Research in Pharmaceutical Sciences, August 2012; 7(3): 133-140
Received: Nov 2011
Accepted: Feb 2012

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Original Article

Assessment of selective toxicity of insect cell expressed recombinant A1-GMCSF protein toward GMCSF receptor bearing tumor cells

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Abstract

One of the emerging therapeutic strategies for targeted treatment of most cancers is the use of immunotoxins which are fusion proteins consisted of a targeting and a toxic moieties. We previously showed that the recombinant A254-GMCSF fusion protein selectively kills acute myeloblastic leukemia cells which harbor a large number of granulocyte-macrophage colony stimulating factor (GMCSF) receptors. Since further *in vitro* and preclinical studies require large amounts of this fusion protein free from any troublesome material like lipopolysacharide, we selected the insect cell expression system. Thus, the coding sequences of the A254-GMCSF and its truncated form, A247-GMCSF, were cloned and expressed by Sf9 cells. Subsequently, specific cytotoxicity of the purified proteins was evaluated on GMCSF receptor positive cell lines. SDS-PAGE and Western blot analysis of the expressed A254GMCSF and A247GMCSF fragments revealed bands of about 60 kD which were larger than the theoretically predicted size of about 47 kD. Deglycosylation analysis showed that these proteins are N-glycosylated by the insect cells. However, any other post-translation modification of the proteins by insect cells could be the reason for higher molecular weight of the fragments. Cytotoxicity assays showed specific killing activity of these proteins on HL60 and U937 cell lines with IC_{50}s ranging 2-2.5 µg/ml. These IC_{50} values are much higher than those obtained from bacterially expressed A254-GMCSF (80 ng/ml) which could be due to any modification performed by insect cells on the fusion proteins.

Keywords: Granulocyte-macrophage colony stimulating factor; Immunotoxin; Insect cell; Shiga toxin

INTRODUCTION

Acute myeloid leukemia (AML) is a malignancy of the myeloid line of blood cells in which miss-regulation in their differentiation and proliferation results in accumulation of non-functional cells called myeloblasts (1). It is one of the most common leukemia with approximately 13000 new cases and 9000 deaths each year in United States (2). Although there is some chemical therapeutics available for this disease but unfortunately they cannot efficiently target tumor progenitor cells which cause reappearance of tumor cells over time (3). Therefore, most patients succumb to new tumors even following achievement of complete clinical responses. In this regard, new therapeutic strategies which are capable of targeting and destroying these leukemic repopulating cells are necessary. One of the novel therapeutic strategies for efficient targeting of tumor cells is the application of immunotoxins which are fusion proteins consisted of targeting and toxin moieties. The targeting moiety is an antibody or antibody fragment or a ligand against a specific cell surface antigen or of a cell surface receptor which is absent on the surface of normal cells or is highly up-regulated on cancer cells (4). Toxin molecules which are used for preparation of immunotoxins are mostly of plant or bacterial origins like ricin and abrin or diphtheria and pseudomonas exotoxin A, respectively (5). Another bacterial toxin that could be used for this purpose is shiga toxin (StxA) (6). Shiga toxin belongs to the AB

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family of protein toxins which consisted of an enzymatically active part (A) and a binding (B) part that binds to the cell surface. The enzymatically active A fragment itself consisted of A_1 and A_2 subunits which are separated by proteolytic processing and reduction of an internal disulfide bond. The A_1 fragment inhibits protein synthesis after releasing in the cytosol by removing one adenenine from the 28S RNA of the 60S ribosomal subunit. The A1 subunit is a 254 amino acids-length fragment and could be used for construction of the recombinant immunotoxins (7).

