyle, Brady et al. 1998). This could not be because of the absence of helper T-cell responses as the presence of isotype switched antibodies is a good indication that Th responses were also present (Zinkernagel 1996). An explanation might be the that H-2^b Th epitopes of hCEA are poorly presented in this strain of mice. This question could be addressed by using different strains of mice or using model antigens that are known to contain strong class II epitopes. The failure to detect helper T cells despite the presence of isotype switched antibodies might be because small numbers of T-cell clones that were undetectable, could provide sufficient signals for the B cells to switch isotypes.

In a variety of models using different antigens, it has been observed that clones of CD8^+ cells expand more than CD4^+ cells in response to the same antigen (Maini, Casorati et al. 1999). In other words, helper T cells do not expand to the same level as the effector cells, i.e. CTL. Helper T cells may not need to expand to the same degree as the effectors as by interacting with more than one effector cell the response can be amplified. This situation is analogous to the above observation that after protein challenge, increased antibody responses could be detected without detectable helper T-cell responses.

The observation that protein antigen may have caused expansions of B cells and not T cells could be explained by the mode of antigen recognition by T and B cells. B cells can detect antigen in its native form whereas T cells can only
see antigen in the context of MHC molecules. As adjuvant was not used in the protein challenge experiment, it is possible that dendritic cells that took up antigen either did not migrate to the T cell areas of the draining lymph nodes (De Smedt, Pajak et al. 1996) or if they did, they were not capable of antigen presentation due to their immaturity (Banchereau and Steinman 1998).

The factors that influence the magnitude of antibody and T-cell responses after DNA immunisation are likely to be different. Boyle et al. have demonstrated that i.m. injection of DNA encoding a secreted form of ovalbumin (sOVA) resulted in stronger antibody responses than when membrane bound OVA (mOVA) or cytoplasmic OVA (cOVA) were coded by the plasmid vector. Strongest CTL responses, however, were obtained after i.m. injection of DNA encoding sOVA or mOVA. Furthermore, intradermal DNA immunisations followed the same patterns for antibody responses whereas the pattern of CTL responses differed such that they were also optimal in response to cOVA immunisations (Boyle, Koniaras et al. 1997). The magnitude of antibody responses after DNA immunisation, therefore, appear to depend on the availability of secreted antigen regardless of the route of immunisation. The disparity between the CTL responses to cOVA when injected i.m. or i.d. may be due to the availability of antigen to professional APCs. Intradermal immunisations could result in transfection of skin Langerhan's cells that would prime CTLs whereas in muscle the cytoplasmic form of the antigen would be sequestered from APCs. It is interesting that when DNA is injected i.d., despite the transfection of APCs, the antibody response to cytoplasmic or membrane bound forms of the antigen remain weak.

The magnitude of the antibody response, therefore, appear to be limited by the availability of antigen at sites distant from the place of injection and not by antigen availability to APCs. This may indicate that the magnitude of the
antibody response depends on the amount of native antigen seen by B cells in secondary lymphoid organs. On the other hand, the magnitude of T-cell responses may be limited by the accessibility of antigen to APCs as increasing this by intradermal injection of DNA resulted in stronger CTL responses. Therefore, as mentioned earlier, it is possible that in the protein challenge experiment B cells were stimulated by the injected native antigen whereas because the APC were not given any adjuvant signals the T-cell responses were not boosted.

As discussed previously, the tag sequence may have enhanced responses to hCEADo either through its nucleotide or peptide sequence. It has been shown that inclusion of certain hexameric DNA motifs in vaccination vectors can enhance antibody responses (Sato, Roman et al. 1996). The Tag sequence also contains a hexameric CpG motif. On the other hand, the peptide sequence of Tag may contain a Th epitope or may help to direct the antigen to a lymphoid compartment. It has been shown that both antibody and Th responses to antigen can be enhanced by fusion of the antigen gene with CTLA4 or L-selectin (Boyle, Brady et al. 1998). These fusion proteins would be expected to be targeted to APC and lymph nodes, respectively. This would increase the effective concentration of the antigen. It is interesting that these manoeuvres particularly in the case of CTLA4 resulted in faster seroconversion, higher antibody titres and demonstration of lymphoproliferative responses. Moreover, these results were shown not to be due to the immunomodulatory properties of CTLA4 since CTLA4 was only effective if it was fused to the protein. Hence, CTLA4 did not alter the immune responses when it was coinjected with the antigen whether they were injected in protein or DNA vaccine forms.

In this chapter, therefore, immune responses against the hCEA were weak and un reproducible. An explanation for this could be that the
immunisation regiments did not result in threshold levels of activation. A set of experiments are described in the next chapter that were designed to augment costimulatory signals as the means of reaching the activation threshold.
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The effect of LPS and tetanus toxoid fragment-c on DNA immunisation against hCEA
Introduction

In the previous chapter a set of experiments on DNA immunisation against hCEA were described. Variable and weak immune responses were the features of those experiments. The work described in this chapter sought to explore the reason(s) for the poor responses described in the light of present understanding of T and B cell activation. According to the current paradigm of T cell activation, both antigen specific and costimulatory signals are required for activation of naive T cells. The second costimulatory signals for T cell activation, are provided by ligation of T cell surface molecules such as CD28 or cytokines such as IL-2 that function in an autocrine or paracrine fashion (Jenkins and Johnson 1993). Two types of signals are also required for B cell activation to T-dependent antigens. A model of B cell activation is based on positive signals from activated Th cells via CD40 ligation that in turn induces Fas expression on B cells and could result in negative signals and apoptosis. The negative signals are overridden by triggering of the membrane Ig (mIg) when native antigen binds to mIg in germinal centres. B cells that have received CD40 and mIg signals would differentiate in response to cytokines such as IL-4, IL-13 and IL-10 (van Kooten and Banchereau 1997). Activation of T cells to express CD40 ligand (L) and secrete cytokines for B cell proliferation and differentiation depends on accumulation of positive intracellular signals to a threshold level through antigen specific (TCR) and costimulatory (CD28) pathways (Klaus, Pinchuk et al. 1994). The absence of detectable responses in some of the animals immunised in our studies might have been because an activation threshold had not been reached. Experiments described in this chapter were designed to augment the costimulatory signals by the use of lipopolysaccharides (LPS) or tetanus toxin fragment-c (Fr-c).

It has been demonstrated that LPS can act as an adjuvant for a variety of different antigens. LPS can cause an increase in antibody responses against
Echis ocellatus venom or sheep red blood cells in mice and tetanus toxoid in rats (Laing and Theakston 1993). This may have been mediated by TNF-α in the rat system. In a study by Parks et al. it was shown that LPS converted the tolerogenic antigen, deaggregated human gamma globulin (DHGG), to an immunogen (Parks, Walker et al. 1981). In this study, cell transfer experiments showed that the use of LPS resulted in activation of antigen specific Th and B cells. In vitro studies have demonstrated that LPS activated B cells can act as antigen presenting cells. In one study it was shown that LPS activated B cells can act as accessory cells for a con-A response or present chicken ovalbumin to Th cells (Krieger, Grammer et al. 1985). In another study LPS activated B cells induced CTL responses against vesicular stomatitis virus (VSV) in vitro (Ciavarra and Burgess 1988). Therefore, LPS enhances immune responses and this could be by increasing the second activation signal either by enhancing the antigen presentation ability of B cells or by other means such as stimulating the secretion of cytokines. Hypothetically also in the presence of antigen, antigen specific B cells may become preferentially selected to expand during LPS-induced polyclonal B cell activation.

As mentioned before, one possible explanation for the weakness of immune responses to hCEA may have been the lack of T cell help. Therefore we set out to investigate whether Fr-c could alter the immunogenicity of hCEA. Tetanus toxin (TT) has been used as a carrier for a synthetic peptide epitope of Plasmodium falciparum circumsporozoite (CS) protein (Herrington, Clyde et al. 1987). Various studies have shown that TT contains T helper epitopes that can be recognised by human T cell clones of varied specificity and MHC II restriction (Panina Bordignon, Tan et al. 1989; Reece, Geysen et al. 1993). Conjugation of a "universal" T cell determinant from TT was shown to result in induction of antibody responses to a Plasmodium falciparum peptide epitope that was not otherwise immunogenic in six strains of mice with different H-2 haplotypes (Kumar, Arora et al. 1992). In another study two other TT epitopes were conjugated to a Plasmodium falciparum epitope from CS protein and were
shown to result in augmentation of antibody responses against the protein (Valmori, Pessi et al. 1992). The non-toxic fragment-c of TT that encompasses some of the T helper cell determinants of TT has been studied as a vaccine against TT. It has been demonstrated that immunisation with a DNA construct containing the gene for Fr-c resulted in high anti-Fr-c antibody titres and protection against lethal challenge with TT (Anderson, Gao et al. 1997). Finally, in a DNA vaccination study, Spellerberg et al. showed augmentation of anti-Id antibodies against a single chain Fv (scFv) as a result of fusion of Fr-c with the scFv (Spellerberg, Zhu et al. 1997). On the basis of these observations we chose Fr-c to increase immunogenicity of hCEA and made an immunisation construct that contained hCEAfollowed by the gene for Fr-c.

We investigated humoral and cellular immune responses in all the immunisations described in this chapter. Therefore magnitude, frequency and kinetics of antibody responses to hCEA and in the case of the Fr-c experiments to both hCEA and Fr-c were studied. As in our previous experience Th recall responses to hCEA could be demonstrated, however, antibody isotypes were determined as a means of investigating the type of the immune response induced by different immunisation constructs. Two types of immune responses are thought to be mediated by Th1 and Th2 cell subsets. In mice Th1 cells predominantly secret IL-2 and IFN-γ and are associated with IgG2a secretion, whereas, Th2 cells secrete IL-4 and IL-5 and are associated with production of IgG1 (Mosmann, 1986; Stevens, 1988). Therefore, in order to determine what type of Th cell may be involved in an immune response, the T cells are restimulated in vitro and the profile of the cytokines secreted is determined by ELISA or ELI-spot assays. The cytokine profiles of Th cells could not be determined, since in vitro restimulation of Th responses after DNA vaccination proved to be difficult. Alternatively, determination of the antibody isotypes could indirectly point towards the Th type involved in the immune response. In some strains of mice such as C57BL/6 that contain the Igh1-b allele, the gene for IgG2a is deleted (Martin, Brady et al. 1998). These mice express a different
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isotype called IgG2c. In our experiments, for isotype detection we used a commercial antibody that was raised against IgG2c (see material and methods). However, it is not known whether isotype switching to IgG2c occurs as a result of IFN-γ signalling or any other events that occur as part of a Th1 response. In our experiments the mice produced either predominantly IgG1 or IgG2c with different treatments. This could be reasonably interpreted as two different types of response but cannot be categorised definitively as Th1 and Th2.

Finally, in this chapter the necessity of fusion of the antigen to the immunomodulatory Fr-c was studied by comparing the immunisation of the fusion construct to co-immunisation of separate hCEADo and Fr-c constructs. In addition, an experiment was performed that demonstrates priming of CTL in response to DNA immunisation and provides support for the hypothesis that the failure of CTL detection after our DNA immunisations may be due to a low frequency of CTL precursors.
Methods

Vaccination constructs

For the experiments on the adjuvant effect of LPS, pVAC/empty and pVAC/hCEA that were described in chapter 3 were used. pcDNA3/empty, pcDNA3/hCEADo and pcDNA3/hCEADoFr-c were used in the remaining experiments of this chapter. hCEADo fragment was cut out of pVAC/hCEADo using Hind III and Xba I restriction sites and inserted into the same sites of the pcDNA3 vector. hCEADoFr-c fusion was achieved in two stages. First, hCEADo and Fr-c were separately amplified. The 3' primer for hCEADo and the 5' primer for Fr-c had complementary sequences that extended beyond the sequences of their corresponding target genes. The complementary overlapping nucleotides were designed to code for a linker peptide of Gly-Pro-Gly-Pro sequence. In a second round of amplification the two PCR products were mixed after gel purification and a set of primers were used that bound to the 5' end of hCEADo and the 3' end of Fr-c genes. This fusion gene was cut by Hind III and Not I restriction enzymes, gel purified and inserted into the corresponding sites of pcDNA3. hCEADo and hCEADoFr-c were sequenced in order to detect possible mutations introduced by the PCR procedure, however, such mutations were not detected. Expression of protein by pcDNA3 vectors was not tested in vitro, however, in the experiments described in this chapter it will become apparent that all these constructs, regardless of the presence of Fr-c, induced antibody responses specifically against hCEA. This indicates that the proteins coded by these vectors were expressed, in vivo.
**Immunisation schedule**

Mice were immunised in the quadriceps muscles under anaesthesia. For the experiments on the adjuvant effect of LPS, control mice were given a single dose of 100 µg DNA at 1 mg/ml in saline. The experimental groups were injected with the same dose of DNA, which had been spiked with 5 or 10 EU of LPS. The batch of DNA used in this experiment was prepared using a commercial kit that incorporated an endotoxin removal step (see material and methods). For the experiments on the adjuvant effect of tetanus toxoid fragment c (Fr-c) mice were immunised with 50 µg and boosted twice with 50 µg of DNA at three weeks intervals. This protocol was used by Spellerberg *et al.* for immunisation with a scFv-Fr-c fusion construct (Spellerberg, Zhu *et al.* 1997).

