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Ανάπτυξη DNA εμβολίου για την ανοιξιάτικη ιογενή ιαιμία του Κυπρίνου

Θ.Σ. Κανέλλος^{1,2}, Ε.Μ. Ξυλούρη¹, Russell P.H.²

ΠΕΡΙΛΗΨΗ. Η ανοσοποίηση ενός οργανισμού με το πλασμιδιακό DNA αποτελεί έναν πολλά υποσχόμενο εναλλακτικό τρόπο για να προκληθεί ανοσία έναντι λοιμωδών παραγόντων. Σκοπός της παρούσας έρευνας ήταν να μελετηθεί ένα DNA εμβόλιο έναντι της Ανοιξιάτικης ιογενούς ιαιμίας του κυπρίνου (Spring Viraemia of Carp Virus, SVCV), οφειλόμενης σε ραβδοϊό, μελετώντας τη γλυκοπρωτεΐνη G του ιού. Το γονίδιο G ενσωματώθηκε στο πλασμιδιακό DNA και κλωνοποιήθηκε. Η έκφραση του γονιδίου πραγματοποιήθηκε με τη βοήθεια των παρακάτω ενισχυτών: 1) του κυτταρομεγαλοϊού, 2) του simian virus 40, και 3) της χοίρειας βαριάς αλυσίδας μυοσίνης II A. Το κλωνοποιημένο γονίδιο G ανασυνδυάστηκε είτε με το γονίδιο της *E coli*, LacZ (ως ανοσοενισχυτικό των Τ λεμφοκυττάρων), είτε με την οδηγό αλληλουχία Ig k-chain για την παραγωγή της εκκριτικής μορφής της πρωτεΐνης G. Μύες BALB/c ανοσοποιήθηκαν με τα προαναφερθέντα ανασυνδυασμένα πλασμίδια και προκλήθηκε έντονη κυτταρική ανοσολογική απάντηση (*T-cell proliferative responses*) στον SVCV, εν απουσία ανιχνεύσιμων αντισωμάτων για τον ιό. Η ομάδα των ποντικών που έδωσε εντονότερη ανοσολογική απάντηση ήταν εκείνη που είχε λάβει το γονίδιο LacZ και έτσι αυτό το γονίδιο ελέγχθηκε αργότερα με την παράλληλη έγχυση ενός πλασμιδίου που εξέφραζε την κυτταροκίνη Granulocyte Macrophage Colony Stimulating Factor (GMCSF). Έτσι, η παράλληλη χορήγηση του πλασμιδίου έκφρασης GMCSF είχε ως αποτέλεσμα την υψηλή ανοσολογική απάντηση έναντι του ιού της Ανοιξιάτικης ιογενούς ιαιμίας του κυπρίνου, από τα λεμφοκύτταρα της σπλήνας του ποντικού, με αύξηση στην παραγωγή και των δυο πρωτεϊνών που είχαν υποστεί ανασυνδυασμό (*fusion*). Αυτά τα αποτελέσματα δείχνουν τη λειτουργικότητα των DNA εμβολίων που εξετάστηκαν και την πιθανότητα τα εμβόλια να αναπτύσσουν ανοσολογική προστασία στους ιχθύες.

Λέξεις ευρετηρίασης: Ανοιξιάτικη ιογενής ιαιμία του κυπρίνου, DNA εμβόλια

The development of a DNA vaccine for the Spring Viraemia of Carp Virus (SVCV)

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ABSTRACT. DNA immunization is a promising approach to induce immunity/protection against infectious agents. In the present study a DNA vaccine to Spring Viraemia of Carp Virus (SVCV) G glycoprotein was investigated. The G gene was cloned intact with several promoters namely: 1) the immediate early promoter/enhancer of the human cytomegalovirus; 2) the simian virus 40 promoter; and 3) the porcine myosin heavy chain II A promoter. In fusion constructs, the G gene was fused either upstream to the LacZ gene or downstream to the murine Ig k-chain leader sequence. BALB/c mice immunized with the aforementioned plasmids produced strong *T-cell proliferative responses* to the SVCV, in the absence of any detectable antibody response. The group that responded most robustly was the one that had received the LacZ fusion gene and therefore, this construct was further tested in mice with the addition of a plasmid expressing the murine cytokine GMCSF. Co-administration of the GMCSF expressing plasmid resulted in high SVCV spleenoproliferative responses in mice and the development of antibodies to both fused proteins. These results indicate the functionality of the constructs and the possibility of giving protection in fish.

Key words: Spring Viraemia of Carp Virus, DNA vaccine, Fish

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INTRODUCTION

Nucleic acid immunization has been characterized as "The third revolution in vaccinology" (Robinson, 1997), because of its unique way to stimulate both arms of the immune system. This vaccine technology has already got approval for clinical trials in humans (Donnelly et al., 1997), underlying the potential and the safety (Robertson, 1994) of this technology to prevent or even to cure humans from emerging infectious diseases or even diseases like cancer, allergy and autoimmunity (Tighe et al., 1998). However, other species of significant economic value, besides humans, may reap the benefit of DNA vaccines, such as fish.

Aquaculture is now the most rapidly growing area in animal production and presently accounts for more than 8% of the total food production worldwide. However, because of the fact that these animals are reared intensively, they are even more vulnerable to infectious pathogens. With regard to fish viral vaccines, due to the poor antiviral immunity encountered with inactivated preparations and the lack of widespread acceptance of attenuated strains as vaccines, the use of genetic immunization appears as an attractive alternative to control fish diseases (Leong et al., 1997).

