AD_____

Award Number: DAMD17-98-1-8172

TITLE: Novel Vector System for Breast Cancer Therapy

PRINCIPAL INVESTIGATOR: Robert I. Garver, M.D.

CONTRACTING ORGANIZATION: The University of Alabama at Birmingham
Birmingham, Alabama 35294-0111

REPORT DATE: October 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2000	Annual (1 Oct 99 - 30 Sep 00)

4. TITLE AND SUBTITLE
Novel Vector System for Breast Cancer Therapy

5. FUNDING NUMBERS
DAMD17-98-1-8172

6. AUTHOR(S)
Robert I. Garver, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Alabama at Birmingham
Birmingham, Alabama 35294-0111

E-MAIL:
robgarver@sprintmail.co

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; Distribution unlimited

12b. DISTRIBUTION CODE

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

This project is directed towards the development and testing of a novel, controlled release formulation of tumor necrosis factor alpha (TNFK) in combination with a partially replication defective adenovirus for the regional treatment of breast cancer. In the past year, we have refined our formulations of TNFKso as to be more suitable for ultimate human use. We have also assessed the effects of TNFKwith or without adenovirus on the growth of breast cancer cells *in vitro*. Pilot animal experiments with free TNFKshowed that our flank tumor nodule model did respond to this treatment, and therefore could be used to test the novel therapies developed here. An alternative, novel immunotherapy has been developed whereby plasmids encoding chemokines, agents that attract immunomodulatory cells to tissue sites, were shown to be effective *in vivo*. This strategy will be modified to encapsulate plamsids encoding the adenoviral E1A proteins, and tested head-to-head against the intact adenovirus in the animal experiments planned for the final year of this project.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	11
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

Introduction

This project seeks to explore the feasibility of a novel local-regional therapy for breast cancer that could ultimately be applied for disseminated disease as well. In brief, we are evaluating the combination of a locally administered, controlled release formulation for tumor necrosis factor alpha (TNFK) in combination with adenovirus E1A gene products. The rationale for using a locally administered, controlled release formulation of TNFKis based on the observation that clinical applications of systemic TNFKhave been unable to achieve therapeutic levels within neoplastic cells without unacceptable systemic toxicity [1-3]. However, local infusions of TNFKhave been show to be efficacious in melanoma [4]. Therefore, it seems reasonable to infer that an alternative means of achieving locally high levels of TNFKin breast cancer could be an important advance. The rationale for using adenoviral E1A gene products is based on observations that cells containing adenoviral E1A protein are sensitized to the toxic effects of TNFK[5-10]. The scope of this project is limited to the development of the two reagents (TNFKand appropriate means of delivering E1A), and testing the formulations in breast cancer tumor nodules engrafted on mice.

Body

Task #1: Construct and characterize microspheres that contain and release TNFKover an extended period of time

Additional experiments were undertaken to optimize the coacervate microspheres containing TNFK. These experiments examined several different proteins based on a coacervate formulation from complexation of human serum albumin (HSA) and heparin. Anticipating that these microspheres might be useful in co-delivering drugs and immunoadjuvants in a single vehicle, we first studied the entrapping and release kinetics of drugs with a wide range of molecular weight (Mw=262~67000). Figure1 shows the microsphere formation at different pH of the HSA solution and different dilutions of the heparin solution. No coacervation occurred above pH 3.9 as HSA was not sufficiently protonated. Below pH 2.5 the particle yield was close 100%. At this pH the particle yield was proportional to the heparin concentration. Approximately 35 mg of microparticles was obtained from 1 ml of 2.5% HSA solution and 1 ml of original heparin solution. Some additives and salts included in the clinical preparation of human serum albumin and heparin were also encapsulated into the microparticles. The coacervates were irregular microparticles as observed under microscope and SEM, with an average size around 5 um.