**Measurement of antibody levels**

An Enzyme Linked Immunosorbant Assay (ELISA) was used to measure anti-hCEA or anti-Fr-c antibody levels. The ELISA plates were coated with hCEA or Fr-c protein in PBS. The mAb units were based on the A5B7 mAb. OD\(_{450}\) curves for different dilutions of the standard and the samples were constructed. The A5B7 standard curves were constructed such that the optical density obtained with 1mg/ml of A5B7 in each ELISA plate was taken to be equivalent to 1000 mAb units (see the methods chapter). An OD\(_{450}\) reading of a sample dilution that was in the linear part of the dilution curve was used to read the mAb units for that OD\(_{450}\) in the standard curve and then multiplied by the sample dilution to obtain the mAb units of anti-hCEA antibody in the serum.

The antibody isotypes were determined as above except that HRP-conjugated anti-mouse isotype specific instead of anti-mouse polyclonal Ig antibodies were used for detection. In C57BL/6 mice the IgG2a gene is deleted (Martin, Brady *et al.* 1998). In our experiments, a set of commercially prepared
antibodies that were specifically developed against antibodies of various isotypes of C57BL/6 mice origin, were used.

All the anti-Frc ELISA assays were performed by Dr. C.A. King at the Molecular Immunology Group, Southampton University Hospital, UK.
Results

LPS as an adjuvant for DNA vaccination

This experiment was performed to assess whether LPS can act as an adjuvant. The plasmid DNA used in this experiment was prepared using a commercial kit that had an LPS extraction step. After LPS extraction, different amounts of LPS were added in a fixed volume to aliquots of the same batch of DNA. Three groups of eight mice were injected in the quadriceps muscles with 100 μg of pVAC/hCEA mixed with 0, 5 or 10 endotoxin units (EU) of LPS and boosted 2 weeks later with 50 μg of the same preparation. For control pVAC/empty mixed with varying levels of LPS was injected in the same way, in to groups of 4 or 6 mice.

At four weeks post immunisation there were not any detectable anti hCEA antibody responses. Figure 4.1 shows a low level of anti hCEA response in all the groups immunised with pVAC/hCEA at six weeks post immunisation. Positive responses were considered to be the ones that were more than two standard deviations above the controls. There were 2, 5 and 2 responders in the group that received 0, 5 or 10 EU of LPS, respectively. One of the mice that were injected with 10 EU of LPS died after the first injection.
Comparison of the average antibody responses in the responder populations (fig. 4.2a) or all the mice in each group (fig. 4.2b) shows that LPS did not result in potentiation of the antibody responses. Therefore, LPS neither increased the number of responders nor potentiated antibody levels.

Finally, all the anti-hCEA antibodies detected were of IgG2c isotype.
Figure 4.2: a) Mean mAb units of the responding mice. There were 2, 5 and 3 responders in the groups that received 0, 5 and 10 EU of LPS, respectively. b) Mean anti hCEA levels of all the mice. The error bars represent standard error of the means.
Comparison of responses to hCEADo with hCEADoFr-c fusion protein

To compare the immunogenicity of hCEADo with that of hCEADoFr-c, three groups of 8 mice were immunised with pcDNA3/empty or pcDNA3 that contained the genes for hCEADo or hCEADoFr-c. The mice were boosted twice at three weeks intervals and bled 21, 42 and 63 days after the initial immunisation.

Anti hCEA antibody responses

Figure 4.3 shows anti hCEA responses of mice that were immunised with the above vectors during a 63 days period. This experiment clearly demonstrates that antibody responses to hCEA are stronger when hCEADo is fused with the tetanus toxoid fragment c (Fr-c). At day 21, six of the eight mice in the Fr-c group show weak anti hCEA responses. All the mice in the Fr-c group produced high levels of anti hCEA antibodies by day 42 compared to 1 mouse that produced low levels of antibodies in the group immunised with hCEADo. Therefore, the time taken for seroconversion is reduced and the antibody levels are higher when hCEADo is fused with Fr-c. Moreover, the number of responders was increased when this form of antigen was used.

A similar set of results was obtained when this experiment was repeated in groups of 12 mice (fig 4.4) and Fr-c again induced an increased number of responders. However, the antibody levels of responders were not very different between the two groups during the first 63 days. The difference in the levels of antibodies became apparent later in this group of mice (fig 4.5).
Figure 4.3: Anti hCEA responses of groups of eight mice immunised with a vector containing two N-terminal domains of hCEA (pcDNA3/hCEADo) or one coding for the two domains followed by the gene for fragment-c of tetanus toxoid (pcDNA3/hCEADoFr-c).
Figure 4.4: Anti hCEA responses of groups of twelve mice immunised with a vector containing two N-terminal domains of hCEA (pcDNA3/hCEADo) or one coding for the two domains followed by the gene for fragment-c of tetanus toxoid (pcDNA3/hCEADoFr-c).
Figure 4.5 shows that on day 110, the average of anti-hCEA antibody levels in the group immunised with the Fr-c fusion gene was higher than in the group immunised with hCEADo gene. This was the case both in the responding mice (fig. 4.5a) and the group as a whole (fig.4.5b).

**Figure 4.5:** 
**a)** Average anti hCEA antibody level in the mice that responded to immunisation with pcDNA3/hCEADo or pcDNA3/hCEADoFr-c. 
**b)** Average anti-hCEA antibody level of all the mice in groups of 12 that were immunised with pcDNA3/hCEADo or pcDNA3/hCEADoFr-c.

In contrast, in a third experiment the differences between responses to Fr-c containing and CEA alone constructs were less impressive and were only statistically significant when the responses of the whole group were considered (fig.4.6b). Nevertheless, even in this experiment there was a clear difference in
the frequency of responders in the group immunised with pCDNA3/hCEADo (2/10 responders) compared to pcDNA3/hCEAFr-c (6/10 responders).

**Figure 4.6:** a) Average anti hCEA antibody level in the mice that responded to immunisation with pcDNA3/hCEADo or pcDNA3/hCEADoFr-c. b) Average anti-hCEA antibody level of all the mice in groups of 10 that were immunised with pcDNA3/hCEADo or pcDNA3/hCEADoFr-c.

**Anti Fragment c antibody responses**

Only the mice that were immunised with the vector containing Fr-c developed anti Fr-c antibody responses (fig. 4.7). All the mice in this group developed anti Fr-c antibodies by day 21. The antibodies reached a peak level between days 21 and 63 and were declining on day 63.
Figure 4.7: Average of anti-Fr-c antibody responses in groups of 8 mice immunised with pcDNA3/empty, /hCEADo or /hCEADoFr-c. All the mice in the latter groups produced anti-Fr-c antibodies by day 21.

Comparison of the level of antibodies raised against hCEA with those against fragment-c in individual mouse sera shows that there is not an obvious relationship between these values (fig. 4.8). In particular two of the sera contain very high levels of either anti hCEA or anti Fr-c antibodies and very low levels or no antibodies to the other antigen (fig. 4.8). The majority of the samples, however, contain similar low levels of both antibodies. The absence of a pattern was also observed when the antibody levels to the two antigens were compared in day 63 sera (data not shown).
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Comparison of anti hCEADo to anti Fr-c responses on day 42

![Comparison graph](image)

**Figure 4.8:** Comparison of anti hCEADo with anti Fr-c antibodies in individual sera of 10 mice immunised with pcDNA3/hCEADoFr-c vector.

*Does fusion of hCEADo to Fr-c change the type of effector response*

In the previous section it became clear that Fr-c fusion could augment the antibody response. This could occur as a result of simple amplification of the clones of responding cells or in association with qualitative changes in the effector arm of the immune response. Such qualitative changes might shed some light on the mechanism through which Fr-c augments the immune response.

In one experiment where groups of 10 mice had been used, most of the mice immunised with the Fr-c vector produced anti-hCEA of IgG1 isotype and very few also produced small amounts of IgG2c. In contrast all the responding mice of the group that received hCEADo produced IgG2c antibodies (Table 4.1). Moreover, new isotypes emerged with time such that the hCEADo immunised mice produced IgG1 as well as IgG2c by day 63.
Table 4.1: Percentage of responding mice producing a particular antibody isotype.

Evaluation of the isotype profile of a group of mice that were immunised with the hCEADoFr-c vector in a different experiment showed that these mice did mainly produce IgG1 during the initial stages of the response (fig. 4.9). As the response evolved IgG1 levels decreased whereas IgG2b increased (fig. 4.9). Moreover, IgG2c antibodies appeared later and in lower quantities than the other isotypes. Anti hCEA IgG3 antibodies could not be detected at least until day 63. In this experiment there were only two mice that responded to immunisation with hCEADo. In these mice the anti hCEA antibodies that could be detected were of IgG2c isotypes (data not shown).
Figure 4.9: Different isotypes produced against hCEA in the pooled sera of 8 mice immunised with pcDNA3/hCEADoFr-c vector.

The same isotype profiles that developed against hCEA also developed against the Fr-c portion of the hCEA/Fr-c fusion protein (fig. 4.10). The data in figures 4.9 and 4.10 imply that a type of immune response developed against hCEADoFr-c antigen that in its initial stages was predominantly associated with IgG1 secretion. Since this isotype occurs in Th2 type responses, it can be assumed that the response to this antigen may be a Th2 response.
Isotypes of anti Fr-c antibodies in sera pooled from 8 mice immunised with hCEAFc vector

Figure 4.10: Different isotypes produced against Fr-c in the pooled sera of 8 mice immunised with pcDNA3/hCEADoFr-c vector.

The importance of fusion of Fragment-c to hCEADo

In order to further evaluate the mechanism of augmentation of anti hCEA response we set out to question whether fragment-c has to be fused to hCEADo or can function as a separate protein. Three groups of five mice were injected with hCEADo, hCEADoFr-c or co-injected with hCEADo and Fr-c constructs. Figure 4.11 shows that by day 21 anti hCEA responses could only be detected in the mice immunised with the vector containing the fusion gene. However, anti Fr-c responses had developed when the mice were injected with vectors containing either the Fr-c gene alone or as a hCEADo fusion construct.
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Anti Fragment-c antibody response

Anti hCEA antibody response

Figure 4.11: Anti hCEA and anti Fr-c responses in mice immunised with hCEADoFr-c fusion vector or separate hCEADo and Fr-c vectors.

Therefore, Fr-c could augment anti hCEA antibody responses only if it was fused to the antigen.

CTL responses against Fr-c

In chapter 3, there was evidence for a low frequency of CTLs responding to intramuscular DNA immunisation resulting in an inability to demonstrate such responses. In the experiments of this chapter, specific CTLs against hCEA or tetanus toxoid fragment c (Fr-c) could not be demonstrated following re-stimulation of splenocytes with hCEA transfectants or a class-I peptide epitope of Fr-c. We set out to investigate whether the absence of detectable CTLs was due to a low frequency or the absence of primed cells. In other words, did DNA vaccination prime naive CTL against the epitopes coded by the gene? To
address this question CTL responses of splenocytes from C57BL/6 mice to a known H-2^b class I binding peptide were studied. The use of a peptide epitope instead of transfectants would rule out the absence of CTL due to the inability of transfectants to present CTL epitopes.

Mice were injected intramuscularly and boosted twice at three weeks intervals with pcDNA3/hCEADoFr-c or pcDNA3/empty. Two weeks after the final injection responder splenocytes were cultured for six days with irradiated syngeneic splenocytes as feeder cells. The feeders were pre-incubated with Fr-c 7 peptide from fragment-c of tetanus toxoid and washed twice with medium before incubation with the responders. The medium was not supplemented with any cytokines but contained 10% FCS. On day six, some of the cells were used in a CTL assay and the rest were incubated with fresh peptide pulsed feeder cells for another six days for a second CTL assay. EL4 cells (syngeneic T-cell lymphoma) were used as target cells in the CTL assays. They were prepared by pre-incubation with Fr-c 7 or an irrelevant H-2^b binding peptide, the SV-9 Sendai virus peptide, and washed four times before the assay.

After six days of restimulation no CTL activity against Fr-c 7 could be detected in splenocytes from pcDNA3/empty or hCEADoFr-c immunised mice (fig. 4.12a). The splenocytes from pcDNA3/hCEADoFr-c immunised mice that were restimulated for 12 days, however, lysed a significantly higher proportion of the target cells at all E:T ratios than those from pcDNA3/empty immunised mice (fig. 4.12c). After 12 days of restimulation all the splenocytes lysed control target cells to an equal level at all E:T ratios (fig 4.12d). The small percentage of anti-Fr-c 7 cytotoxicity of pcDNA3/empty immunised splenocytes may be explained by in vitro priming against Fr-c 7. This ought to have occurred in the CTL population from pcDNA3/hCEADoFr-c immunised mice too; i.e. some of the lysis by pcDNA3/hCEADoFr-c splenocytes should also be due to T cells that were primed in vitro. The differences in the cytotoxicity, however, indicates that the hCEAFr-c splenocytes contained CTLs that were more responsive to restimulation; i.e. in vivo primed CTL.
On the other hand, the high percentage of lysis of EL4/SV9 cells by both populations could be attributed to in vitro priming against other epitopes present such as FCS or endogenous epitopes. This experiment, nevertheless, demonstrates that immunisation with pcDNA3/hCEADoFr-c resulted in priming of naive CTL against at least one of the epitopes coded by the vector.