Recently, encouraging reports appeared in the literature showing protection of salmonids fish from viral pathogens, such as Infectious Haemopoetic Necrosis Virus (IHNV) (Anderson et al., 1996) and Viral Haemorrhagic Septicaemia Virus (VHSV) (Lorenzen et al., 1998), following vaccination with naked plasmid DNAs encoding proteins of these pathogens. Furthermore, studies employing reporter genes have shown that fish respond very well to DNA immunization (Russel et al., 1998).

In the present study a DNA vaccine was developed for a severe viral disease of the cyprinids, namely Spring Viraemia of Carp (SVC). The disease is widespread throughout Europe. In the UK, it is a notifiable disease, with numerous outbreaks occurring in 1988 (Dixon et al., 1994) and 1994 (Roberts, 1994). The disease is caused by a rhabdovirus (SVCV), which infects several species of carp during spring. Outbreaks of SVC usually can result in mortality rates of between 40-90% and currently there is no available vaccine to control the disease (Dixon, 1997).

The glycoprotein (G) gene of SVCV, which forms its surface peplomers, was chosen because it is the target of immunoprophylaxis in all other known members of the family Rhabdoviridae (Coll, 1995) for cloning into several mammalian expression cassettes. The G gene was cloned intact with different viral or mammalian promoters, in order to evaluate their efficacy for gene

immunization. The human cytomegalovirus immediate early promoter/enhancer (CMV) and the simian virus 40 early proteins (SV40) were chosen as viral promoters, because both were shown to be very potent in fish (Hansen et al., 1991, Anderson et al., 1996). In addition, the mammalian porcine myosin heavy chain II A promoter (MyHC) (Chang, 2000) was used because it is a muscle specific promoter and therefore, it does not carry the risk of inappropriate or harmful gene expression in other tissues.

The glycoprotein (G) gene of two fish rhabdoviruses namely; Infectious Haemopoetic Necrosis Virus (IHNV) (Robinson, 1997) and Viral Haemorrhagic Septicaemia Virus (VHSV) (Donnelly et al., 1997), are effective as DNA vaccines when driven by the human cytomegalovirus immediate early promoter/enhancer (CMV). In other studies the full length G gene (G_F) of the Spring Viraemia of Carp rhabdovirus (SVCV), as well as a truncated form of the G gene (G_{54}), lacking the transmembrane region of the protein, were placed into a CMV promoter driven plasmid. *In vivo* analysis of these plasmids demonstrated that 1 out of 10 mice with the truncated gene and 2 out of 10 with the complete gene seroconverted to the encoded glycoprotein (Robertson, 1994).

In the present study we further tested the above plasmid encoding the complete SVCV-G protein, in addition to four novel constructs. Two of the new constructs expressed the G gene under the control of either the simian virus 40 early proteins (SV40) promoter or the mammalian porcine myosin heavy chain II A (MyHC) promoter, both of which promote CAT expression in fish (Tighe et al., 1998). A third plasmid was constructed to express the G gene as a fusion upstream to the LacZ gene, under the control of the CMV promoter, in order for the LacZ gene product (β -galactosidase) to give T help to the G protein in a way akin to the hapten-carrier effect (Leong et al., 1997).

This would also direct the expressed G protein towards the cytoplasm, because β -galactosidase accumulates in this cellular compartment (Anderson et al., 1996). Finally, the G_F gene was placed downstream of the murine Ig k chain leader sequence, in order to allow secretion of the expressed protein. The efficacy of these DNA constructs was evaluated by their ability to induce humoral and cellular immune responses in mice.

Furthermore, the G gene was fused upstream to the LacZ gene in order for LacZ to give T help to the G protein in a way akin to hapten-carrier (Mitchison, 1971). This would also direct the expression of the G protein towards the cytoplasm, because β -galactosidase accumulates in that particular cellular compartment

Πίνακας 1. Χαρακτηριστικά των SVCV-G έκφρασης πλασμιδίων για την ανοσοποίηση. Η έκφραση των SVCV-G είχε ρυθμιστεί από τους CMV, SV40 και MyHC, αντίστοιχα. Το SVCV-G γονίδιο είχε συγχωνευθεί με το LacZ ή με το Igk στο ίδιο κεντρικό πλασμίδιο (pcDNA3) και η έκφραση είχε καθοδηγηθεί από τον CMV εκκινητή.

Table 1. Characteristics of the SVCV-G expression plasmids used for immunization. The expression of SVCV-G was controlled by CMV, SV40 and MyHC, respectively. The SVCV-G gene was also fused either to the LacZ gene or to the murine Igk leader sequences into the same plasmid backbone (pcDNA3), and expression was driven by the CMV promoter.

| Construct Name | Promoter | SVCV-G gene | Antibiotic Resistance gene | Additional Properties |
|-----------------------------|----------|-------------------|----------------------------|--|
| pcDNA3/G _F | CMV | Full length | <i>ampR</i> gene | Cytomegalovirus promoter |
| pcSV40/G _F | SV40 | Full length | <i>ampR</i> gene | Simian Virus 40 promoter |
| pMyHC/G _F | MyHC | Full length | <i>ampR</i> gene | Porcine myosin heavy chain promoter |
| pcDNA3.1/G-LacZ | CMV | G protein chimera | <i>ampR</i> gene | Intact Kozak sequence Chimera with B-galactosidase. Cytoplasmic expression |
| pSecTagB/His-G _F | CMV | Full length | <i>ampR</i> gene | Murine Ig kapper chain leader sequence to allow secretion. Intact Kozak sequence. |
| pCMVi-GMCSF | CMV | | <i>kanR</i> gene | Expressing the murine Granulocyte Macrophage Colony Stimulating Factor |

(Davis et al., 1997). The G gene inserted also downstream to the murine Ig k-chain leader sequence in order to allow secretion of the fusion protein. All the fused genes were under the control of the CMV promoter.