Granulocyte-macrophage colony-stimulating factor (GMCSF) is a cytokine responsible for the growth, differentiation, and functional enhancement of granulocytes and macrophages. Human GMCSF is a 127 amino acids long protein that binds to the hematopoietic cells via high-affinity receptors composed of α and β subunits (8-9). It has been shown that the amount of GMCSF receptor (GMCSF-R) is increased on the surface of a majority of leukemic cells while it is almost absent on the surface of normal hematopoietic progenitors (10). Previous *in vitro* studies on a recombinant fusion protein consisted of StxA and GMCSF fragments revealed specific cytotoxicity to the GMCSF-R positive hematologic cell lines, HL-60 and U937 (11). However, to perform more detailed *in vivo* and *in vitro* preclinical studies, large amounts of the recombinant fusion protein, purified to homogeneity and free from any unwanted impurities such as lipopolysaccharides (LPS) is required. Baculovirus/insect cell expression systems have been widely used for the production of a variety of recombinant proteins with diagnostic or medical applications. Insect cells perform most, if not all, of the post translational modifications (12). In addition, insect cells do not contain pyrogens or endotoxins from microbes or contaminants from mammalian sources (13). Therefore, the baculovirus/insect cell expression systems could be efficiently and safely used for the production of recombinant proteins with therapeutic applications. Previously, we attempted to express the recombinant A1 derived fusion proteins by the baculovirus expression vector system. However, the A1 fusion proteins showed to have an inhibitory effect on the baculovirus particle formation (data not shown). Therefore, a non-lytic insect cell expression system (14) was evaluated for its capability to produce large amounts of the fusion protein. We also included the expression of a fusion protein containing a shorter fragment of the A1 toxin that includes the first 247 amino acids of the full A1 which is consisted of 254 amino acids as it has been shown that fusions of the shorter fragment exert cytotoxicities almost equal to those of the full length fragment (15). Following expression and purification of the mentioned recombinant proteins, their specific cytotoxicity was evaluated on two human leukemic cell lines, HL60 and U937, which both highly express the GMCSF receptor on their surface (16).

MATERIALS AND METHODS

Strain, plasmid and reagents used

The pMIB/V5-His C vector was from Invitrogen (Carlsbad, CA). Blasticidine S. HCl was obtained from Invivogen (San Diego, California, USA) and used for selection of stable cell lines. FastDigest™ restriction endonucleases were from Fermentas (Fermentas; Vilnius, Lithuania) and the cloning procedure was performed in Top 10 *E. coli* strain. All other chemicals were obtained from other commercial sources and were of the molecular biology grade.

Construction of the expression plasmids

The coding sequences of the first 247 amino acids of the A1 toxin and the GMCSF fragment were obtained from our previous pBAD-A1-GMCSF construct (17) through overlap PCR. To do this, the ATFr and A47(GM)Rv primers (Table 1) were used for amplification of the A247 fragment. Afterwards, the GM(A47)Fr and GMRv primers (Table 1) were used for amplification of the GMCSF fragment. The amplified fragments were fused via overlap PCR as described earlier (17) using ATFr and GMRv primers. The A254-GMCSF fragment was also amplified using primer pairs ATFr and GMRv through PCR with the pBAD-A1-GMCSF construct as template. The PCR condition

Table 1. Sequences of the oligonucleotides used for amplification or fusion of the fragments

Primer name	Primer sequence	Primer features[a]
ATFr	5'- CG**GGATCC**GATGGAATTTACCTTAG-3'	*Bam*HI site
A47(GM)Rv	5'-CAGCGGGCGGGTGC**TCGCGATGCATGATG**-3'	Overlap sequence with GMCSF
GM(A47)Fr	5'-**CATGCATCGCGA**GCACCCGCCCGCTGC-3'	Overlap sequence with the A247
GMRv	5'- CCG**CTCGAG**GCTCCTGGACTGGCTCCC -3'	*Xho*I site

[a]The primer features are represented in bold

included a primary denaturation step of 5 min at 95ºC followed by 30 cycles at 95ºC for 45 s, 55ºC for 45 s and 72ºC for 80 s, and a final extension time of 10 min at 72ºC. Following amplification, the fragments were *Bam*HI and *Xho*I digested and cloned in similarly digested pMIB/V5-His C plasmid and named pMIB-A247GMCSF and pMIB-A254GMCSF plasmids. Finally, the fidelity of the cloning was evaluated by DNA sequencing.

