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Gene therapy offers an exciting new approach to treating cancer, in particular by introducing cytokine genes into tumor cells. This strategy of enhancing the host immunity has been extensively studied in animal models and has provided the groundwork for the 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. 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. A DNA binding domain will be added to the gene encoding the heavy chain, so that upon expression of the modified heavy chain with its partner light chain, an antibody will be produced capable of binding DNA. We expect such an antibody complexed with DNA will be largely transported to lysosomes after internalisation, so a number of strategies will be examined to facilitate the entry of the DNA into the cytoplasm, transport and retention in the nucleus for successful prolonged expression. Subsequently, the antibody/DNA complexes will be tested in vivo.

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Peter J. Curtis 11/27/98

PI - Signature Date

TABLE OF CONTENTS

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 optimise 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.

Statement of Work, covering the first year.

Technical Objective 1

Task 1. Months 1-6. Clone, sequence and express Her81 as a Fab.
Task 2. Months 6-10. Produce 425fosLZ/Her81junLZ bAb and study its binding to tumor and normal cells.

Technical Objective 2 and 3

Task 3. Months 1-2. Produce 425LZ.DBD and transfect human breast cell lines.
Task 4. Months 2-16. Optimize expression of DNA by:
a) addition of flu fusion peptide
b) addition of nuclear localization signal.
c) selection of replicating plasmid.
d) analysis of putative tumor specific promoters.

Task 1.

Since cloning and expression of a Fab can encounter unexpected difficulties we obtained a second hybridoma, Her66, which also binds erbB2, from Dr. E. Vitetta. RNA prepared from the hybridomas Her66 and 81, was used as template to clone the light and heavy chain cDNAs by RT-

PCR using oligonucleotide primers, which introduce restriction sites to facilitate subcloning into pFab. pFab is a derivative of pUC19 with the multicloning site replaced by restriction sites suitable for the cloning and assembly of IgG cDNAs. The light and heavy chains of Her66 and 81 have been cloned and sequenced.

Task 2.

The light and heavy chains of Her66 and Her81 have not been subcloned yet into the baculovirus transfer vector and have not been expressed.

Task 3.

DNA encoding the DNA binding domain(DBD) of histone H1 was cloned into the C terminal codon of 425/fosLZ cDNA and then transferred into a recombinant baculovirus(rBEV). Co-infection of insect cells with rBEV.425K with rBEV.425/fos/DBD resulted in a 425 Fab with a DBD tail. The production and purification of 425fos/DBD has been improved considerably from the initial submission of this proposal.

The amount of 425fos/DBD found in the supernatant of infected cells was surprisingly low. It was realised that 425fos/DBD contains many positive charges, so that the secreted DBD might bind to the surface of the infected insect cells, which has a net negative charge. Washing infected cells with 1.0M NaCl released much more 425fos/DBD than found in the supernatant. Since the cell surface is limited and bound 425fos/DBD might be internalised, addition of negatively charged beads might increase the yield of 425fos/DBD. Further improvement was made by noting that maximal expression of 425fos/DBD was achieved only when an excess of 425 kappa chains was produced, but the excess kappa chains saturate the anti-mouse kappa affinity column. Therefore an ion exchange column was used prior to the affinity column to eliminate the excess kappa chains.

The procedure now used is as follows. Twenty four hours post infection, a slurry of sephadex SP-50, equilibrated with the insect medium, Express 5,is added to the infected cells, and the flasks are gently rocked to ensure mixing of the beads with the cells. After 50-60 hours the cells and beads are harvested by centrifugation, the pellet suspended in 0.15M NaCl, 10mM Tris pH 7.5, 10mM EDTA, 100ug/ml PMSF and then adjusted to 1.0M NaCl. Cells and beads are removed by centrifugation. The supernatant, after being diluted to 0.3M is passed over a sephadex SP-50 column, which is washed with 0.3M NaCl and tris, EDTA and PMSF, followed by 1.0M NaCl to elute 425fos/DBD, with very little 425 kappa chain.

In the next step the 1.0M eluant is applied undiluted to the anti-mouse kappa affinity column. The reason for applying 425fos/DBD in 1.0M NaCl is because molecules capable of inhibiting the binding of DNA by 425fos/DBD, possibly sulphated mucopolysaccharides, are eluted from the insect cells by 1.0M NaCl; any polyanions will associate with the 425fos/DBD at low salt. After washing with 1.0M NaCl the column is washed with a low salt buffer, followed by elution by a neutral buffer(buffer C, provided with the column by Sterogene, Calif). This step eliminates any DBD cleaved from the Ab, as well as any inhibitory polyanions. The eluting buffer is diluted to less than 0.5M salt before applying to a HiTrap SP column(Pharmacia). The 425fos/DBD is eluted by 1.0M NaCl followed by dialysis against PBS.