The immunogenicity of these constructs was evaluated in a mouse model, because T cell responses to DNA vaccines are well described in these animals.

MATERIALS AND METHODS

Vaccination Vectors

The Characteristics of all plasmids used in these studies are shown in table 1.

Molecular cloning procedures were essentially as described by Sambrook, et al. (Sambrook, et al., 1989). Two incomplete genes (pSegG₂₅, pSegG₅₄) of the SVCV G glycoprotein as it was confirmed by sequencing (results not shown) were kindly provided by Dr Bjorklund H., Abo Akademi University, Turku Finland. Both genes were inserted by blunt end cloning into the CMV mammalian expression vector pcDNA3 (Invitrogen) to create the plasmids pcDNA3/G₂₅ and pcDNA3/G₅₄. The complete SVCV-G gene was prepared by removing the 1020 bp 5' end of the pcDNA3/G₅₄ gene by digestion with *Hind*III and *Xcm*I and then by inserting it into compatible digested sites of the pcDNA3/G₂₅. The resulting plasmid was designated pcDNA3/G_F.

The SV40 promoter from the plasmid pCAT (Promega) was -digested with *Hind*III and *Bgl*II and inserted into compatible restriction sites of the pcDNA3

in place of CMV to produce the pSV40 plasmid. Subsequently, the full length G (G_F) gene was removed by digestion with *Bam*HI and *Apa*I from the pcDNA3/G_F and transferred into the pSV40 plasmid by sticky end cloning. The resulting plasmid was designated as pSV40/G_F. The muscle specific promoter MyHC was inserted by semi-directional cloning to the pcDNA3/G_F after removing the CMV promoter with the restriction endonucleases *Bgl*II and *Apa*I. The resulting plasmid was named pMyHC/G_F.

The pcDNA3/G_F was digested with *Hind*III and *Psh*AI in order to release part of the G_F gene (1424 bp) that lacked the stop codon at the 3' end. Subsequently, the pcDNA3.1/HisB/LacZ was digested with *Kpn*I, blunt ended with calf intestinal alkaline phosphatase, and re-digested with *Hind* III following phenol/ chloroform/ Isoamyl alcohol extraction and ethanol precipitation in order to release the 6 histidines and the enterokinase tag. The 1.4 kb fragment of the G_F was then inserted by semi-blunt ended cloning into the aforementioned plasmid in order to create the plasmid pcDNA3.1/G-LacZ.

The G_F gene was removed from the pcDNA3/G_F by digestion with *Hind*III and *Xba*I and inserted in frame to a same enzymes digest of pSegTag B (Invitrogen), which contains the murine Ig k chain leader sequence upstream of the multiple cloning site to allow secretion of the SVCV-G gene.

All plasmids were sequenced after three repeated runs by cycling sequencing to confirm that they were

intact to promoters or in frame with the fusion genes (results not shown).

The plasmid pCMVi-mGMCSF, encoding the murine cytokine Granulocyte Macrophage Colony stimulating Factor (mGMCSF) under the control of the CMV, was generously donated from professor Johnston SA, Southwestern medical centre, USA.

All plasmids were purified by alkaline lysis of ampicillin treated cultures followed by anion-exchange chromatography (Giga-Prep, Qiagen) and then resuspended at 1mg/ml in endotoxin-free saline (Sigma) for injection.

Transient Transfection

To determine the expression of the G_f gene in the aforementioned plasmids, Epithelioma Papulosum Carpino cells (EPC) were transfected in a 12 well cell culture plate (Nunc) using Lipofectamin (Gibco-BRL, Life technologies) and cultured for 3 days. The cells were then fixed with acetone and stained with an anti-SVCV rabbit polyclonal antibody R116, generously donated by Dr Dixon, MAFF, Weymouth, which has neutralizing activity against SVCV, and with goat anti-rabbit IgG labelled with HRP (Dako). Transfected cells were then visualized with a chromogen (HRPO) (Czerkinsky et al., 1988) under an inverted microscope.

For the plasmid encoding the fusion Gf-LacZ gene, an additional *in situ*, staining with X-gal was performed in order to determine the expression of the β -galactosidase (Wells et al., 1997).

TNT® Coupled Reticulosyte Lysate Systems

The ability of the pcDNA3 vector to express SVCV glycoprotein was determined using a TNT® coupled reticulocyte lysate system (Promega), which utilizes the T7 RNA polymerase to transcribe the inserted gene.

Translation reaction products were resolved by reducing SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) on a 15% gel, and then electro-blotted onto nitrocellulose membrane (Sambrook et al., 1989). The membrane was probed with the R116 anti-SVCV rabbit antiserum, and then probed with an anti-rabbit/alkaline phosphatase conjugated secondary antiserum. Finally, the secondary antibody was detected by NBT/BCIP staining as per manufacturer's instructions (Promega).