Insect and mammalian cell culture

Unless otherwise indicated, all cell lines were obtained from national cell bank of Iran (Pasteur Institute, Iran). *Spodoptera frugiperda* (Sf9) insect cells were obtained from Invitrogen and cultivated at 27°C in Excell™ 420 serum free insect cell culture medium (Sigma, Germany) supplemented with 100 U penicillin/ml and 100 mg streptomycin/ml (Biosera, UK). GMCSF receptor bearing human leukemia cell lines HL60 and U937, were cultured in RPMI medium containing 20 or 10% FBS, respectively, in the presence of 100 U penicillin/ml and 100 µg streptomycin/ml. Vero cells, a GMCSF receptor negative cell line, were also cultivated under the same condition and in the presence of 10% FBS and used as negative control. All the mammalian cell lines were grown at 37ºC and in the presence of a CO_2 atmosphere of 5%.

Transfection of insect cells and preparation of stable cell lines

Transfection of the recombinant pMIB plasmids into the Sf9 cells was carried out by cellfectin II transfection reagent as described by the manufacturer (Invitrogen). Three days post transfection, the culture medium was replaced with fresh medium containing 80 µg/ml blasticidin S HCl to induce integration of the plasmid elements into the genomic DNA of the cells. The culture medium was replaced with fresh medium containing blasticidin S HCl (80 µg/ml) every 4 days until sufficiently large isolated colonies were obtained and the high producer colonies were expanded stepwise to larger monolayer cultures.

Evaluation of the recombinant proteins expression by Western blot analysis

The culture supernatant was concentrated and evaluated for expression of the recombinant proteins via Western blot analysis. To carry out the experiment, the protein samples were separated by 12% (v/v) SDS-PAGE and subsequently transferred to nitrocellulose membrane. The membrane was blocked with 5% (v/v) skimmed milk containing 0.1% Tween 20 for 90 min. Then, the membrane was three times washed with wash buffer (PBS containing 0.05% Tween 20) and incubated with primary rabbit anti human GMCSF antibody (Abcam, MA, USA) for two h. Afterwards, the membrane was washed three times with the wash buffer and incubated with secondary HRP conjugated goat anti rabbit antibody (Sigma) for 90 min. Finally the membrane was washed three times with the wash buffer and the reaction was developed by 3,3 diaminobenzidine (DAB) solution.

Purification of the expressed proteins

High yield expression of the recombinant proteins were carried out in suspension cultures at a density of about $4\text{-}6\times10^6$ cells/ml and the presence of 10 µg/ml blasticidin S for maintenance of the integrated genes. The culture medium was subjected to purification by ion metal affinity chromatography using Ni-NTA agarose (Qiagen, Valencia, CA) as described elsewhere (18). Finally, the eluted proteins were dialyzed against PBS to remove imidazole and evaluated by SDS-PAGE and subsequently confirmed by Western blot analysis.

Glycosylation analysis

In order to check whether the expressed

proteins are glycosylated, the purified recombinant fusion proteins were subjected to deglycosylation by Endoglycosidase H treatment as instructed by the supplier (New England Biolabs, Germany). Consequently, the proteins were evaluated by SDS-PAGE analysis.

MTT assay

To assess the cytotoxicity of the fusion proteins, MTT assay was performed. HL60 (Human promyelocytic leukemia) and U937 (Human leukemic monocyte lymphoma) cell lines were used as GMCSF receptor positive cell lines and Vero cell line lacking human GMCSF receptor was used as negative control. The cell lines were incubated in the presence of various concentrations of the recombinant proteins ranging from 40 ng/ml to 4 µg/ml for different time lengths of 24, 48 or 72 h. In this regard, 1×10^4 cells were cultivated in each well of a 96-well plate. 24 h following seeding, different concentrations of the fusion proteins were added to each well and incubated for the mentioned time period. Then, MTT was added to each well at a final concentration of 0.5 mg/ml and incubated for further 4 h at 37ºC. Finally, the medium was replaced with 100 µl DMSO to dissolve the formazan crystals and absorbance was read at 570 nm.