**Figure 4.12:** Cytotoxicity of splenocytes from pcDNA3/empty or /hCEADoFr-c immunised C57BL/6 mice against EL4 cells coated with Fr-c 7 (a,c) or SV-9 (b,d) peptides 6 days (a,b) or 12 days (c,d) after in vitro re-stimulation with Fr-c 7 peptide pulsed irradiated syngeneic splenocyte feeders. Each value represents the mean % cytotoxicity of triplicates. (E:T= Effector to target ratio).
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Discussion

As reviewed in the introductory section of this chapter, LPS has been shown to render immunogenic a variety of non-immunogenic antigens. Parks et al. demonstrated that the use of LPS resulted in activation of T\textsubscript{H} cells that were crucial for B cell activation and production of antibodies to the DHGG antigen (Parks, Walker et al. 1981). Moreover, these T\textsubscript{H} cells were capable of providing help for a hapten conjugated to DHGG. Therefore, in the presence of sufficient amounts of antigen, LPS can act as an adjuvant to result in activation of both T\textsubscript{H} and B cells. However, in our system LPS did not appear to increase the number of responding mice. Hence, following the same line of argument, lack of costimulation or T cell help does not seem to be the limiting factor in our experiments. Although, the initial endotoxin content of our DNA preparation was, according to the manufacturer of the plasmid preparation kit, less than 1 EU/preparation, this value was never formally measured in our experiments. Therefore, the initial endotoxin content of our preparations could have been high enough to make the spiking of the samples futile. This would mean that there was initially enough endotoxin in the samples to provide a maximal adjuvant effect so the absence of an increase in the number of responding mice could have been due to factors other than lack of costimulatory signals. Alternatively, there is another explanation for the ineffectiveness of LPS in our system based on the differences in our method of immunisation with that of the reviewed literature. In all the systems that used LPS to augment or induce immune responses, the antigen injected was a protein. Therefore, the antigen and LPS were encountered by the immune system at the same time. However, in our experiments the antigen would have been synthesised by the transfected cells after a delay that may have been long enough to allow clearance of LPS from the system. This could be tested by injecting LPS at various times after DNA immunisation.
The presence of only IgG2c antibodies in all the group receiving LPS may be indicative of a Th1 type of response. In the literature on DNA vaccination there are consistent reports of Th1 responses as a result of intramuscular immunisation (Raz, Tighe et al. 1996; Carson and Raz 1997; Feltquate, Heaney et al. 1997). Moreover, the use of LPS may have resulted in stimulation of the innate immune machinery to drive the response towards a Th1 type through signals such as IL-12.

In the Fr-c experiments the number of responders was consistently increased to almost 100% as a result of using Fr-c. There could be a variety of explanations for this observation. Firstly, Fr-c could be providing T helper epitopes. If this is the explanation, Fr-c should only work as part of the antigen molecule, given the demonstration of linked help by Mitchison et al. (Boak, Mitchison et al. 1971; Britton, Mitchison et al. 1971; Mitchison 1971). Indeed, the final experiment of this chapter shows that Fr-c alters the immunogenicity of hCEADo only when it is an integral part of the antigen. In order to demonstrate whether Th cells were induced to Fr-c, Fr-c primed T cells could be tested to see whether they could provide help for hapten primed B cells in a cell transfer assay.

On the other hand Fr-c might have increased the effective concentration of the antigen by targeting the antigen to the lymphoid compartment or APCs. As demonstrated by Boyle et al. fusion of an antigen to CTLA4 or L-selectin can enhance immunogenicity of the antigen by targeting it to APC or the lymphoid compartment respectively (Boyle, Brady et al. 1998). In those experiments targeting of the antigen to APC resulted in production of IgG1 antibodies whereas all the other forms of the antigen stimulated IgG2a synthesis. This was also the case in our experiments and would make the hypothesis that Fr-c targets the antigen to APC more probable. This hypothesis can be further tested by purification of hCEADo and hCEADoFr-c proteins and comparison of their abilities to localise to APC.
Finally, the DNA sequence of Fr-c could have had immunostimulatory properties. However, this is unlikely since co-immunisation of hCEADo and Fr-c constructs did not result in potentiation of anti-hCEA antibody responses. It has been shown that immunostimulatory sequences can enhance immunogenicity by co-administration with the antigen (Roman, Martin Orozco et al. 1997). Moreover, when immunostimulatory DNA sequences are used as adjuvants, Th1 type of immune responses associated with IgG2a antibodies are induced. In our experiments the use of Fr-c resulted in IgG1 synthesis. Therefore, it is unlikely that Fr-c mediated enhancement of hCEADo immunogenicity was due to immunostimulatory properties of the DNA sequence of Fr-c. The Fr-c protein is most likely to potentiate anti-hCEA responses by providing Th epitopes or targeting antigen to APC. Under any of these circumstances helper T cells would be more efficiently recruited which in turn provide above threshold signals for activation of B cells resulting in sufficient expansion of B-cell clones that antibody becomes detectable.

Another feature of the Fr-c experiments was the variations in the antibody levels and the kinetics of the responses within and between the experiments. Figure 4.3 shows that the level of anti-hCEA antibodies is highly variable between the animals. This could be due to variations in the injection technique resulting in varied effectiveness of transfection of antigen synthesising cells since the same batch of DNA was used for immunisation of all the animals in each group. On the other hand there seems to be a difference in the average of antibody levels obtained in different experiment. Different batches of DNA were used for different experiments and therefore factors such as salt concentration and level of LPS contamination may have been responsible for the inter-experimental variation. The difficulty with controlling the injection procedure and the fact that in each experiment the same DNA was used, argues for developing methods that maximise the transfection of DNA into various antigen synthesising cells. Different groups have studied the use of cationic lipids for this purpose. Ishii, Davis and Gregoriadis et al. have reported that
cationic lipids can increase immune responses to DNA vaccination (Davis, Brazolot Millan et al. 1997; Gregoriadis, Saffie et al. 1997; Ishii, Fukushima et al. 1997). Yokoyama et al., however, have shown that although the use of cationic lipids could increase antigen expression, this does not necessarily result in a stronger immune response (Yokoyama, Zhang et al. 1996). Therefore, observations on the effect of cationic lipids in DNA vaccination are inconsistent, however, the effectiveness of better transfection has not been yet ruled out. The observations of Yokoyama et al. might be explained by the biological effect of the lipids they used or the tissues in which antigen expression was measured.

In the Fr-c experiments, anti-Fr-c antibody levels could not be related to the anti-hCEA antibody levels. In most of the animals there were similar antibody responses to both parts of the antigen and in a few animals the response was polarised to either the hCEADo or the Fr-c part. In other words, the antibody responses to different B cell epitopes of the same antigen when the same T helper epitopes were present occurred in an apparently random fashion. One feature of the polarised responses was that the magnitude of polarised antibody responses was higher than that in the non-polarised animals. This may reflect the capacity of the lymphoid compartment where a fixed number of Th cells are engaged in providing help for activation of different B cell clones.

Despite our attempts to demonstrate anti-hCEA Th responses at different time points, no such responses were demonstrated. Some explanations for difficulties in demonstrating T cell responses in DNA vaccination are given in chapter 3. The final experiment of this chapter demonstrates that CTL responses to an epitope that was coded by a pcDNA3/hCEADoFr-c could only be demonstrated after 12 days of in vitro culture, perhaps indicating that primed CTL precursors (CTLp) were at a low frequency. In addition, this experiment demonstrates the development of memory CTLp. This experiment is an example of weak effector responses after DNA vaccination and will be discussed further in chapter 7.
The augmented anti-hCEA antibody responses as a result of fusion of hCEADo to Fr-c may be sufficient to elicit a detectable response against tumours that express hCEA. This possibility is being investigated in tumour challenge experiments by our collaborators at Southampton University Hospital, UK.
Chapter 5

The effect of CpG DNA on antigen presentation
Introduction

The literature and our experiments provide evidence that immune responses to DNA immunisation differ from those to conventional protein immunisation. Sato et al. showed that an important component of a DNA vaccine is sequence motifs that contain unmethylated CG nucleotides flanked by two 5' purines and two 3' pyrimidines (Sato, Roman et al. 1996). Other workers have shown that these sequences cause Th1 skewing of the immune response (Raz, Tighe et al. 1996; Roman, Martin Orozco et al. 1997). We decided to further investigate these sequences with a view to gaining more insight into the mechanisms which resulted in such characteristic responses.

In recent years evidence that CG containing oligodeoxynucleotides have multiple effects on immune cells in vitro has been accumulating. CG oligodeoxynucleotide (ODN) has been shown to cause proliferation of splenocytes (Messina, Gilkeson et al. 1991) and purified B cells (Krieg, Yi et al. 1995), to provide costimulation for antigen specific activation of a B-cell line (Krieg, Yi et al. 1995), to induce splenocytes to produce IFN α/β and augment NK cell activity (Yamamoto, Yamamoto et al. 1992), to be taken up and activate macrophages (Stacey, Sweet et al. 1996) and to induce IL-6, IL-12 and IFN γ secretion by mouse splenocytes (Klinman, Yi et al. 1996). Therefore, CG ODN appears to cause activation, proliferation and cytokine secretion by a variety of immune cells. An integrated picture of the mode of interaction of CG ODN with the immune system, however, has not yet emerged despite the availability of numerous reports on its effects on different compartments of the system.

One interesting evolutionary aspect of unmethylated CG dinucleotides is that they are more commonly found in bacteria than in mammals. There is a lower frequency of CG dinucleotides in vertebrate DNA than expected to occur
by chance, whereas in bacteria, they are present at their expected frequency (Pisetsky 1996). In addition to "CG supression", 80% of CG dinucleotides are methylated in vertebrates. The immunostimulatory motifs have been shown to be hexameric with an unmethylated central CG (Yamamoto, Yamamoto et al. 1992). Methylated CG mutate at an accelerated rate and it is thought that they may be lost from the vertebrate genome by methylation in the germ line and mutation to TG or CA (Antequera and Bird 1993). On this basis, it has been speculated that these hexameric motifs may be recognised by vertebrate immune system as foreign in the same way as exclusively bacterial molecules such as LPS. The innate immune system can recognise unique bacterial molecules by specialised receptors refered to as pattern recognition receptors. Signalling through these receptors can alter the course of the adaptive immune response (Medzhitov and Janeway 1997). These observations suggest that the immune system may establish the source of an antigen via molecules found uniquely in the associated microorganism in order to raise appropriate responses against the pathogen. Unmethylated CG ODN may play such a role, taking into account its ability to stimulate Th1 responses against co-administered antigens and its relative exclusiveness to bacteria.

For our studies we chose an oligodeoxynucleotide that contained the CG motif and had been shown by Krieg et al. to provide costimulation for antigen specific activation of a B-cell line (Krieg, Yi et al. 1995). In our preliminary studies the effect of this oligodeoxynucleotide on splenocyte and mixed lymphocyte reaction (MLR) cultures was studied in order to observe detectable changes in function and composition of a population of immune cells in vitro. These experiments suggested a role for CG ODN in antigen presentation to T cells. In the following experiments the effect of this oligodeoxynucleotide on Bone Marrow derived Dendritic Cells (BMDC) was investigated.
Methods

**Splenocyte cultures**

Single cell suspensions of splenocytes were made by macerating mouse spleens through a plastic mesh in MEM plus 2% FCS. The cells were washed once with 10 ml of RPMI plus 10% FCS and were cultured in 96 well plates at 2.5x10^5 cells/well in 100 μl of RPMI supplemented with 10% FCS, 2ME and p/s. To study the effects of various reagents on splenocytes, 100 μl of medium alone or medium containing the reagents was added to triplicate wells.

**MLR cultures**

One way MLRs were set up as follows. The stimulator splenocytes were treated with 20 μg/ml mitomycin-c (MMC) for 20 minutes at 37°C then washed and resuspended in RPMI supplemented with 10% FCS, 2ME and p/s at 2x10^5 cells/ml. The responder cells were single cell suspensions of splenocytes prepared in the same way as splenocyte cultures but resuspended in medium at 10^7 cells/ml. 100μl aliquots of a mixture of equal volumes of stimulators and responders were placed in 96 well plates. To study the effects of various reagents on MLR cultures, 100μl of medium with or without these reagents was added to triplicate wells.

**CTL cultures**

Cytotoxic T Lymphocyte cultures were set up the same way as the MLR cultures except that different stimulator to responder ratios were cultured in 10
ml of medium in T25 flasks. Three stimulator to responder ratios of 1:50, 1:25 and 1:2 were used.

**Bone marrow derived dendritic cells**

BMDC were prepared according to the method described by Inaba et al with minor modifications (Inaba, Inaba *et al.* 1992). Muscle tissue was removed from mouse femoral bones and the end were cut off with sharp scalpels to leave the bone shaft. Bone marrow was flushed out of the femoral shafts with MEM plus 2% FCS using a syringe and a 25 gauge needle. A single cell suspension was made by vigorous pipetting in 20 ml of MEM plus 2% FCS. The suspension was left to stand for 2 minutes to allow fragments of bone and muscle to settle at the bottom of a universal tube then sucked slowly into a 10 ml pipette taking care to leave out the debris. The cells were recovered by centrifugation and resuspended in IMDM supplemented with 10% FCS, 5x10^{-5} M 2-ME, p/s and 5ng/ml of recombinant mouse GM-CSF (Autogen Bioclear, UK) at 3.33x10^5 cells/ml. The bone marrow cells were incubated at 37°C and 5% CO_2 horizontally in T25 flasks in 10 ml volumes. The flasks were gently shaken and the supernatants containing the non-adherent cells were removed on day 3. Fresh medium supplemented with GM-CSF was added to the flasks taking care not to disturb the monolayer. After 3 more days of culture, non-adherent cells had "immature" DC characteristics but became "mature" if they were recovered from the supernatants and re-cultured overnight with fresh medium and GM-CSF.

**Dendritic-cell stimulation of lymphocytes**

BMDC, on day six, were treated overnight with 1 µM CG or GC ODN or were left untreated then washed three times with 20 ml of medium before
Chapter 5

incubation with responders. The responder splenocytes were prepared in the same way as for the splenocyte cultures. In studies where "mature" dendritic cells were used, day six BMDC were harvested and reincubated overnight with granulocyte/monocyte-colony stimulating factor (GM-CSF) and the reagent of interest in fresh medium.

