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TITLE

THE USE OF VIRUS ATTACHMENT PROTEINS FOR THE TARGETING OF LIPOSOME ENCAPSULATED MATERIALS TO HOST CELLS

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The Use of Virus Attachment Proteins for the Targeting of Liposome Encapsulated Materials to Host Cells

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The Use Of Virus Attachment Proteins for the Targeting Of Liposome Encapsulated Materials to Host Cells

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Submitted to: Biomedical Defence Section
Defence Research Establishment Suffield

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OBJECTIVE

The objective of the study was to develop a protocol to isolate adequate amounts of sigma 1 protein, the protein which is responsible for the specific recognition and attachment of the virus to the host cell. The sigma 1 protein could then be conjugated to the exterior of liposomes, to specifically deliver the contents of the liposome to a host target cell. Antibodies and other ligands had been used in the past for specific targeting of liposomes and other antibody conjugates. This was a novel method of the use of the cell attachment proteins of viruses to target delivery to host cells. The advantage of this method over other means, is that virus attachment proteins retain fusogenic activity, possibly allowing greater efficiency in delivery of the contents of the liposome inside the cell.

FINAL REPORT, CONTRACT NO. W7702-9-R151 XSG89-00232-(602)

"The Use of Virus Attachment Proteins for the Targeting of Liposome Encapsulated Materials to Host Cells."

INTRODUCTION

Reoviruses are nonenveloped, icosahedral viruses with a double capsid that is composed of eight structural proteins. The genome is enclosed within the inner protein shell ("core") and consists of 10 segments of double-stranded RNA that are tightly packaged. The 10 genomic segments fall into three size classes designated L, M, and S, which encode proteins that are also grouped into three size classes, λ, μ, and σ. Each segment encodes a single protein product, with the exception of the S1 gene segment which encodes two proteins in alternative reading frames (1).

Based on hemagglutination and antibody-neutralization tests, mammalian reoviruses have been divided into three serotypes, 1, 2 and 3. These serotypes manifest distinct patterns of disease in mice. Through the use of genetic reassortants (generated by coinfection of cells with two different serotypes) possessing gene segments from different parental strains, it has been possible to link particular viral proteins with specific disease manifestations. These studies have led to the revelation that each of the three outer-capsid proteins μ1C, σ1, and σ3, plays a distinct role in pathogenesis (1). Of these three proteins, the σ1 protein, which is the cell attachment protein, is the most thoroughly studied and extensively characterized.

Protein σ1 is a fibre-with-knob structure located at the 12 vertices of the icosahedral virus (2,3). Direct evidence that protein σ1 is the cell attachment protein has come from the demonstration that of all the soluble reovirus-specified proteins present in the infected cell lysate, protein σ1 alone possesses the capacity to bind to host cells (2). Such binding is specific since it is blocked in the presence of excess reovirus. This observation complements the earlier finding, from genetic reassortment studies, that the S1 gene segment (encoding σ1) is responsible for the hemagglutinating function of the virus (4). Genetic reassortment studies have also revealed that the S1 gene defines reovirus tissue

tropism (5), and dictates the pathway of virus spread in the host (6). These observations are compatible with the receptor-binding function of σ1. Protein σ1 has also been found to be the major antigen for neutralizing antibody production (7), despite the fact that it is a minor viral capsid protein. There is also evidence that σ1 triggers cellular immune responses, resulting in the generation of cytotoxic T lymphocytes and suppressor T cells and the development of delayed-type hypersensitivity (5). Other functions attributed to σ1 have included the interaction with the host cell microtubules (8) and the inhibition of host cell DNA replication (9). In view of the important role of σ1 in reovirus infection, considerable effort has been made to probe the structure-function relationship of this protein. These studies have been facilitated by the observation that σ1 proteins expressed in both prokaryotic and eukaryotic systems (10-14), as well as *in vitro* (15,16), are all functional (i.e., capable of binding to cell receptors).

The observation that σ1 can bind to cell receptors by itself has led to the interesting possibility of using σ1 as targeting agents. This can be achieved by incorporating σ1 into liposomes containing specific drugs. With this goal in mind, we set out to purify this protein.