From 6 T175 flasks seeded with 30 x 106 cells per flask, about 430 ug purified 425fos/DBD was recovered, as estimated by OD280 and by comparison with known amounts of mAb17-1A on a SDS gel stained with coomassie blue(Fig. 1).

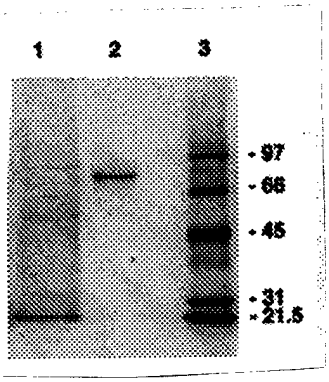


Figure 1. SDS PAGE of 425Fab.DBD. Lane 1, Cells and slurry 1.0M wash, 20ul. Lane 2, purified 425Fab.DBD, 5ul. Lane 3, Protein size markers. Stained with Coomassie Blue.

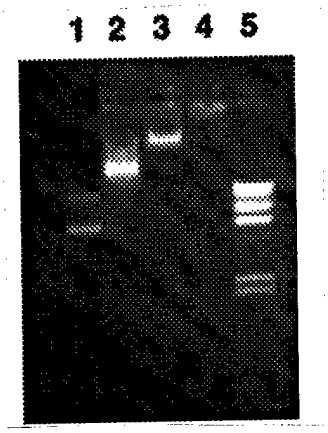


Figure 2. Agarose gel of 425Fab.DBD/DNA. Lane 1, pCMVLuc. Lane 2, Ab/DNA ratio 0.8:1.Lane 3, 1.7: 1. Lane 4, 3.3:1. Lane 5, λHindIII size marker

The ability of 425Fab.DBD to bind DNA was demonstrated by gel electrophoresis of different ratios of 425Fab.DBD to plasmid(Fig. 2).

The specificity of 425fos/DBD binding was demonstrated by FACS analysis, using human A431 cells and mouse CT26 cells which do not bind mAb425. No significant binding to the CT26 cells was detected by the Ab alone or complexed with DNA(Table 1).

Table 1

		Mean Fluorescence Intensity	
425DBD ug/ml	DNA ug/ml	CT26 Cells	A431 Cells
0	0	3.9	5.7
1.4	0	3.9	-
4.6	0	4.3	-
14.0	0	4.8	-
1.4	1.8	4.2	160.3
4.6	5.5	4.1	249.7
14.0	16.6	4.2	166.9

These complexes were added to human A431 cells, which express EGF-R abundantly, and 983-B cells, which express a much lower level of EGF-R, at different concentrations at 37°C for 4 hours in the presence of chloroquine(100uM) followed by 24 hours in medium alone, after which the cells were trypsinized, washed, lysed and assayed for luciferase. The results showed that the Ab/DBD concentration must be at least 4 ug/ml, and complexes with a higher ratio of Ab/DNA resulted in more luciferase. In experiment 2, DNA was reduced by a factor of 4 with Ab/DBD kept constant without significantly diminishing luciferase activity(Table 2).