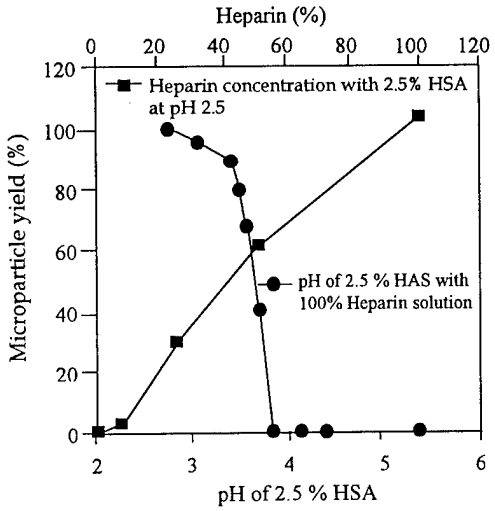
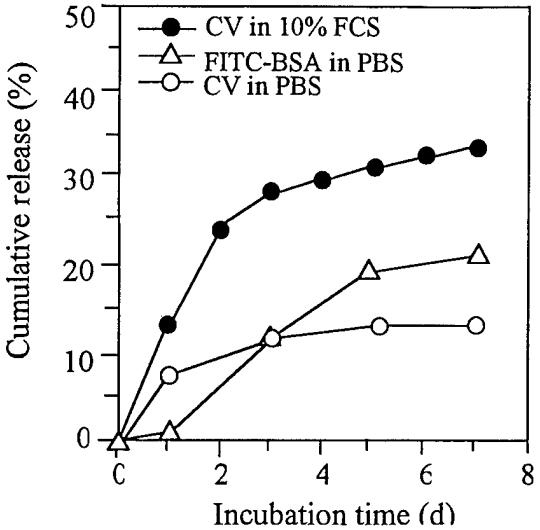


Figure 1

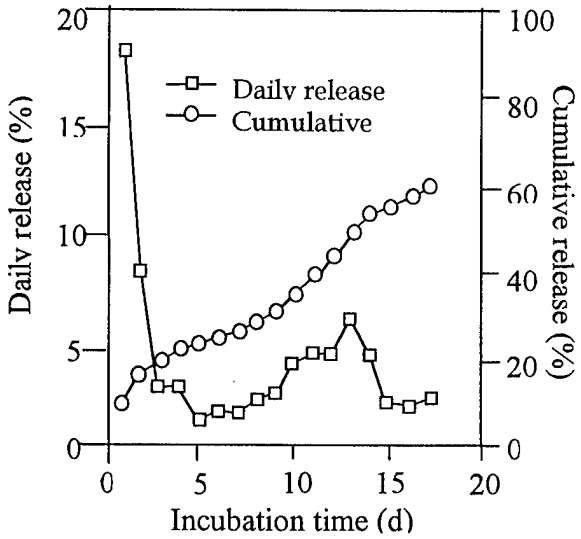
To obtain drug-loaded microparticles, the encapsulants (IL-2, 100000 U/mL; FITC-BSA, 2 mg/mL; CV, 2 mg/ml and doxorubicin, 100 μg/mL) were added to heparin solution immediately before coacervation. HSA solution (3 mL) was added into the vortexing heparin solution (3mL) and kept vortexing for 10 sec. Crosslinking reagent, 1-ethyl 3 (3-propylamino) carbodiimide hydrochloride (EDC) was added to a final concentration of 0.75 to 3 mg/mL, vortexed and kept for 15 min at room temperature. To quench the extra EDC, 0.1 M glycine (7mL) was added, and the solution let stand for another 15 min. The encapsulation efficiency was almost 100% for proteins (IL-2, BSA, and GM-CSF), and over 85% for low-molecular compounds (crystal violet, doxorubicin, 5-Flurorouidine and cyclophosphamide). The release kinetics of these microparticles in PBS solution (Fig. 2a, b, c, d) is relatively sustained. Release was more rapid because of the proteolytic degradation of the microspheres. A burst is typically observed in 10%

FCS. Bioactive cytokines, such as GM-CSF and IL-2, as determined by a cell proliferation assay, could be released for over one month, although the release rate declined in a near exponential manner. This drop in release rate is typical of such microparticle delivery system because of the small particle size and the combination of diffusion and matrix-degradation in effecting the drug release.

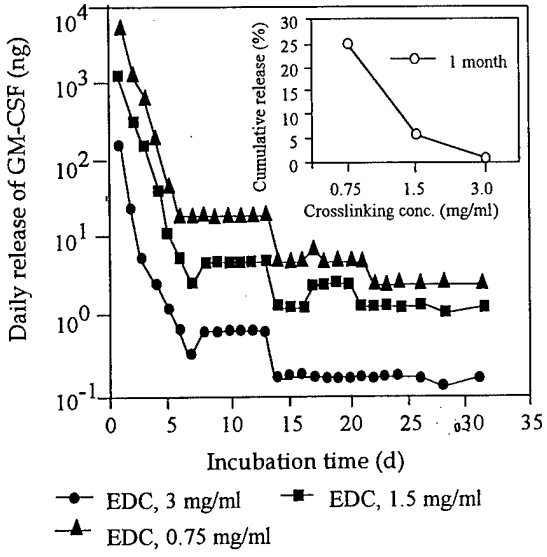
a.



b



c.



d.

