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FINAL REPORT
Period of Funding 12/1/95-11/30/04
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Title: Genetically engineered multivalent single chain antibody constructs for cancer therapy

P**rincipal Investigator**: Surinder k. Batra, Ph.D

Project history: The grant was funded at the University of Nebraska Medical Center to **Dr. David Colche**r in 1995. Dr Batra joined the project as a co-Investigator in late **1996** when he moved from Duke University Medical Center, NC, where he was collaborating with Dr. Michael Zalutskey and Dr. Bigner on the development of 81C6 antibody constructs funded by the similar DOE program.

Dr. Batra participated on the competitive renewal of this proposal in **1998** where Dr. Colcher was the PI. Dr. Colcher left UNMC to join a Senior Position in a pharmaceutical company in **1999**. At the time of his departure, Dr. Batra credentials were evaluated by the DOE program officials at Washington, DC, head office. Dr. Batra made presentation and hd discussion with Program Director. Dr. Batra was appointed as the Principal Investigator of the project in the middle of 1999. He led the project successfully for two years (generated multivalent antibodies-see the report below) and competed for its renewal at the end of **2000**. The project was successfully renewed in 2001 and was given final extension in **2003** for another one year till **2004**. No cost extension was also given till 2005 for the completion of the studies.

REPORT

1The major goal of our study was to develop specific molecular tracers for the diagnosis and treatment of solid tumors. Radiolabeled monoclonal antibodies (MAbs) directed against tumor associated antigens (TAAs) have been used as radioimmunoimaging (RII) and radioimmunotherapeutic (RIT) agents in a variety of diseases including carcinomas. Radiolabeled MAbs can assist in the early diagnosis of neoplasia when used for external imaging and can be useful for tumor therapy when labeled with the appropriate radionuclide. The MAb CC49 is a second generation murine monoclonal antibody that binds an epitope (sialyl-Tn) on the TAG-72 antigen expressed by a majority of human adenocarcinomas. CC49 has shown in preclinical studies a three to five-fold improvement in therapeutic efficacy over the first generation monoclonal antibody B72.3. A major limitation for systemic administration of intact CC49 radiolabeled MAb has been the hematological toxicity. This limits the maximum tolerated dose and dose escalation essential for solid tumors. Moreover, intact CC49 showed poor heterogeneous tumor penetration.

In order to improve its therapeutic efficacy, we engineered a range of smaller fragments (monovalent, divalent and tetravalent) single chain Fv (scFv) forms using DNA recombinant techniques. Generally, the scFvs show very rapid blood clearance, the excellent penetration into a tumor from the vasculature, higher tumor:normal tissue ratios, and reduced immunogenicity than corresponding IgG in *in vivo* studies. These scFv forms are being analyzed for their biodistribution and therapeutic efficacy in athymic mice bearing human LS-174T colon carcinoma cell line xenografts. The monovalent scFv could be expressed and purified in sufficient amounts from *E.coli.* However, multimeric scFvs, including covalent dimeric proteins, showed problems associated with poor expression, aggregation and folding. Therefore, we used methotrophic yeast, *Pichia pastoris,* to express monomer and multimer

scFv. The Fv region of murine MAb CC49 was cloned under the regulation of alcohol oxidase 1 (AOX1) promoter. For achieving efficient purification of scFvs by immobilized metal-ion affinity chromatography (IMAC), a His-tag was placed either at the C-terminal (scFv-His_6) or N-terminal (His_6-scFv) of the coding sequence. The immunoreactivity of the radiolabeled scFv proteins was determined by solid phase RIA using the bovine submaxillary gland mucin (BSM), which contains the epitopes recognized by MAb CC49, and BSA as a negative control, attached to Reacti-Gel beads. scFv-His_6 showed only 20-25% binding whereas both His_6-scFv and scFv (no His-tag) showed 60-65% binding. Surface plasmon resonance studies by BIAcore revealed the binding affinity constant (K_A) for His_6-scFv and scFv as 1.19 x 10^6 M^-1 and 3.27 x 10^6 M^-1, respectively. Comparative homology modeling for scFv and scFv-His_6 demonstrated that the C-terminal position of the His-tag partially covered the antigen-binding site of the protein. Therefore, the C-terminal position of His-tag on the CC49 scFv was found to adversely affect the binding properties of the construct.

