Antibodies induced in mice by a DNA-construct coding for the elastase of *Schistosoma mansoni* recognize the enzyme in secretions and preacetabular glands of cercariae

M. BAHGAT[1]†‡, K. CHLICHLIA[1,2]‡, V. SCHIRRMACHER[2] *and* A. RUPPEL[1]*

[1] *Department of Tropical Hygiene, University of Heidelberg, Im Neuenheimer Feld 324, 69120 Heidelberg, Germany*
[2] *Division of Cellular Immunology, German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany*

(*Received 30 July 2001; revised 5 October 2001; accepted 12 October 2001*)

SUMMARY

A DNA-construct coding for the elastase of the parasite *Schistosoma mansoni* was prepared from adult *S. mansoni* worm RNA which was reverse transcribed into cDNA. The gene coding for the elastase was amplified using primers specific for the sequence of cercarial elastase and was cloned into a mammalian expression vector. Expression of the elastase gene at the transcriptional level was achieved for the first time in transfected mammalian cells (COS-7) and was also successful in muscle tissue of mice injected with the DNA-construct. These mice developed antibodies recognizing in Western blots the elastase from cercarial secretions. Also, these antibodies reacted in immunofluorescence tests with the preacetabular glands of cercariae, i.e. the site of origin for elastase. Thus, the DNA-construct induced the expression of elastase in mice and formation of antibodies that recognized the native antigen.

Key words: *Schistosoma mansoni*, elastase, transfection, DNA vaccination, immune response.

INTRODUCTION

For successful invasion, cercariae release secretions rich in proteolytic enzymes which can cleave proteins of connective tissue such as collagen, gelatin, keratin and extracellular matrix (Lewert & Lee, 1956; Dresden & Asch, 1972; Campbell, Frappaolo & Stirewalt, 1976). Of these proteinases, the predominant activity is a 28–30 kDa serine protease, the cercarial elastase (CE), which was first purified from cercarial secretions (CSs) by McKerrow *et al*. (1985). The release of this enzyme from cercariae is stimulated by fatty acids (Shiff *et al*. 1972) and lipids (Stirewalt, 1978) on host's skin, and in particular by linoleic acid (McKerrow *et al*. 1983). CE degrades connective tissue and, thereby, facilitates cercarial penetration (McKerrow *et al*. 1983). The major role of the CE is underlined by the ability of various serine protease inhibitors to block cercarial penetration by 80–100%, with a corresponding reduction in worm burden and liver egg counts (Cohn *et al*. 1991; Lim *et al*. 1999).

The cDNA coding for CE of *S. mansoni* was sequenced and includes a 5′-untranslated region of 254 base pairs (bp), an open reading frame of 810 bp and a short 3′-untranslated region (Newport *et al*. 1988). The deduced amino acid sequence indicates that the CE is synthesized as a preproenzyme of 267 amino acids including a signal peptide of 27 amino acids. The enzyme has a catalytic triad of amino acids characteristic for serine proteases, i.e. histidine (68), aspartic acid (126) and serine (218) (Newport *et al*. 1988). The gene of CE includes 3 exons and 2 introns (Price, Doenhoff & Sayers, 1997) and is transcribed in several stages of the life-cycle including cercariae, schistosomula and adult worms, although the protein was not detectable in adult worms (Pierrot *et al*. 1996).

A monoclonal antibody directed against the CE was cytotoxic for *S. mansoni* cercariae *in vitro* (Pino-Heiss *et al*. 1986) and antibodies against CE raised in rats mediated *in vitro* macrophage cytotoxicity against *S. mansoni* schistosomula (Pierrot *et al*. 1996). CE is not immunogenic in mice, rabbits or humans after natural infection (Darani *et al*. 1997; Bahgat *et al*. 2001). However, immunization with recombinant fragments of the CE elicited a strong antibody response in rabbits (Price *et al*. 1997). Immunization with gel-purified CE induced antibody formation in mice, although not in all animals, and the responder mice were partially protected against a challenge infection with *S. mansoni* (Darani *et al*. 1997). Thus, the CE is considered as a candidate molecule to develop an anti-schistosome vaccine (Doenhoff, 1998). The aim of the present study was to evaluate the potential of a DNA-construct coding for the elastase as a new antigen

* Corresponding author: Tropenhygiene, Im Neuenheimer Feld 324, D-69120 Heidelberg, Germany. Tel: +49 6221 56 5044. Fax: +49 6221 56 5948. E-mail: Andreas.Ruppel@urz.uni-heidelberg.de
† Present address: Department of Medicinal Chemistry, National Research Center, Dokki, Cairo, Egypt.
‡M. B. and K. C. contributed equally to this work.