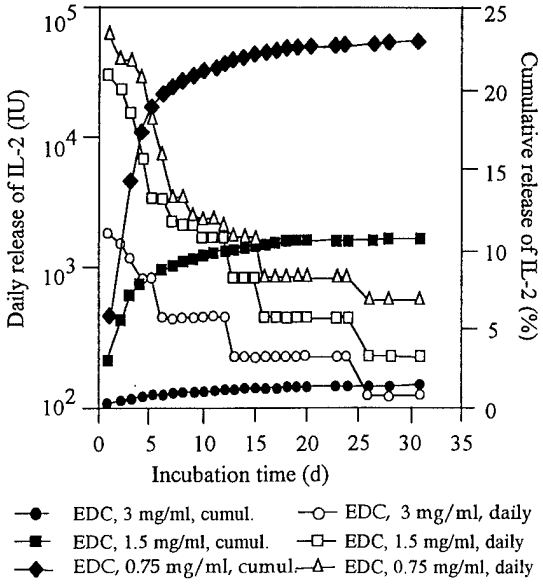


Figure 2

Based on these background studies, it was possible for us to synthesize microspheres containing TNFKwith the release profile shown in figure 3.

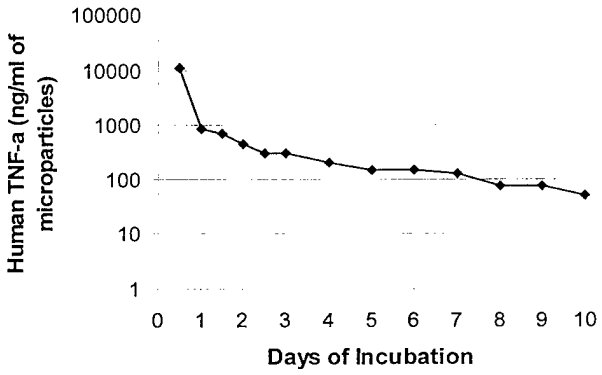


Figure 3

Release of human TNF-a in 10% FBS medium was sustained over 10 days. Human TNF-a concentration in the supernatant was measured by ELISA assay.

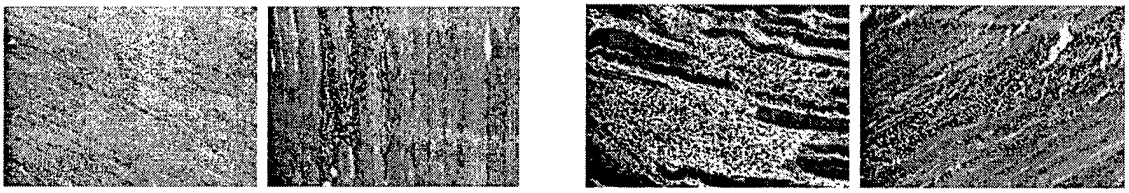
Task 2: Identify a conditionally replicative adenovirus suitable for use in combination with the extended release TNFKformulation

In last year's report, we described our progress in obtaining the partially defective adenovirus *dl*338 that contained the intact E1A transcription unit. The virus identity was confirmed by partial sequencing, and the virus amplified for use. We have also developed a novel adenovirus containing and E1A transcriptional unit with a transgene encoding the HSVTK product that sensitizes cells to ganciclovir, AdE1A-tk. The construction and characterization of this virus is detailed in the publication attached in the appendix. In brief, this virus has limited replication in neoplastic tissue, and was shown to produce bioactive E1A. The potential advantage this virus holds over the *dl*338 virus is the absence of any E1B-encoding sequences as an added safety feature, and the inclusion of the HSVTK sequences that provide a further means of killing the infected cells.

As a further innovation, we have examined the development of nanospheres containing plasmids encoding immunologically active proteins: chemokines. These proteins are not directly relevant to this task, but the development and feasibility demonstration have laid the background for analogous nanospheres to be constructed containing E1A-encoding plasmids.