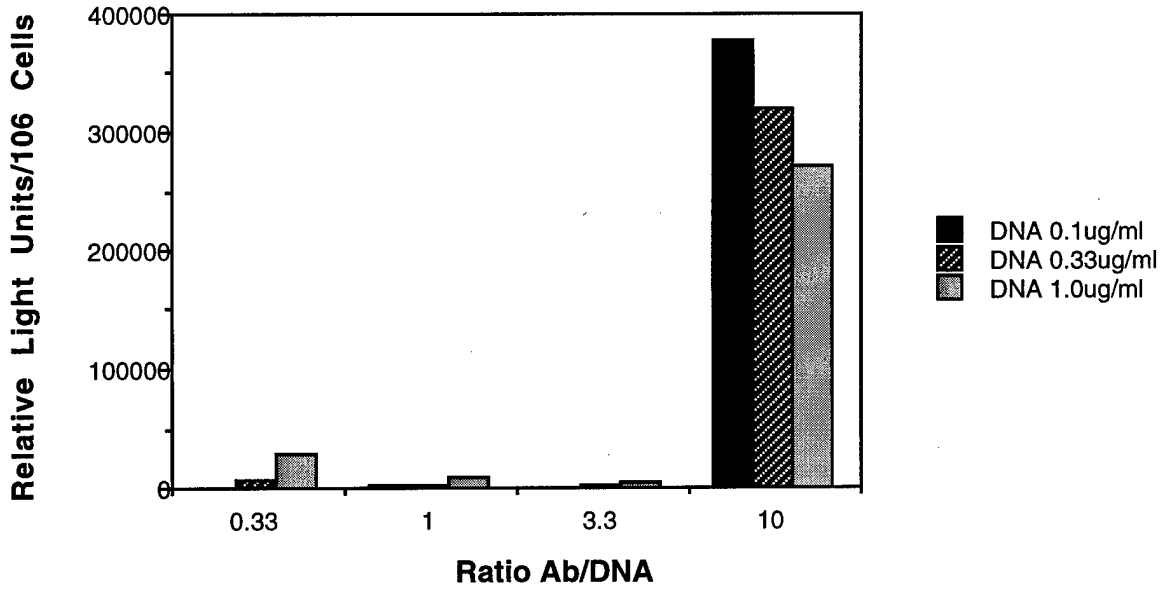
Table 2

	Ab : DNA (ug/ml)	RLU/106 cells
Expt. 1-A431 cells	0.8 : 6.0	0
	0.8 : 1.2	0
	4.0 : 30.0	0
	4.0 : 6.0	3120
983-B cells(-EGF-R)	4.0 : 6.0	220
Expt. 2	6.0 : 7.2	10,720
	6.0 : 3.6	10,720
	6.0 : 1.8	10,360

Task 4.

Further experiments were performed to identify the conditions to achieve optimal transfection. 425fos/DBD was mixed with DNA at different ratios, diluted upto 10 fold, and added to human A431 cells in the presence of chloroquine(100uM). A very high level of luciferase was detected using a ratio of Ab/DNA of 10. It was not concentration dependent over the range tested, as the same result was obtained with a concentration of 1.0ug/ml Ab as well as 10ug/ml(Fig. 3).

Figure 3.

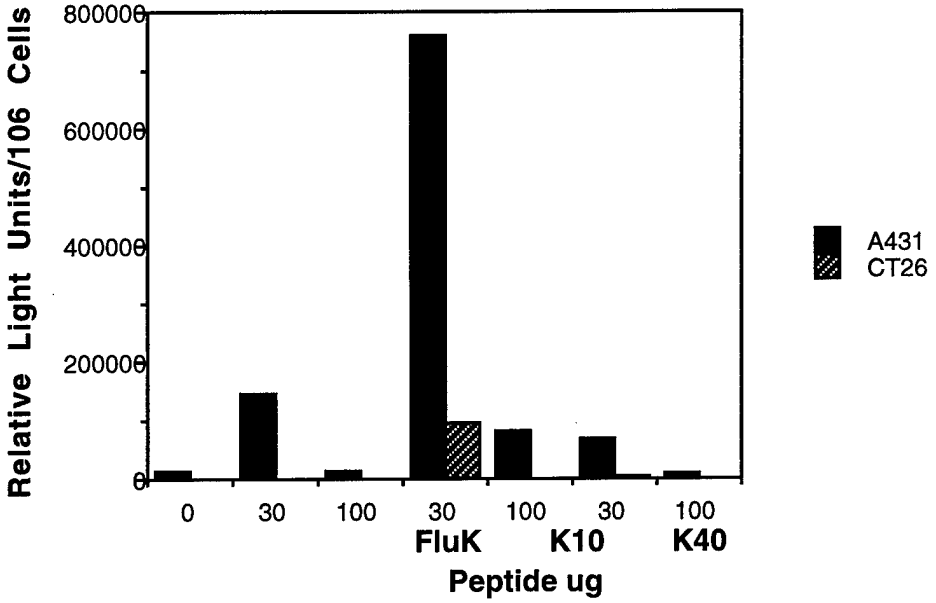


This result suggested that covering the DNA negative charges may be important to achieve efficient transfection. If this is the explanation, it should be possible to substitute polylysine for some of 425fos/DBD, which will also reduce the size of the complex. To avoid crosslinking to more than one DNA molecule, short polylysines were used, K10-10 lysines, andK40-40 lysines. In addition, the flu fusion peptide was synthesized with a C-terminal tail (KKKP)3. Each peptide

bound DNA, as judged by gel mobility shift on an agarose gel, and these complexes were stable to prolonged dialysis.

For this experiment, the Ab was mixed with DNA at a ratio of 2.2:1. It was found that only concentrations of the peptides much higher than needed to cover the DNA negative charge were effective. The highest level of luciferase was obtained using 0.15mg/ml K10(Fig 4). Under these conditions the complex still targeted specificially human A431 cells. However the flu peptide did not improve luciferase expression.

