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Full Length Research Paper

An interesting design of fusion protein to kill glioma cell line of U-87 MG cells

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According to the American Brain Tumor Association, primary brain tumors occur at a rate of 12.8 per 100,000 people. However, tumors growing in the brain are difficult to treat. Now, researchers have found that antibodies utilize complementarity-determining regions (CDRs) of their variable domains to bind antigens with high affinity and specificity. Therefore, we established an antibody mimetic fused with diphtheria toxin for targeting glioma cell line of U87 MG. V_HCDR1 and V_LCDR3, together with 5 amino acid residues on both side of the CDRs, through a cognate framework region (V_HFR2) yielded a mimetic of BT32/A6 (United States Patent number: 5639863). We fused the mimetic with the first 388 amino acid residues of diphtheria toxin through a linker of GGGS. *Escherichia coli* strain BL21 (ED3) was used to express the soluble immunotoxin DTLMG. The killing activity of U-87 MG cells by this fusion protein was examined *in vitro*. The immunotoxin DTLMG alone did not kill Raji up to the maximal concentration tested (10^{-6}M) *in vitro*. By contrast, concentrations ≥10^{-9}M, of the fused DTLMG killed more than 95% of U-87 MG cells. It is suggested that the mimetic maintained the synergic interactions and high-affinity associated with the parent antibody. This construct holds promise for targeting aimed cancer epitopes and raised hopes for their more effective and much wider use in the future.

Key words: Glioma cell, fusion protein, U87 MG.

INTRODUCTION

According to the American Brain Tumor Association, primary brain tumors occur at a rate of 12.8 per 100,000 people. Although people of any age can develop a brain tumor, the problem seems to be most common in children ages 3 to 12 and in adults ages 40 to 70. In the United States, approximately 2,200 children younger than age 20 are diagnosed annually with brain tumors. Gliomas account for 30 to 40% of all intracranial tumors (Zülch, 1986). With the development of application of monoclonal antibodies (mAbs) to oncology, available clinically useful mAbs typically use a combination of mechanisms in directing cytotoxic effects to a tumor cell. However, those antibodies can't get into the brain because of a special filtering mechanism in the body (called the blood-brain barrier) or approach their targets through neoplastic “binding site barriers” owing to their greater molecular weight (Fujimori et al., 1991). Recently, Qiu et al. (2007) and Zhen et al. (2009) found that such antibodies utilize CDRsof their variable domains to bind antigens with high affinity and specificity. But, previous studies ignored binding residues located within their framework regions(FRs) to form reasonable antigen recognizing site (Qiu et al., 2007) and the interaction between mimetic and toxic molecular (Qiu et al., 2007; Zhen et al., 2009).

So, we fused two CDRs, V_HCDR1 and V_LCDR3, together with 5 amino acid residues on both side of the CDRs, through a cognate framework region (V_HFR2) that possesses mimetic properties. Then, it was genetically

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Abbreviations: CDRs, Complementarity-determining regions; **mAbs,** monoclonal antibodies; **FRs,** framework regions; **DNA,** deoxyribonucleic acid; **PI,** propidium Iodide; **VH,** heavy chain; **VL,** light chain.

fused with the first 388 amino acid residues of diphtheria toxin, through a linker of GGGS, to form a mimetic-toxin (immunotoxin) which retaines the antigen recognition of the parent antibodies and reacts only with targeted gliomas cells of U87.

MATERIALS AND METHODS

Bacterial strains and plasmids

We used *E. coli* strain JM109 to amplification of plasmids and cloning. Furthermore, *E. coli* strain BL21 was applied to expression of soluble immunotoxin. The plasmid pGEX-T containing the DT388 fragment was fused by our laboratory.

Preparation of mimetics and fusion protein

The deoxyribonucleic acid (DNA) sequences of a human monoclonal antibody specific to the cell cycle independent gliomas surface antigen of BT32/A6 were published previously (United States Patent number: 5639863). The genetically engineered plasmid fusion, expression and purification were described previously (Qiu et al., 2007; Zhen et al., 2009). The amino acid sequence for the 5aa-V_HCDR1-5aa-V_HFR2-5aa-V_LCDR3-5aa mimetic of the parent molecules were:
5aa-V_HCDR1-5aa-V_HFR2-5aa-V_LCDR3-5aa:
GFTFSSYAMHWVRQAAPGKGLE Y VSGVYYCMQRIEFPFTFGGGT. The amino acid sequence for the linker: GGGS.