Chemokines are highly inflammatory molecules that act primarily as chemoattractants of various leukocytes. They are particularly important in the regulation of leukocytes trafficking from the blood and lymph vessels to the peripheral sites. For instance, monocyte chemotactic protein-1 (MCP-1) induces monocytes to migrate from the blood to the tissue and become tissue macrophages. The recruitment of dendritic cells to the tumor site is of great interest because they are the most potent professional antigen-presenting cells. Monocyte-derived immature Daces and monocytes express receptors for some inflammatory chemokines such as MCP-1, MIP-1α, and RANTES, but not for MIP-3α or MIP-3β. Immature Daces derived from CD34+ hematopoietic progenitor cells (HPCs) are most sensitive to MIP-3α, and also to RANTES and MIP-1α, while mature Daces lose their response to these chemokines but become sensitive to a single chemokine, MIP-3β. To test the effects of these different chemokines in enhancing APC recruitment and activation we have cloned murine MCP-1,

MIP-1α, MIP-3α and MIP-3β plasmids into the pDNA3.1 or pRK backbone. After demonstrating the bioactivity of these plasmids in transfecting HEK293 cells in culture, we tested the chemoattractant effects of these chemokines in the intramuscular space of mice (Figure 4). Compared to the PBS control with only the backbone of the construct, all three chemokine plasmids (b) MIP/pcDNA3.1, (c) MIP-3a/pcDNA3.1, (d) MIP-3b/pcDNA3.1 showed significant infiltration of immune cells into the injection site. This preliminary result demonstrates the bioactivity of these plasmids in vivo.



(a) (b) (c) (d)

Figure 4. Histology of muscle injected with (a) pcDNA3.1 construct in PBS, (b) MIP/pcDNA3.1, (c) MIP-3a/pcDNA3.1, (d) MIP-3b/pcDNA3.1. (2 μg of DNA per animal; Day 3)

To further characterize these plasmids, we performed a chemotactic assay on dendritic cells isolated from spleens of mice. Figure 5 shows the significant chemotactic effect of MIP-3b on dendritic cells from splenocytes. The constructs with the RK backbone appear to be more potent that that with pcDNA3.1. This correlates with the higher expression of the chemokines with the pRK backbone in cell culture.

