Cytotoxic Activity of a Recombinant Fusion Protein between Insulin-like Growth Factor I and Pseudomonas Exotoxin

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ABSTRACT

A chimeric toxin in which the cell binding domain of Pseudomonas exotoxin was replaced with mature human insulin-like growth factor I (IGF-I) was produced in Escherichia coli. This protein, IGF-I-PE40, was cytotoxic to human cell lines derived from a variety of tumor types, with a breast carcinoma line (MCF-7) and two hepatoma lines (HEP3B and HEPG2) showing the highest sensitivity to the toxin. The specificity of IGF-I-PE40 cytotoxicity was confirmed through competition with excess IGF-I and through blockage of toxin binding using an antibody specific to the type I IGF receptor. A potential interaction between the toxin and soluble IGF-binding proteins was also demonstrated. IGF-I-PE40 may be useful in the selective elimination of cells bearing the type I IGF receptor.

INTRODUCTION

PE2 is a 66,000-Da protein toxin, produced by the Gram-negative bacterium Pseudomonas aeruginosa, which kills eukaryotic cells that bear specific receptors for the toxin (1, 2). The toxin enters the cell via receptor-mediated endocytosis and is translocated into the cytosol where it catalyzes the ADP ribosylation of elongation factor 2 which leads to cell death. The three-dimensional structure of PE has been solved (3), and the functions of the domains of PE have been established by studying Escherichia coli-expressed proteins derived from various portions of the toxin. In this way, it was demonstrated that domain Ia is responsible for cell recognition and binding, domain II for translocation across the cell membrane, and domain III for the ADP ribosylation activity (4). No role for domain Ib has been determined (5).

By deleting the cell recognition domain (Ia), a 40-kDa protein (termed PE40) is produced (6) which, although retaining domains II and III, is not cytotoxic to cells. We have constructed a number of chimeric toxins of differing specificities by fusing cDNAs encoding TGFα (7, 8), IL-2 (9), IL-4 (10), IL-6 (11), and CD4 (12) to the gene segment encoding PE40. These chimeric proteins are specifically toxic to cells expressing receptors or which bind these ligands (for review of chimeric toxins please see Refs. 13 and 14).

IGF-I (also known as somatomedin C) is a 70-amino acid peptide. It is a member of a family of structurally related peptides which includes insulin and IGF-II (15). These factors could displace bound IGF-I, we conclude that the cytotoxic effects were blocked by excess IGF-I or by a monoclonal antibody specific to the type I IGF receptor, and because the toxin could displace bound IGF-I, we conclude that the cytotoxic effect of IGF-I-PE40 is mediated via the type I IGF receptor.

MATERIALS AND METHODS

Reagents. Chemicals and enzymes were purchased from standard suppliers as described previously (9). 125I-IGF-I (2000 Ci/mmol) and [3H]leucine (125 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, IL). Human recombinant IGF-I (Thr59) analogue was purchased from Amgen Biologicals (Thousand Oaks, CA) and United States Biochemical Corp. (Cincinnati, OH). Mouse monoclonal immunoglobulin α-IR-3 (95% IgG1) (50, 51) was purchased from Oncogene Sciences, Inc. MOPC-21 (IgG2a) was obtained from Cappel Laboratories (Westchester, PA). The Gene-Amp PCR kit was purchased from Perkin-Elmer/Cetus or United States Biochemical Corp. Plasmids, Bacterial Strains, and Cell Lines. Plasmid pUC38HR(+)/T carried the DNA encoding for the chimeric toxin TGFα-PE40 (8, 52). This plasmid was designed such that the DNA encoding for TGFα is
bunded by a Ndel site at the 5' end and a HindIII site at the 3' end. The plasmid pGF-I, containing the CDNA for human IGF-I (53), was received as a gift from Dr. M. Rechler, NIH. E. coli strain BL21 (DE3) has been described previously (54). The following human cell lines were obtained from the American Type Culture Collection unless otherwise stated: prostate carcinoma lines DU145, LNCap, PC-3; breast carcinoma lines BT-474, MCF-7, MDA-MB-468; colon carcinoma lines HT-29, LS 174T, KM12C (from J. Fidler, M. D. Anderson Hospital); kidney carcinoma lines A-498, A-704, Caki-1; Wilms' tumor line SK-NPE-1; hepatocellular carcinoma lines HEP-3B, HEP G2, PLC/PRF/5; melanoma lines FEM-X (obtained from M. G. Heaman, NIH), SK-MEL-2, SK-MEL-28; osteosarcoma lines HOS, MG-63; ovarian carcinoma lines OVCAR-3, OVCAR-4; rhabdomyosarcoma lines RD, RH30 (obtained from P. Houghton, St. Jude's Children's Research Hospital); TC212 (obtained from M. Tsokos, NIH); neuroblastoma lines LAN-1 (obtained from J. Bielder); SK-N-BE2 (obtained from C. P. Reynolds, Los Angeles Children's Hospital); epidermoid carcinoma line A431; and neuroepithelialoma line TC32 (obtained from C. P. Reynolds). L929 is a murine fibroblast cell line.