Statistical analysis

Cytotoxicity assay was performed in three independent experiments of triplicates and the results were measured as the percent of viability of the treated cells over the non-treated or PBS treated controls. When needed, $P<0.05$ is considered to be significant. IC_{50}s were calculated by linear regression method using Microsoft Excel software. Error bars represent standard deviation (SD).

RESULTS

Expression and purification of the recombinant proteins

The PCR amplified fragments were cloned into the plasmid pMIB/V5 His which adds the V5 and 6X histidine tags to the C terminus of the recombinant proteins. Following confirmation of the recombinant plasmids by DNA sequencing they were transfected to Sf9 cells. The integration of the plasmid into the genomic DNA was induced in the presence of 80 µg/ml blasticidine S HCl. Western blot analysis of concentrated culture media of some stable cells confirmed the expression of the recombinant proteins (Fig. 1).

Finally, of the obtained positive colonies the high producer colonies were expanded to larger monolayer cultures and the stable cell lines were named Sf9-AG254 and Sf9-AG247. Following verification of the expression of the recombinant proteins, the stable cell lines were subjected to suspension cultures for higher production of the proteins followed by protein purification. In this regard, the cells cultivated in suspension cultures in the presence of 10 µg/ml blasticidine reaching a density of $4\text{-}6\times10^6$ cells/ml, the cell medium was replaced and then collected 72 h post refreshment. The collected medium was divided into 100 ml aliquots and the recombinant proteins were purified using Ni-NTA agarose and analyzed by SDS-PAGE and Western blot analysis which revealed specific bands of about 60 kDs for both A247-GMCSF and A254-GMCSF fragments (Fig. 2).

As it is shown, there is an obvious size difference between the theoretically predicted size of the recombinant proteins which are about 46.4 and 47.1 kD for A247-GMCSF and A254-GMCSF, respectively (including the size of V5-His tag and vector derived amino acids). To better elucidate this observation, the purified A247-GMCSF and A254-GMCSF fragments were analyzed by SDS-PAGE alongside the bacterial expressed A254-GMCSF recombinant protein. This revealed a significant size difference between these fragments (Fig. 3).

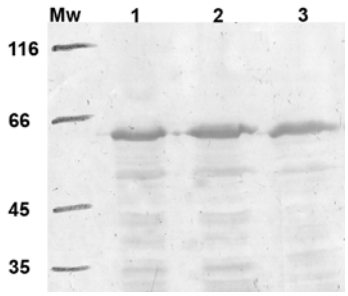

Fig. 1. Western blot analysis of the expressed fusion proteins. Concentrated culture medium of the A254-GMCSF (1 and 2) and A247-GMCSF (3) producing cell lines were evaluated by Western blotting using primary rabbit anti human GMCSF antibody and secondary HRP conjugated goat anti rabbit antibody. Bands of about 60 kDs confirmed expression of the recombinant proteins. Mw, is Protein molecular weight marker.

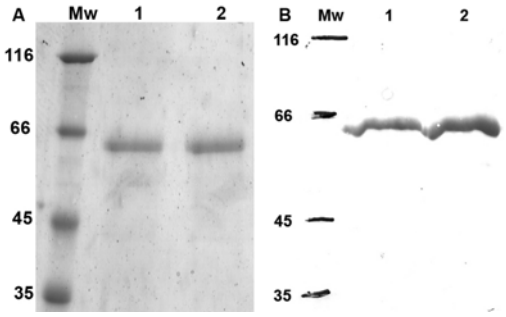

Fig. 2. SDS-PAGE (A) and Western blot (B) analysis of the purified proteins. The purified A254-GMCSF (1) and A247-GMCSF (2) proteins were evaluated by 12% SDS-PAGE end subsequent Western blotting.