Northern Blot Analysis

Total RNA was extracted from transfected cells (RNeasy kit Qiagen,) and following electrophoresis, RNA was fixed to a Hybond-N+ membrane (Amersham, Life Science) (Wells et al., 1997). The blot was subsequently hybridized with a specific SVCV-G probe

overnight at 60°C. The probe was prepared by excising the 1.6kb G_f gene from the pcDNA3 G_f with *Hind*III and *Xba*I. Labelling of probe and subsequent hybridization detection were performed by an ECL random prime labelling and detection system, version II kit membrane (Amersham, Life Science).

Intramuscular Injection

Experiment 1: Male BALB/c mice aged 6-8 weeks were obtained from Harlan Olac, UK. Groups of 6 mice received either 50 µg of the empty plasmid (pcDNA3) or 50 µg of plasmids encoding the SVCV-G protein either under the control of different promoters (pcDNA3/ G_f , pSV40/ G_f , pMyHC/ G_f) or as fusion gene (pSecTagB/ G_f , pcDNA3.1/ G_f -LacZ). Half the plasmid was injected into the middle of each BaCl₂ pretreated²¹ anterior tibial muscle (TA) using an insulin syringe fitted with a 28/29g needle, under general anaesthesia. The mice were re-immunized 4 times at monthly intervals. Tail blood was collected at 4, 8, 12, 16, 20 weeks after the first injection.

Experiment 2: Two groups of five female BALB/c mice were injected either with 100 µg empty plasmid (pcDNA3) or with 100 µg of an equal mixture of the plasmids pcDNA3.1/ G -LacZ and pCMVi-mGMCSF. DNA injections were performed as before, except that this time recipient TA muscles had not received any pretreatment. One month after the injections, mice were euthanised and serum samples were tested for antibody, while their spleens were processed for T-cell stimulation.

Anti-β-Galactosidase ELISA

Serum samples were analyzed by ELISA against purified *E. coli* β-galactosidase VIII protein (Sigma) as described previously (Russel et al., 1998). In brief twofold dilutions of mice sera at 1:25 to 1:12800 were reacted for 1 hour at 37°C and binding was detected using a rabbit anti-mouse conjugate (Dako). The serum titers calculated as the reciprocal of the serum dilution that gave an OD₄₉₂ of three standard deviations above that of the negative control mice. A mouse monoclonal anti-β-galactosidase antibody (Sigma) was used as a positive control with an end point of 10⁻⁶.

Anti-SVCV ELISA

In principals the anti-SVCV ELISA was identical to the aforementioned anti-β-gal ELISA with the only exception that the coating antigen was concentrated SVCV from supernatant of infected cells, which had been centrifuged at 20.000rpm for 2 hours. The rabbit antiserum R116 (positive control) reacted to a titer of 10⁻⁵.

Anti-SVCV Antibody Assay

Sera from DNA injected mice were analyzed for antibodies to SVCV by indirect Monolayer Immunoperoxidase Assay (IPMA) (Hansen et al., 1991) on overnight infected EPC cells with 10^2 pfu SVCV (American type culture collection: ATCC, VR-1390). Cells were then fixed, blocked (PBST-B) and test serum of three fold dilution starting at 1/25 was added. Chromatogenic detection with a rabbit anti-mouse IgG-HRP conjugate was as earlier described. The rabbit antiserum R116 (positive control) stained foci to a titer of 10^{-5} .

T Lymphocyte Proliferation Assay

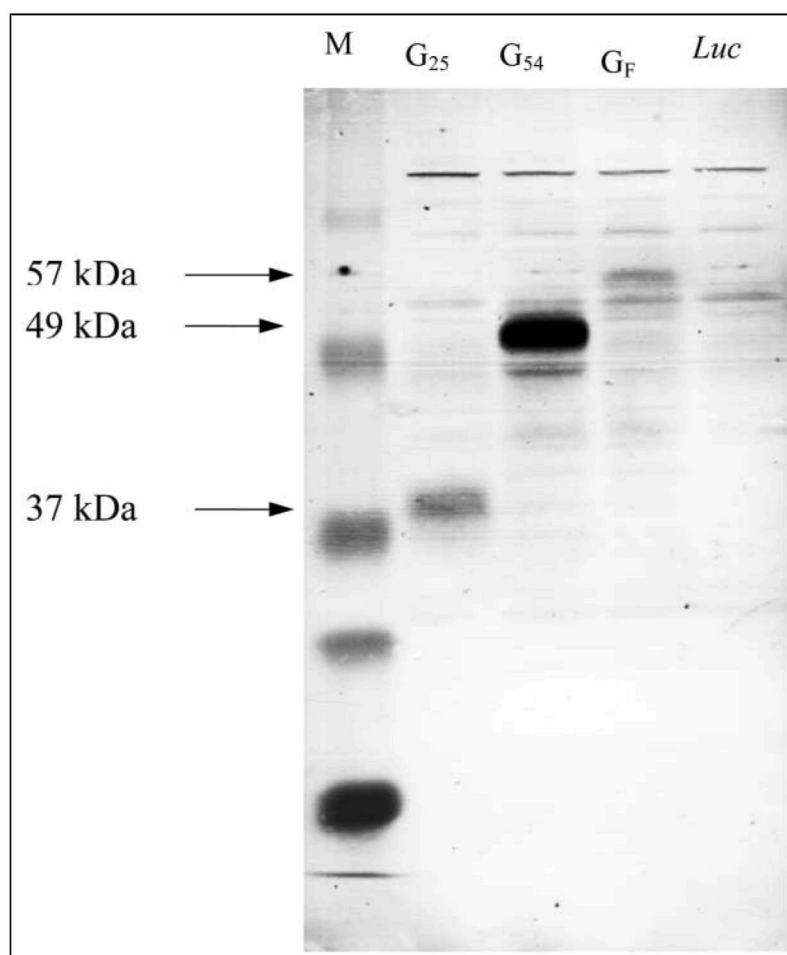
Mouse spleens were aseptically removed and were made to a single cell suspension in a supplemented RPMI-1640 medium (Gibco, BRL) (Partidos and Stewart, 1990). Cultures were then stimulated either with 100μl (neat) supernatant of SVCV infected EPC cells or with 10μg recombinant β-galactosidase. Controls included cells stimulated with medium or 0.5μg/ml phytohaemagglutinin (PHA) (Sigma). The stimulation index was defined as the ratio of counts per minute (cpm) of stimulated and medium-only cultures.