Plasmid Construction. Oligonucleotides were prepared as described previously (9). Plasmid DNA was prepared as described (9) or using QIAGEN columns (QIAGEN, Inc., Studio City, CA) according to the manufacturer's instructions. The chimeric gene encoding IGF-I-PE40 under the control of the bacteriophage T7 promoter was constructed as shown in Fig. 1. First, we created a Ndel recognition site at the 5' end and a HindIII site at 3' end of the mature processed IGF-I coding sequence by PCR (55, 56) using oligonucleotide primers with recognition sites for the enzymes. The sequences of the primers were designed as follows: primer 1 is complementary to the 3' region of the antisense strand of the IGF-I cDNA; primer 2 is complementary to the 3' region of the sense strand of the IGF-I CDNA. The primers were chosen to correspond to the sequence encoding the mature, active peptide. Introduction of the Ndel site using primer 1 resulted in the change of six bases and in the creation of the initiation codon. ATG. Thus, the first residue of the IGF-I moiety on the chimeric toxin is methionine, followed by the first residue of the mature, native peptide, glycine. Use of primer 2 resulted in the addition of three residues between the IGF-I and PE40 moieties. After 30 cycles of PCR using primer 1 and primer 2 (1 µmol each) and 10 ng of the IGF-I cDNA plasmid as the template, a 250-base pair DNA fragment was amplified. After purification on 1.5% (w/v) low-melting-point agarose (SeaPlaque GTG Agarose; FMC BioProducts, Rockland, ME), the amplified fragment was digested with Ndel and HindIII, and the resulting 217-base pair DNA fragment purified from low-melting-point agarose. Plasmid pVC38Hf(+)T was prepared by digestion with Ndel and HindIII and dephosphorylated with calf intestinal alkaline phosphatase. The 4.2-kilobase band was separated and purified from a 1% (w/v) low-melting-point agarose. The DNA fragments were ligated and the recombinants were screened by restriction digestion analysis. Several positive clones were checked for protein expression. Plasmid pTP39 was identified as carrying the gene for the chimeric toxin IGF-I-PE40.

Expression and Localization of IGF-I-PE40. E. coli strain BL21 (DE3) was transformed with plasmid pTP39, grown in 35 ml of Luria-Bertani broth with ampicillin (100 µg/ml) until the absorbance at 650 nm was 0.4. Protein expression was induced with IPTG at 1 mm, and the culture was grown for a further 90 min. At this time, the cells were collected by centrifugation and the expressed recombinant was localized as described previously (11).

Gel Electrophoresis and Immunoblotting. SDS-PAGE was performed under reducing conditions using 10% (w/v) gels as described by Laemmli (57). For Western blotting, electrophoresed samples were transferred to nitrocellulose paper and immunoblotted using rabbit anti-PE antiserum as described (4).