Proliferation and cytotoxicity assays

Proliferation was measured using a standard $^3$H-thymidine assay (see methods). A standard chromium release assay was used to measure cytotoxicity (see methods). The target cells used were the RMA T-cell lymphomas (H-2$^b$) and P815 mastocytoma (H-2$^d$).

Staining for cell surface antigens

All cell surface staining was performed as described in the materials and methods chapter. Monoclonal antibodies (mAb) M1/70 and N418 were used for detection of CD11b and CD11c, respectively. Anti-CD4 and CD8 mAbs were directly conjugated, hence as a negative control unstained cells were used. Positive cells were taken to be the cells that showed a stronger fluorescence than that obtained from splenocytes stained with isotype matched irrelevant immunoglobulins. The B220 antibody was not directly conjugated; cells that were stained with the second layer alone, therefore, were used as negative control for B-cell staining.

Detection of IL-12

A sandwich ELISA assay was developed for detection of interleukin-12 in supernatants of BMDC (see material and methods chapter). This assay had a sensitivity of 16 ng/ml.
Results

**CG oligodeoxynucleotides may interact with APCs**

This section describes the preliminary experiments that lead to forming the hypothesis that CG oligodeoxynucleotides may interact with antigen presenting cells to potentiate T-cell responses. The following sections are devoted to testing the above hypothesis.

Krieg *et al.* showed that oligodeoxynucleotides that contained unmethylated CG sequences which were flanked by two 5' purines and two 3' pyrimidines, with either phosphodiester or a nuclease resistant phosphorothioate back bone caused proliferation of B cells both *in vitro* and *in vivo* (Krieg, Yi *et al.* 1995). The sequence of one of these oligodeoxynucleotides was; 5'GCATGACGTTGAGCT3'. This oligodeoxynucleotide was used in all the experiments of this chapter. As a control an oligodeoxynucleotide was used that had the same sequence apart from the central CG nucleotides that were changed to GC; i.e. 5'GCATGAGCTTGAGCT3'. An experiment was performed to establish that the CG ODN caused proliferation of B cells in our hand and the GC oligo acted as a control. Single cell suspensions of spleen cells from C57BL/6 or BALB/c mice were incubated at 2.5x10^5 cells per well with 1μM of CG or GC ODN or medium alone. The cells were pulsed with ^3^H-thymidine for 14-16 hours and their proliferation levels measured at 24, 48, 72 and 96 hours (fig. 5.1).

Figure 5.1 shows the time course of proliferation of C57BL/6 splenocytes in response to oligodeoxynucleotides and B or T-cell mitogens. Splenocytes proliferated in response to the CG and not GC ODN and reached a peak at 72 hours. In other experiments the peak of proliferation was reached earlier for all
the mitogens when the background levels were high (e.g. fig. 5.2). BALB/c splenocytes proliferated to comparable levels in response to CG ODN with similar kinetics (data not shown).

**Figure 5.1:** Time course of proliferation of C57BL/6 splenocytes in response to CG (1μM) ODN, Con-A (5μg/ml) or LPS (0.5μg/ml).

**Figure 5.2:** Proliferation of splenocytes in response to three doses of CG ODN over a period of 96 hours.
The dose range of activity for the oligodeoxynucleotide was studied in the experiment shown in figure 5.2. This shows that CG ODN does not result in proliferation of splenocytes at a 0.001 μM concentration.

As mentioned before, the CG ODN used here was shown by Krieg et al. to cause mouse B-cell proliferation. Other CG oligodeoxynucleotides that contained the hexameric motif have also been shown to cause proliferation of human B cells (Liang, Nishioka et al. 1996). To establish what cells were proliferating in our system, single cell suspensions of splenocytes were stained with mAbs against CD4, CD8 and B220 surface molecules after incubation with oligodeoxynucleotides for 48 hours.

<table>
<thead>
<tr>
<th></th>
<th>no treatment</th>
<th>CG</th>
<th>GC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>37</td>
<td>13</td>
<td>28</td>
</tr>
<tr>
<td>CD8</td>
<td>8</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>B220</td>
<td>41</td>
<td>91</td>
<td>45</td>
</tr>
</tbody>
</table>

Table 5.1: Percentages of cells stained with antibodies against B and T cells in splenocytes that were treated for 48 hours with CG or GC ODN or were left untreated.

Therefore, table 5.1 shows that the percentage of B cells increased when they were incubated with CG oligodeoxynucleotide and not GC. This was accompanied by increased $^3$H-thymidine uptake (as in fig. 5.1) and an increase in the number of viable cells (table 5.2) as measured by trypan blue dye exclusion.

The increase in the percentage of B cells as a result of incubation with CG ODN was observed in three repeat experiments. There was, however, no
change in the percentages of different cell types when oligos were added to MLRs (table 5.3).

<table>
<thead>
<tr>
<th>Time</th>
<th>no treatment</th>
<th>CG</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.5x10^5</td>
<td>2.5x10^5</td>
</tr>
<tr>
<td>24</td>
<td>7.6x10^5</td>
<td>9x10^5</td>
</tr>
<tr>
<td>48</td>
<td>6.4x10^5</td>
<td>1.8x10^6</td>
</tr>
<tr>
<td>72</td>
<td>all dead</td>
<td>1.48x10^6</td>
</tr>
</tbody>
</table>

Table 5.2: The number of live cells/well in CG ODN treated or untreated splenocytes over a time period of 72 hours. Viable cells were counted by the trypan blue dye exclusion method.

<table>
<thead>
<tr>
<th></th>
<th>no treatment</th>
<th>CG</th>
<th>GC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>14</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>CD8</td>
<td>19</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>B220</td>
<td>43</td>
<td>48</td>
<td>36</td>
</tr>
</tbody>
</table>

Table 5.3: Percentages of cells stained with antibodies against T and B cell markers at 48 hours of allo-MLR cultures treated with CG or GC ODN or left untreated.

To test whether CG ODN had any effect on the outcome of MLR, allospecific cytotoxicity in the presence or absence of oligodeoxynucleotides was measured. MLRs with different stimulator to responder (S:R) ratios were set up in the presence of 1μM of CG or GC ODN or with no treatment (see methods). Figure 5.3 shows that CG and not GC ODN increased alloreactive cytotoxicity of splenocytes when sub-optimal numbers of stimulators were used.
Figure 5.3: Anti-H-2b cytotoxicity of BALB/c splenocytes cultured with MMC treated C57BL/6 splenocytes for 6 days in the presence or absence of CG or GC ODN. S:R = Stimulator : Responder, E:T = Effector : Target.
At a S:R ratio of 1:2 all the effector cells had maximum cytotoxicity regardless of the treatment. At a S:R ratio of 1:50 only the CG ODN-treated effectors were able to lyse allospecific target cells. The cytotoxicity of untreated effectors was less than the CG ODN-treated but more than the GC ODN-treated effectors at a 1:25 S:R ratio.

The percentage of T and B cells in MLR cultures, therefore, did not change, despite the development of allo-specific CTL. On the other hand B cells, but not T cells, proliferated in splenocyte cultures. Considering that effector CTL activity requires activation and proliferation of T cells, the absence of a change of percentages in MLRs might have been due to proliferation of both T and B cells. CG ODN, therefore, caused proliferation of B but not T cells in splenocyte cultures whereas in MLR cultures it enhanced T-cell activity. The latter might have been through the influence of CG ODN on the process of antigen presentation rather than as a result of direct action of ODN on T cells. Experiments to investigate the interaction of CG ODN with antigen presenting cells are described in the following section of this chapter.

Do CG oligodeoxynucleotides interact with DCs?

In the last section a series of experiments were described which raised the question whether CG oligodeoxynucleotides interacted with APC. To address this question, Bone Marrow derived Dendritic Cells (BMDC) were used. These cells were grown by treatment of mouse bone marrow cultures with Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF). This system was set up in our laboratory and had been shown to result in development of populations of cells with DC markers and characteristics (Drakesmith, PhD thesis). Thus, the BMDC expressed: CD11c, a molecule expressed by DCs and not other members of the monocyte/macrophage family;
high levels of MHC class I and II when at their mature stage; CD11b, expressed by ex-vivo macrophages and in vitro BMDC and B7-2. The B and T-cell markers B220 and CD3 were not expressed on these BMDC. These cells morphologically resembled DCs and stimulated allo-CTL and MLR at low numbers. At day six of the culture, they appeared to have immature DC characteristics such as low MHC class II levels and an inability to present antigen but could acquire the mature phenotype if they were re-incubated with fresh GM-CSF over night (Drakesmith, PhD thesis).

Effect of CG ODN on antigen presentation by mature BMDC

The effect of CG ODN on the ability of mature DCs to stimulate allogeneic and syngeneic MLR was examined in this experiment. Day six immature DCs were incubated overnight with GM-CSF and CG oligodeoxynucleotide at 1 μM then washed with 20 ml of medium three times and co-incubated for four days with non-adherent splenocytes at different DC to splenocyte ratios. The cells were pulsed with $^3$H-thymidine for the last 14-16 hours of the culture before proliferation was measured.

Figure 5.4 shows proliferation of BALB/c or C57BL/6 splenocytes when stimulated with BALB/c BMDC. The proliferation values shown here are averages obtained from triplicate cultures. The DC dose-response curve has shifted to the left in both syngeneic and allogeneic MLRs as a result of treatment of mature DCs with CG ODN. In both allogeneic and syngeneic MLRs, at a responder to DC ratio of 15:1, the stimulation indices of CG ODN treated DCs were approximately two fold higher compared with GC ODN treated or untreated controls. As shown in figure 5.2 splenocytes do not proliferate in response to CG ODN at a 0.001μM concentration. As DCs that were incubated with 1μM of ODN were extensively washed before setting up
the MLR, the responses observed here are unlikely to be as a result of direct stimulation of T or B cells by ODN. This experiment, therefore, provides some evidence for the potentiation of the antigen presentation capacity of DCs in response to CG ODN.

![Graph](image)

**Figure 5.4:** Proliferation of BALB/c or C57BL/6 splenocytes after 4 days of incubation with BALB/c BMDC treated on day 6 with GM-CSF or GM-CSF plus CG or GC ODN, overnight. DCs incubated on their own gave cpm values of <800.
Effect of CG ODN on antigen presentation of immature BMDC

Immature DCs, in vivo, are capable of capturing and processing antigen but become able to present antigen only when they mature in response to inflammation (Banchereau and Steinman 1998). In the previous experiment CG ODN was incubated with DCs that were receiving maturation signals from the GM-CSF in the medium. The next experiment was designed to question whether CG ODN can enable immature DCs to present antigen; i.e. functionally mature the immature DCs.

As mentioned before, BMDC have an immature phenotype on day six of culture with GM-CSF and remain so unless they are cultured overnight with fresh GM-CSF. In this experiment oligodeoxynucleotides were added at 1µM to day six BMDC cultures. The next day the DCs were washed with 20 ml of medium three times and set up with syngeneic and allogeneic non-adherent splenocytes.

Figure 5.5 shows that immature DCs pre-treated with CG ODN can stimulate strong MLRs. The proliferation indices, compared to untreated or GC treated DCs, are 6 and 13 fold higher for syngeneic and allogeneic MLRs respectively, at a responder to DC ratio of 5:1. This experiment was reproducible when BMDC from BALB/c, CBA or C57BL/6 mice were used. Therefore, CG ODN can cause functional maturation of BMDC and potentiate their ability to stimulate allogeneic and syngeneic splenocytes.

As a result of maturation, DCs are known to upregulate expression of MHC class II and costimulatory molecules. This was examined in the next experiment.
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**Figure 5.5:** Proliferation of BALB/c or C57BL/6 splenocytes after 4 days of incubation with BALB/c BMDC that were treated on day 6 with CG or GC ODN, or left untreated overnight. DCs incubated on their own gave cpm values of <800.

**Phenotype of BMDC treated with CG ODN**

The previous experiment demonstrated that immature BMDC became functionally mature as a result of treatment with CG ODN. In this experiment the possible mechanisms behind the functional maturation were investigated. Antigen presentation involves direct interaction of APC with the responding...
cells as well as paracrine signals, some of which are produced by the APC. According to the current paradigm, the presenting molecules (MHC class I and II), intercellular adhesion molecules (ICAMs), costimulatory molecules (B7-1 and 2) and cytokines such as IL-12 could potentiate antigen presentation. We used the available panel of antibodies in our laboratory to investigate possible changes to the expression levels of these molecules after CG ODN treatment. As in the previous experiment immature DCs were treated overnight with 1μM CG, GC ODN or were left untreated. The DCs were stained with antibodies against MHC class I and II, B7-1 and 2, CD11b and CD11c and were also set up in MLR assays. There was not a significant difference between B7-1, B7-2 or MHC class I expression on CG ODN treated or control DCs. Approximately 50% of the cells expressed B7-2 whereas 100% of the cells in all three groups stained for MHC class I and B7-1. The percentage of cells stained for CD11b, CD11c and MHC class II, however, increased in the populations treated with CG ODN, in three repeat experiments (Table 5.4). These DCs were also functionally mature in the MLR assays.

Figure 5.6 shows typical histograms of MHC class II staining of DCs. Examination of these histograms and comparison of mean fluorescent intensities shows that the number of cells expressing MHC class II has increased but the average level of expression has stayed the same. In one experiment out of six, however, the percentage of DCs expressing MHC class II and the mean fluorescent intensity of staining for MHC class II increased in the populations treated with CG ODN (fig. 5.7).
Figure 5.6: A higher percentage of bone marrow derived dendritic cells stains for MHC class II when treated over night with CpG DNA.