The CC49 divalent scFv [V_L-linker-V_H-linker-V_L-linker-V_H-His_6] was engineered and characterized *in vitro* and *in vivo*. Expression of sc$(Fv)_2$ in *Pichia pastoris* resulted in yields 30-40 fold greater than those from *E. coli*. The biodistribution and tumor-targeting characteristics of radiolabeled CC49 scFvs produced in yeast demonstrated similar results to the divalent forms produced in *E. coli*. Upon expression, the sc$(Fv)_2$ associated spontaneously to form non-covalent tetrameric scFv {[sc$(Fv)_2]_2$}. The binding affinity constant (K_A) for the [sc$(Fv)_2]_2$ and CC49 IgG were similar, i.e., 1.02 x 10^8 M^-1 and 1.14 x 10^8 M^-1, respectively, and were four-fold higher than its divalent scFv [sc$(Fv)_2$, 2.75 x 10^7 M^-1]. Biodistribution studies were performed in athymic mice bearing LS-174T human colon carcinoma xenografts. At 6 h post-administration, the mean percent injected dose accumulated per gram of LS-174T colon carcinoma xenografts (% ID/g) was 21.3, 9.8 , and 17.3 for radioiodinated [sc$(Fv)_2]_2$, sc$(Fv)_2$, and IgG, respectively. Pharmacokinetic analysis of blood clearance studies showed the elimination half-life for [sc$(Fv)_2]_2$, sc$(Fv)_2$, and IgG as 170, 80, and 330 min, respectively. [sc $(Fv)_2]_2$ appeared as an important reagent for cancer therapy and diagnosis in MAb-based radiopharmaceuticals.due to the gain in avidity resulting from multivalency along with an improved biological half-life.

The therapeutic efficacy of [sc$(Fv)_2]_2$ and sc$(Fv)_2$ was investigated in athymic mice bearing subcutaneous LS-174T human colon carcinoma xenografts. Mice received 1000 microCi of ^{131}I-[sc$(Fv)_2]_2$ or ^{131}I-sc$(Fv)_2$, either as a single injection on day 0 or as four injections (250 microCi each) on days 0, 1, 2, and 3. The median survival for the control group was 20 days. Comparisons of single and fractionated therapeutic regimes showed median survival as 26 ($P < 0.001$) and 47 days ($P < 0.0001$), respectively for [sc$(Fv)_2]_2$ and 20 ($P > 0.5$) and 32 days ($P < 0.0001$), respectively for sc$(Fv)_2$ when compared to the control groups. The mean time for the quadrupling of tumor volume for single and fractionated therapeutic treatments were: 9.0 and 21.1 days respectively for sc$(Fv)_2$; 16.6 and 32.9 days respectively for [sc$(Fv)_2]_2$; and 8.3 and 8.4 days respectively for the control group. No ^{131}I-systemic toxicity was observed in any treatment groups. The results show that RIT delivery for sc$(Fv)_2$ and [sc$(Fv)_2]_2$ in a fractionated schedule clearly presented a therapeutic advantage over single administration.

The diagnostic potential of sc$(Fv)_2$ and [sc$(Fv)_2]_2$ was evaluated in athymic mice bearing LS-174T colon carcinoma xenografts. ScFvs were radiolabeled with 99m-technetium using the bifunctional chelator succinimidyl-6-hydrazinonicotinate hydrochloride (SHNH) and tricene as the transchelator. In RIA, both (scFv)$_2$ and [sc$(Fv)_2]_2$ showed 75-85% immunoreactivity with non-specific bindings between 0.8-1.2%. HPLC size exclusion chromatography showed sc$(Fv)_2$ and [sc$(Fv)_2]_2$ as 60 and 120 kDa proteins. Blood clearance studies showed the elimination half-life of ^{99m}Tc-labeled sc$(Fv)_2$ and [sc$(Fv)_2]_2$ as 144 and 307 min, respectively. Whole body clearance studies confirmed the rapid elimination of scFvs from the body with >90% of sc$(Fv)_2$ and [sc$(Fv)_2]_2$ clearing by 24 h post administration. At 6 h post administration, the tumor

localization with ^{99m}Tc-sc(Fv)$_2$ and ^{99m}Tc-[sc(Fv)$_2$]$_2$ was 7.2 and 17.1 percent injected dose per gram of tumor (%ID/g), respectively. The uptake of the radiometal in liver, kidneys and spleen was found to be elevated. Macroautoradiography performed at 6 and 16 h post administration detected the tumor clearly with both sc(Fv)$_2$ and [sc(Fv)$_2$]$_2$. These reagents, therefore, hold importance in clinical imaging studies of cancer in the field of nuclear medicine.