Purification of reovirus type 3 and reovirus type 1

Reovirus type 3 (strain Dearing) and type 1 (strain Lang) were from laboratory stocks. Purification followed published procedures [17] with minor modifications. Mouse L fibroblasts were grown in Joklik's modification of Eagle minimal essential medium (MEM) containing 5% fetal calf serum (FCS) (Gibco), 2 mM L-glutamine, 1 U of penicillin per ml, and 1 µg of streptomycin per ml. 4×10^9 actively growing cells were pelleted and resuspended in the medium at a concentration of 2×10^7 cells per ml. Virus from cell lysate stocks was then added to give a multiplicity of infection of 1 to 5 plaque forming units (PFU) per cell, and allowed to attach for 1 hr at room-temperature. The infected cells were then diluted to 1×10^6 cells per ml in complete MEM and incubated in spinner culture for 2 - 3 days at 35°C. Cells were then harvested by centrifugation and suspended in homogenization buffer (10 mM tris [pH 7.5], 250 mM NaCl) at a concentration of 2×10^7 cells per ml. Virus was then extracted from the infected cells by sonication and Freon

extraction. Cells were kept cooled on ice between all steps in the procedure. Cells in homogenization buffer was first disrupted by sonication. Sodium deoxycholate was then added to a final concentration of 0.1%, and the mixture was incubated for 30 min on ice. An equal volume of Freon was then added, and the mixture was again sonicated to form a complete emulsion. The aqueous and organic phases were separated by centrifugation, and the aqueous phase was decanted into a new tube. An equal volume of Freon was again added to the aqueous phase, and the mixture was sonicated to form an emulsion and subjected to centrifugation to separate the two phases. The aqueous phase was collected with a Pasteur pipette and layered on top of a 7-ml preformed CsCl gradient (1.25 to 1.45 g/cm^3). Centrifugation was carried out at 4°C for 2 hr in a Beckman 28.1 rotor (26,000 rpm). Virion bands were harvested by puncturing the wall of the tube with a needle. Harvested bands were extensively dialyzed against phosphate-buffered saline (0.14 M NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4 [pH 7.2]) and stored at 4°C. Virion particle concentrations were determined from optical density measurements at 260 nm by using the conversion factor of Smith *et al.* (17).

Isolation of biologically active reovirus type 3 Sigma 1 protein

We have been attempting to purify the reovirus cell attachment protein, σ1, which will then be encapsulated in liposomes for tissue-targeting purposes. The σ1 protein is present in small amounts in reovirus-infected L cells. While it is possible to purify σ1 from this source, large scale purifications would be costly and time-consuming. Similarly, while the *E. coli* expression vector has been invaluable for analytical purposes, it is of no use for preparative procedures in its present form. In order to produce mg quantities of purified σ1 protein, we have used two expression vector systems: the baculovirus system and the glutathione S-transferase expression system.

The baculovirus system is a helper-independent recombinant virus vector derived from the insect virus, *Autographa californica* nuclear polyhedrous virus (AcMNPV). Genes from bacteria, viruses, plants and mammals have been expressed using this system at levels ranging from 1 - 500 mg/liter in insect cell culture suspensions. The baculovirus vector utilizes the highly expressed and regulated AcMNPV polyhedrin promoter which has been

modified for the insertion of foreign genes. The proteins may be expressed in a fused or non-fused form. Since the polyhedrin gene is non-essential for virus production, it can be replaced by a foreign gene to yield a recombinant virus; cells can be infected instead of transfected, a considerable advantage. One of the major advantages of this expression vector system is the high level expression of recombinant proteins which are antigenically, immunogenically, and functionally similar to their authentic counterparts.

We have expressed the σ1 protein of reovirus type 3 using the baculovirus expression vector system. The baculovirus shuttle-vector employed to introduce the S1 gene into AcMNPV was pAC700, obtained from Max Summers, Texas A & M University. This vector contains the *Eco*RI I fragment of AcMNPV (containing the polyhedrin gene and promoter plus flanking sequences) cloned into pUC8 for propagation in *E. coli.* A large poriton of the polyhedrin coding sequences were deleted and a unique *Bam*HI site was introduced immediately following the initiation codon of the polyhedrin gene. Due to impurities in the *Bam*HI linker preparation, an additinoal 18 nucleotides are present in the 5'untranslated region of the polyhedrin gene, the significance of which will be discussed below. The S1 gene was obtained by digestion of pSP4 with *Bam*HI and *Eco*RI. *Bam*HI cleaves immediately after the initiation codon of the S1 gene. The Bam-Eco fragment was inserted into the *Bam*HI site of pAC700 (using an Eco-Bam linker). Ribosomes translating mRNA transcribed from this polyhedron promoter will initiate at the polyhedrin initiation codon and proceed immediately in-phase into the σ1 protein coding region resulting in the production of authentic σ1 proteins. The S1 gene under control of the polyhedrin promoter was inserted into AcMNPV by cotransfection of recombinant pAC700-S1 plasmid DNA and AcMNPV virion DNA into *Spodoptera fruqiperda* insect cell cultures (the SF109 cell line was obtained from Dr. Peter Faulkner, Queen's University) A rare double-crossover event can occur between the regions flanking the polyhedrin gene, resulting in the substitution of the polyhedrin coding regions with the σ1 coding regions. Recombinant virions were identified by their ability to produce occlusion body-negative plaques (the polyhedrin protein is responsible for occlusion-body formation). Two mutants were isolated from approximately 5000 plaques screened.