Figure 5.7: A higher mean fluorescent intensity (MFI) was obtained in one of six experiments where CpG treated bone marrow derived dendritic cells were stained for MHC class II expression.
Table 5.4: Percentage of cells stained for CD11b, CD11c or MHC class II in immature BMDC cultures untreated or treated overnight with CG or GC ODN.

Table 5.4 shows that not all the cells in the population stain for the DC marker CD11c (Steinman 1991). It is possible that as a result of CG ODN treatment cells other than DC acquired class II expression. This possibility was investigated by double staining for MHC class II and CD11c. Table 5.5 and figure 5.8 show the results of this experiment.

Table 5.5: Percentage of immature BMDC, treated overnight with CG ODN, that express MHC class II and CD11c alone or together.

The data in table 5.5 shows that 66% (55/82%) of the cells expressing class II MHC are also CD11c positive. This value was 51% for GC ODN treated and 20% for untreated cultures. CG ODN, therefore, increased the percentage of MHC class II expressing cells that had a DC marker. In addition, the previous experiment showed that the number of cells expressing MHC class II or the DC marker CD11c increased as a result of CG ODN treatment (table 5.4). CG ODN
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treatment, therefore, resulted in more cells acquiring the DC marker CD11c as well as MHC class II.

A characteristic of mature DCs is their ability to secrete IL-12 which could contribute to their ability to stimulate proliferation of responders in a MLR (Banchereau and Steinman 1998). This was investigated by measuring IL-12 levels in supernatant of DCs using an ELISA assay. Three aliquots of a single cell suspension of bone marrow in medium supplemented with GM-CSF were cultured in three T25 flasks. On day 3 the supernatants containing the non-adherent cells were discarded and 10 ml of fresh medium and GM-CSF was added to each flask. On day 6, CG or GC was added to two of the flasks at a final concentration of 1μM. The next day the cells were centrifuged and the supernatants were used in an IL-12 ELISA assay (see methods). In two out of 6 experiments the cultures treated with CG ODN contained 39 ng/ml and 65 ng/ml of IL-12. IL-12 could not be detected in the supernatants of untreated or GC ODN treated cells.

Cells stimulated by CG ODN-treated DCs

In the preliminary experiments of this chapter it was observed that when splenocytes were treated with CG ODN the B cells proliferated. This was detected by 3H-thymidine uptake and an increase in the percentage of B cells in the splenocyte population. However, when an MLR culture was treated with CG ODN the percentages of T and B cells did not change despite the presence of proliferating cells. A possible explanation for this observation is that T and B cells were both proliferating in the MLRs. The reason for T cells not proliferating in splenocyte cultures may be that they do not respond directly to ODN and that the percentage of DCs in these cultures is very low, hence, in the MLR cultures they proliferated in response to APCs which were presenting the
highly immunogenic alloantigens. On the basis of this reasoning the hypothesis that CG ODN may potentiate the process of antigen presentation in MLR was formed and it was shown that CG ODN does potentiate the antigen presentation capacity of BMDC. The following experiment was done to further test the explanation for the absence of change in the percentages of B and T cells. If the explanation is that in MLR cultures B cells proliferate directly to ODN but T cells proliferate in response to APCs then purified T and B cells should behave in this manner.

![Proliferation of enriched T or B cells in response to syngeneic dendritic cells pre-treated with oligonucleotides](image)

**Figure 5.8:** Proliferation of $10^5$ syngeneic T or B cell enriched populations, 4 days after incubation with $3 \times 10^4$ or $1.5 \times 10^4$ DCs which were untreated or pretreated with CG or GC ODN.

Immature DCs were treated with ODN or were left untreated overnight, then washed and set up with enriched populations of T or B cells in an syngeneic MLR assay. B-cell enrichment was performed by positive selection using B220 mAb in a magnetic bead system. The negative fraction after
repeated rounds of passage through the magnetic column was taken as the T cell fraction. In the B-cell fraction a total of 5% of cells stained for CD4 and CD8 whereas 2.3% of cells were stained with B220 in the T-cell fraction. Figure 5.9 shows that maximum proliferation could be detected in the T-cell fractions that were stimulated by DCs pre-treated with CG ODN.

There was a small level of proliferation in response to CG treated DCs in the B cell fraction. This may be attributed to the 5% T cells that could be detected in this fraction. Staining the proliferating cells for B and T cell markers could have resolved this issue. It appears, nevertheless, that CG ODN-treated DCs cause proliferation of T cells but not B cells.
Discussion

Studies on CG ODN have provided evidence that support a role in modulation of immune responses for these molecules. In vivo studies have shown that CG ODN acts as an adjuvant (Sato, Roman et al. 1996; Roman, Martin Orozco et al. 1997) that induces Th1 responses (Raz, Tighe et al. 1996; Roman, Martin Orozco et al. 1997). A possible mechanism by which CG ODN could act as an adjuvant is through provision of costimulatory signals during antigen presentation. This explanation was investigated in this chapter by studying the effect of CG ODN on APCs.

Preliminary experiments showed that B cells proliferate in response to CG ODN. Assuming that cultured splenocytes represent a model of the immune system in a secondary lymphoid organ where induction of the immune response is thought to occur, B-cell proliferation alone does not explain potentiation and Th1 skewing of the immune responses by CG ODN. Moreover, in the preliminary experiment an effector response of T cells, namely CTL activity, was demonstrated to be potentiated by CG ODN. There was, however, no evidence of direct proliferation of T cells in response to CG ODN. Proliferation is not the only response of T cells to stimuli but other parameters of T cell activity such as cytokine secretion and modulation of expression of cell surface molecules were not studied here. Nevertheless, there was no evidence that potentiation of T cell responses may be as a result of direct action of CG ODN on T cells. In keeping with this finding Liang et al. have also demonstrated that human T cells (>95% purity) did not proliferate directly in response to ODN whereas >90% pure human B cells proliferated as a result of incubation with ODN (Liang, Nishioka et al. 1996).
To evaluate what cells proliferate directly to CG ODN, highly purified B and T cell fractions depleted of APCs are required. Using the same method of enrichment as the last experiment, both T and B cell fractions proliferated to CG ODN and the proliferating cells in both fractions were B cells. As APCs were not depleted, it still remains unclear whether B cells can proliferate directly in response to CG ODN. On the other hand, as a result of CG ODN treatment the percentage of T cells did not change in T-cell enriched populations. This does not rule out direct proliferation of T cells to CG ODN. As these cultures were not completely depleted of B cells and B cells proliferate strongly to CG ODN, small levels of T-cell proliferation would not alter the percentage of T cells and would remain undetected.

A very important component of an immune response is the antigen presenting cell. Dendritic cells not only activate antigen specific responding cells but also have the potential to influence the type of response (Banchereau and Steinman 1998). The APC, therefore, could be a candidate for transducing the effect of CG ODN. In our experiments, it was shown that treatment of BMDC with CG ODN resulted in potentiation of MLRs. Evidence was also provided that this was due to a significant potentiation of T cell proliferation in response to pre-treatment of BMDC with CG ODN. This effect of CG ODN was more marked on "immature" BMDC. Dendritic cells are considered to be immature when they express low levels of class II MHC and costimulatory molecules, however, average expression levels of these molecules by individual cells was not increased in most of our experiments. In our system more cells in the BMDC population expressed MHC class II, CD11b and CD11c molecules. Therefore, more cells in the population acquired BMDC phenotype and the population as a whole was functionally more mature.
The functional maturation of BMDC cultures is most likely to be due to the DCs and not other cell types. Culturing bone marrow cells with GM-CSF results in the growth of neutrophils, macrophages, B cells and DCs (Inaba, Inaba et al. 1992). Macrophages are strongly adherent and would not be expected to contaminate the BMDC used in MLRs (Inaba, Inaba et al. 1992). Neutrophils and B cells are non-adherent in this set up and are eliminated on the third day of bone marrow culture by replacing the supernatant with fresh medium (Drakesmith, PhD thesis) (Inaba, Inaba et al. 1992). In addition, if B cells were present in the BMDC population, they would be expected to proliferate directly to CG ODN, whereas, in all our experiments BMDC alone did not proliferate.

The observation that DCs did not show phenotypic maturation (e.g. upregulation of class II MHC) but were functionally mature still remains unexplained. A possible explanation could be that phenotypic maturation would become detectable at a later time after setting up the MLR. Kinetic studies should have been performed to clarify this point. In one of six experiments, on the other hand, class II MHC was upregulated on CG-treated DCs compared to the control DCs. This may indicate that the BMDC culture conditions in most cases may have induced maximal expression of class II MHC on a population of DCs without the need for extra GM-CSF or CG treatment. In other words, on day 6 in five of the six experiments the cultures did contain some BMDC with mature phenotypes and the CG-treatment increased the number of these mature cells. In any case, the presence of a higher percentage of cells that express MHC class II could to some degree explain potentiation of MLR; in a given number of BMDC more cells become capable of presenting antigen. CD11b upregulation could also contribute to stronger cell-cell adhesion as it has been shown that CD11b can bind to ICAM-1 (Diamond, Staunton et al.)
CD11b is found on PMNs and BMDC and these cultures are bound to be contaminated by PMNs. Double staining experiments should have been performed to establish what cells were expressing the higher levels of CD11b.

The fact that CG ODN induces Th1 skewing of immune responses could be explained, if DCs secreted IL-12 in response to CG ODN. In our experiments, however, this was detected in only two out of six experiments. This could have been due to a failure of the experimental design. The IL-12 measurements were done on the supernatants of day 6 cells cultured overnight with CG ODN and IL-12 secretion was measured only during the first 16 hours after treatment with CG ODN but IL-12 production may have slower kinetics.

The mechanism of potentiation of antigen presentation by CG ODN remains unclear. The modest changes in phenotype probably do not account for the effects and the IL-12 measurements are inconsistent. An important parameter that was not measured and may have had a profound effect on antigen presentation is CD40 expression. Other aspects of DC physiology such as antigen processing may have altered without detectable changes in surface markers. Antigen processing by DC's may be altered by cytokines. For instance, it has been shown that IL-6 treatment of DC's could result in presentation of subdominant rather than dominant epitopes of ovalbumin (Drakesmith, O'Neil et al. 1998).

Finally to establish whether CG ODN can potentiate antigen presentation in vivo, further experiments need to be conducted. The observation of potentiation of T-cell stimulatory effect of BMDC is an in vitro phenomenon observed in cells that do not exist in vivo. BMDC are very similar to in vivo DCs, however, through their development are conditioned by their in vitro environment. A source of in vivo immature DCs is skin Langerhans cells. These DCs could be isolated and tested for their ability to stimulate T cells or become...
phenotypically mature in response to ODN. Langerhans cells could be also pulsed with ODN and antigen in the skin to study whether ODN has any effect on antigen presentation by these cells *in vivo.*
Chapter 6

The target molecules of CpG DNA
Chapter 6

Introduction

There is a body of evidence in the literature suggesting an immunostimulatory role for unmethylated CG containing oligodeoxynucleotides (see introductory section of chapter 5). The experiments described in the previous chapter suggested that a CG oligodeoxynucleotide (ODN) can potentiate the antigen presentation capacity of mouse bone marrow derived dendritic cells (BMDC) and directly cause proliferation of mouse B cells. The mechanism of interaction of ODNs with BMDC or B cells is still unclear. Liang et al. have suggested that ODNs bind a surface receptor on human B cells to cause proliferation and IgM secretion (Liang, Nishioka et al. 1996). In these experiments, although B cells proliferated in response to immobilised ODN, they did not reach the maximum proliferation levels achieved in response to the soluble form of the same ODN. In addition, moderate B-cell proliferation was also observed when non-CG ODNs were used. This suggests that ODN may bind non-specifically a surface receptor on B cells to cause moderate proliferation but sequence-specific mechanisms may also be involved for the maximum response to occur. In accordance with this explanation, Krieg et al. have demonstrated that the surface of mouse B cells is stained to an equal extent with stimulatory and non-stimulatory ODNs (Krieg, Yi et al. 1995).

In B cells, cross linking of surface Ig could be a possible route for induction of proliferation, and as ODNs are highly charged it is possible that non-specific ODNs cause moderate proliferation of B cells by this mechanism. The integrin Mac-1 (CD11b/CD18) binds in a sequence-independent manner to ODNs (Benimetskaya, Loike et al. 1997). This integrin is found on BMDC, however, the fact that it binds non-specifically to ODNs would rule out its role
as the transducer of the stimulatory signals of CG ODN. Considering that ODNs are highly charged, it is unlikely that they would diffuse through the lipid bilayer. Therefore, there may either be an unidentified specific cell surface receptor for CG ODN or one that non-specifically binds ODNs and internalises to deliver them to their specific targets. On the other hand, immature DC may take up ODN by phago/pinocytosis, however, the likelihood of degradation in acidic compartments argues against this route.