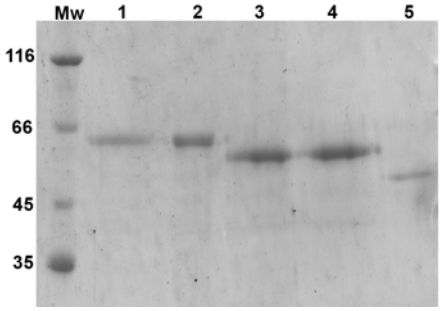

Fig. 3. Deglycosyaltion of the recombinant proteins. Considering the significant size differences between the insect cells expressed A254-GMCSF and A247-GMCSF (1 and 2) comparing to the bacterially expressed A254-GMCSF (5), the purified proteins were treated with Endoglycosidase H. This resulted in smaller bands (2 and 3) which were still larger than the bacterially expressed protein (5).

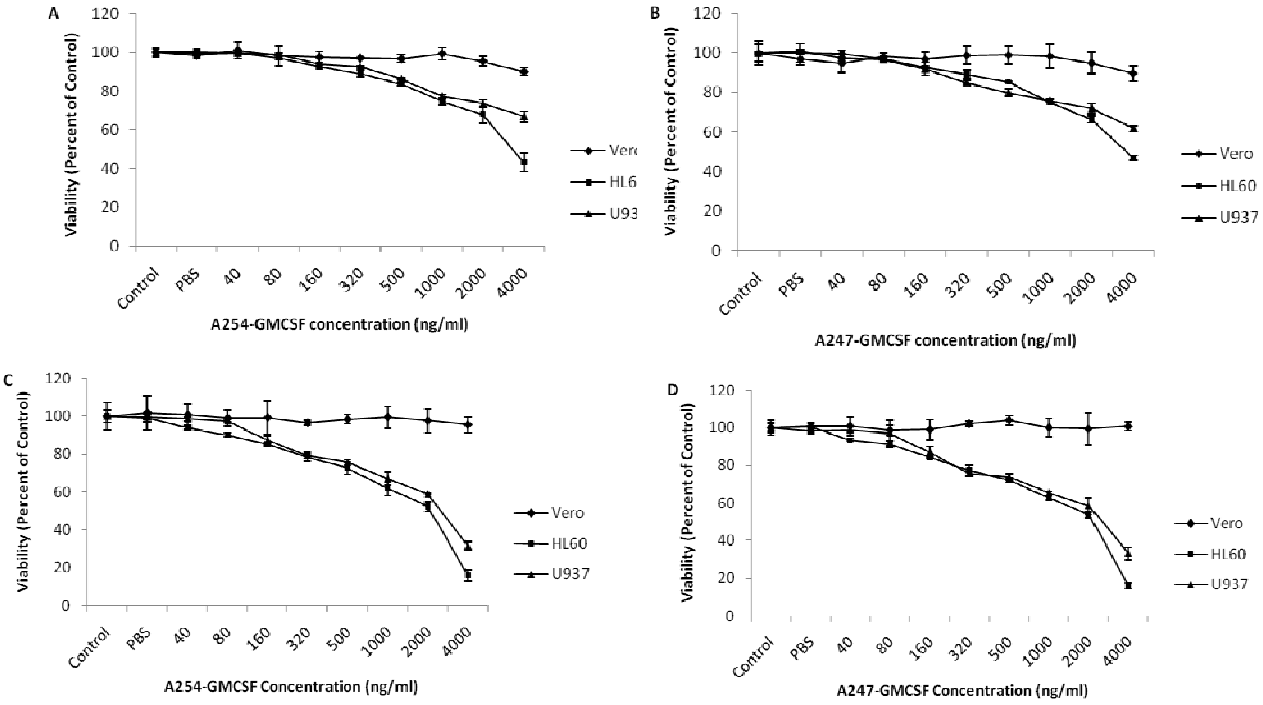

Fig. 4. Cytotoxicity assay of the recombinant fusion proteins. Treatment of Vero cells, which are devoid of GMCSF receptor, with the fusion proteins did not show any significant toxicity to these cells (A). Treatment of the GMCSF receptor bearing cell lines HL60 and U937 with the recombinant A254-GMCSF (A) and A247-GMCSF (B) for 24 h resulted in selective but slight decrease in the viability of the cells. Extension of the treatment time length to 48 h resulted in the highest observed cytotoxicities for both A254-GMCSF (C) and A247-GMCSF (D) proteins. HL60 cells showed to be more sensitive than the U937 cells to the applied treatments. Data represent the mean percent of three independent experiments of triplicates. Error bars represent SD.