Restriction endonuclease analysis of recombinant viral DNA confirmed the presence of the S1 gene; however, one of the mutants appeared to have crossed over within the S1 gene, leading to the production of a truncated σ1 protein. The second recombinant directed the production of authentic σ1 protein in SF109 cells. Estimates from small scale monolayer cultures suggested the equivalent of 10 - 30 mg of σ1 produced per liter of suspension culture cells.

We have since upscaled our production of baculovirus-expressed σ1 using larger tissue culture flasks. Briefly, a liter of infected cells (6.5×10^9 cells) were harvested at 65 hr postinfection, and were resuspended in hypotonic buffer. After two cycles of freeze thawing to rupture the cells, solid urea was added to a final concentration of 8 M. After incubation at 4°C for 30 min, phosphate buffer (10 mM, pH 6.0) was added such that the final concentration of urea was 2 M. After removal of nuclei and large aggregates by low-speed centrifugation, sequential ammonium sulfate precipitation was carried out such that ~90% of the total σ1 was recovered. The precipitates were then pelleted, resuspended, and dialyzed against 10 mM phosphate and subjected to DEAE-Sephacel chromatography. The eluted σ1 was then concentrated and allowed to pass through a Sepharose S-300 column. If necessary, an additional Sepharose CL-6B chromatographic step was also included. The σ1 yield after this final step was approximately 60 - 70% and is over 99% pure as determined by silver staining of polyacrylamide gels.

It needs to be mentioned that it has taken us considerable time and effort to perfect the σ1 purification procedure. We indeed prepared a good batch of σ1 (25 - 30 mg) intended to be delivered to DRES. In order to precisely determine the amount of σ1 obtained, the protein was lyophilized. Very unfortunately, upon resuspension in a smaller volume and re-analyzed by SDS-PAGE, the σ1 protein, though extremely pure, was found to be cleaved in the middle (a typical proteolytic cleavage of σ1). We consider this a very major loss. Although we did save 1 mg of σ1 before the lyophilization process, this amount was obviously insufficient for the kind of studies intended. Unfortunately, at the same time, our virus titer declined and the yield of σ1 diminished accordingly. We repeatedly tried to isolate new plaques in order to increase the titer, but our attempts were, for unknown reasons, futile. It was time to move on and try another expression system.

Recently the glutathione S-transferase (GST) expression system has been very popular. These plasmids (pGEX-1N, pGEX-2T and pGEX-3X [18]) have been constructed to give a fusion polypeptide with the C-terminus of the *Schistosoma japonicum* glutathione S-transferase protein. In most cases fusion proteins are soluble in solutions and can be purified from crude bacterial lysates under non-denaturing conditions by affinity chromatography on immobilized glutathione. Yields of up to 15 mg protein/liter of culture have been reported. The glutathione S-transferase carrier can be cleaved from fusion proteins by digestion with site-specific proteases such as thrombin (pGEX-2T) and blood coagulation factor Xa (pGEX-3X). The carrier and any uncleaved fusion protein can then be removed by adsorption on glutathione agarose.

To express σ1 using the GST system, the S1 gene was first isolated from the pGEM clone pUC-S1 by restriction with *Bam*HI followed by agarose gel purification of the linear DNA. The ends of the DNA fragment were then filled in and the fragment was then cloned into the pGEX-3X vector (cut with SmaI) by blunt-end ligation within the multiple cloning site according to the methods of Maniatis *et al.* [Molecular Cloning, a Lab Manual section 1.6].