RESULTS

In Vitro Expression of DNA Plasmids Encoding the SVCV-G Glycoprotein

Initial attempts to express the aforementioned constructs *in vitro* by employing the liposome mediated transfection method were without apparent success, because the rabbit antiserum stained normal cells by the indirect monolayer peroxidase assay and reacted to many proteins in cells and foetal calf serum by western blotting. Therefore, the lack of a monospecific serum to the SVCV-G protein made problematic the detection of the expressed protein either *in vitro* or *in vivo*. The positive control plasmid that encoded the *Escherichia coli* LacZ gene was highly expressed as reported before (Russel et al., 1998).

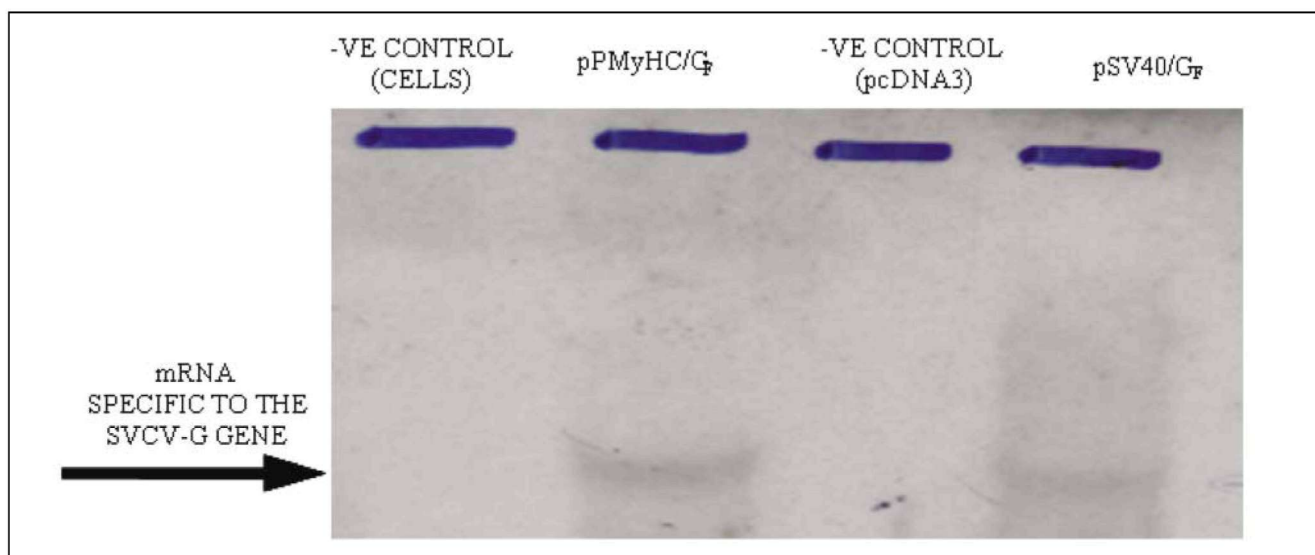
The functionality of these plasmids was determined *in vitro* by employing several diverse approaches. The efficiency of the pcDNA3/G vectors to express the



Εικόνα 1. *In vitro* έκφραση των τριών προϊόντων pcDNA3/G₂₅, pcDNA3/G₅₄ και pcDNA3/G_F, που κωδικοποιούν είτε τις δύο ατελείς μορφές της SVCV-G πρωτεΐνης (G₂₅ και G₅₄) είτε την πλήρη μορφή της (G_F), χρησιμοποιώντας το ενδοθηλιακό σύστημα λύσης μεταγραφής-μετάφρασης TNT. Από την ανάλυση των προϊόντων αντίδρασης με τη μέθοδο Western blot φάνηκε η έκφραση των διαφορετικών μεγεθών της SVCV-G πρωτεΐνης στα προβλεπόμενα μοριακά βάρη. Το γονίδιο της λουσιφεράσης χρησιμοποιήθηκε ως αρνητικός μάρτυρας.