Purification of IGF-I-PE40. Purification of IGF-I-PE40 was achieved as described previously (11) with some modification. Briefly, an induced culture of strain BL21 (DE3) containing plasmid pTP39 was obtained from a 1-liter culture grown in Super broth (Quality Biologicals, Inc., Gaithersburg, MD): protein expression was induced with 1 mm IPTG as described above when culture absorbance (650 nm) was 1.2. A pellet containing insoluble inclusion bodies was collected (11), dissolved, and denatured in extraction buffer (7 M guanidine hydrochloride-5 M EDTA-100 mM Tris-HCl, pH 8.0) by sonication (6 x 30 s at 100 W with a 30-s cooling period between each sonication). The sonicated material was mixed by inversion for 1 h at 4°C and centrifuged at 100,000 x g (r.,) for 30 min at 4°C. The supernatant was collected by centrifugation and the expressed recombinant was localized as described previously (11).

The renatured material was dialyzed against 1 M NaCl-10 mM sodium phosphate, pH 7.4, and stirred gently for 16 h at 4°C. The renatured material was dialyzed against 2 x 4 liters of Buffer A, the mixture was clarified by centrifugation (100,000 x g, 30 min, 4°C), and the supernatant was loaded onto an 8-ml Q-Sepharose (Fast-Flow) column (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) previously equilibrated with Buffer A. A linear gradient 0–500 mM NaCl in Buffer A was applied to the column; IGF-I-PE40 eluted as a broad peak between 220 and 300 mM NaCl. The peak was diluted 30-fold with Buffer A and applied to a Mono-Q-FPLC column (Pharmacia/ LKB, Ltd.) as described for the Q-Sepharose column. The fractions containing cytotoxic activity were pooled, concentrated, applied onto a TSK-G3000 SW gel filtration column, and eluted with PBS at a flow rate of 0.5 ml/min.

Protein Synthesis Inhibition Assays. The cytotoxic activity of IGF-I-PE40 was determined by a protein synthesis inhibition assay (4). The cells were plated in 24-well tissue culture dishes at a density of 1 x 10^6/well 24 h before assaying. Unless otherwise stated, the medium was removed and replaced with 1 ml of fresh media well immediately prior to the addition of IGF-I-PE40. The chimeric toxin was diluted in phos-
Concentration, and applied to a TSK G3000SW gel filtration column. These fractions were pooled, diluted, and loaded onto a Mono-Q column (HR 10/10) (Fig. 3B); fractions 23 and 24 containing IGF-I-PE40 were identified on SDS-PAGE (data not shown). The protein eluted in a broad band corresponding to the expected molecular mass of IGF-I-PE40 (Fig. 2) and which reacted with anti-PE antiserum (Fig. 2B), was identified in the total cell pellet. Further fractionation indicated that this protein was primarily located in inclusion bodies.

Purification of IGF-I-PE40. Since a protein was identified which reacted with antibodies to PE and which corresponded in molecular mass to that expected for IGF-I-PE40, we concluded that the cloning was successful and that IGF-I-PE40 was being produced. We then attempted to purify this protein to facilitate studies of its biochemical action. The material collected from the renatured inclusion bodies (see "Materials and Methods") was applied to an 8-ml Q-Sepharose Fast-Flow ion exchange column (Fig. 3B). This additional Chromatography step (8-11) removes the bulk of the contaminating material which can clog the finer Mono-Q column, thus prolonging its life. The column was eluted as described and the fractions containing IGF-I-PE40 were identified on SDS-PAGE (data not shown). The protein eluted in a broad band from fractions 16 to 29 (corresponding to 220 to 300 mM NaCl). These fractions were pooled, diluted, and loaded onto a Mono-Q column (HR 10/10) (Fig. 3B); fractions 23 and 24 contained the majority of the cytotoxic activity and were pooled, concentrated, and applied to a TSK G3000SW gel filtration column (Fig. 3C). Most of the cytotoxic activity eluted in fractions 43 and 44. As shown in Fig. 2A, lane 5, pooled fractions contained a 47-kDa protein that was approximately 90% pure and that reacted with antibodies to PE (Fig. 2B, Lane 5). Further, this protein possessed ADP ribosylation activity at levels similar to those of purified PE40 (data not shown).