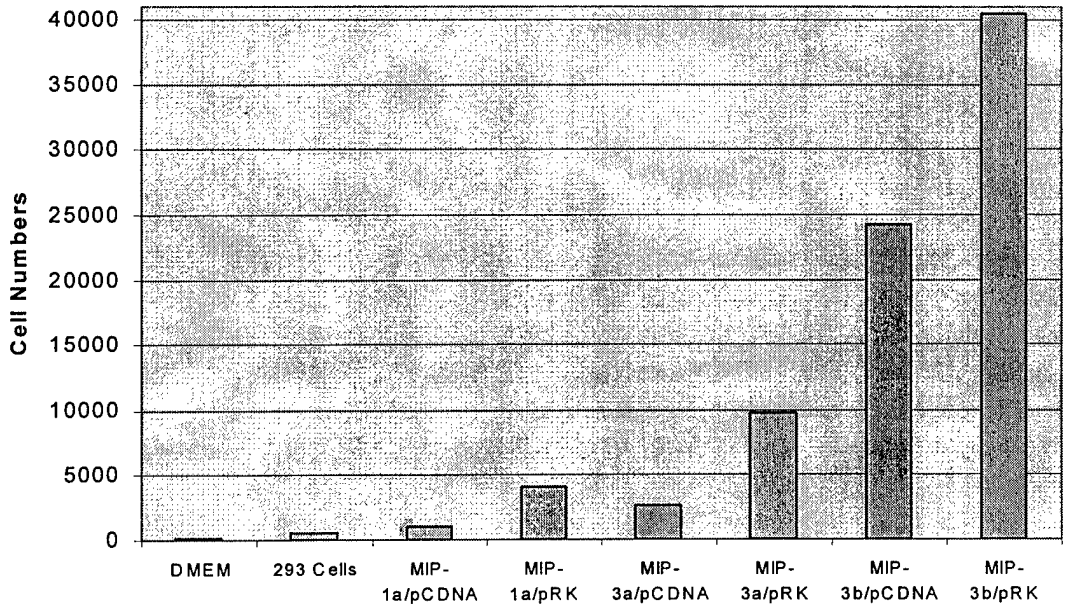


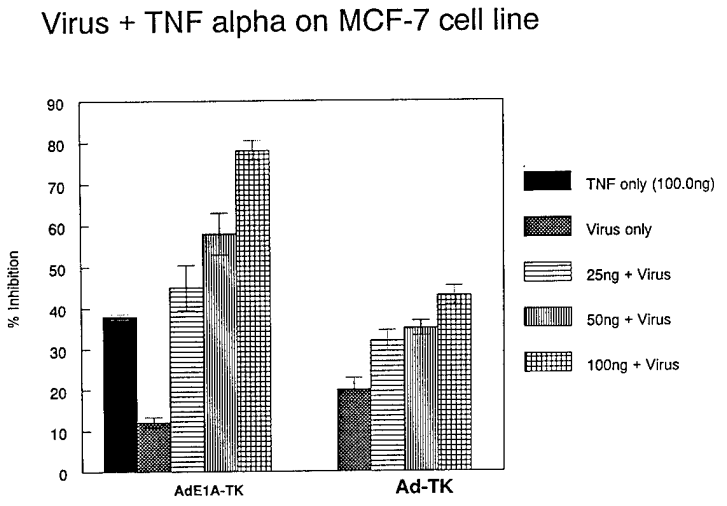
Figure 5. Chemotactic assay performed in a diffusion chamber.

We have obtained plasmids that direct the transcription of either both 12S and 13S E1A transcripts, or the 12S transcript selectively. These plasmids will be tested in vitro by transfection, followed by TNFKexposure in order to select the optimal plasmid for encapsulation.

Task #3: Evaluate individual and combined activity of AdE1A-tk virus and TNFKin vitro

In last progress report, we had tested the combination of the *dl*338 virus with TNFKin a single lung cancer cell line. We have extended these experiments by testing two human breast cancer cell lines with TNFKonly, the AdE1A-tk virus only, or the AdTK virus which does not contain and E1A sequences (fig. 6). In these experiments, the addition of the E1A-encoding virus at an moi=1 with increasing doses of TNFKshowed at least an additive toxicity in the MCF-7 human breast ca cell line. Infection with the AdTK virus which did not contain E1A sequences did not enhance the activity of the TNFK The amount of toxicity achieved with either agent alone, or in combination, was significantly less in the MDA-MB-231 cell line.

a.



b.

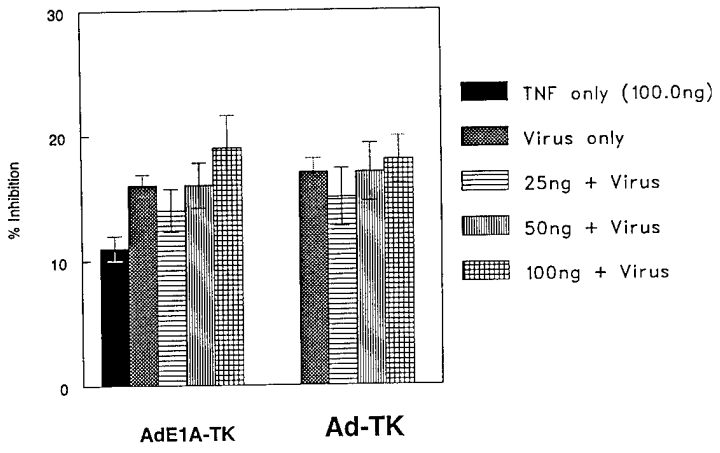


Figure 6

Task #4: Evaluate the combined activity of *dl*338 virus and TNFK in vivo

This task has just been started by testing the impact of TNFK only on the growth of tumor nodules engrafted in mice (figure 7). A pilot experiment with A549 tumor nodules utilized the intratumoral administration of unencapsulated TNFK (10 μg) followed by serial tumor nodule measurements. These experiments have shown that TNFK can be locally administered with a measurable effect on tumor growth. Additional, more comprehensive animal experiments using the microsphere formulations are now underway.