DOI: 10.1017/S0031182001001226

delivery system to induce an antibody response in mice.

MATERIALS AND METHODS

Construction of DNA vectors expressing elastase

The full-length cDNA coding for elastase was obtained from *S. mansoni* adult worm RNA by RT-PCR as previously described (Chlichlia *et al*. 2001). The primers were designed according to the published nucleotide sequence of the *S. mansoni* cercarial elastase (Genbank accession number: J03946; Newport *et al*. 1988). The nucleotide sequences were for the sense primer: 5′-ACC ATG GAG TCG AAC CGA TGG AGG-3′ and the antisense primer: 5′-GTT CAA ATA TTG GAG CGT ACA AAA-3′. For optimal full-length expression of the DNA sequences, a Kozak consensus sequence (A/GnnATGG) was inserted in the sense primer flanking the start codon (Kozak, 1986). The amplified RT-PCR products were purified using the PCR purification kit (Qiagen, Hilden, Germany) and then cloned into the pcDNA3.1/V5/His-TOPO vector using the TOPO TA cloning kit (Invitrogen). Following restriction endonuclease treatment with *Hin*dIII/*Xho*I the positive clones containing the *S. mansoni* worm elastase (SmWE) cDNA inserts were sequenced (MWG Biotech, Ebersberg, Germany). Sequence alignments were performed using HUSAR software. Subsequently, the SmWE-cDNA was subcloned into the mammalian pRK7 expression vector using *Bam*HI and *Xba*I restriction sites for direct cloning. The DNA construct (pRK7-SmWE) was then used for transformation of DH5α *E. coli* grown in LB broth containing 100 μg/ml ampicillin. Large-scale purification of pRK7-SmWE was performed using the Qiagen Endofree Plasmid Maxi Kit (Qiagen) according to the manufacturer's protocol. Purified plasmid DNA was dissolved in PBS (pH 7·2) at a concentration of 1 mg/ml and stored at −20 °C.

Transfections and slot-blots

COS-7 cells were cultured and transfected with 50 μg of pRK7-SmWE using the calcium phosphate co-precipitation method as described (Chlichlia *et al*. 2001). Forty-eight h later, the cells were harvested and total RNA prepared for slot-blot hybridization. Total RNA from transfected cells was prepared using the RNA clean kit (Hybaid, Heidelberg, Germany) according to the manufacturer's instructions and applied to slot blots as described (Chlichlia *et al*. 2001).

Injections of mice with DNA constructs

Pathogen-free female NMRI mice were purchased from Charles River (Sulzfeld, Germany) and used at 6–12 weeks of age. pRK7-SmWE was adjusted to a final concentration of 1 mg DNA/ml in PBS and was injected (50 μl/dose) into the ear pinna of anaesthesized animals using 29-gauge needles as described (Chlichlia *et al*. 2001). Blood samples were obtained from the tail veins and sera were prepared and stored at −20 °C until use.

For testing *in vivo* transcription of the elastase, pRK7-SmWE (1 mg DNA/ml in PBS) was injected (100 μl/dose) into the tibialis anterior muscle of anaesthesized mice using 27-gauge needles. Twelve days after a single injection, muscle tissue was excised from the sacrificed mice, frozen in liquid nitrogen and stored at −80 °C. cDNA synthesis from the frozen muscle was performed by RT-PCR as described (Chlichlia *et al*. 2001) and using the primers mentioned above.

Antibody detection in sera of DNA-injected mice

Cercariae of *S. mansoni* were collected from infected *Biomphalaria glabrata* by exposing the snails to light for 1 h. Cercarial secretions (CSs) were prepared, separated by SDS–PAGE and tested with mouse sera by Western blotting as described (Bahgat *et al*. 2001). Infected *B. glabrata* were embedded in paraffin, cut with a microtome (6 μm; cryostat CM 3050; Leica, Solms, Germany) and used for immunofluorescence test (IFT; Bahgat *et al*. 2001) with mouse sera. The immune reactions were observed by a fluorescent microscope (Axioskope; Zeiss, Jena Germany). A green-yellow fluorescence was considered as positive reaction against the red background.