In the last chapter it was shown that BMDC became functionally mature in response to a 15 base long oligodeoxynucleotide. The immortalised dendritic cell line CB1 of DBA/2 mouse origin have been demonstrated to be functionally immature (Paglia, Girolomoni et al. 1993) and may make a good model for studying the molecular basis for interaction of CG ODN with dendritic cells. CB1 cells have been generated by immortalisation of DBA/2 splenocytes with the M1B-ΨN11 retroviral vector that carries the avian v-myc oncogene. The CB1 cells have dendritic morphology and display other characteristics of dendritic cells such as motility, constitutive expression of MHC-I, MHC-II, costimulatory molecules B7-1, heat shock antigen (HSA) and ICAM-1 and expression of CD11c that is a typical marker of splenic DC’s. CB1 cells do not express the B cell marker B220 and are also negative for the T cell markers; Thy1, CD3-e, TCR, CD4 or CD8. Expression of MHC class-II is constitutive on CB1 cells, however, it is markedly increased in response to GM-CSF. Paglia et al. have shown that CB1 cells ability to stimulate antigen specific responses by T cell hybridoma and allogeneic responses by splenocytes is greatly enhanced by preincubation with GM-CSF. In addition, the ability of CB1 cells to prime responses against hapten antigens has also been shown to be enhanced by preincubation with GM-CSF. Therefore, GM-CSF results in increased levels of MHC class-II expression parallel with enhanced antigen presentation ability of
CB1 cells. These results strongly suggest that CB1 cells may represent an early or intermediate stage of DC development. In the experiments of this chapter we set out to investigate the mechanisms by which CG ODN effect dendritic cells by studying localisation and identity of their target molecules in CB1 cells.
Methods

**Proliferation of splenocytes in response to biotinylated oligodeoxynucleotides**

These experiments were performed exactly as described in chapter 5 except that instead of ODNs the biotinylated forms of these molecules were used.

**Staining CB1 cells with oligodeoxynucleotides**

Biotinylated oligodeoxynucleotides were used for staining CB1 cells exactly in the same way as antibodies would be used. Hence, $2 \times 10^5$ CB1 cells were placed in flexible 96-well plates and washed three times with medium then incubated with medium alone or medium containing 1 μM of biotinylated oligodeoxynucleotides for 30 minutes. All the reactions were carried out on ice in the presence of 10% FCS to reduce non-specific interactions. After washing, cells were incubated for another 30 minutes on ice with FITC- or Cy-5-conjugated streptavidin. After another three washes the stained cells were analysed by FACScan or confocal microscopy.

For intracellular staining the cells were stained exactly as above apart from an initial permeabilisation step. This was done by incubating the cells for 40 minutes at room temperature in the dark with 50 μl of a commercial reagent called ORTHO PermeaFix™ diluted 1:2 in PBS. This treatment resulted in fixation and permeabilisation of the cells. Before staining the cells were washed four times with 150 μl of medium (See Materials and Methods chapter).
**Preparation of the cytoplasmic fraction of CB1 cells**

The culture medium was removed from sub-confluent monolayer cultures and the cells were washed twice with cold PBS. The cells were scraped and pelleted by centrifugation at 4°C. The pellet was rapidly resuspended in 5X the packed cell volume (PCV) of cold hypotonic buffer (10mM HEPES pH 7.9 @ 4°C, 1.5mM MgCl₂, 10mM KCl and prior to use added 0.2mM protease inhibitor mixture (Boehringer Mannheim BmbH-Germany), 0.5mM DTT) and spun at 2500 rpm at 4°C. The supernatant was decanted and the pellet was resuspended in cold hypotonic buffer to a final volume of 3X the original PCV. The cells were left on ice for 10 minutes allowing them to swell. A cold Dounce homogeniser was used to homogenise cells. This process was continued until over 90% of cells were homogenised. The percentage of lysed cells was monitored by addition of trypan blue to a small aliquot of cells. The nuclei were removed by centrifugation of lysed cells at 1850 g at 4°C. The supernatant was diluted in 0.11 volume of 10X cold cytoplasmic extract buffer (0.3M HEPES, 1.4M KCl, 0.03M MgCl₂) and mixed thoroughly then centrifuged at 100,000 g at 4°C for one hour. After this step the supernatant was dialysed for 1 hr. and 20 min. in cold dialysis buffer (20mM HEPES, 20% glycerol, 100mM KCl, 0.2 mM EDTA and prior to use added 0.2mM mixture (Boehringer Mannheim BmbH-Germany), 0.5mM DTT) at 4°C. The dialysed cytoplasmic fraction was centrifuged for 20 min. at 25000 g at 4°C, if any precipitates could be detected. Aliquots of the cytoplasmic fraction were frozen rapidly using a dry ice and 100% ethanol bath and stored at -80°C.

The protein concentration in the cytoplasmic extract was determined using a Bio-Rad® protein assay kit (BIO-RAD laboratories GmbH, Germany).
Mobility shift DNA-binding (band shift) assay

These assays were performed in 20 μl volumes containing a high ionic strength reaction buffer (see materials and methods chapter), 5 μg of cytoplasmic extract, 1 μg of double stranded poly(dI.dC)-poly(dI.dC) as non-specific competitor, an amount of the Complete™ protease inhibitors cocktail recommended by the manufacturers (Boehringer Mannheim GmbH-Germany), and 20 p moles of a probe labelled with γ32P-ATP. All the components apart from the hot probe were assembled first then hot probe was added to the mixture. After 30 minutes of incubation at room temperature, 2 μl of 10X loading buffer was added to the reactions before it was analysed by non-denaturing polyacrylamide gel electroporesis (PAGE). The gels contained 4% acrylamide and were run at 10 volts/cm for 30 minutes before and 1.5 to 2 hour after loading the samples. Where a competitor oligodeoxynucleotide was used, it was added to the reaction mixture five minutes before the addition of hot probe.

Isolation of proteins bound to oligodeoxynucleotides

The proteins bound to CG or GC ODN were isolated in reactions similar to the band shift assays by using biotinylated ODN instead of radiolabelled probe. These reactions were performed in a total volume of 500 μl containing 125 μg of the cytoplasmic extract, 4 μl of 125 μM free CG ODN, 50 μg of double stranded poly(dI.dC)-poly(dI.dC) as non-specific competitor and 2.5 p moles (12.5 μg) of biotinylated CG or GC ODN in reaction buffer (see material and methods chapter). The streptavidin-conjugated magnetic beads (Dynabeads® M-280) were washed three times in 200 μl and resuspended in 10 μl of the reaction buffer. The beads were then incubated at room temperature on a rotor with 25 μl of 4 μM biotinylated CG or GC ODN and washed three times in the
reaction buffer before addition to the reaction for 30 minutes at room temperature. All the other components were incubated at room temperature for 5 minutes before the addition of the magnetic beads. The extracted proteins were either eluted using a high salt elution buffer (see materials and methods chapter) or the beads were washed 3 or 6 times before the elution step with the reaction buffer containing a concentration of free CG ODN 100X that of biotinylated ODN.

**Western blot analysis**

The proteins bound to CG or GC ODN were extracted as above and separated on a sodium dodecyl sulphate (SDS)-PAGE gel. Using a blotting apparatus the separated bands of proteins were transfered to PVDF membranes (Hybond-P, Amersham Life Science, UK) prewetted with 100% methanol and washed in water for 5 minutes. Non-specific binding sites on the membranes were blocked with 1% BSA in TBS-Tween for 1 hour then washed 3 times for 10 minutes with TBS-Tween. The primary antibody was diluted to a concentration recommended by the manufacturer in TBS-Tween and 1% BSA and incubated with the membrane for 1 hour at room temperature on an orbital shaker. The membrane was washed three times in TBS-Tween for 30 minutes before the secondary antibody for detection was added. The detection step was performed using a commercial detection kit based on a chemi-luminesence system (ECL detection kit, Amersham Life Science, UK) that included the secondary antibody and the substrates for the light reaction.
Chapter 6

Results

The binding site of CB1 cells for CG-ODN

This experiment was performed to investigate the specific site of CG-ODN interaction with CB1 cells. For this purpose a biotinylated phosphorothioate oligodeoxynucleotide with the same sequence as CG-ODN (b-CG ODN) was prepared. This was used to enable detection of bound ODN with streptavidin conjugated to marker reagents. As a control a biotinylated phosphorothioate oligodeoxynucleotide with the same sequence as the GC ODN was utilised. To ensure that biotinylation did not inhibit the immunostimulatory function of these oligodeoxynucleotides they were tested for their ability to stimulate proliferation of splenocytes (see chapter 5). b-CG ODN stimulated proliferation of splenocytes almost as well as CG ODN whereas b-GC ODN did not have any stimulatory effects (fig. 6.1).

Figure 6.1: Time course of proliferation of non-adherent splenocytes in response to 1\mu M of CG, GC, biotinylated-CG (b-CG) or biotinylated-GC (b-GC) ODN. The error bars represent standard deviation of the triplicates.
Biotinylated ODN, therefore, behaved in a similar way to non-biotinylated ODN causing proliferation of splenocytes where the immunostimulatory motifs were present. The maximum proliferation reached in response to b-CG ODN was lower than those for CG ODN, however, as the pattern of splenocyte response to ODN did not change it can be assumed that b-CG interacted with its target in the same way as CG ODN. As b-CG ODN was to be used for establishing the site of interaction of CG ODN with CB1 cells, it would have been more appropriate to test whether biotin altered the effect of CG ODN on CB1 cells. However, considering that the immunostimulatory motif is under study here, it can be assumed that using the biotinylated oligodeoxynucleotides, the site of interaction of CG-ODN motif and not biotin with CB1 cells can be studied. This assumption is made on the following basis; firstly, biotinylation did not alter the proliferative effect of the motif on splenocytes (fig.6.1) and secondly, biotinylated-GC would provide a control for interactions of biotin with irrelevant target molecules.

In order to determine whether the CG motif interacted specifically with a cell surface molecule, CB1 cells were stained with the biotinylated oligodeoxynucleotides using the same method as for cell surface staining with antibodies, using b-GC ODN as irrelevant first layer (see methods). The data for cell surface staining of CB1 cells with oligodeoxynucleotides is shown in figure 6.2.
Figure 6.2: Cell surface staining of CB1 cells with CG or GC ODNs showing that there is no difference in the binding of CG or GC ODN to the cell surface of the cells.

As shown in figure 6.2 equal percentage of cells were stained with b-CG and b-GC, however, the mean fluorescent intensity of staining was higher where b-CG was used as the first layer. This could mean that the b-CG ODN binds with a higher specificity to the CB1 cell surface or there are more receptors on CB1 cell surface that can bind the CG ODN.

The following experiment was performed to establish if a specific interaction occurred intracellularly. In this experiment, CB1 cells were permeabilised (see method) then extensively washed and stained with oligodeoxynucleotides as above. Permeabilised CB1 cells were stained more strongly with b-CG ODN than with b-GC (fig. 6.3). The control ODN stained 29% of permeabilised CB1 cells whereas 84% of these cells were stained with b-CG ODN.
Figure 6.3: Intracellular staining of CB1 dendritic cells with CG or GC ODNs showing that more cells are stained with CG ODN than with GC ODN.

The above experiment was repeated four times. CG-ODN, therefore, interacts specifically with an intracellular component of CB1 cells. The intracellular interaction may occur in the cytoplasm and/or the nucleus of the cells. The next experiment was designed to clarify this point. Hence, the cells were treated exactly as the previous experiment apart from using Cy-5-conjugated streptavidin as the second layer and the cells were analysed by confocal microscopy (fig. 6.4).
Figure 6.4: Confocal micrographs of CB1 cells stained with ODNs. This figure shows that CG ODN and not GC ODN strongly bind to a cytoplasmic component of the CB1 dendritic cells.

Figure 6.4 shows that Cy-5-staining of the cytoplasm of CB1 cells is stronger where the first layer used was b-CG ODN and not b-GC, confirming the results of the previous experiment. In addition, the nuclei of the cells were not stained indicating that the specific interaction of CG ODN with its target occurred in the cytoplasm and not the nucleus.
Analysis of CG ODN interaction with cytoplasmic molecules

**CG ODN induces appearance of a high affinity target in cytoplasmic extract**

In order to further analyse the target molecule/s in cytoplasm with which CG ODN interacts, mobility shift DNA-binding (band shift) assays were performed. ODNs were labelled with $\gamma^{32P}$-ATP to study their interaction with cytoplasmic DNA-binding proteins. An excess amount of double stranded poly(dI.dC)-poly(dI.dC) was used as non-specific competitor. Radiolabelled CG ODN (hot probe) was incubated at room temperature for 30 min. with 5µg of the cytoplasmic extract of CB1 cells and the non-specific competitor. Mobility of the hot probe was retarded by two protein species of different sizes, resulting in two distinct bands (bands A and C) when analysed using non-denaturing PAGE (fig. 6.5 lanes 1 and 2). In order to determine which one of the protein species had a higher specificity for the sequence of the probe (which might be more functionally relevant), prior to addition of the hot probe, increasing amounts of unlabelled (cold) probe was added to the reaction and left at room temp. for 5 min.. The band representing the protein species with a lower affinity for the probe would be expected to be competed out with lower amounts of the cold probe. Surprisingly, however, a new band (band B) was detected when specific competitor was used (fig. 6.5 lanes 3-8). Band B was competed out with more cold competitor than band C and less competitor than band A. This would indicate that band B has an intermediate affinity for the probe compared to the other two bands. This conclusion, however, is paradoxical since if band B did represent a target molecule with higher affinity than band C, it should have appeared in lane 2 in preference to band C. Moreover, if a target with lower affinity is found in relative excess to the other target, affinity would not be the
limiting factor for detection of one band in preference to another. In order to make affinity the limiting factor, considering that in a crude extract the relative concentrations of different proteins cannot be altered, the availability of hot probe can be made limited.

Figure 6.5: Mobility shift DNA-binding analysis of the cytoplasmic extract of CB1 dendritic cells using the immunostimulatory CG ODN as the radioactive DNA probe.

Figure 6.6 lanes 2 and 8-14 show that when the amount of hot probe is limited to 1/10 of that in the last experiment, first band A and then bands C and B are competed out with increasing levels of specific competition. Moreover, both figures 6.5 and 6.6 show that band B increases in intensity to a maximal
level in response to increasing amounts of the cold probe and then is gradually competed out by any further additions. Band B, therefore, represent a target with the highest affinity and band A one with the lowest for the probe. In addition, lanes 1-7 in figure 6.6 show that, in the absence of cold competitor, changing the concentration of hot probe in a range between 1/10 to 60 times the amount used in the last experiment does not result in detection of band B, whereas, band B is detected in the same experiment when similar amounts of combined hot and cold probes were present (lane 8 fig. 6.6). In summary, band B represents a target with higher affinity for the probe but is detected only if the cytoplasmic extract is exposed to the cold probe prior to the addition of the hot probe. This finding might be explained as follows; as a result of addition of cold probe to a critical level, the conformation or composition of a target molecule or complex was changed to expose a high affinity binding site corresponding to band B. However, the fact that band B was not detected when the concentration of hot probe was increased to those 'critical' levels remains unexplained. Nevertheless, figure 6.6 and repeat experiments using limited amounts of hot probe clearly demonstrated that band B represented the most specific target for CG ODN compared to bands A and C.
Figure 6.6: Mobility shift DNA-binding analysis of the cytoplasmic extract of CB1 dendritic cells using different concentrations of a radioactive CG ODN (lanes 1-7). Binding of the radioactive probe was competed out with cold CGODN (lanes 8-14).

In the previous experiment addition of an amount of hot probe, equal to that of the cold probe which caused appearance of a new high affinity band, did not result in formation of the new band. As a result of labelling with radioactive ATP, the hot probe contains an extra phosphate group compared to the cold one. In addition, during the labelling process the hot probe is mixed in with ATP and T4 kinase and is passed through a polyacrylamide column. Therefore, the inability of the hot probe at the critical concentrations to induce the new band might be due to the extra phosphate group (which could for instance compete with a phosphatase substrate), inhibition by contaminating ATP or T4
kinase or a substantial reduction in the concentration of the probe during the
labelling process resulting in overestimated calculated concentrations of the hot
probe. In order to clarify these points the next experiment was set up. In this
experiment four different competitor probes were used all of which had been
through the labelling process but one was labelled with cold ATP, a second one
was incubated with the kinase without the ATP, another incubated with ATP
and not the kinase and the final one was put through the process of labelling
without any substrate or enzyme additions. A band shift assay was performed
and as a positive control, cold competitor that was diluted from stock was
added to a final concentration of 30X the hot probe. This as expected resulted in
formation of a new band, however, none of the other four probes induced
appearance of this band (data not shown). As the probe that was only put
through the labelling process also did not induce formation of the new band, it
can be concluded that the process of labelling itself is responsible and probably
not the presence of an extra phosphate group or contaminating ATP or kinase.
This might occur by the loss of some probe in the polyacrylamide column to an
extent that the calculated concentrations would be less than the actual levels of
probe required for induction of the new band.

CG ODN may interact with NF-κB signalling pathways

In the previous chapter it was shown that CG ODN caused functional
maturation of BMDCs resulting from MHC class II upregulation and possible
increases in IL-12 secretion. Signalling through CD40 has also been shown to
result in increased expression of class II MHC, ICAMs and costimulatory
molecules, increased levels of IL-12 secretion and functional maturation of
dendritic cells (van Kooten and Banchereau 1997), Ligation of CD40 or CG-
ODN treatment, therefore, result in similar responses by dendritic cells. In
addition, CD40 signalling causes activation of nuclear factor (NF)-κB (Berberich, Shu et al. 1994; Rothe, Sarma et al. 1995). Staining CB1 cells with ODN showed that CG ODN interacts with cytoplasmic targets. Components of the NF-κB pathway, therefore, could be possible candidates for the cytoplasmic targets of CG ODN. The next set of experiments were designed to further investigate the identity of the cytoplasmic targets of CG ODN by isolation of these target molecules.

In the previous experiment it became apparent that a high affinity binding site became exposed to CG ODN as a result of incubation of the cytoplasmic extract of CB1 cells with cold probe prior to addition of the hot probe. In this experiment the exact reaction conditions that resulted in appearance of the high affinity band were used apart from using biotinylated CG or GC ODN instead of the hot probe and scaling the reaction up by 250 fold. After 30 minutes of incubation at room temperature the biotinylated ODN and the bound material were extracted using magnetic beads. The bound material was eluted in a high salt buffer and analysed on SDS-PAGE. Figure 6.7 shows that similar bands can be seen from the materials extracted by CG or GC ODN, however, some bands present in the CG fraction are missing from the GC fraction and the bands in the GC fraction are in general weaker. This finding is not surprising, considering that the two oligodeoxynucleotides differ only in the position of one base and are exactly identical in other respects; i.e. these oligodeoxynucleotides have very similar affinities for common target molecules.
Figure 6.7: SDS-PAGE electrophoresis of the cytoplasmic extract of CB1 dendritic cells that bound biotinylated CG or GC ODN. Magnetic beads conjugated to streptavidin were used to extract the protein/ODN complex. The gel was silver stained. (The arrow shows a band that is only present in the CG fraction.)

In the next experiment, to isolate target molecules with higher affinities for CG ODN, the bound material with lower affinities were competed out using non-biotinylated CG ODN. Hence, before the elution step, biotinylated ODN and the bound material was washed in a set volume of the reaction buffer containing free CG ODN at a concentration 100 fold higher than the biotinylated ODN. Figure 6.8 shows that the more the washing process was repeated the more bands were lost preferentially from the GC fraction. Therefore the remaining band that could only be found in the CG fraction can be considered to have a high degree of specificity for CG ODN.
Figure 6.8: SDS-PAGE electrophoresis of material bound to CG or GC ODN after three (low stringency) or six (high stringency) times washing with the reaction buffer containing CG ODN at a 100X concentration of biotinylated CG ODN. (The arrow shows a band that is only present in the CG fraction.)

In order to establish whether CG ODN interacted with the NF-κB pathway, the fractions that were isolated after 3 washes were separated on SDS-PAGE and transferred to blotting membranes for western blot analysis. Western blot analysis was performed using mAb to the p50, p52 and p65 members of the NF-κB family of proteins. This showed that the CG and not the GC fraction contained NF-κB p65 (fig. 6.9). NF-κB p65, therefore, is precipitated with CG ODN, however, it is not clear whether it binds directly to CG ODN or via another molecule. Therefore, CG ODN does interact with the NF-κB pathway.
Figure 6.8: Western blot analysis of material bound to CG ODN using a NF-κB p65 specific antibody. Sp1 protein was used as a control. Using NF-κB p50 or p52 specific antibodies or the material extracted by GC ODN did not result in detection of any bands. The figure above is a picture taken from an autoradiograph superimposed on the original gel in order to determine the size of the band. The marker used was "Rainbow" marker (BioRad, UK) that is prestained.
Discussion

The initial experiments of this chapter showed that the mean fluorescent intensity of CB1 cell surface staining was higher for CG ODN, however, equal percentages of cells were stained with CG and GC ODN. There may be, therefore, a cell surface component that interacts with CG ODN. The integrin Mac-1 could be a candidate cell surface receptor for these ODNs. It has been shown that DNA can bind Mac-1 and become internalised (Benimetskaya, Loike et al. 1997). The oligonucleotides should have been used in cell surface binding experiments in competition with mAbs against MAC-1. Alternatively, other cell surface receptors may be present that bind CG ODN and transduce intracellular signals that result in DC activation. The identity of such receptors may have been investigated by competition staining with antibodies to known cell surface receptors or by using the cell membrane fraction for purification of the putative CG ODN receptor.

Intracellular staining experiments clearly demonstrated that CG ODN specifically binds to a cytoplasmic component of CB1 cells. The identity of the target molecule/s for CG ODN, however, is still unclear. The functional effects of CG ODN and CD40 on DC are similar. Ligation of CD40 results in a cascade of events that in turn cause NF-κB activation. The precipitation of NF-κB p65 by CG ODN and its detection by western blotting, indicates that CG ODN either binds to this protein or another protein that can bind NF-κB. This could be one of the proteins that is involved in the intracellular signalling pathway of CD40. The CD40 signal is transmitted by at least three of the TNF receptor-associated factor (TRAF) proteins (Rothe, Sarma et al. 1995; van Kooten and Banchereau 1997). These proteins contain a RING and a zinc finger domain (Rothe, Wong et al. 1994) which have DNA binding properties. The
other proteins involved in CD40 mediated NF-κB activation that could be candidates for binding to CG ODN include the inhibitor of NF-κB (I-κB) and I-κB kinases (IKK).

Does the new high affinity band in the band shift assays represent any of the members of NF-κB family? In order to investigate this, a supershift assay with mAbs to NF-κB p50, p52 or p65 was performed. Antibodies at high concentrations are normally used for these assays, however, concentrated antibodies were not available and low concentrations that had been used for western blot analysis were also used here. Addition of these antibodies did not alter the mobility of any of the three bands seen in the band shift assays. The fact that the bands did not shift any further when mAbs were added could mean that these bands did not represent the NF-κB proteins, however, as positive controls were not available such conclusion could not be made.

Comparison of the sequence of CG ODN with that of NF-κB response element (NF-κB-RE) shows a high degree of homology between these two molecules. The core sequence of NF-κB-RE is 5'GGGRNNYYCC3' (Baldwin 1996), where R is purines, Y is pyrimidines and N could be any deoxynucleotide. The sequence of CG ODN is 5'GCATGACGTTGAGCT3' where the bases that could be homologous to NF-κB-RE are shown in bold letters. As mentioned previously, this hexameric homology has also been described as an immunostimulatory motif. Could the immunostimulatory effect of CG ODN be due to activation of NF-κB? If NF-κB was activated by CG ODN, could the homology of the immunostimulatory motif with NF-κB-RE be responsible for that? In the band shift assays a high affinity band emerged when a certain amount of CG ODN was added to the reaction. If the appearance of this band is related to the functional effect of CG ODN and the homology with NF-κB-RE is responsible for this effect, the NF-κB-RE should
also cause the formation of the same band. To address this question, an oligodeoxynucleotide with the same sequence as NF-κB-RE (5'GGGACTTTCC3') was used. Before addition of the hot probe (CG ODN) a range of concentrations of cold NF-κB-RE, CG ODN or an irrelevant 15 bp long ODN were added to the reaction mixture for 5 minutes at room temperature. Addition of either CG ODN or NF-κB-RE but not the control ODN resulted in emergence of band B (fig. 6.10).

Figure 7.11: Mobility shift DNA-binding analysis of the cytoplasmic extract of CB1 dendritic cells using radioactive CG ODN. A high molecular weight band emerged when cold CG ODN or NF-κB response element were added to the reaction. Addition of an irrelevant 15 bp long control ODN did not result in emergence of band B.
Therefore, the immunostimulatory motif of CG ODN that is homologous to NF-κB-RE caused appearance of a new high affinity band. Activation of NF-κB results from dissociation of I-κB from NF-κB homo/heterodimers resulting in exposure of NF-κB nuclear localisation signal and the DNA binding site. Does the immunostimulatory motif cause dissociation of I-κB and activation of NF-κB? The western blot data and the literature evidence implicate NF-κB in the immunostimulatory mechanism of CG ODNs. For instance, Stacey et al. have shown that the level of NF-κB in the nuclear extract of bone marrow-derived macrophages increased after one hour of incubation with plasmid DNA (Stacey, Sweet et al. 1996). In addition, there are also some indirect evidence of NF-κB activation by CG ODNs. Yi et al. have shown that anti-IgM-induced apoptosis of the B cell line WEHI-231 is inhibited by CG ODNs via upregulation of bcl-x (Yi, Hornbeck et al. 1996). This effect through bcl-x upregulation can also be achieved by ligation of CD40 (Wang, Karras et al. 1995). Moreover, it is known that CD40 ligation induces activation of NF-κB. Therefore, there is a good case for further investigations of the possible role of CG ODN in activation of NF-κB.
Chapter 7

Final Discussion
**DNA immunisation**

As discussed in chapter 3 antibody responses in DNA vaccination are generally weak and show slower seroconversion times compared to those resulting from conventional methods of immunisation. Moreover, despite the abundance of reports on DNA immunisation against a variety of antigens, evidence of helper T cell responses is scarce. There are not many studies that directly compare DNA with protein immunisation, however, the few available reports demonstrate variable (Peet, McKeating *et al.* 1997) and low antibody responses compared to protein immunisation even where protein is used without adjuvants (Anderson, Gao *et al.* 1997; Boyle, Silva *et al.* 1997). In a study by Boyle *et al.* a direct correlation between antigen dose and the level of antibody response is demonstrated when protein antigen is injected without adjuvant into muscle (Boyle, Silva *et al.* 1997). In some models the data suggest that the antigen dose correlate with the magnitude of both cellular and humoral responses. For instance in one study, cytotoxic T lymphocytes (CTL) against MUC-1 antigen could not be detected after DNA immunisation, unless the animals were challenged with MUC-1 expressing tumours (Graham, Burchell *et al.* 1996). The same phenomenon was observed for CTL raised in mice by DNA immunisation against lymphocytic choriomeningitis virus (LCMV) (Martins *et al.*, 1995). In another report antibodies against the haemagglutinin of influenza virus were not detected after DNA immunisation but the vaccinated animals were protected against lethal challenge and had detectable antibody after viral challenge (Webster, Fynan *et al.* 1994).

A possible explanation for the features mentioned above is that the response to DNA immunisation may be a characteristic homeostatic response to low antigen dose; a low dose of antigen triggers a response with a large enough magnitude to neutralise the antigen and avoid unwanted pathology and
expenditure of energy. Hence, low antigen dose would trigger suboptimal responses that result in small or no expansion of the responding clones so that these remain undetectable by methods used for demonstration of effector cells. The responding clones would not be tolerant as a second antigen challenge results in their expansion and makes the effector function detectable. According to this hypothesis, challenge of DNA immunised animals showing low antibody responses with antigen, should result in production of large quantities of antibodies. Additionally, antibody responses should become detectable, at least in some animals that have not responded to DNA vaccination, when a regimen of antigen challenge is used that does not result in induction of immune responses in naive animals. Likewise, responding T cells that are not detectable ought to be present in the lymphoid system and may expand and be detected after antigen challenge.

