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A Mutant Streptokinase Lacking the C-Terminal 42 Amino Acids Is Less Reactive with Preexisting Antibodies in Patient Sera

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Streptokinase (SK) is an efficacious thrombolytic drug for the treatment of myocardial infarction. Because of its immunogenicity, patients receiving SK therapy develop high anti-SK antibody (Ab) titers, which might provoke severe allergic reactions and neutralize SK activity. In this report we studied the reactivity of a synthetic 42-residue peptide resembling SKC-2 C-terminus with patient sera. SKC-2(373–414) peptide was recognized by 39 and 64% of patients, before and after SKC-2 therapy, respectively. An SKC-2 deletion mutant (mut-C42), lacking the same 42 C-terminal residues, was constructed and expressed in *Escherichia coli.* Recognition of mut-C42 by preexisting Abs from patient sera was 51 and 68% of reactivity to SKC-2, as assessed by direct binding and competition assays, respectively. For most of the patients, mut-C42-neutralizing activity titer (NAT) significantly decreased with respect to SKC-2-NAT. This study opens the possibility of producing a less immunogenic variant of SK, which could constitute a preferred alternative for thrombolytic therapy.

Streptokinase (SK) is a 47-kDa protein produced by various strains of haemolytic streptococci and is a potent activator of the fibrinolytic enzyme system in humans (1, 2). SK binds to both circulating and thrombus-bound plasminogen (Plg) to form the SK-Plg activator complex. This complex activates other Plg molecules by cleaving a peptide bound in Plg, to form plasmin (Pl), an active enzyme that degrades the fibrin component of thrombi (3). SK is in widespread clinical use to treat acute infarction because of its function as an activator of vascular fibrinolysis, wherein it has been demonstrated to be virtually as efficacious as its more expensive alternatives, namely urokinase and tissue-type Plg activator (4).

SK is a bacterial protein and therefore, antigenic in humans. Since streptococcal infections are common, normal individuals are immunized with SK and antibodies (Abs) to SK can be detected in most of them (2). Patients receiving SK as thrombolytic therapy are also immunized with SK, and anti-SK Abs begin to rise approximately five days after administration, peaking in several weeks and declining by six months (5). This therapy generates significant T-cell responses to SK (6–9) and the neutralizing capacity of the Abs rise significantly (10–15). Neutralizing Abs reduce the efficiency of thrombolytic therapy (16) and may cause allergic reactions including fever, rash, nausea, immune complex disease and anaphylactic shock in up to 20% of the patients (17–20).

The widespread use of SK in humans makes its antigenicity an important clinical problem. The recent publication of X-ray crystallographic data of SK-Pl catalytic complex (21) permits the analysis of SK antigenic regions elucidated by complementary approaches. Previous reports have shown five antigenic regions in SK, mapped with soluble recombinant SK fragments and anti-SK Abs from human sera from patients treated with SK (15). They comprise amino acids 1–13, 1–253, 120–352 (containing two distinct, non-overlapping epitopes) and 353–414.

The skc-2 gene was cloned from *Streptococcus equisimilis* group C ATCC 9542 (22). The nucleotide sequence differs from previously reported genes, and contains five differences at the amino acid level with respect to the reported SKC protein (23): residues Ser-71, Asn-210, Arg-244, Arg-253 and Asp-303, in SKC

Abbreviations used: SK, streptokinase; Plg, plasminogen; Pl, plasmin; Ab, antibody; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PCR, polymerase chain reaction; IDA–Sepharose, imidoacetic acid–Sepharose; ED50, effective dose 50%; NAT, neutralizing activity titer; Sak, staphylokinase.

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are changed to Pro, Thr, His, Glu and Asn respectively in SKC-2. Heberkinase (Heber Biotec SA, