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13. ABSTRACT ***(Maximum 200 Words)***

Gene therapy offers an exciting new approach to treating cancer, in particular by introducing cytokine genes into tumor cells, which has been extensively studied in animal models. Ideally it would be desirable to have a reagent that could be injected into the patient and would target tumor cells.

The main objective of this proposal is to develop monoclonal antibodies as cell specific targeting vectors so that they are able to bind and carry DNA into cells. The molecularly engineered antibody targeted DNA to tumor cells, but the expression of the DNA was low. Different strategiers are being examined to improve the expression. The condensation of DNA has been shown to be important to efficient DNA uptake. A detailed study of conditions to achieve DNA condensation under physiological salt concentration was performed. Though DNA condensation was achieved, it did not result in an improvement. The entire domain containing the influenza hemagglutinin fusion peptide has been engineered to bind DNA in order to facilitate DNA entry into the cytoplasm. Two different strategies are being pursued to overcome the nuclear membrane barrier.

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Peter J Curtis

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5. Introduction

Breast cancer, like most cancers, develops as a result of several mutational events. These events result in tumor cells capable of considerable phenotypic variability, so that within a large population there are cells that are able to evade the immune system and frequently are capable of spreading to distant tissues to form metastatic lesions. Yet patients do produce some immune response to their tumors as indicated by recovery of tumor specific cytotoxic lymphocytes and occasionally spontaneous regression. The strategy of transfecting cytokine genes into tumor cells to enhance the host immunity has been extensively studied in animal models and has provided the groundwork for human clinical trials. However these approaches rely on ex vivo modification of tumor cells, which is difficult, time consuming, expensive and dependent on research facilities. Vectors capable of targeting a particular cell type, i.e tumor cells, are urgently required to achieve tumor cell modification in vivo.

In this proposal we intend to develop a breast tumor-specific targeting vector by combining two different monoclonal antibodies, mAb425, which binds human epidermal growth factor receptor(EGFR) and Her66 and 81, which bind to human erbB2, both of which are growth factor receptors abundantly expressed on a subset of breast tumors. One arm of this bispecific antibody will be further modified by molecular engineering to contain DNA encoding a DNA binding domain into the gene encoding the heavy chain, so that the bispecific antibody will be capable of binding DNA. A number of strategies, designed to optimize entry into the cytoplasm, transport to the nucleus and expression for a prolonged period of time, will be combined to produce a novel breast tumor specific targeting vector capable of delivering DNA into tumor cells.

6. Body

The success of the project depends completely upon achieving efficient transfection and expression of the targeted DNA, as identified in Task 4. Though some progress was reported in last year's report towards this goal, it was not sufficient. Therefore all of the effort described in this report has been directed at achieving efficient expression of the transfected DNA, using a plasmid containing the luciferase gene as a reporter.

The previously reported results had not shown linearity or proportionality of the response to varying the Ab/DNA complex added to the target cells, human A431 . An explanation for this nonlinearity could be that DNA condensation, which has been shown to be very important for efficient uptake of DNA[1] [2], occurs only under specific conditions. In the report of Perales et al.(1997) [1] the complex was made in 1M salt and injected into an animal. The use of physiological salt conditions would be preferable.

DNA condensation results in the formation of a toroid, about 50 nm in diameter, which with time forms larger aggregates. Attempts were made to identify conditions for DNA condensation using 425Fab.DBD, complexed with DNA, by electron microscopy, but viewing of grids showed always a variety of structures in addition to objects that had the dimensions expected for condensed DNA.

A simpler way of assessing DNA condensation is filtration through a 0.2um filter, combined with testing for DNAse I resistance, a property of condensed DNA.

Initially, polylysine with 10, 40 and 180 residues was added to DNA at 0.15 M NaCl, but the resulting complexes appeared to stick to plastic tubes. This was not a problem using the Ab/DNA complex. Polylysine , average length 10, 40 and 180 was added to the Ab/DNA complex at different concentrations either in one or many steps over different periods of time. The Ab/DNA complex was used at low concentrations on the assumption that its lower concentration would tend to minimize the process of aggregation. It was found that optimal conditions for DNA condensation and DNAse I resistance was achieved using the Ab/DNA complex at 0.5 ug/ml and polylysine, average length 10, added to a final concentration of 6 ug/ml over a period of 1 hour at 37°C(figure 1). The Ab/DNA /polylysine complex was stable for 3-4 hours before aggregation occurred.

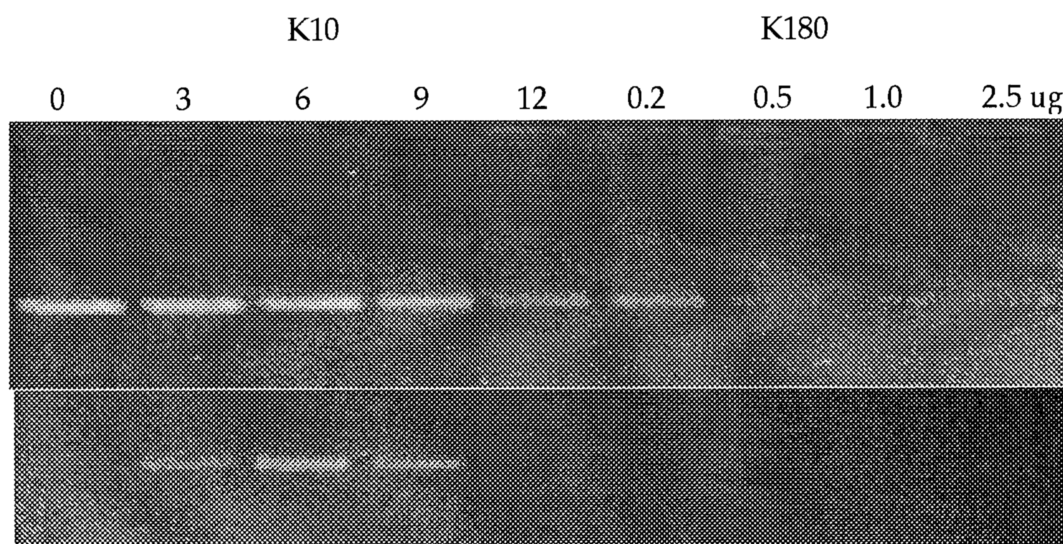

Figure 1. Agarose gel electrophoresis of DNA recovered from Ab/DNA complexes with polylysine-10 residues(K10), or 180 residues(K180) after filtration. Lower panel, DNA recovered after filtration and DNAse I.

However the use of such complexes on the target cells, human A431, did not result in any improvement in the expression of the DNA. Presumably other barriers to the expression of the DNA must exist.

To identify the fate of the bound complex, a comparison was made between complexes assembled with 425fos/DBD and with Superfect(an oligodendrimer, Qiagen) upon transfection of human A431 cells. The 425fos/DBD complex was bound to cells at 4^{0}C for 2 hours, so that the amount bound could be estimated in the absence of internalization and degradation. After 2 hours at 4^{0}C, one plate was used to isolate bound plasmid, while two plates were shifted to 37^{0}C for 3 and 20 hours,at which time cells were trypsinized and internalized plasmid was isolated by the alkaline lysis method. Plasmid DNA was estimated by Southern blot in comparison with known amounts of plasmid. The Superfect/DNA complex was placed on the cells at 37^{0}C for 45 minutes. Plasmid was isolated from trypsinized cells incubated for a further 3 and 20 hours, as above(Table 1).

Table 1.

Complex	Estimated DNA ug/10^6 cells 4^{0}C	3 hrs	20 hrs	RLU/10^6 cells
425fos/DBD	0.3 (10%)	0.03 (1%)	0.005 (0.15%)	10^5
Superfect	-	0.3 (3%)	0.3 (3%)	1-5x10^7

For the 425fos/DBD complex upto 10% of the input DNA was bound to the cells, but most of the DNA was lost after 20 hours. The same result was obtained using a complex of 425fos/DBD and DNA mixed with polylysine under conditions where most of the complex is filterable. With Superfect, DNA appeared to be more stable inside the cell, at least upto 20 hours. The amount found after 20 hours corresponds to about 60,000 DNA molecules/cell, yet if human A431 cells were transfected in the same way with a β-galactosidase plasmid, less than 10% of the cells were stained. This result suggests that very few DNA molecules are actively transcribed, probably because they do not enter the nucleus.

The major barriers that the DNA must cross to achieve expression are the cytoplasmic and nuclear membranes. The influenza hemagglutinin(HA) has been shown to be the only protein necessary for entry of the viral particle into the cytoplasm. It is a very efficient fusogen, which has been extensively studied. It contains two domains, HA1 which is the binding domain for sialic acid resdues, and HA2, whose N terminus initiates fusion. The X-ray structure of HA1 and 2 have been determined not only at neutral pH but also at acid pH where extensive rearrangement of their structure occurs which facilitates fusion of the viral membrane with the cytoplasmic membrane[3]. At the acid pH, HA1 does not undergo any major change, but HA2 changes dramatically, so that its N-terminal fusion domain projects outward at the end of a long coiled coil. The N-terminus inserts into the cytoplasmic membrane, while its C-terminus including the transmembrane domain moves closer to the N-terminus by a fold back mechanism, thus bringing the two membranes close together[4, 5]. The other domains of HA2 in addition to the fusion peptide clearly play an important role increasing the efficiency of fusion

The DNA encoding the HA2 domain lacking its transmembrane and cytoplasmic domains has been cloned by PCR and joined with a signal peptide derived from mouse kappa cDNA, which has been used for the expression of several proteins in the baculovirus/insect cell system. The cloning was accomplished by use of overlapping PCR to achieve fusion of HA2 DNA to that of the signal peptide and introducing alanine residues to the -3 and -1 positions of the signal peptide to conform with the most frequently observed residues at the signal peptide cleavage site[6]. It was found that it was very difficult to obtain stable clones in pUC19 , but possible in pUC 8. This was presumably because leaky expression of the HA2 peptide was possible from the lac promoter in pUC 19 and expression of the HA2 peptide is lethal. Next the DNA encoding the DNA binding domain was added to the HA2 DNA. To achieve expression of the fused HA2, the HA2 DNA will be transferred to pVL1393 to make a recombinant baculovirus. Once the HA2 peptide can be made, it is critical to establish that the N-terminus of HA2 is glycine. Two other forms of the HA2 DNA will be made; 1) the external domain and transmembrane domains, 2) the complete HA2 with the three domains.

For efficient expression of transfected DNA nuclear localization is important. Though DNA transfected into cells in various ways is expressed, the efficiency is very variable, and probably largely dependent on DNA gaining access to the nucleus during cell division, when the nuclear membrane disassembles. In general transfection efficiency is known to depend upon cells plated at less than 100% confluency so that they can continue to divide. DNA microinjected into the cytoplasm is not expressed [7].

The nuclear localization signal(NLS) has been well defined in SV40 proteins, large T antigen, Vp2 and Vp3.[Dingwall, 1986 #45} and consists of the sequence PKKKRKV. NLS peptides have been linked to DNA in different ways with variable results. In one study, a single NLS peptide was covalently linked to a plasmid, and resulted in a dramatic increase in the efficiency of expression[8]. The authors suggested that DNA containing multiple NLS might become bound by more than one pore. In another study, at least 101 NLS peptides/1kbp of DNA was necessary to achieve nuclear transport. In this case perhaps the NLS peptides, because of its positive charges, interact electrostatically with the DNA phosphate backbone and are mainly unavailable to bind to α-importin.

DNA encoding NLS has been fused to the C-terminus of mouse gamma signal peptide and to the N-terminus of c-fos LZ linked to DBD, and will be expressed as a recombinant baculovirus.

The presence of c-fos LZ between NLS and DBD should prevent the NLS from binding to DNA and being inaccessible. The c-fos LZ has an excess negative charge, as well as hydrophobic residues and would be expected to be repelled by the negative charge of the DNA.

A few studies have shown that naked DNA is not transported into the nucleus. However naked SV40 DNA, but not pBR322, is imported into the nucleus via α-importin and dependent on transcription[9]. The SV40 sequences necessary for importing were the origin of replication, parts of the early and late promoters and the enhancer. A 100 fold increase of gene expression was also obtained by adding Epstein-Barr viral origin of replication as well as EBNA1[10].

To check on the role of DNA containing origins of replication on nuclear transport, four constructs were compared. Each construct contained the luciferase gene driven by the CMV early promoter. In addition, plasmids contained the human BKV origin and T antigen(BKV is very similar to SV40), or the human Epstein-Barr viral origin and its EBNA I, or a 250 bp DNA fragment containing the dyad symmetry element from the Epstein-Barr origin. Equal amounts of each plasmid was transfected using Superfect(Qiagen) into human A431 cells, and after 24 hours, the cells were lysed and assayed for luciferase(Table 2).

Table 2.

DNA Sequences	RLU/10^6 cells
-	2.2×10^7
BKV ori + T antigen	3.0×10^8
E-B ori + EBNA I	4.5×10^8
E-B dyad symmetry element	3.4×10^8

The presence of the origins increased the expression by at least 10 fold, and the 250 bp fragment was sufficient. Clearly this small element will be added to plasmids used for transfections. It has been suggested that newly translated transcription factors in the cytoplasm bind to sequences in the DNA origins and as a result the complexes are transported into the nucleus via the NLS in the transcription factor. It will be interesting to determine if addition of NLS containing peptides to Ab/DNA complexes will further improve their expression.

7. Key Research Accomplishment

- Monoclonal antibodies that recognize human epidermal growth factor receptor(EGFR) and erbB2 have been cloned and engineered to contain a DNA binding domain(DBD).

- Anti-human EGFR with a DBD has been expressed abundantly in the baculovirus/insect cell system, from which it has been purified.

- Anti-human EGFR with a DBD, complex with DNA, delivered the DNA specifically to the target cells, a human adenocarcinoma, which expresses high levels of human EGFR.

- Strategies are being pursued to increase the efficiency of targeting and expressing the DNA in the human tumor cells.

8. Reportable Outcomes

- Manuscripts etc. Antibody directed Gene Therapy – Abstract, Era of Hope meeting, Atlanta, June 8-12, 2000.

- Patents. None.

- Funding applied for:

a). Title – Targeting of a DNA Vaccine – American Cancer Society – rejected.

b). Title – Targeting a DNA Vaccine – NIAID RO3-AI45699 – funded.

c). Title – DNA Targeting of Cancer Cells by an Engineered Antibody – NCI RO1-CA85625-01 – rejected.

9. Conclusions

The cDNAs for mAb425 have been genetically engineered to contain a DNA binding domain(DBD), so that co-expression of the light and heavy chain cDNAs as recombinant baculoviruses in insect cells results in the production of a Fab with a DBD that bound DNA as well as its target cell, human A431. The Ab/DNA complex specifically targeted human A431 cells resulting in the expression of the reporter gene, luciferase. However more than 99% of the targeted DNA was rapidly degraded, presumably by being fusing with lysosomes. Incorporation of the influenza fusion

peptide into the Ab/DNA complex did not facilitate entry of the DNA into the cytoplasm. Though DNA condensation had been shown to greatly increase expression of transfected DNA, it was not effective in our system. However there are DNA sequences in viral origins of replication that facilitate transport of DNA into the nucleus. A 250 bp fragment containing the dyad symmetry element from human Epstein-Barr viral origin was sufficient for this transport.

To achieve more efficient entry of targeted DNA into the cytoplasm, the HA2 domain from the influenza hemagglutinin gene has been engineered so that it can be incorporated into the Ab/DNA complex. In addition DNA encoding a NLS has been joined to DNA encoding the DBD, so it also can be incorporated into the same complex to facilitate the transport of the DNA into the nucleus.

The major problem with cancer is the spread of metastatic lesions. Chemotherapy and radiation are clumsy and inefficient tools to combat these lesions. The ability to target DNA to tumor cells in vivo is an exciting new approach. DNA encoding one or more cytokine targeted to tumor cells can induce an immune response to the tumor cells, particularly cytotoxic T cells, and once induced the CTLs would monitor the whole body for remaining tumor cells. Therefore the targeting of DNA to tumor cells in vivo is a very important goal. Viral vectors, such as adenovirus and retroviruses, are not suitable for in vivo use, since they infect a variety of cells. In addition many people have antibodies to adenovirus, which will limit the effectiveness of this vector, and if not adenovirus induces usually a strong immune response. Effects to retarget these viral vectors have so far not been successful.

The approach described in this project is to develop a nonviral vector that will target specific cells as determined by the antibody's specificity. Though there are technically difficult goals to achieve, it is extremely important to have a therapeutic agent that could be injected into a cancer patient, target the DNA to tumor cells to induce an immune reponse to the tumor cells. Such an immune response should be able to seek out any surviving tumor cells.

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11. Appendices – see next page.

ANTIBODY DIRECTED GENE THERAPY

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The major problem with cancer is the spread of metastatic lesions. Chemotherapy and radiation are clumsy and inefficient tools to combat these lesions and inflict considerable damage to the patient.. Gene therapy offers an exciting new approach to treating cancer by targeting cytokine genes into tumor cells.

The approach of this project is to use a molecularly engineered monoclonal antibody to target DNA to tumor cells. Many studies have shown that tumor specific monoclonal antibodies can target tumors selectively. We have genetically modified a monoclonal antibody with specificity for some human breast cancers, so that the antibody can bind DNA as well as target tumor cells. The complex formed between the monoclonal antibody and DNA binds and transfects selectively the appropriate tumor cells, but the efficiency is low as judged by using the beta-galactosidase gene as a reporter. The majority of the bound DNA is transported to lysosomes.

The major barrier to efficient expression of targeted DNA after binding is entry into the cytoplasm. The incorporation of the small influenza fusion peptide into the complex did not enhance DNA expression. However physical studies of the influenza virus hemagglutinin indicates that the entire C-terminal region including the fusion peptide plays a critical role in membrane fusion. Therefore the C-terminal region has been redesigned to contain a DNA binding domain and a leader signal sequence. Once expressed and purified this hemagglutinin domain will be incorporated into antibody/DNA complexes, and the efficiency of DNA entry into the cytoplasm will be determined.

A second important barrier to efficient expression is the nuclear membrane. A nuclear localization signal has been engineered to contain a DNA binding domain, so that it also can be incorporated into antibody/DNA complexes.

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