Clones were screened using mini-plasmid prep (Promega) of 10 ml overnight cultures grown in LB Amp broth followed by digestion with Pst1 and EcoRV restriction endonucleases. Bands of the appropriate apparent size corresponding to recombinants of the correct orientation were observed on 1% agarose gel + EtBr. Further screening was performed by growing an overnight culture in LB-Amp broth with IPTG inducement of expression (0.1 mM final) followed by cell harvesting (centrifuged 2500 rpm for 10 min, 4°C in a Beckman J6B centrifuge with JS 4.2 rotor) three hours later. Harvested cells from a 100 ml culture were resuspended in 15 ml of PBS (pH 7.4) + 1.5% sarcosyl (Sigma), then sonicated for 1 min. The lysate was centrifuged at 1800 g for 15 min to pellet. One ml of supernatant was then incubated with 5.0 µl of glutathione beads for 1.0 hr on ice with periodic shaking, pelleted briefly in a table-top centrifuge at 10 K rpm, then washed either (a) 2X with Equilibrium buffer (eq) alone (pH 7.60, 100 mM KCl, 2.0 mM $MgCl_2$, 20 mM Tris), (b) 2X eq buffer, then 2X eq buffer + 0.1% Triton X-100, or (c) wash "b" and 2 washes including 2.0 M NaCl. The pelleted beads from each of the three washes were

resuspended in protein sample buffer and loaded on a 10% mini-polyacrylamide gel and electrophoresed for 1 hr at 35 mA at room temperature against a prestained molecular weight marker.

The resultant gel was electroblot-transferred overnight at 50 mA to a nitrocellulose filter. The filter was then probed for the presence of σ1 using an anti-type 3 sigma 1 polyclonal Ab followed by an anti-IgG horseradish peroxidase conjugate then visualized using ECL reagents and x-ray film (30-second exposure) [Amersham RPN 2106]. A band of apparent molecular weight of 80 kd remained intense at all stringencies of washing with most other bands waning.

To further demonstrate that the 80 Kd protein in fact represents the GST-σ1 fusion product, proteins bound to glutathione beads were treated with factor Xa. A protein migrating at 45 Kd was found to be cleaved off the beads, and was confirmed to be σ1 by Western blotting. Production of σ1 has since been scaled up using one-litre cultures (see flow chart appended). According to our estimation, 2 - 5 mg of σ1 can be isolated from a 1-litre culture (Table 1). Preliminary evidence suggests that this σ1 is functional, i.e., capable of binding to cell receptors.

Generation of polyclonal anti-σ1 antibodies

Female New Zealand white rabbits (5 to 6 lbs) were inoculated subcutaneously and intramuscularly with purified σ1 (100 μg) in Freund's complete adjuvant for the initial dose. Two subsequent boostings (same amount of antigen but prepared in Freund's incomplete adjuvant) were made at 14 and 28 days. Sera were collected 7 days after the third inoculation.

DISCUSSION

Isolation of protein σ1 was initially achieved by disruption of reovirions followed by differential precipitation and chromatography. The protein thus isolated retains its trimeric form and is capable of binding to cell receptors. However, the fact that σ1 is present in the virion to no more than 1% makes such an approach less than ideal. The baculovirus system subsequently adopted was superior in terms of yield and is no doubt the system of choice

for σ1 expression. Unfortunately, for reasons still unknown, the titer of our baculovirus stock dwindled and we eventually lost our σ1-expressing baculovirus. Fortunately at that time, the *E. coli* GST system was gaining popularity and we decided to give it a try. The cloning and screening of positive GST clones is straightforward but it suffers from the drawback that the expressed protein is in the form of a fusion product with the desired protein fused to the C-terminus of glutathione S-transferase. Although most GST fusion proteins are used without further processing, for our purpose it is important to obtain σ1 in the unfused form. An added complication is the need for σ1 to be in the trimeric state in order to be functional (i.e., capable of binding to cell receptors). The possibility of the GST portion being a hindrance to the trimerization process was a real concern. The final requirement would be the retention of σ1 trimeric state and function after enzymatic treatment to remove the GST. Our results indicate that all the above conditions have been met. The final σ1 product appears to be pure and functional. Considering the amount of σ1 fusion product expressed, the yield has not been impressive. This is most likely due to the relative insolubility of the protein. The use of stronger chaotropic agents (such as urea) to break up aggregates should result in higher yield.

Deliverables

We have previously delivered 15 mg of purified reovirus type 1 and 20 mg of reovirus type 3. The present shipment contains 15 mg of purified, biologically active reovirus type 3 σ1 protein, 3 mg of ^{35}S-labelled reovirus type 3 σ1, 40 mg of gradient purified roevirus type 3, and 0.5 ml of polyclonal antibodies against reovirus type 3 σ1. The contractual agreement is now considered fulfilled.