Deglycosylation of the purified fusion proteins

To verify if the observed size variation is a consequence of protein glycosylation, the purified recombinant proteins were subjected to deglycosylation by Endoglycosidase H. As it is shown in Fig. 3, the endoglycosidase H treatment of the A254-GMCSF and A247-GMCSF fragmnents resulted in production of smaller fragments, but still larger than the bacterially expressed recombinant A254-GMCSF fragment. This could possibly be a consequence of any other modifications performed by the eukaryotic insect cells.

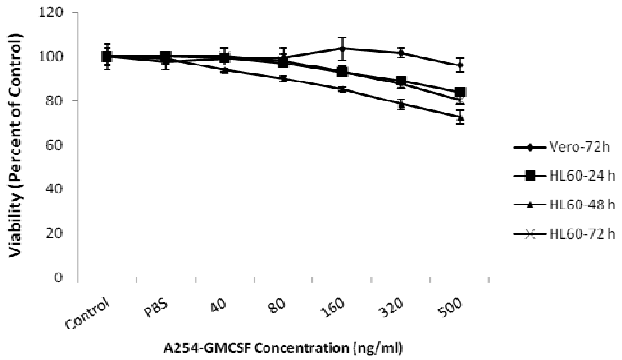

Fig. 5. Assessment of the A254-GMCSF cytotoxicity in different time courses. As it is shown, extension of the treatment time length did not affect the viability of Vero cells. It also did not exert significant reductions in viability of the HL60 cells comparing to those obtained following increasing the protein concentration. Data represent the mean percent of three independent experiments of triplicates. Error bars represent SD.

Cytotoxicity assessment of the recombinant fusion proteins

None of the examined recombinant proteins revealed significant cytotoxic effects on Vero cells which lack human GMCSF receptor except 24 h after their exposure to the highest examined concentration of 4µg/ml which caused cell viability reductions of about 10 percent (Fig. 4, A and B). Treatment of the HL60 cells with different concentrations of the recombinant fusion proteins after 24 h caused gradual cell death (Fig. 4, A and B); however, extension of the incubation time to 48 h resulted in cell viability reduction to less than 20 percent for both recombinant fusion proteins (Fig. 4, C and D).

In case of U937 cells, the highest cytotoxicity was also observed following incubation of the cells with 4 µg/ml of the fusion proteins. Considering these data, the IC_{50}s of both A254-GMCSF and A247-GMCSF were about 2 µg/ml for HL60 cells and 2.5 µg/ml for U937 cells. In order to verify the effect of time extension on the viability of the cells, HL60 cells were also treated with lower concentrations 40 to 500 ng/ml) of the A254-GMCSF fusion protein but for longer time (72 h). However, no significant reduction in cell viability was observed comparing to those obtained after 24 or 48 h (Fig. 5).

DISCUSSION

We previously reported the bacterial expression and production of the recombinant fusion protein A1-GMCSF containing the first 254 amino acid sequences of the shiga toxin 1 (A1) fragment and the GMCSF protein sequence for targeting the fusion toxin toward those tumor cells which highly expressed GMCSF receptor (17). Cytotoxicity studies of the constructed protein on different GMCSF receptor positive cell lines revealed reasonable specific cell cytotoxicities (10-11). Therefore, for conducting further studies on different aspects of the fusion protein e.g. its specific and non-specific cytotoxicities, a large amount of the protein, free from any unwanted substances like bacterial lipopolysacharide (LPS) was required. In this regard, a non-viral, plasmid based insect cell expression system was chosen. Insect cells have been shown to perform most, if not all, of the post-translational modifications similar to those known to occur in mammalian cells (19). Glycosylation is one of the most usual modifications which are performed by insect cells, however in different patterns comparing to that of mammalian cells (20). In the present study we found that the expressed fragments are heavier than the theoretically expected sizes of 46.4 and 47.1 kD (including the size of V5-His tag and the vector derived N-terminal amino acids) for A247-GMCSF and A254-GMCSF, respectively. Evaluation of the amino acid sequences of the fusion proteins showed that there are three potential N-glycosylation sites within the structure of the proteins at Asn residues 83, 273 and 283 for A247-GMCSF and Asn residues 83, 280 and 290 in case of A254-GMCSF. Furthermore, there are the possibility of O-glycosylation at Ser or Thr residues within the fusion sequences, and GMCSF has been