The clinical utilization of murine MAbs is also limited by induction of a human anti-murine antibody (HAMA) response. The HAMA response may elicit allergic reactions in patients, and it can alter the rate of anti-tumor antibody clearance from serum. To minimize the immunogenicity of murine CC49, we genetically humanized these antibody constructs, in which non-human regions were replaced by corresponding human ones. Preliminary results of the clinical trials involving the administration of the murine MAb CC49 indicate that the patients' anti-idiotypic responses are directed mainly against CC49 light variable region. Therefore, we targeted the murine CC49 variable light chain to minimize the immunogenicity of CC49 constructs. A new humanized CC49 scFv (hu/mu scFv) construct was developed using the method of gene shuffling, where murine CC49 variable light chain was entirely replaced by a homologous human light chain (the human subgroup IV germline variable light chain; Hum4 V_L). The properties of hu/mu scFv were compared to murine CC49 scFv (mu/mu scFv) to determine if the replacement would affect its antigen-binding, pharmacokinetics, and biodistribution. The gene-shuffled hu/mu scFv formed stable three dimensional structure with high affinity to TAG-72 antigen with an association constant of 1.1 x 10^6 M^-1 as measured by surface plasmon resonance analysis. The hu/mu scFv showed a faster dissociation rate (4.01 x 10^-3) than murine scFv (1.83 x 10^-3). The association constant for mu/mu scFv construct was 1.4 x 10^6 M^-1. The scFvs showed similar binding to BSM (60%-66%).

Pharmacokinetic studies of radiolabeled humanized CC49 scFvs showed a rapid blood and whole body clearance. Since hu/mu scFv showed a faster dissociation rate than mu/mu scFv using BIAcore analysis, it was essential to analyze the capability of hu/mu construct to localize human tumor xenografts *in vivo*. At various times after injection, blood, tumor, and normal organs were analyzed to determine the amount of each radionuclide retained per gram of tissue. The hu/mu scFv demonstrated equivalent tumor targeting properties in comparative dual label studies with mu/mu scFv with 1.95 %ID/g and 1.68 %ID/g, respectively, at 6 h post administration. The levels of the radiolabeled scFvs in normal organs reflected their concentration presented in blood and did not showed statistical differences in the biodistribution in normal organs with exception of kidneys when compared to murine scFv. Overall, the pharmacokinetic and biodistribution studies of hu/mu CC49 scFv molecule exhibited excellent tumor targeting properties and rapid clearance. The human subgroup IV light chain can be used to combine with any V_H capable of forming a three dimensional structure with ability to bind to TAG-72 to produce humanized or human antibody constructs. The V_L gene can be also used to isolate a gene encoding human V_H having the capability to bind TAG-72 from a combinatorial library.

To further minimize HAMA responses in patients, CC49 MAb was humanized by grafting CDRs of CC49 variable heavy chain region onto human frameworks of MAbs 21/28' CL. This human MAb was most homologous to CC49 in the framework residues crucial to maintaining the combining-site structure with 28 of the 40 critical framework residues being identical. The CC49 CDRs grafted onto variable chain V_H frameworks of MAb 21/28' CL were combined with the human subgroup IV kappa variable light chain to assemble the second humanized CC49 scFv construct (hu/CDR scFv). The mouse (mu/mu scFv), the human light chain-shuffled scFv (hu/mu scFv) and humanized scFv (hu/CDR scFv) were expressed in *E. coli*, and recombinant proteins were purified and compared in SDS-PAGE, HPLC, and ELISA analyses. The immunoreactivity of the scFv forms was tested in a competition ELISA. CC49 scFvs were able to compete for 100% of the binding to the epitopes recognized by MAb CC49 on the TAG-72

with biotinylated CC49 IgG. The immunoreactivity of the labeled forms was determined by solid phase RIA using proteins attached to Reacti-gel beads. BSM was used as a positive control and BSA as a negative control. The mu/mu scFv and the hu/mu scFv demonstrated again similar binding (66%); however, the hu/CDR scFv showed decreased binding to BSM (40%) with only 0.5-3% binding to negative control BSA for all tested CC49 scFv constructs.