REFERENCES

1. Schiff, L.A. and Fields, B.N. (1990). Reovirus and their replication. In *Fields Virology.* (eds. B.N. Fields and D.M. Knipe), pp. 1275-1306. Raven Press.
2. Lee, P.W.K., Hayes, E.C. and Joklik, W.K. (1981). Protein σ1 is the reovirus cell attachment protein. *Virology* 108, 156-163.
3. Furlong, D.B., Nibert, M.K. and Fields, B.N. (1988). σ1 protien of mammalian reoviruses extends from the surfaces of viral particles. *J. Virol.* 62, 246-256.
4. Weiner, H.L., Ramig, R.F., Mustoe, T.A. and Fields, B.N. (1978). Identification of the gene coding for the hemagglutinin or reovirus. *Virology* 86, 581-584.
5. Sharpe, A.H. and Fields, B.N. Pathogenesis of viral infections. Basic concepts derived from the reovirus model. *N. Engl. J. Med.* 312, 486-497.
6. Tyler, K.L., McPhee, D.A. and Fields, B.N. (1986). Distinct pathways of viral spread in the host determined by reovirus S1 gene segment. *Science* 233, 770-774.
7. Weiner, H.L. and Fields, B.N. (1977). Neutralization of reovirus: The gene responsible for the neutralizaiton antigen. *J. Exp. Med.* 146, 1305-1310.
8. Sharpe, A.H., Chen, L.B. and Fields, B.N. (1982). The interaction of mammalian reoviruses with the cytoskeleton of monkey kidney CV-1 cells. *Virology* 120, 399-411.
9. Sharpe, A.H. and Fields, B.N. (1981). Reovirus inhibition of cellular DNA synthesis: role of the S1 gene. *J. Virol.* 38, 389-392.
10. Masri, S.A., Nagata, L., Mah, D.C.W. and Lee, P.W.K. (1986). Functional expression in *Escherichia coli* of cloned reovirus S1 gene encoding the viral cell attachment protein σ1. *Virology* 149, 83-90.
11. Pelletier, J., Nicholson, R., Bassel-Duby, R., Fields, B.N. and Sonenberg, N. (1987). Expression of reovirus type 3 (Dearing) σ1 and σ1 polypeptides in *Escherichia coli.* *J. Gen. Virol.* 68, 135-145.
12. Atwater, J.A., Munemitsu, S.M. and Samuel, C.E. (1987). Efficiency of expression of cDNAs of the reovirus S1 and S4 genes in transfected animal cells differ at the level of translation. *Virology* 159, 350-357.

13. Banerjea, A.C., Brechling, K.A., Ray, C.A., Erickson, H., Pickup, D.T. and Joklik, W.J. (1988). High-level synthesis of biologically active reovirus protein σ1 in a mammalian expression vector system. *Virology* 167, 601-612.

14. Fajardo, E. and Shatkin, A.J. 1990. Expression of the two reovirus S1 gene products in transfected mammalian cells. *Virology* 178, 223-231.

15. Duncan, R., Horne, D., Strong, J.E., Leone, G., Pon, R.T., Yeung, M.C. and Lee, P.W.K. 1991. Conformational and functional analysis of the C-terminal globular head of the reovirus cell attachment protein. *Virology* 182, 810-819.

16. Leone, G., Maybaum, L. and Lee, P.W.K. (1992). The reovirus cell attachment protein possesses two independently active trimerizaiton domains: Basis of dominant negative effects. *Cell* 71, 479-488.

17. Smith RE, Zweerink HJ and Joklik WK. (1969) Polypeptide components of virions, top component, and cores of reovirus type 3. *Virology* 39:791-810.

18. Smith DB and Johnson KS. (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* 67:31-40.

FLOW CHART

Sigma 1 Purification Scheme (GST System)

1 L culture of *E. coli* containing recombinant pGEX-S1 plasmid

↓

pellet and resuspend in PBS and sarkosyl

↓

sonication and centrifugation

↓

supernatant mixed with glutathione agarose beads

↓

beads collected and washed

↓

elute GST-σ1 fusion protein from beads with glutathione

↓

digest eluted protein with factor Xa

↓

remove carrier (GST) and uncleaved protein with glutathione agarose beads

↓

purified σ1

Table 1

Sigma 1 Purification Summary (GST System) (1 litre)

Fraction	Total protein (mg)	Sigma 1 (mg)	Sigma 1 (%)
Disrupted cells	4200	137	3.3
PBS-sarkosyl supernatant	1350	29	2.1
Bound to glutathione beads	16	6.7	42
Eluted from glutathione beads	5.7	5.3	93
After second glutathione bead adsorption	4.2	4.2	>99

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