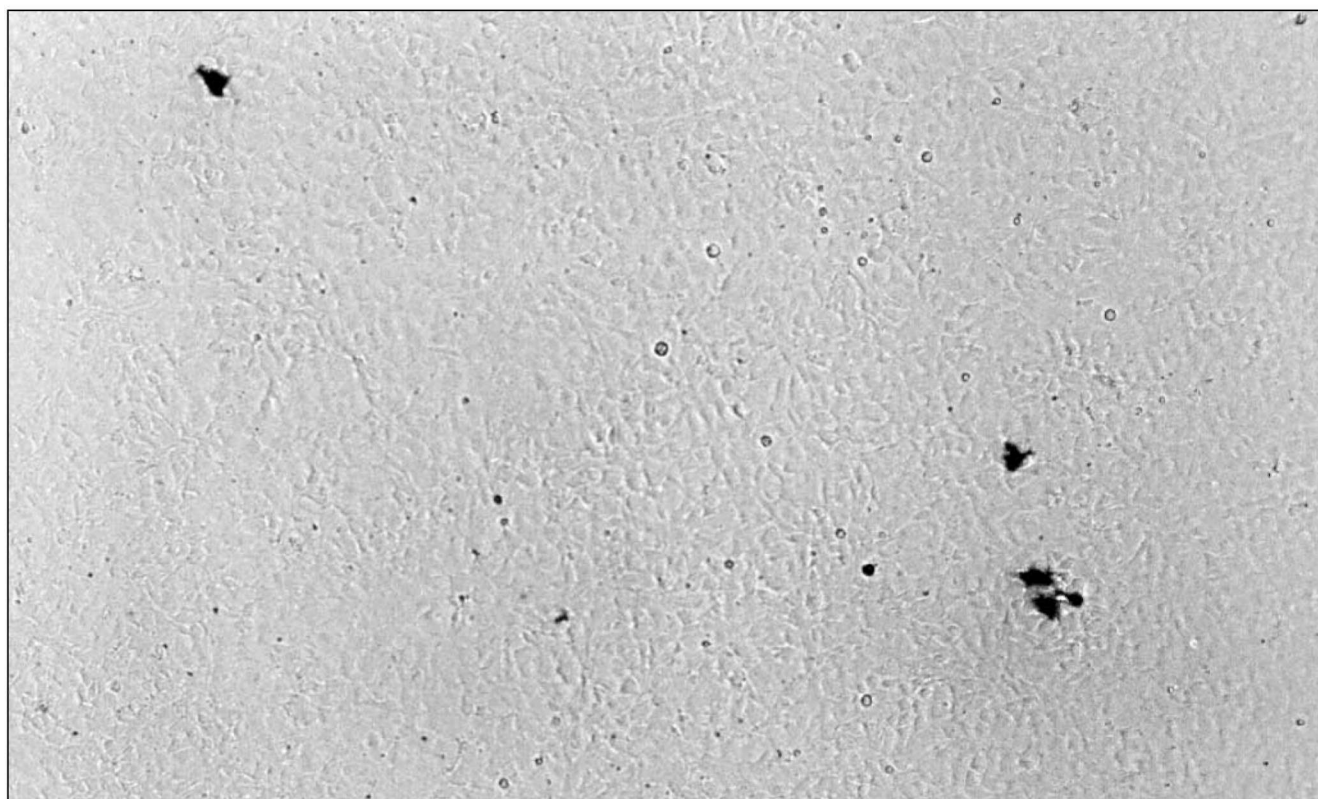
Figure 1. *In vitro* expression of the three constructs designated as pcDNA3/G₂₅, pcDNA3/G₅₄ and pcDNA3/G_F, encoding either the two incomplete forms of the SVCV-G protein (G₂₅ and G₅₄) or its full length (G_F) by using the coupled transcription/ translation reticulocyte lysate system TNT. Western blot analysis of the reaction products revealed the expression of the different lengths of the SVCV-G protein at the predicted molecular weights. Luciferase gene was used as negative control.

several lengths of the G gene was determined by using a rabbit reticulocyte lysate system. As shown in figure 1, the G protein was expressed at the expected molecular weight and was recognized by the virus-specific serum R116. For plasmids that did not contain the T7 promoter, G gene transcriptional activity was determined by detecting mRNA expression following transfection (figure 2). Moreover, the limited expression of the LacZ gene from the plasmid expressing the fusion gene G-



Εικόνα 2. Ανάλυση με τη μέθοδο Northern blot των συνολικών RNA δειγμάτων, που απομονώθηκαν από τα μετασχηματισμένα κύτταρα, στα οποία είχαν εισαχθεί τα πλασμίδια pPMYHC/G_F και pSV40/G_F.

Figure 2. Northern blot analysis of total RNA samples extracted from transfected cells with the plasmids pPMYHC/G_F and pSV40/G_F.



Εικόνα 3. *In situ* X-gal χρώση των μετασχηματισμένων EPC κυττάρων με το πλασμίδιο pcDNA3.1/G_F-LacZ, το οποίο εκφράζει και τα δύο ανασυνδυασμένα γονίδια (G_F-LacZ).

Figure 3. *In situ* X-gal staining on transfected EPC cells with the plasmid pcDNA3.1/G_F-LacZ, which expresses both genes as a fusion.

LacZ was apparent after transfection studies (figure 3), attesting that the G gene, which was upstream of the LacZ, was indeed expressed but it could not be detected.

Antibody Responses

At the end of the first experiment, antibody to SVCV-G protein and b-galactosidase was detected in



Εικόνα 4. Ανοσοποιητική απάντηση του μυός, μετά από ενδομυϊκή έγχυση του pcDNA3.1/G_r-LacZ, ήταν ειδική στον ιό, όπως επιβεβαιώθηκε με IPMA.