Figure 4.



The above results did not show linearity or proportionality of the response to varying the Ab/DNA complex. An explanation for this nonlinearity could be that DNA condensation occurs only under specific conditions. It has been shown that DNA condensation increases the efficiency of DNA uptake and expression[1][2]. DNA, polylysine and salt concentrations are critical to achieve DNA condensation. Attempts were made to identify conditions for DNA condensation using 425Fab.DBD 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. Supercoiled plasmid DNA passed through such a filter, but addition of increasing amounts of polylysine resulted eventually in no filterable DNA. Filtered DNA alone or DNA plus a low ratio of 425Fab.DBD was completely sensitive to DNase I, while filtered 425Fab.DBD/DNA/polylysine complex was partially resistant to DNase I, a reliable measure of condensed DNA.
Further studies are clearly necessary to define the conditions to achieve DNA condensation and DNase I resistance with 425fos/DBD.

In order to achieve prolonged expression of transfected DNA, plasmids have been constructed that should be episomally replicated in different human tumor cells. The BKV origin of replication was cloned into pUC19 together with the BKV T antigen gene driven by a CMV

promoter. Different orientations were identified. To detect replication, plasmids were transfected by DEAE-dextran into a human adenocarcinoma HT29 cells. After 3 days, Hirt supernatants were prepared, digested with Dpn I, to destroy plasmid methylated in E.coli., i.e. unreplicated DNA, and transfected into E.coli. The results of two experiments are shown in Table 3. As expected the T antigen has to be present to obtain replication in the human cells.

Table 3

	Ori T ag	Total no. colonies
1	->	0 , 0
2	<-	0 , 0
3	-> ->	1150 , 1690
4	-> <-	2760 , 5600
5	<- ->	2490 , 980
6	<- <-	2050 , 2760

Transfection of SV40-based plasmids into COS cells, expressing SV40 T antigen results in plasmid replication upto 50,000 copies per cell over a period of 3-4 days, but expression is lost after this time, possibly due to selection against cells containing large numbers of plasmids.

Mutagenesis was performed on the BKV origin of replication in order to select a BKV-based plasmid that would be capable of being maintained for a long period of time in human HT29 cells. The BKV origin of replication was amplified by PCR in the presence of dITP to promote random mutagenesis. The PCR fragment was cloned and individual clones were sequenced. On average each clone contained 2-3 base changes. Plasmid was prepared from about 10,000 colonies. The fragment containing the origin of replication was excised and cloned into pUC19 containing BKV T antigen, driven by a CMV promoter. Plasmid , prepared from 30,000 colonies was transfected into human HT29 cells. After 2 and 3 weeks, plasmid was extracted from the HT29 cells, digested with DpnI and transfected into E.coli. Fifty six colonies were obtained from plasmid harvested after 2 weeks and 27 colonies after 3 weeks. Plasmid, prepared from the pools of colonies as well as the unselected pool was transfected into human HT29 cells. Plasmid was harvested from the cells after 1, 2 and 3 weeks, and transfected into E.coli. before and after digestion with DpnI(Table 4).

Table 4

		Number of Colonies		
Plasmid	DpnI	1 Week	2 Week	3 Week
Unselected	-	420	140	20
	+	140	72	4
2 week	-	3000	1750	20
	+	220	60	12
3 week	-	4800	1250	70
	+	448	108	4

The results did not show more replicated plasmid from HT29 cells transfected with 2 week or 3 week selected plasmid than with the unselected plasmid pool, so there is no evidence of selection of a plasmid capable of being maintained over several weeks. In all cases however replicated plasmid was found after 2 and even 3 weeks. The other noteworthy result is that even

after 2 weeks the majority of plasmid recovered from the HT29 cells has not been replicated, indicating that a majority of the plasmid may not have entered the nucleus.

Currently, different forms of the human EGFR promoter are being used to replace the CMV promoter, which drives T antigen transcription. The EGFR promoter should result in more expression of the T antigen and therefore more plasmid replication in breast cancer cells overexpressing EGFR than in normal cells, providing another level of selection for tumor cells in addition to the selection by the antibody.

Conclusions

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.

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 β-galactosidase gene as a reporter.

The major barrier to efficient expression of targeted DNA is entry into the cytoplasm. The incorporation of the flu fusion peptide into the complex did not enhance DNA expression. It was expected that the flu fusion peptide would facilitate the entry of DNA through the cytoplasmic membrane. Other studies have identified the importance of DNA condensation for efficient DNA uptake and expression(1, 2). Therefore we have begun studies to identify the conditions necessary to achieve DNA condensation on our system and in the presence of the flu fusion peptide.

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