RESULTS

Construction of a DNA vector expressing SmWE

cDNAs coding for the *S. mansoni* elastase were obtained from adult worm RNA by RT-PCR using specific primers according to the published nucleotide sequence of the CE. An 810 bp cDNA was obtained representing the full-length transcript of the adult worm elastase (SmWE) (not illustrated). This cDNA was cloned into a pcDNA3.1-based vector and then subcloned into the mammalian pRK7 expression vector, generating the DNA construct pRK7-SmWE. The cloned cDNA was verified by sequencing and showed 99·4 % identity at the nucleotide level to the sequence published for the CE (Newport *et al*. 1988). Three changes were found, replacing amino acids Lys 100, Ala 133 and Leu 226 with Glu, Thr and Pro, respectively, but the sequence contained the catalytic triad His 68, Asp 126 and Ser 218, which is characteristic of serine proteinases.

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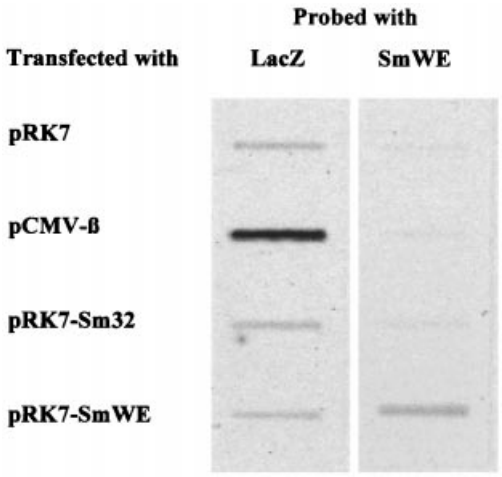

Fig. 1. Slot-blot showing *in vitro* expression in mammalian cells of the elastase from *Schistosoma mansoni* worms (SmWE). COS-7 cells were transfected with either the empty pRK7 vector or with the DNA constructs coding for *lac*Z (pCMV-β), or for *S. mansoni* asparaginyl endopeptidase (Sm32) or for SmWE. Two days after transfection total RNA was prepared, 5 μg were transferred into slot-blots, and the filters hybridized with digoxigenin-labelled DNA probes specific for *lac*Z or SmWE. Visualization of bands was performed with alkaline phosphatase-conjugated anti-digoxigenin and the colorigenic substrates nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate.

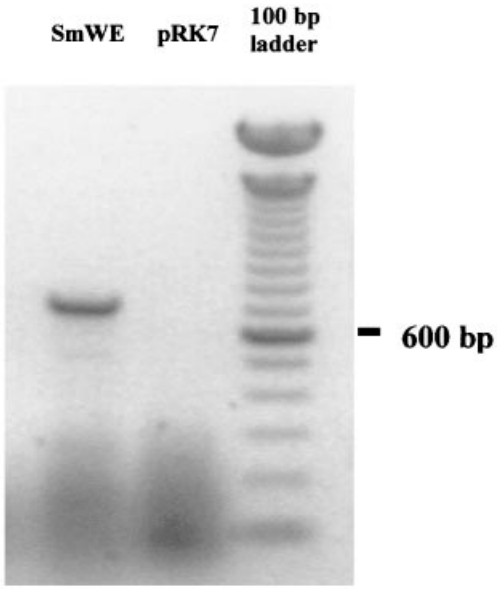

Fig. 2. *In vivo* expression of elastase of *Schistosoma mansoni* worms (SmWE) in muscles of mice after injection with pRK7-SmWE. Total RNA was extracted from the muscles of 12 days following intramuscular injection of 100 μg of either pRK7-SmWE or empty pRK7. RT-PCR was performed with primers specific for the elastase gene. PCR products were separated on an agarose gel. The lanes show a 100 bp DNA ladder (right), control RNA from mice injected with empty pRK7 (centre) and amplification products of RNA from mice injected with pRK7-SmWE (left).