Immunogenicity of CC49 scFvs constructs were evaluated using human sera obtained from patients with colon cancer treated by radiolabeled murine CC49 IgG (0.6-20 mg) in therapy trials. In order to evaluate the anti-idiotypic response, the circulating antigen TAG-72 and the anti-isotypic/- anti-allotypic response in sera had to be eliminated. The Reacti-gel was coated with MAb B72.3 that recognized the different epitop of TAG-72 antigen and can bind free TAG-72 antigen without absorbing the human anti-idiotypic immunoglobulins against CC49 IgG in patient serum. MAbs CC49 and B72.3 are murine IgG_1, therefore, the B72.3 IgG was also used to absorb the anti-isotypic and anti-allotypic immunoglobulins from human serum. The Reacti-gel coated with BSA as a negative control and with MAb CC49 IgG were also prepared. We have used the double-antigen (DAB) assay, in which the antibodies present in a patient's serum bind to the solid-phase antigen (catcher antibody) as well as to the labeled antibody in the detection step. The advantage of the DAB assay is that nonspecific human immunoglobulins present in serum will not be detected. The plates were coated with MAb CC49 IgG and reactive human immunoglobulins from sera were detected by radiolabeled scFvs. All the sera showed the highest reactivity against the mu/mu scFv. Most of patients' sera demonstrated decreased reactivity (50-74%) to constructs with scFvs with the human light chain; however, the different serum reactivities against hu/CDR scFv and hu/mu scFv were observed. One serum showed lower reactivity against hu/mu scFv (74%) than hu/CDR scFv (50%). This finding is supported by other current studies with different humanized MAbs suggesting that replacing antibody CDRs could remove existing immunogenic epitopes, but could also generate new ones. Furthermore, humanized MAbs are potentially immunogenic since murine CDRs are coding for non-human variable region sequences and may still elicit an anti-idiotypic response. Therefore, it is most beneficial to acquire fully human variable regions capable of binding to a selected antigen. We have continued preparing the human monoclonal antibodies specific to the Sialyl-Tn epitope of tumor cell mucins using phage display technology. We have obtained peripheral blood from patients that were immunized with Sialyl-Tn-KLH. The samples showing positive response to Sialyl-Tn were selected for the generation of phage display clones. The heavy chain variable region genes for IgG and IgM were separately amplified from mRNA using a collection of primers that hybridize to the conserved DNA sequences with the first framework (5'-Primers) and a carboxy-terminal portion (3'-Primers) C_{H1}-hinge junction for heavy chain. We prepared the light chain Fd DNA by combining the human subgroup IV germline variable light chain-Hum4 V_L from our humanized scFv construct and constant k light chain region amplified by PCR via *Bcl 1* restriction site. The constant k light chain region was amplified from mRNA obtained from the lymphocytes of Sialyl-Tn-KLH immunized patients. The light chain Fd DNA is subcloned into the pComb 3 phagemid vector (Dr. Richard Lerner, The Scripps Research Institute, La Jolla, CA). PCR-amplified heavy chain Fd DNA were digested with *Xho 1* and *Spe1.* and cloned into the pComb 3-light chain phagemid vector. After several cycles of panning and amplification, individual clones will be isolated and the selected clones will be used for construction of fully human antibodies.