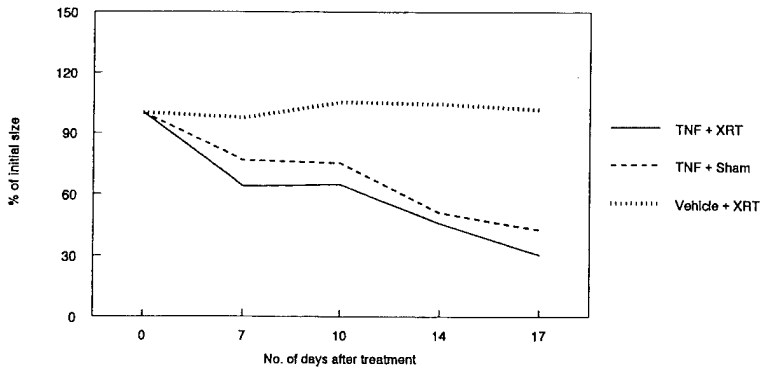


Figure 7

Key Research Accomplishments

- optimization of controlled release TNFK microspheres
- testing feasibility of nanonspheres containing plasmids expressing immunomodulatory molecules, like E1A or TNF
- demonstrating efficacy of new adenovirus (AdE1A-tk) in combination with TNFK in vitro
- establishing that tumor nodule model can be used to measure TNFK efficacy in vivo

Reportable Outcomes

- abstract presented at Era of Hope Meeting: "Progress Towards Developing a Novel Strategy for Intratumoral Breast Cancer Therapy"
- grant application (VA Merit Review) submitted 6/2000: "Preclinical Assessment of Regional TNFK for NSCLC"

Conclusions

Significant progress has been made in the past year on Tasks 1-3. Task 4 is underway, and Task 5 will be completed in the final year of the contract. These results thus far support the original rationale and feasibility of using a controlled release formulation of TNFK for the treatment of local regional breast cancer. The addition of E1A protein, either by virus or plasmid, is expected to further enhance efficacy. The animal experiments will be an important next step to better evaluate the preclinical efficacy of this therapeutic strategy, and if successful, may lead to initial human clinical trial planning in the near future.

References

1. Fajardo, L. F., Kwan, H. H., Kowalski, J., Prionas, S. D., and Allison, A. C. Dual role of Tumor Necrosis Factor-a in angiogenesis. Am. J. Pathol., 140:539-544, 1992.
2. Eggermont, A. M. M. TNFK in isolated perfusion systems: success in the limb, developments for liver credits, debits and future perspectives. Anticancer Res., 18:3899-3906, 1998.
3. Mueller, H. Tumor necrosis factor as an antineoplastic agent: pitfalls and promises. Cellular and Molecular Life Sciences, 54:1291-1298, 1998.
4. Eggermont, A. M. M. TNFK in isolated perfusion systems: success in the limb, developments for the liver credits, debits and future perspectives. Anticancer Res., 18:3899-3906, 1998.
5. Chen, M.-J., Holskin, B., Strickler, J., Gorniak, J., Clark, M. A., Johnson, P. J., Mitcho, M., and Shalloway, D. Induction by E1A oncogene expression of cellular susceptibility to lysis by TNF. Nature, 330:581-583, 1987.
6. Duerksen-Hughes, P. J., Hermiston, T. W., Wold, W. S. M., and Gooding, L. R. The amino-terminal portion of CD1 of the adenovirus E1A proteins is required to induce susceptibility to tumor necrosis factor cytolysis in adenovirus-infected mouse cells. J. Virol., 65:1236-1244, 1991.
7. Hashimoto, S., Ishii, A., and Yonehara, S. The E1b oncogene of adenovirus confers cellular resistance to cytotoxicity of tumor necrosis factor and monoclonal anti-Fas antibody. International Immunology, 3:343-351, 1991.
8. Ames, R. S., Holskin, B., Mitcho, M., Shalloway, D., and Chen, M.-J. Induction of sensitivity to the cytotoxic action of tumor necrosis factor alpha by adenovirus E1A is independent of transformation and transcriptional activation. J. Virol., 64:4115-4122, 1990.
9. Wold, W. S. Adenovirus genes that modulate the sensitivity of virus-infected cells to lysis by TNF. J. Cell. Biochem., 53:329-335, 1993.
10. Tsuji, Y., Ninomiya-Tsuji, J., Torti, S. V., and Torti, F. M. Augmentation of IL-1 alpha of tumor necrosis factor-alpha cytotoxicity in cells transfected with adenovirus E1A. J. Immunol., 150:1897-1907, 1993.