Protein Synthesis Inhibition by IGF-I-PE40. To test the cytotoxic activity of IGF-I-PE40, we initially used the human breast carcinoma line MCF-7. MCF-7 has been shown previously to express type I IGF receptors and to respond to IGF-I (22, 37). The cells were treated with toxin for 20 h after changing the media. Fresh medium was used in these studies since some cell lines, including MCF-7 (59), have been shown to secrete IGF-I binding proteins (17) into the medium; these binding proteins may bind the chimeric toxin and thus interfere with cytotoxic action. As shown in Fig. 4, IGF-I-PE40 inhibited protein synthesis in MCF-7 cells; the ID₅₀ for IGF-I-PE40 was
cytotoxic effect of IGF-I-PE40 using this antibody. The results (Fig. 5) show that α-IR-3 can protect MCF-7 cells from IGF-I-PE40. As a further control, we repeated the above experiment using the monoclonal antibody MOPC-21, which is the same isotype as α-IR-3 but does not react with the type I IGF receptor. As expected, MOPC-21 could not block the action of IGF-I-PE40.

Cytotoxicity of IGF-I-PE40 on Various Cell Lines. As discussed under "Introduction," a number of carcinomas have been shown to be responsive to either IGF-I or IGF-II. Thus, we screened a number of human cell lines for their sensitivity to the chimeric toxin IGF-I-PE40 (Table 1). IGF-I-PE40 was toxic to a number of cells including MCF-7, PLC/PRF/5, HEP3B, HEPG2, MG-63, RH30, and RD, all of which have

14 ng/ml. Treatment of MCF-7 with PE40, which lacks a cell-binding domain, had little effect on protein synthesis (ID$_{50}$ > 1000 ng/ml).

Effect of IGF-I-PE40 on Protein Synthesis Is Specific. To demonstrate that the cytotoxic effects of IGF-I-PE40 on the cell line MCF-7 were specific we used two approaches. First, we used recombinant human IGF-I to block IGF-I-PE40-dependent cytotoxicity (Fig. 5). In each experiment, 1 μg of recombinant human IGF-I was added to the cells immediately prior to addition of the toxin. Recombinant IGF-I partially protected MCF-7 (Fig. 5) from the toxin.

Since the murine monoclonal antibody α-IR-3, which is specific for the type I IGF receptor, can block the mitogenic effect of IGF-I on MCF-7 cells (37), we also attempted to block the
Table 1  Cytotoxic activity of IGF-I-PE40

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>ID_{50} (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DU 145</td>
<td>Prostate carcinoma</td>
<td>270</td>
</tr>
<tr>
<td>LNCap</td>
<td>Prostate carcinoma</td>
<td>39</td>
</tr>
<tr>
<td>PC-3</td>
<td>Prostate carcinoma</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>BT-474</td>
<td>Breast carcinoma</td>
<td>200</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Breast carcinoma</td>
<td>14</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>Breast carcinoma</td>
<td>430</td>
</tr>
<tr>
<td>HT-29</td>
<td>Colon carcinoma</td>
<td>24</td>
</tr>
<tr>
<td>LS174T</td>
<td>Colon carcinoma</td>
<td>90</td>
</tr>
<tr>
<td>KM12C</td>
<td>Colon carcinoma</td>
<td>420</td>
</tr>
<tr>
<td>A-498</td>
<td>Kidney carcinoma</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>A-704</td>
<td>Kidney carcinoma</td>
<td>800</td>
</tr>
<tr>
<td>Caki-1</td>
<td>Kidney carcinoma</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>SK-NEP-1</td>
<td>Wilms' tumor</td>
<td>90</td>
</tr>
<tr>
<td>PLC/PRF-5</td>
<td>Hepatoma</td>
<td>70</td>
</tr>
<tr>
<td>HEP3B</td>
<td>Hepatoma</td>
<td>5</td>
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<tr>
<td>HEPG2</td>
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<tr>
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<tr>
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<tr>
<td>FEM-X</td>
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</tr>
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<td>HOS</td>
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</tr>
<tr>
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<td>560</td>
</tr>
<tr>
<td>OVCAR3</td>
<td>Ovarian carcinoma</td>
<td>&gt;1000</td>
</tr>
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<td>OVCAR4</td>
<td>Ovarian carcinoma</td>
<td>900</td>
</tr>
<tr>
<td>RH30</td>
<td>Rhabdomyosarcoma</td>
<td>50</td>
</tr>
<tr>
<td>RD</td>
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<td>60</td>
</tr>
<tr>
<td>TC3212</td>
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</tr>
<tr>
<td>LAN-1</td>
<td>Neuroblastoma</td>
<td>&gt;300</td>
</tr>
<tr>
<td>SK-N-BE2</td>
<td>Neuroblastoma</td>
<td>&gt;300</td>
</tr>
<tr>
<td>TC32</td>
<td>Neuroepithelioma</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>A431</td>
<td>Epidermoid carcinoma</td>
<td>56</td>
</tr>
<tr>
<td>L929</td>
<td>Murine fibroblast</td>
<td>390</td>
</tr>
</tbody>
</table>