During the last cycle of the grant, our studies were aimed at evaluating and improving tumor-uptake and the retention of scFv-based radiopharmaceuticals exhibiting favorable pharmacokinetics for therapy and diagnosis of solid tumors. Innovative technologies were employed to modulate the tumor vascular permeability and enhanced tumor penetration of the radiopharmaceuticles for therapeutic dose delivery and deposition. To understand the short

plasma half-life of multivalent single-chain antibody fragments, the pharmacokinetic properties of covalent dimeric scFv [sc(Fv)2], noncovalent tetrameric scFv {[sc(Fv)2]2} and IgG of MAb CC49 were examined. The scFvs displayed an ability to form higher molecular aggregates in vivo. At 2h post-administration, nearly 25% of the circulating dimer aggregated to yield a molecular spcies comparable to tetrameric scFV, while another 15% aggregated to higher molecular weight forms. Similarly, 20% of the circulating tetramer associated to yield high molecular weight complexes. A specific proteolytic cleavage of the linker sequence of the covalent dimeric or a deterioration of the noncovalent association of the dimeric scFv into tetravalent scFv constructs was not observed. In conclusion, sc(Fv)2 and [sc(Fv)2]2 are stable in vivo and have significant potential for diagnostic and therapeutic applications.

Previously we studied the therapeutic potential of ^{131}I-labeled dimer and tetramer in animal models and demonstrated that tetramer is more efficacious. As a next step we explored the compatibility of tetramer with therapeutically relevant radiometal Lutetium-177 (^{177}Lu). ^{177}Lu is a radionuclide of interest for RII and RIT on account of its short half-life (161 h) and the ability to emit both beta and gamma radiation. Conjugation and labeling conditions of multivalent scFv with 177Lu were optimized without affecting integrity and immunoreactivity. The scFv construct and IgG form of CC49 were conjugated with a bifunctional chelating agent, ITCB-DTPA, and labeled with 177Lu and their comparative biodistribution, blood clearance, and tumor-targeting characteristics were studied. Approximately, 90% of 177Lu incorporation was achieved using ITCB-DTPA chelator, and the labeled immunoconjugates maintained integrity and immunoreactivity. Blood clearance studies demonstrated an alpha half-life (t1/2alpha) of 177Lu-labeled [sc(Fv)2]2 and IgG of CC49 at 4.40 and 9.50 min and a beta half-life (t1/2beta) at 375 and 2,193 min, respectively. At 8 h post administration, the percent of the injected dose accumulated/gram (%ID/g) of the LS-174T tumor was 6.4+/-1.3 and 8.9+/-0.6 for 177Lu-labeled [sc(Fv)2]2 and IgG of CC49, respectively, in the absence of L-lysine. The corresponding values were 8.0+/-0.6 and 8.4+/-1.2 in the presence of L-lysine. Renal accumulation of [sc(Fv)2]2 was significantly ($p<0.005$) reduced in the presence of L-lysine. The results of this study demonstrated that the ITCB-DTPA conjugation and 177Lu-labeling of scFvs are feasible without influencing the antibody characteristics. 177Lu-labeled [sc(Fv)2]2 showed faster clearance and equivalent tumor uptake at 8 h compared with its IgG form, with a markedly reduced renal uptake in the presence of L-lysine. Therefore, 177Lu-labeled [sc(Fv)2] 2 may be a potential radiopharmaceutical for the treatment of cancer.

High interstitial fluid pressure and poor tumor vascular flow are major impediments for the the delivery of radiopharmaceuticles to solid tumors; therefore, the co- administration of angiotensin II (AngII) can result in an increased uptake of drugs into the tumor interstitium. We have engineered a dimeric sc(Fv)2-AngII fusion construct that combines the superior kinetics of covalent dimeric scFvs [sc(Fv)2], recognizing the pancarcinoma tumor-associated antigen 72 (TAG-72), with the advantageous intrinsic activity of AngII. The binding characteristics of the fusion construct were unaltered by the addition of the AngII sequence [affinity constant K (A) 1.18 x 10^7 M^-1 and 8.42 x 10^6 M^-1 for sc(Fv)2 and sc(Fv)2-AngII, respectively]. The binding of the fusion construct to the angiotensin receptor (AT-1) was similar to AngII, and the arterial contraction was 16 +/- 1% of the response observed with norepinephrine. In animal studies, the radiolabeled sc(Fv)2-AngII construct exhibited similar uptake and a more homogeneous distribution within the tumor as compared to sc(Fv)2.

In order to improve tumor uptake and/or to enhance the tumor retention time of scFvs, we explored the possibility of using cell penetrating peptide sequences, penetratin and Tat. These cationic peptide sequences have been reported to transport cargoes ranging from small inhibitory peptides to liposomes, across cell membranes in a receptor and energy independent manner. Penetratin increased the tumor retention of the scFvs without affecting the peak dose accumulation. The percentage of the doses retained in tumors at 24 hours post-administration

with a control (no peptide), penetratin, and TAT were 27.25%, 79.84%, and 48.55%, respectively, of that accumulated at 8 hours postinjection. The tumor-to-blood ratios at 24 hours post-administration were 7.14, 19.53, and 16.48 with control, penetratin, and TAT treatment, respectively, whereas the pharmacokinetics were unaltered. Coinjection with TAT, however, resulted in increased uptake of the radioconjugate by the lungs. Autoradiography of the excised tumors indicated a more homogenous distribution of the radiolabeled scFv with both penetratin and TAT in comparison with the control treatment. Real-time whole-body imaging of the live animals confirmed improved tumor localization with penetratin without any increase in the uptake by normal tissues.

In summary, we generated and characterized a series of monovalent {scFv}, divalent {sc $(Fv)_2$} and tetravalent {$[sc(Fv)_2]_2$} single-chain Fvs of MAb CC49 recognizing a tumor associated glycoprotein-72 (TAG-72). In the animal model bearing tumor xenografts, these novel scFv constructs have exhibited improved pharmacokinetics and biodistribution in comparision to intact IgG. Using ^{131}I- and ^{99m}Tc-labeled constructs, therapeutic and diagnostic potential of the scFv constructs were demonstrated. Multivalent scFvs, therefore, exhibited a gain in avidity with an improved biological half-life. For both divalent and tetravalent scFvs, RIT delivered in a fractionated schedule clearly presented a therapeutic advantage over single administration. The treatment group receiving radioiodinated $[sc(Fv)_2]_2$ demonstrated statistically significant prolonged survival with single and fractionated doses suggesting a promising prospect of this reagent for cancer therapy. Macroautoradiography performed at 6 and 16 h post administration of labeled ^{99m}Tc-$sc(Fv)_2$ and ^{99m}Tc-$[sc(Fv)_2]$ clearly detected the tumors in mice. The multivalent scFvs, therefore, hold importance in clinical imaging studies of cancer. A significant improvement in the tumor retention of sc(Fv)2 was achieved by administration of penetratin. Overall, the combination of penetratin and scFvs has the potential of improving the utility of MAb-based radiopharmaceuticals. The new generation of multivalent optimized recombinant antibody constructs with desired pharmacokinetic and decreased immunogencity is finding a unique place as radiopharmaceuticals for the diagnosis and therapy of solid tumors.

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1. Davda, J.P., Jain M., Batra, S.K. and Gwilt, PR. Characterization of the biodistribution of CC49 monoclonal antibody using a physiologically based pharmacokinetic model. Submitted, **J. Nuclear Medicine**, 2006
2. Jain M., Venkatraman G and Batra S.K. New generation of genetically engineered antibody constructs for the diagnosis and therapy of solid tumors. Near submission, 2006.
3. Venkatraman G, Jain M., and Batra S.K. Recent advances in oncologic imaging. Near submission, 2006

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3. De Pascalis, R., Gonzales, N.R., Padlan, E.A., Schuck, P., Batra, S.K., Schlom, J., and Kashmiri. S.V.S. Generation of minimally immunogenic high affinity variant of humanized anti-carcinoam antibody HuCC49V10 by an vitro affinity maturation. ***Proc. Amer. Assoc. Can. Res*****,** Vol. 44, 1284, 2003
4. Wittel, U.A., Kurizaki, T., Goel, A., Booth, B.J.M,. Agrawal, D.K., Baranowska-Kortylewicz, J., and Batra S.K. Engineering and characterization of a CC49 single-chain Fv angiotensin Ii fusion construct for improved targeting. **Proceedings of the Annual Meeting American Pancreatic Association**. *Pancreas*, 23, 467, 2001
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6. Goel, A., Colcher, D., Baranowska-Kortylewicz, J., Augustine, S., Booth, B.J., Pavlinkova, G and Batra S.K**.** Genetically engineered multivalent single chain antibody constructs for the diagnosis and therapy of cancer. **European Consortium Meeting 2000**, Porto, Portugal. September 17, 2000.
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