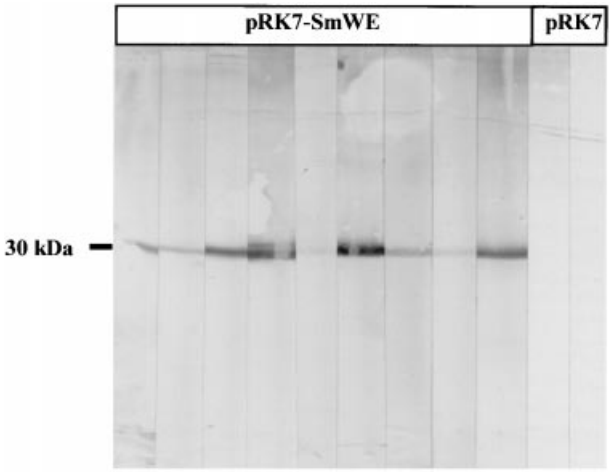

Fig. 3. Western blot showing the antibody response of SmWE-DNA-vaccinated mice against the 30 kDa elastase in *Schistosoma mansoni* cercarial secretions. Mice were vaccinated twice with 50 μg of either the construct coding for elastase (pRK7-SmWE) or the empty vector (pRK7). Sera were collected 3–4 weeks after the last injection and applied at a dilution of 1 : 500. Immunodetection was performed with a peroxidase conjugate against mouse IgG. The immune reaction was visualized with 3,3′ diaminobenzidene.

Transcription of SmWE in transfected mammalian cells and DNA-injected mice

In order to test whether pRK7-SmWE is able to express the elastase *in vitro* in mammalian cells, COS-7 cells were transfected with this construct. COS-7 cells were also transfected with a construct coding for the *S. mansoni* asparaginyl endopeptidase (Sm32) as a control antigen. The empty pRK7 vector and the *lac*Z-expressing pCMVβ vector were further included in this experiment as negative and positive controls, respectively. Probes specific for *lac*Z and SmWE were allowed to hybridize to RNA extracted from the transfected cells. Figure 1 shows that RNA from cells transfected with any given construct was only recognized by the respective probe. The SmWE-specific probe detected only RNA from SmWE-transfected cells, thus demonstrating that SmWE was successfully transcribed in mammalian cells.

Transcription of SmWE *in vivo* was studied in mice injected intramuscularly with pRK7-SmWE. Twelve days later, RT-PCR was performed with RNA extracted from the injected muscle. A specific amplification product of the expected size (0·9 kb) was obtained, whereas no band was detected with muscle tissue from mice injected with the empty pRK7 vector (Fig. 2). Thus, SmWE was transcribed *in vivo* in mouse muscle.

Antibody response against SmWE in DNA-immunized mice

To investigate the potential of pRK7-SmWE to

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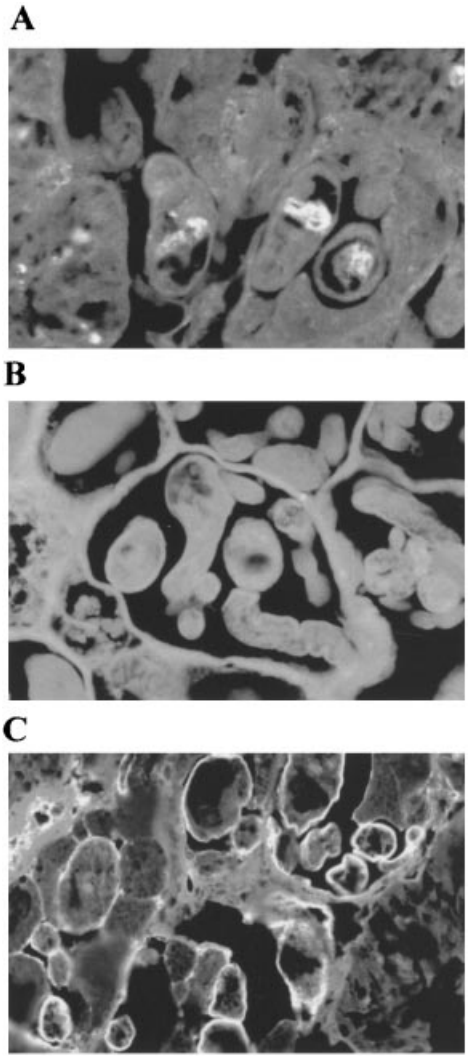

Fig. 4. Localization of the cercarial elastase in the preacetabular glands of cercariae by immunofluorescence. Paraffin sections of *Schistosoma mansoni*-infected *Biomphalaria glabrata* snails were tested with mouse sera (dilution of 1:10). Sera were from (A) a mouse injected with the SmWE construct and which showed reactivity with the cercarial elastase in Western blots, (B) a naive mouse and (C) from a mouse 6 weeks after injection with *S. mansoni*. The visualization was performed using a FITC-conjugate against mouse IgG.

induce a specific antibody response *in vivo*, 9 mice received 2 injections a week apart into the ear pinna, each containing 50 μg of pRK7-SmWE. At 3–4 weeks after the last immunization sera were collected and tested in Western blots using *S. mansoni* cercarial secretions (CSs) as antigen (Fig. 3). All sera of these immunized mice reacted with a 30 kDa protein corresponding to the CE, but sera from mice immunized only with the empty pRK7 vector did not show any reactivity to the CSs. Thus, production of antibodies against SmWE was induced by the DNA injection.