Fig. 6. Displacement of bound IGF-I by IGF-I-PE40. RD cells (1 x 10⁶) were incubated for 18 h at 0°C with [¹²⁵I]-IGF-I (20,000 cpm) and various amounts of either recombinant human IGF-I (•) or IGF-I-PE40 (■). Cell-associated radioactivity was determined.

DISCUSSION

We have constructed the chimeric toxin IGF-I-PE40 by fusing the cDNA encoding human IGF-I to a gene encoding for the translocation (domain II) and ADP-ribosylating (domain III) activities of PE. This hybrid toxin was produced in E. coli in insoluble inclusion bodies and purified from this source. IGF-I-PE40 is toxic to cell lines known to express IGF-I or type I IGF receptor (MCF-7, PLC/PRF/5, HEP3B, HEPG2, MG-63 (24, 35, 37). In addition, the rhabdomyosarcoma cell lines RD and RH30, in which IGF-II acts as an autocrine growth and mobility factor (42), were also sensitive to the chimeric toxin.

The cytotoxic activity of IGF-I-PE40 appears to be mediated via a type I IGF receptor. First, excess recombinant human IGF-I could protect the cells from the toxin. The murine monoclonal antibody α-IR-3, which is specific for the type-I IGF receptor, but not the antibody MOPC-21, which is the same isotope, also could block the cytotoxicity. Finally, there was no observed cytotoxicity (ID_{50} > 1000 ng/ml) when the breast carcinoma line MCF-7 was treated with the protein.

Effect of Washing Cells Prior to Treatment. As stated above, some cell lines are known to secrete soluble IGF-binding proteins into the media (17, 59). In order to investigate the possible effects of IGF-binding proteins on the cytotoxic activity of IGF-I-PE40, we determined the ID_{50} of the chimeric toxin on MCF-7 cells on which the medium was replaced with fresh medium or on which the medium was not changed (Fig. 7). The results were determined using two different toxin preparations and with treatment performed on several days. The ID_{50} of IGF-I-PE40 on cells incubated with fresh media was 14 ± 2 (SD) ng/ml (n = 6); the ID_{50} on cells incubated with conditioned media was 39 ± 7 ng/ml (n = 7). The difference in ID_{50} is significant (P > 0.0002, Student’s t test). In order to determine the effect of changing the media on protein synthesis, the amount of [³H] leucine incorporated by the cells also was compared. Cells in fresh media incorporated 16,811 ± 1,082 cpm (n = 6) and 17,895 ± 1,014 cpm (n = 5) in conditioned media; this difference in leucine incorporation is not significant (p = 0.2).

Fig. 7. Effect of washing cells prior to treatment with IGF-I-PE40. MCF-7 cells were prepared as described in the legend to Fig. 4. The medium was either replaced (○) or not changed (■) with fresh medium immediately prior to treatment with various amounts of IGF-I-PE40. After 20 h incubation, protein synthesis was measured; results are expressed as the percentage of the protein synthesis activity of the cell incubated without toxin.
PE40, which lacks any intrinsic cell binding capability.

Many carcinomas contain high numbers of the type I IGF receptor or overexpress IGF-I (18-35). A number of cell lines derived from these and other tissues were screened for their sensitivity to IGF-I-PE40 (Table 1). Sensitivity to the toxin varies widely between tissue types and between specific cell lines derived from the same tissue. Significantly, the hepatoma lines HEP3B and HEPG2 were the most sensitive cell lines to IGF-I-PE40. The presence of significant levels of the type I IGF receptor in fetal liver tissues (61, 62) and hepatocellular carcinomas (24, 61) has been observed; adult liver has been reported to contain few receptors (61-63), although Venkatesan and Davidson (64) recently reported substantial IGF-I binding in rat liver. These observations are particularly striking since liver is a major site of IGF-I synthesis and possesses a high tissue concentration of this factor (see Ref. 63 for review). It is interesting to speculate whether tumorigenesis in this tissue may involve deregulation of the type I IGF receptor mitogenic pathway. Also significant is the observation that several cell lines (RD and RH30) in which IGF-II exerts a mitogenic effect via the type I IGF receptor (42) are sensitive to IGF-I-PE40, indicating that the toxin may be of use in the treatment of not only IGF-I-producing carcinomas but also IGF-II producing tumors.

Disappointingly, IGF-I-PE40 was approximately 250-fold less effective at displacing radiolabeled IGF-I than was unlabeled factor. This result may explain why ID50 levels with IGF-I-PE40 did not approach the 0.1-1.0-ng/ml range obtained with other chimeric toxins of this type (7-12). The lower binding observed may reflect steric interference between the IGF-I and PE40 moieties; alternatively, the relatively high number of cysteine residues in IGF-I (6 cysteine/70 total residues) and PE40 (4 cysteine residues) may result in incorrect disulfide bond formation. These problems may be overcome by mutating either the IGF-I or PE portions of the toxin; such mutational studies have been undertaken on intact IGF-I, resulting in alteration of binding affinity for IGF receptors (65-67).

We also attempted to see whether IGFBP-binding proteins would bind the IGF-I-PE40 toxin and thus reduce its cytotoxicity. The breast carcinoma cell line MCF-7 has been shown to secrete IGFBPs into the media (59). We treated this cell line with the toxin in both “conditioned,” i.e., containing any secreted proteins, and fresh media. The ID50s obtained under these conditions were significantly (P < 0.0002) different, suggesting that secreted binding proteins are interacting with IGF-I-PE40 and that this effect can be measured directly. We cannot, however, rule out the possibility that changing the concentration of this factor (see Ref. 63 for review). It is interesting to speculate whether tumorigenesis in this tissue may involve deregulation of the type I IGF receptor mitogenic pathway. Also significant is the observation that several cell lines (RD and RH30) in which IGF-II exerts a mitogenic effect via the type I IGF receptor (42) are sensitive to IGF-I-PE40, indicating that the toxin may be of use in the treatment of not only IGF-I-producing carcinomas but also IGF-II producing tumors.

Thus, the chimeric toxin IGF-I-PE40 is a potent cytotoxic agent for cells overexpressing the type I IGF receptor. Since many human carcinomas express IGF-I and/or IGF-II receptors (18-35, 38-46), a molecule composed of IGF-I and a toxin may be of use in the treatment of some cancers. Furthermore, IGF-I-PE40 may be useful in studying IGFBP-binding proteins and their interaction with the type I IGF receptor.

ACKNOWLEDGMENTS

We thank Dr. V. K. Chaudhary and M. G. Gallo for technical advice, Dr. C. Minniti for performing the binding experiments, and M. L. Lanigan and A. Gaddis for typing the manuscript.

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