The final experiments of chapter 3, in which DNA immunised animals were challenged with protein in PBS, provide support for the above hypothesis that in response to low antigen levels antigen specific lymphocytes are primed but since the level of expansion is appropriately low these responses cannot be detected. Demonstration of helper T-cell responses proved to be difficult after DNA immunisation or protein challenge where previously undetectable B cell responses became detectable. As discussed in chapter 3, this might have been due to different modes of antigen recognition by T and B cells resulting in different boosting requirements. Thus, for T cell to expand in response to protein challenge, adjuvant may have been required. These responses, however, may be demonstrated in a functional assay. In order to show whether Th cells were primed against hCEA after primary immunisation or protein challenge, T cells from immunised animals could be transferred to an irradiated animal along with hapten specific B cells and the hapten specific antibody levels could
be measured. This classic technique could be more refined by the exclusive transfer of CD4+ fractions. Using this assay, the question of whether boosting T-cell responses is limited by adjuvant signals and B-cell responses by antigen dose can also be addressed.

The final experiment of chapter 4 demonstrated that CTL had been primed as a result of DNA immunisation, however, they were not detectable unless were allowed to expand in vitro for two weeks. This provides some support for the hypothesis that DNA immunisation result in priming of low frequencies of CTL precursors. The protein challenge experiments, on the other hand, showed that memory B cells existed eight weeks after final injection of DNA despite the absence of detectable effector responses. These experiments, therefore, indicate the development of immune memory as a result of DNA vaccination associated with weak effector responses.

A new model for generation of T-cell memory states that memory T cells develop directly from naive T cells bypassing the effector phase. According to this theory suboptimal signalling through T-cell receptor (TCR) could result in differentiation of T cells into a memory phenotype, whereas, strong signalling through the TCR would result in proliferation and differentiation of naive T cells to effector cells (Farber 1998). This model is based on the differences seen in the mechanistics of memory and effector T cells. For instance, Liu et al. have shown that there may be different pathways for induction of memory and effector T cells; memory T cells can be generated in vivo in either CD28 or heat stable antigen (HSA) knockout mice whereas effector T cells can develop in HSA but not CD28 knockout animals (Liu, Wenger et al. 1997). Furthermore, there appear to be differences in intracellular signalling through memory and effector TCR; memory T cell receptors are hypo-phosphorylated, resembling
TCR phosphorylation as a result of suboptimal signalling by partial peptide agonists (Farber 1998). This model predicts that in situations where antigen dose is low, such as in DNA immunisation, memory T cells can develop in the absence of expanding clones of effector T cells. The preliminary data shown in chapters 3 and 4 are consistent with this model of immunological memory.

The hypothesis that DNA immunisation induces better memory compared to protein immunisation may also be tested at a cellular level. This can be done by phenotypic characterisation of antigen specific cells. Antigen specific T cells may be demonstrated by the heteroduplex analysis that detects clones of responding cells. Thus, clones of T cells responding to DNA and protein immunisation obtained from draining lymph nodes or the spleen could be compared. These clones could be further characterised by analysis of CD4 or CD8 fractions as well as fractions with a memory phenotype (L selectin-ve, CD44 hi, CD45RB Lo). Demonstration of CD4+ T cells from human peripheral blood mononuclear cells (PBMCs) responding to tetanus toxoid has been shown to be difficult unless the CD4 fraction was first restimulated, in vitro (Maini, Casorati et al. 1999). This is not the case for CD8+ cells and has been attributed to small expansions of clones of CD4+ compared to CD8+ cells. However, restimulation of T cells from draining lymph nodes of mice may not be necessary as they might be found in frequencies within detection levels of the heteroduplex technique. Quantitative techniques such as Elispot assays or tetramer binding could also be used. However, the tetramer binding technique has been used successfully for detection of CD8 cells but its use for CD4 cells is still under development.

In chapter 4, it was shown that fusion of hCEADo with Fr-c resulted in an increase in the number of responders and potentiation of anti-hCEA
antibody responses following DNA immunisation. It was argued that the presence of helper epitopes in Fr-c or the ability of Fr-c to target antigen to APCs might be responsible for the effect. This does not contradict the explanation given for irreproducibility and weakness of the responses in our earlier experiments. Targeting antigen to APCs and presence of helper epitopes both lead to a raised effective concentration of antigen at the point of presentation. In addition, activation of more helper T cells would result in an immune response of a larger magnitude, allowing sufficient expansion of B-cell clones so that antibody becomes detectable. On a practical level, for DNA immunisation to be an effective method of vaccination, it is essential to avoid or reduce to a minimum the number of non-responders. Our experiments and the literature show that fusion of the antigen to a molecule that contains strong Th epitopes that may target the antigen to APCs is a good way of ensuring this.

Spellerberg et al. used Fr-c to augment anti-tumour responses in mice against idiotypic determinants taken from three different B cell lymphoma (Spellerberg, Zhu et al. 1997). In these experiments it was observed that without inclusion of Fr-c in the DNA constructs mice hardly generated any responses against the antigens. However, idiootype/Fr-c fusion constructs induced strong antibody responses against both components of the chimeric proteins. The fusion of Fr-c to other antigens, in future, would determine if the effect of Fr-c is universal. Moreover, if Fr-c is to be used in human subjects, it would be interesting to study the response to Fr-c fusion constructs in mice preimmunised with the tetanus toxoid (TT) as infants are regularly immunised with TT vaccines.
Immunostimulatory DNA Sequences

In the second part of this project, it was demonstrated that an oligonucleotide containing an immunostimulatory motif could cause functional maturation of bone marrow derived dendritic cells (BMDC). There are currently two theories that attempt to explain the nature of signals received by the immune system that allow it to become appropriately activated and to use effective defensive strategies. Matzinger argues that the main requirement of the immune system is not to distinguish self from non-self but to be only tolerant against the self antigens presented by professional APCs and recognise "danger" signals that are released when a tissue is being damaged (Matzinger 1994). This theory predicts that the danger signals are received by professional APCs and could be the molecules that are released from cells only when they undergo necrotic rather than the physiological apoptotic damage.

On the other hand, Janeway C. A. Jr. proposes that the innate immune system has inherited a non-clonal set of primitive "pattern recognition receptors" (PRR) that recognise molecular patterns that are unique to and essential for specific groups of organisms (Medzhitov and Janeway 1997). This recognition is translated to signals that result in expression of costimulatory molecules and cytokines by professional APCs which in turn facilitate initiation of adaptive immune responses.

According to both of these theories the professional APCs such as dendritic cells are the cells that receive signals that allow initiation of immune responses. In addition, the Janeway theory predicts that the type of response is also determined by the signals that are transduced through PRR and are received by professional APCs. As mentioned in chapter 5 the hexameric
immunostimulatory DNA motifs are relatively confined to bacterial genomes and they have been shown to cause Th1 skewing of the immune response. These properties fulfil the criteria of a molecule recognised by PRR. The findings of chapter 5 that a CG oligodeoxynucleotide (ODN) caused maturation of BMDCs also support such a role for these molecules. In addition, Janeway has shown that a PRR candidate in humans causes upregulation of costimulatory molecules and cytokines by activation of the NF-κB molecule (Medzhitov, Preston-Hurlburt et al. 1997).

In chapter 6, evidence supporting a specific interaction of CG ODN with the NF-κB pathway was presented, however, it still remains unclear whether this results in activation of NF-κB. In order to investigate this, we used a commercial reporter plasmid containing the L-selectin promoter. This promoter is activated by the active forms of NF-κB and is placed upstream of the green fluorescent protein (GFP) of jellyfish Aequorea victoris. When transiently-transfected CB1 cells were treated with CG ODN, NF-κB-response element or LPS, the mean fluorescent intensity of CB1 cells was moderately increased whereas treatment with GC ODN did not have any effect. The moderate increases in fluorescence could have been an artefact or a sign of activation of NF-κB in a small proportion of cells that were transfected with the plasmid. Due to time constraints stable transfections were not performed to resolve this issue.

It would not be surprising to find that NF-κB was activated by CG ODN as this transcription factor has been found to play a central role in cytokine production and activation of lymphocytes, monocytes and DC’s. The innate immunostimulatory properties of plasmid DNA and skewing of helper T cells to a type 1 phenotype, after DNA immunisation, may be explained by synthesis of cytokine such as IL-2, IL-6, GM-CSF and IL-12 in response to CG sequences,
via the NF-κB pathway. An important question is whether these effects result from CG binding to a cell surface receptor or from internalisation of CG ODN and their direct effect on a cytoplasmic component.

In chapter 5, there was some evidence that CG ODN directly effects dendritic cells and B cells but not T cells. On the other hand the evidence in chapter 6 suggests that CG ODN may cause its effects through binding to a component of the NF-κB pathway. These two sets of data appear contradictory as T cells can also be triggered by activation of NF-κB. This contradiction might be explained by the specific binding of CG ODN to a cytoplasmic component of CB1 dendritic cells. Due to the highly charged nature of DNA it cannot enter cells by diffusion through the cell membrane, however, it may be taken up by B cells and dendritic cells through the same mechanisms used for antigen uptake by these APCs. Therefore, CG ODN would not be able to bind to its cytoplasmic receptor even if it was expressed by T cells as these cells are not known to have a similar uptake mechanisms to APCs.

It has been shown that Mac-1 receptor can bind and internalise DNA in a nonspecific manner (Benimetskaya, Loike et al. 1997). As a result of this interaction reactive oxygen species (ROS) are generated. In another study it was been shown that CG ODN induced IL-6 transcription in murine B cells was mediated through a reactive oxygen intermediate-dependant pathway (Yi, Klinman, et al. 1996). ROS are thought to cause degradation of I-κBα and β resulting in activation of NF-κB (Baldwin 1996). In another study it was shown that plasmid DNA caused activated of murine macrophages resulting in TNF-α and nitric oxide synthetase induction (Stacey, Sweet et al. 1996). In this study plasmid DNA was taken up by macrophages and remained sufficiently intact to code for leuciferase protein. Moreover, in response to bacterial DNA NF-κB was translocated in to the nucleus of macrophages.
These studies as well as our data support, firstly, the involvement of NF-κB in CG induced activation of dendritic cells, and secondly, give weight to the hypothesis that CG interacts with a cytoplasmic molecule to bring about NF-κB activation. Transcription factors are proteins that have evolved to interact with genomic DNA in a sequence specific manner. It may not be just a coincidence that the CG ODN used in our studies has a high sequence homology with NF-κB response element (NF-κB-RE). The NF-κB DNA binding region is masked by a regulatory element I-κB that inhibits nuclear translocation of NF-κB. CG ODN may displace I-κB and allow nuclear translocation (i.e. NF-κB activation). In the nucleus CG ODN would be competed out by NF-κB-RE that has a higher affinity for its binding site than CG ODN.

Considering that CG ODN and CD40 ligation cause similar phenotypic and functional changes in DC's and that CG ODN may bind a cytoplasmic component of DC's, it is possible that CG ODN interact with the down stream signals of CD40 ligand (e.g. NF-κB). As mentioned elsewhere, a feature of DNA immunisation is generation of long lasting memory responses. It has been shown that CD40 is required for generation of memory in B (Gray, Dullforce et al. 1994) and T cell compartments (Borrow, Tough et al. 1998) but generation of effector cell is not influenced by CD40 blockade by antibodies or absence in knockout animals. An interesting question is whether CG sequences in DNA vaccines contribute to their propensity to generate memory.
Summary

Antibody responses in DNA immunisation are generally weak and the evidence for helper T cell responses in the literature is not strong. However, DNA immunisation appears to result in good memory responses. In chapter 4, it was shown that antibody responses could be augmented by addition of tetanus toxoid fragment-c. Fr-c may contain helper T cell epitopes or have the ability to localise the antigen to APCs. The preliminary data in this chapter suggest that the magnitude of antibody responses may be limited by the dose of antigen that is very small in DNA immunisation. Furthermore, at least for CTL, the absence of detectable T-cell responses may be because of the low frequency of primed T cells. In chapters 5 and 6, it was shown that immunostimulatory sequences in DNA potentiate APC function and might do so through NF-κB activation.

A new theory states that low antigen dose may be a stimulus for development of memory. Signalling through CD40 resulting in NF-κB activation has also been shown to be involved in formation of memory. Considering our observations and the recent developments in the mechanisms of immunological memory certain issues arise that may be addressed by employing the following strategies. For a particular antigen, DNA and conventional protein immunisations may be compared directly to establish whether the former results in better memory.

The clones responding to these methods of immunisation may be compared by heteroduplex analysis, Elispot assays and the tetramer binding technique. The magnitude and number of responding clones may be compared to establish if DNA immunisation results in responding clones that are limited
in numbers and/or size. The phenotypes of the responding clones may be studied to look for expression of memory markers.

The mechanism of ISS signalling may be studied further to establish whether ISS activate NF-κB. ISS may be included in conventional protein immunisations to establish whether they contribute to development of a better memory response.

The augmentation of antibody responses by Fr-c need to be studied for other antigens and in mice with different haplotypes to establish whether the effect is universal. A classical Mitchisonian hapten carrier experiment may be used to clarify whether Fr-c augmented antibody responses by providing T helper epitopes. Finally, a marker protein such as the green fluorescent protein could be joined to Fr-c in order to establish whether Fr-c facilitates localisation to the lymphoid compartment.
References


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