Localization of the elastase in the preacetabular glands of cercariae

Immunofluorescence tests were carried out with sections of infected *B. glabrata* and sera from pRK7-SmWE-immunized mice. An intense reaction was obtained inside the heads of the developing cercariae. Fluorescence was limited to 1 or 2 compartments and these corresponded to the preacetabular gland region (Fig. 4A). Neither postacetabular glands nor the tegument of the cercariae showed any fluorescence with sera from SmWE-DNA-immunized mice (Fig. 4A). In contrast, sera from naive mice showed no reaction (Fig. 4B) and sera from infected mice gave fluorescence only with the tegument of the developing cercariae, but not with any of the cercarial glands (Fig. 4C).

DISCUSSION

The elastase of *S. mansoni* had previously been described from cercariae. Here, the gene coding for this enzyme was amplified by RT-PCR using RNA isolated from adult worms. The DNA sequences for the cercarial enzyme (Newport *et al.* 1988) and for the elastase gene from adult worms (this report) showed 99·4% identity including the catalytic triad, His 68, Asp 126 and Ser 218, characteristic for serine proteinases (Newport *et al.* 1988). Our results agree with those of Pierrot *et al.* (1996) who demonstrated that the sequence of the elastase from adult *S. mansoni* worm RNA was identical to that of the cercarial enzyme.

Whereas cercarial elastase (CE) was already successfully expressed in *E. coli* as fusion protein (Pierrot *et al.* 1996; Price *et al.* 1997), this is the first report on expression of elastase in mammalian cells both *in vitro* and *in vivo*. The expression of SmWE in the muscles of mice after injection of pRK7-SmWE is consistent with our previous reports on expression of *lac*Z (Förg *et al.* 1998; Schirrmacher *et al.* 2000) and *S. mansoni* asparaginyl endopeptidase (Sm32) (Chlichlia *et al.* 2001) in muscles of mice immunized with DNA constructs encoding *lac*Z or Sm32, respectively.

Although CE is not immunogenic in mice, rabbits and humans after natural infection (Darani *et al.* 1997; Bahgat *et al.* 2001), our results show that immunization with a DNA construct coding for elastase represents a successful antigen delivery system which resulted in generation of a specific antibody response against elastase. This immune response was induced by injecting the DNA-construct into the ear pinna of mice. This injection site was shown to be the most effective to generate humoral immune responses by DNA vaccination with *lac*Z- (Förg *et al.* 1998) or Sm32- (Chlichlia *et al.* 2001) encoding constructs.

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In immunofluorescence studies, the sera of mice injected with pRK7-SmWE reacted exclusively with the preacetabular glands of the developing cercariae, but neither with the postacetabular glands nor the tegument. These results agree with our previous localization of the CE in the preacetabular glands using antibodies against purified native CE (Bahgat *et al*. 2001). However, Marikovsky, Arnon & Fishelson (1990) detected CE not only in the preacetabular but also in the postacetabular glands and on the surface of cercarial sections using the immunogold technique and monospecific rabbit antibodies against CE. Although the immunogold technique may be more sensitive, it appears from our results that the enzyme is, at least predominantly, associated with the preacetabular glands. By implication, the contents of the preacetabular glands were part of the secretions released by exposure to linoleic acid and containing the CE evidenced in our Western blots. In contrast to the immunized mice, sera from infected animals did not contain antibodies detectably reacting with the acetabular glands, but rather recognizing the cercarial tegument. This is consistent with earlier observations by Darani *et al*. (1997) and by ourselves (Bahgat *et al*. 2001).

In conclusion, immunization of mice with a plasmid coding for elastase induced the production of antibodies which could recognize the native enzyme in both cercarial secretions and preacetabular glands.

This work was supported by the Deutsche Forschungsgemeinschaft, SFB 544 'Control of Tropical Infectious Diseases'. M.B. was recipient of a scholarship from the German Academic Exchange Service (DAAD). We thank Mrs Christa Kirsten, Mrs Judith Kammer and Mrs Mariana Bucur for their excellent technical assistance in immunofluorescence tests, the schistosome life-cycle, and molecular techniques, respectively.

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