Figure 4. The mouse antibody response following the intramuscular administration of the pcDNA3.1/G_r-LacZ was virus specific as it was confirmed by IPMA.

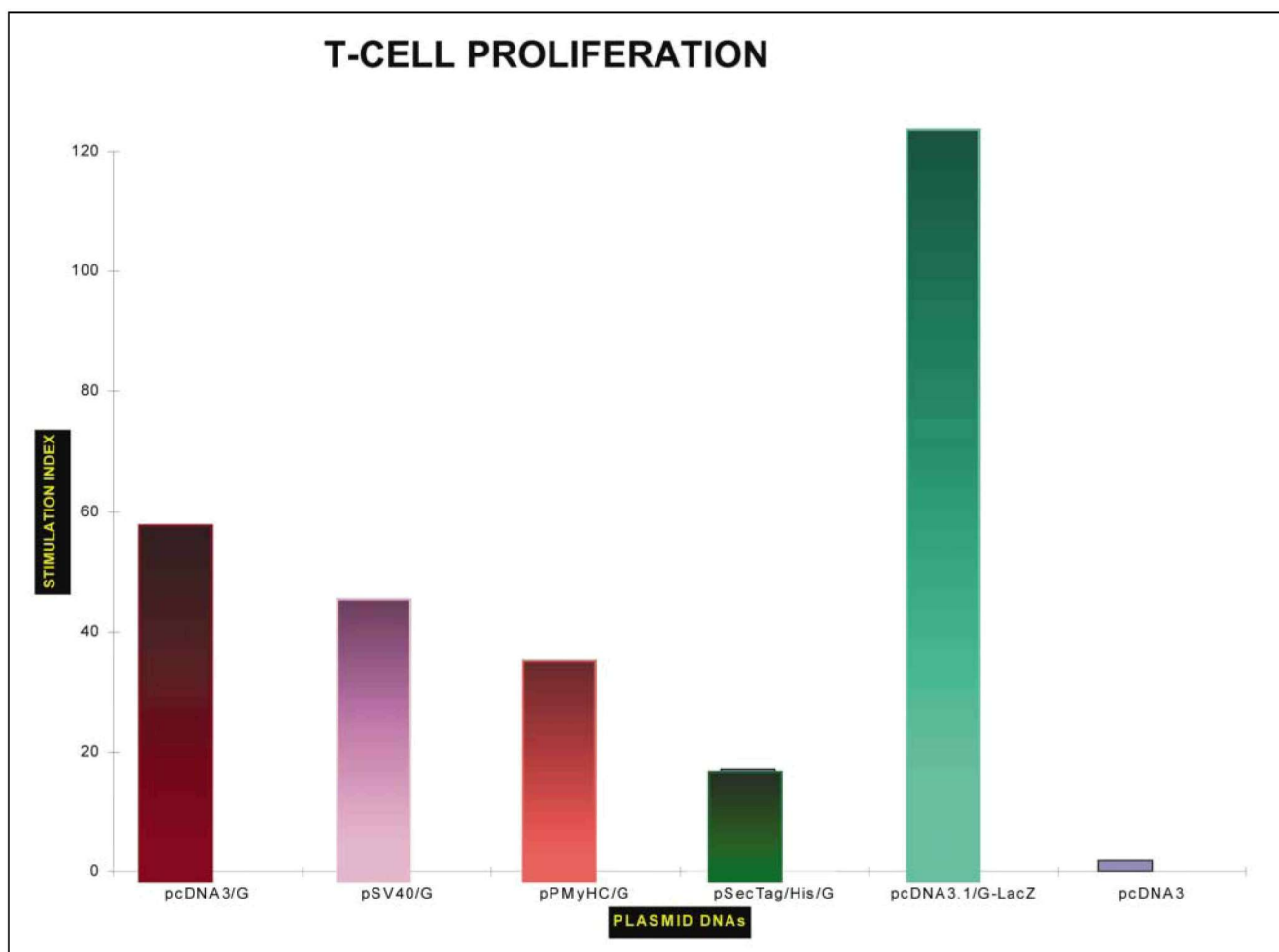
only one out of six mice. The mouse that responded positively was after the last bleeding, from the group which had been immunized with the plasmid pcDNA3.1/G-LacZ. The antibody response was specific to the SVCV at a serum dilution of 1/50 and to the β-galactosidase at a dilution of 1/200 as determined by ELISA.

In the second experiment, where mice co-injected with a mixture of the most prominent plasmid (pcDNA3.1/G-LacZ) and a plasmid encoding the murine cytokine GMCSF, four out of five mice responded positively to the SVCV with a constant serum dilution of 1/25, and to the b-gal in serum

dilutions of between 1/25 - 1/50 with a mean of 1/30. Obviously the co-administration of the mGMCSF ameliorated the antibody response to both proteins after a single injection, although titers were lower than that observed from the only responded mouse in the first experiment.

Proliferative T cell Responses

Surprisingly normal and immune mouse splenocytes proliferated *in vitro* after re-stimulation with supernatant from SVCV infected cells. The overall stimulation indices were higher after injection with plasmid DNAs expressing the SVCV-G protein,



Εικόνα 5. Ανοσοποιητική απάντηση των λεμφοκυττάρων της σπλήνας του μυός που ενοφθαλμίστηκε με pcDNA3/G, pSV40/G, pPMycHC/G, PSecTag/His/G, pcDNA3.1/G-LacZ και pcDNA3 (αρνητικός μάρτυρας). Τα αποτελέσματα εκφράζονται ως ο γεωμετρικός μέσος όρος των καλλιιεργειών εις τριπλούν.