Cell culture and killing activity in vitro

The positive glioma cell line U-87 MG (ATCC HTB-14) and the negative Burkitt's lymphoma (Raji, ATCC CCL-86) were used in our experiments. Both cells were inoculated in Falcon 3046 six-well tissue culture plates (Becton Dickinson Company) with 3 ml RPMI 1640 (GIBCO BRL) at an initial cell density of 5×10^5 cells/ml. All media were supplemented with 10% fetal bovine serum, penicillin (200U/ml) and streptomycin (200 µg/ml), L-glutamine (2 mM), and amphotericin B (Fungizone; 1.5 µg/ml). The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 (Sanyo Electro. Biomed). When the cell fully covered on the tissue culture plates, different concentrations of DTLMG ranging from 10^{-9} M to 10^{-6} M were used in the experiments and physiological saline served as control. Live and dead cells were counted with 50 nM acridinorange (AO)/500 nM Propidium Iodide (PI) double staining after 48 h. Vital staining pictures were collected using an inverted fluorescent microscope (IX-71, Olympus) at 400× with U-MWU2, U-MVB2 and U-MNG2 filters. Each experiment was repeated at least five times.

RESULTS

Preparing the toxin-mimetic fusion peptides DTLMG

Design of the mimetics was guided by two precepts. First, as antigen recognition by intact Fab is synergistically produced by all six CDRs residing in both the V_H and V_L domains, it should contain at least two antigen-binding sites: one from the V_H and the other from the V_L domain. Such synergistic recognition cannot be accomplished if the CDR loops all originate from one variable domain (Ewert et al., 2003; Holliger and Hudson, 2005). Secondly, as the CDR3 loop is the central, most accessible antigenbinding segment in an intact Fab, it should be regarded as an essential component of the mimetic (Ewert et al., 2003; Holliger and Hudson, 2005). Also, the CDR3 loop should be complemented by either the CDR1 or CDR2 loop of the other variable domain, as these are normally the closest to CDR3 in the parental antibody. Importantly, we added the 5 amino acids on both sides of CDRs. The genes of 5aa-V_HCDR1-5aa-V_HFR2-5aa-V_LCDR3-5aa of BT32/A6 and the linker of GGGS were constructed to follow position DT388 by double-stranded oligonucleotide mutagenesis (QuickChange Kit, Stratagene) using the plasmid containing the DT388 gene to form DTLMG. The fusion peptide was expressed and purified, and the silver staining indicated there was a very bulky band about 50KD (Figure 1).

In vitro killing activity of DTLMG fusion peptides

Phosphate buffered saline did not affect the survival rate of either U-87 MG or Raji. These U-87 MG cells were incubated with various concentrations of DTLMG. Raji served as a control. We found that DTLMG could not kill Raji up to the maximal concentration tested (10^{-6}M) (Figure 2). In contrast, at concentrations ≥10^{-7}M, DTLMG killed 95% of U-87 MG cells (Figures 3 and 4).

DISCUSSION

The idea to use antibodies as magic bullets to target toxins or radioisotopes to defined cell types is now 100 years old (Erlich, 1957). A major step toward this goal was made 25 years ago by Kohler and Milstein who developed hybridoma technology, thereby providing a reliable source of mAbs of known specificity (Kohler and Milstein, 1975). However, until very recently, there have been few antibody-based products suitable for therapy available. In 1988, antibody engineering techniques were boosted by the groups of Skerra and Better who demonstrated that active antibody fragments could be expressed in *E. coli.* The smallest such fragment is the Fv fragment, which is obtained by association of the variable domains of the heavy chain (VH) and the light chain (VL) of the antibody (Skerra and Pluckthun, 1988; Better et al., 1988). From then on, researchers began to find out the less antigen recognition element. To our great surprised, Qiu et al., 2007 first demonstrated that a variety of specific antibodies can be tailored to construct mimetics comprising two CDRs, each selected from the V_HCDR1 and V_LCDR3 domain of a Fab, oriented by means of a framework region. Then, Zhen et al., 2009 found that the