shown to be O-glycosylated in different sites (at Ser 22, 24 and 26 and Thr 27) by mammalian cells (21-22) which could be also performed by insect cells. To evaluate the possibility of N-glycosylation of the expressed fragments they were treated with endoglycosidase H. Deglycosylation reactions produced smaller fragments which were still larger than the bacterially expressed A254-GMCSF fragment. These results indicated the presence of other post-translational modifications which could be performed by insect cells. Evaluation of the specific cytotoxicity of the recombinant fusion proteins to the HL60 and U937 cells showed IC_{50}s of about 2 and 2.5 µg/ml to these cell lines, respectively. Our previous study using the bacterially expressed A254-GMCSF fragment revealed an IC_{50} of about 80 ng/ml to the HL60 and U937 cell lines (11). This difference in cytotoxicity could also be observed for the recombinant immunotoxin DT-GMCSF, which in case of its expression by *E. coli* cells has shown an IC_{50} of about 1 ng/ml to the HL60 cells (23). However, in case of its expression by the baculovirus expression system, a significant reduction in its potency to an IC_{50} of about 2.7 µg/ml could be observed (24). This reduced potency could be due to any modification carried out by the insect cells on the toxic fragments as they are of bacterial sources and perhaps represent their most active forms when expressed bacterially.

Furthermore, it has been shown that the glycan groups which are often large, bulky and physically dominant significantly alter the physicochemical properties of glycosylated proteins (25-26). This is the case for glycosylated GMCSF whose activity is significantly reduced due to the reduction of its affinity for binding to cell surface GMCSF receptors comparing to its non-glycosylated form (27).

In the present study, we also expressed a fusion protein consisted of a shorter derivative of the A1 fragment that is 247 amino acids in length which has been reported to exert cytotoxicity to the cells similar to that of the complete A1 fragment which is consisted of 254 amino acids (15). Similarly, cytotoxicity assays performed in the present study revealed almost identical effects following administration of both recombinant fusion proteins. One of the most characteristics of various heterologous expression systems is their production yield. The yields of expression obtained by the none-lytic insect cell expression system have been different for various proteins ranging from milligrams to grams per liter (28). However, in the present study we obtained an expression amount of about 750 µg/L that is much lower than the amount of 1 mg/L we previously obtained from bacterial expression system.

CONCLUSION

Considering the higher yield of bacterial expression of the recombinant protein A1-GMCSF, and also the higher potency of the bacterially expressed fusion protein which is almost 25 folds higher than that of the insect cell expressed proteins, it seems that in case of the A1 derived recombinant fusion proteins, the *E. coli* expression system is a more economic system. Furthermore, because of the higher potency of the bacterially expressed recombinant proteins, lower amount of the proteins are needed for obtaining similar effects, which results in less complicated reactions following its administration for any therapeutic purposes. However, because of the possible non-specific uptake of these toxins by normal cells, e.g. those epithelial cells which are located at the site of injection or normal cells which express much lower amounts of the GMCSF receptors, the lower toxicity of the fusion proteins could be advantageous. This hypothesis must be further evaluated by in vivo and animal studies.

ACKNOWLEDGMENT

We would like to express our gratitude to Dr. Júlia Costa and Dr. Ricardo M. Gouveia (Glycobiology Department, Instituto de Tecnologia Química e Biológica, Portugal) for their invaluable helps and comments on the expression and purification of the proteins. We are also grateful to Dr. Amir Amanzadeh and Mr. Hasan Sanati for their technical assistance on cell culture. This work has been financially supported by the Pasteur Institute of Iran.

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