Figure 5. Proliferative responses of splenocytes from mice vaccinated with pcDNA3/G, pSV40/G, pPMycHC/G, PSecTag/His/G, pcDNA3.1/G-LacZ and pcDNA3 as negative control. Results are expressed as the geometric mean of triplicate cultures.

suggesting the *in vivo* functionality of these constructs. The background observed from the negative control mice was attributed to the presence of mitogenic substances in the supernatant (eg. viral RNA) of the infected cells (figure 5). The cpm values of the untreated cultures were between 160 and 635, while the same values from the mice injected with the negative control plasmid were between 3.316 and 9.310. The cpm figure from the mice immunized with constructs expressing the SVCV-G protein was between 6.985 and 27.530. Concerning the efficacy of the plasmids with the different promoters at inducing proliferative splenocytes, the CMV was better than the other two, although the differences were insignificant. The plasmid pSecTagB/G_p, which secreted the G-protein, produced the weakest response, as was the case with the glycoprotein of the rabies virus where the secreted form

of the protein conferred less protection in mice than the anchored form (Xiang et al., 1995). By far the highest T-cell response was achieved by the plasmid expressing the fusion G_r-LacZ gene and it was double.

In the second experiment, mice splenocytes proliferated again in the presence of the SVCV or after cultivation with the recombinant b-galactosidase. The stimulation indices for the SVCV were lower in relation to results from the first experiment and this was probably due to differences in the immunization regime. The background in that instant was not as high as in the first experiment; mainly due to the repeated freeze-thawed of the SVCV supernatant, which should had resulted in the degradation of the viral RNA. The cpm numbers from the unstimulated cultures was between 456 and 687. The cpm numbers from the negative control wells were between 1.070 and 1.210 when they

stimulated with SVCV and between 450 to 610 when they stimulated with recombinant b-gal protein. The corresponding values from the splenocytes of mice injected with the tested plasmids, were between 4.676 and 6.139 when restimulated with SVCV and 14.213-17.149 when they cultured with β -gal protein .

DISCUSSION

The present study demonstrates for the first time immune response to the SVCV following DNA immunization of mice. The presence of proliferative responses, to the whole virus, in the absence of any consistent or low antibody, is not uncommon for DNA vaccination, since many workers have shown T-cell immunity following by protection in several animal models, although these animals had no or very low antibody titers (Fynan et al., 1993, Martins et al., 1995, Oliveira et al., 1997). More importantly, studies with experimentally inactivated SVCV vaccines have shown that the development of virus neutralizing antibody in fish was not accompanied by protective immunity and therefore T-cell immunity should be critical for the protection of fish from this disease (Fijan, 1988). However, the co-administration of the mGMCSF, as it has been reported from other studies (Gerloni et al., 1998), increased the antibody response to the SVCV-G protein. We have now used mixtures of the above constructs to induce T cell proliferation in fish, and more importantly we have shown that the mCMCF is also active in fish (unpublished observations). In recent studies, similar DNA vaccines were also tested in the target animals with very encouraging results (Kanellos et al., 2006).

SVCV appears mitogenic to normal mouse splenocytes. The mitogenicity of negative stranded RNA viruses to lymphocytes has been reported previously (Russel, 1988) and may be attributable to the virus cross-linking cells or its stable double stranded replicative form inducing cytokines.

The low levels of antibody to the SVCV G-protein are assumed to be due to the poor expression of the G-gene. The low levels of the SVCV-G expression may relate to the toxicity of the gene or to the down-regulation of the host cell function by the protein (Barry and Johnston, 1997). More research will be needed either on the development of a minigene expressing B-

cell neutralizing epitopes or to the sequence optimization of the gene for mammalian expression, as in the case of HIV (Andre et al., 1998). Nevertheless, a challenge experiment in a licensed establishment (since the disease is notifiable for the UK) will clarify the potential of this prototype vaccine. However, when the virus from the supernatant of infected cells was repeatedly freeze-thawed, its mitogenicity diminished, probably because of the RNA degradation.

Davis and co workers had shown that the co-administration of two plasmids, one expressing a low-immunogenic protein, such as the luciferase, and a second one encoding a strong immunogen, like the β -galactosidase, resulted in the early clearance of the myocytes expressing the luciferase gene (Davis et al., 1997). This, they postulated, was due to the local accumulation of cytotoxic T cells against myocytes expressing β -galactosidase. We hypothesized that the direct fusion of problematic genes, such as the SVCV-G gene, with the immunogenic LacZ gene will ensure the co-expression of both genes in the same cell and therefore will increase the immunogenicity of the problematic protein by attracting antigen presenting cells and T cells to the transfected myocytes. Results presented herein demonstrated that the direct fusion of the G-gene with the LacZ gene increased the T-cell response to the SVCV G-protein and it was present for both proteins as clarified from the second experiment. The higher stimulation indices that observed for the β -galactosidase in relation to the SVCV should represent the differences in the purity of the material used for proliferation. This approach therefore can be used as an alternative way to increase the immunogenicity of other genes, where the expression or the antigenicity of expressed proteins is low. Furthermore, the theoretical problem of competition between genes was not detected (unpublished observations) and this has been attested by other researchers (Grifantini et al., 1998).

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