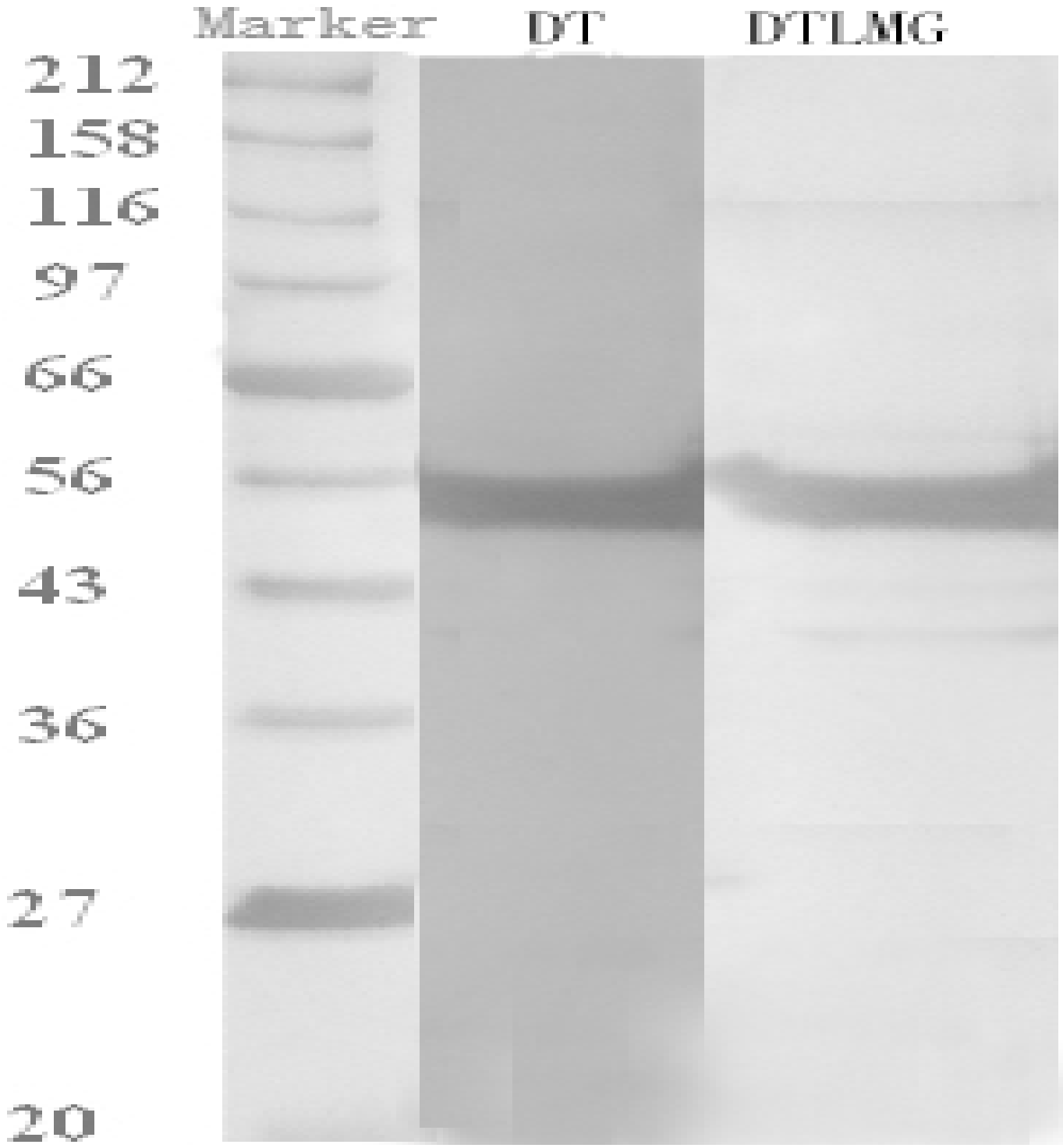
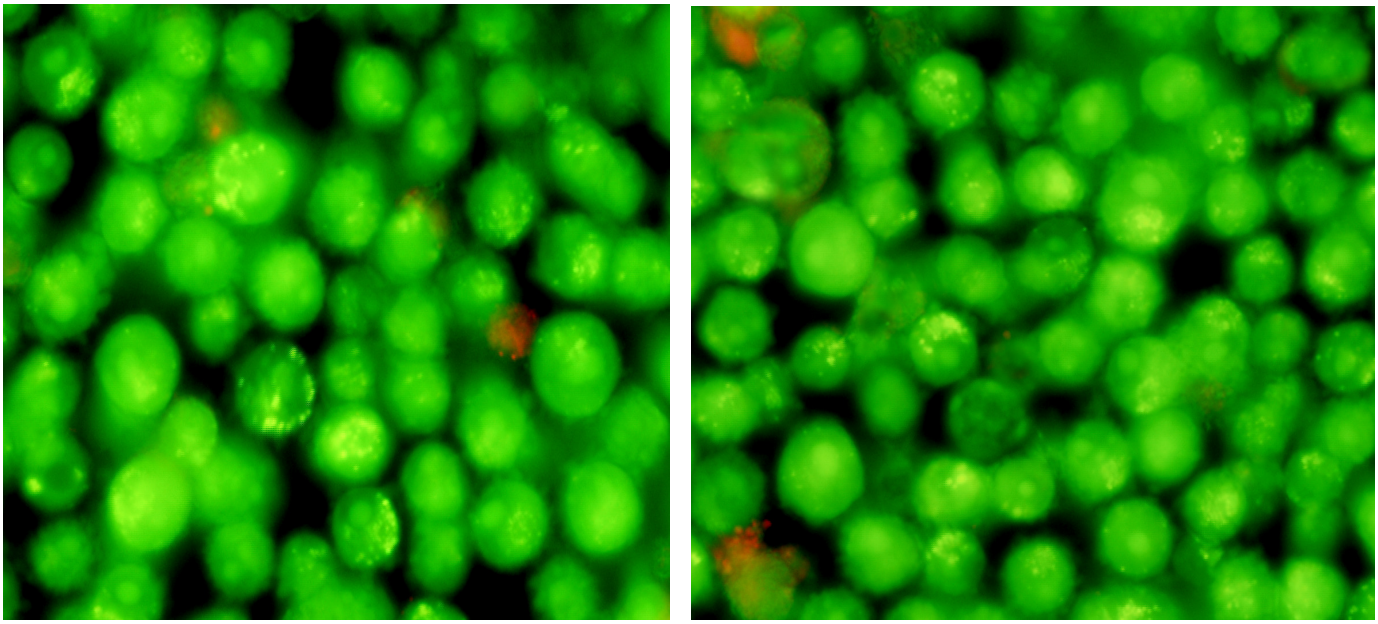


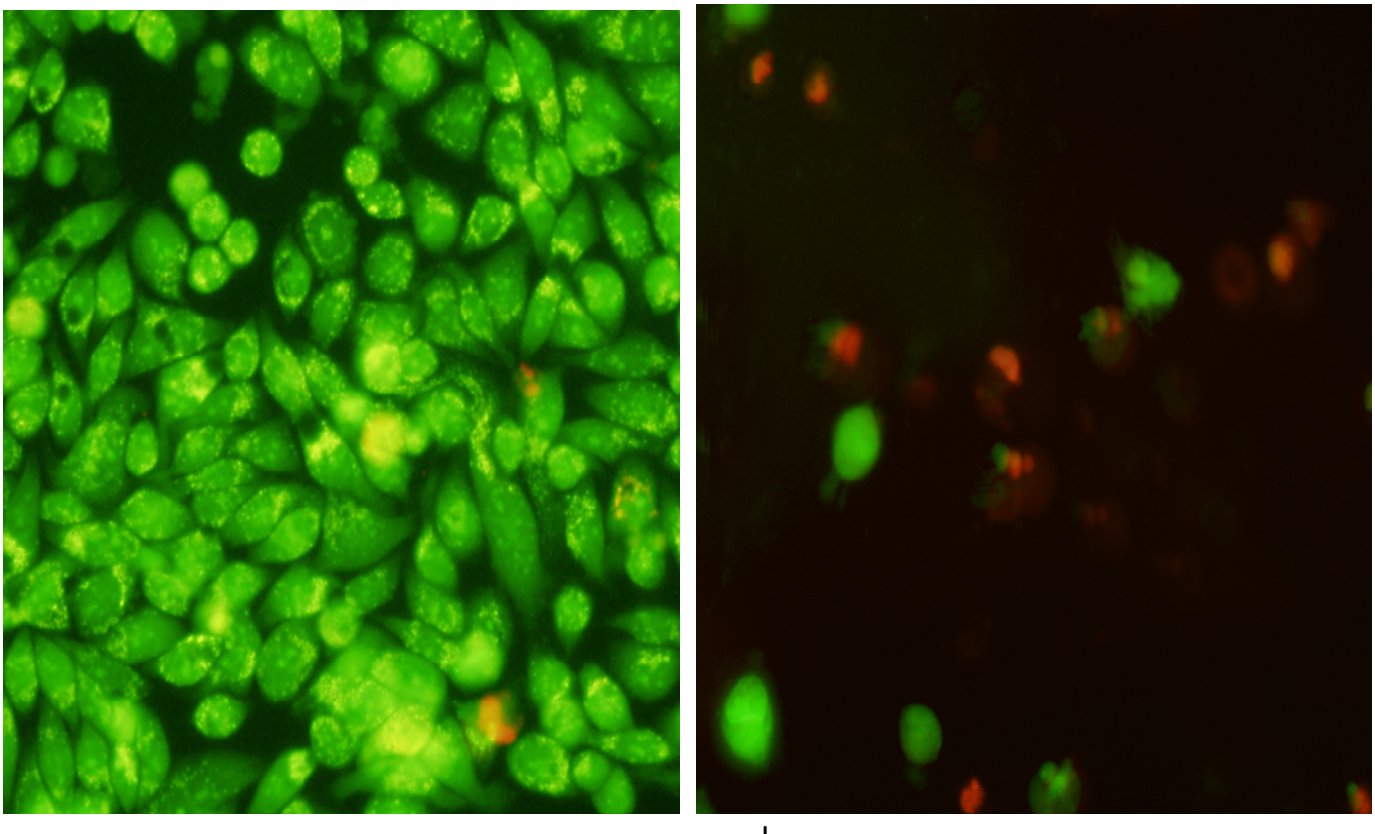
Figure 1. SDS-PAGE of DT and DTLMG fusion protein.

5 amino acid residues on both side of the CDRs played important parts in retain the structure and antigen recognition ability of CDRs. In order to avoid the interaction between the mimetic and toxic molecular, we established the mimetic-linker-toxin complex. We have found this fusion mimetic-toxin kills positive glioma U-87MG cells but not control Raji cells. This killing activity declared that the spatial structure of 5aa-V_HCDR1-5aa-V_HFR2-5aa-V_LCDR3-5aa mimetic was not affect by the toxic molecular because of the linker, and possessed the



a b

Figure 2a. Showed the normal Raji; **b** showed that DTLMG could not kill Raji up to the maximal concentration tested (10^{-6}M) after 48 h



a b

Figure 3a. Showed the normal U87 MG cells before adding DTLMG; **b** showed 10^{-7}M of DTLMG killed 95% of U-87 MG cells after 48 h.

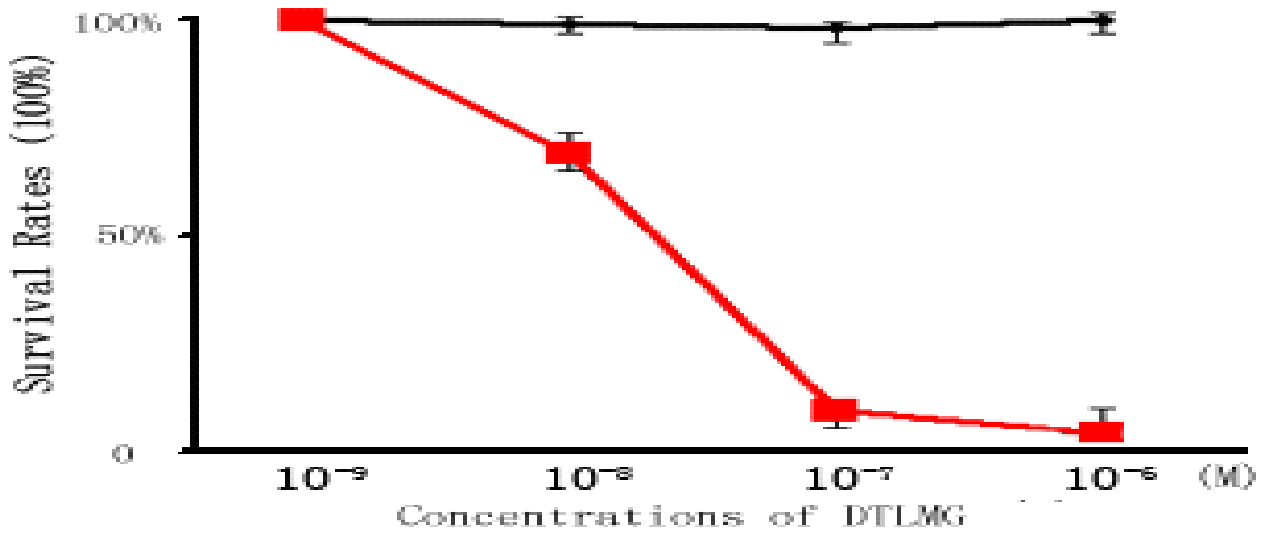


Figure 4. The killing ability of fusion peptides of DTLMG on U-87 MG cells. DTLMG could kill Raji up to the maximal concentration tested (10^{-6}M) after 48 h. While, DTLMG killed more than 95% U-87 MG cells at the concentration of 10^{-7}M after 48 h.

synergic interactions and high-affinity of its parent antibody. Such mimetic may hold promise for markedly improving cancer diagnostics and therapeutics, raised hopes for their more effective and much wider use in the future.

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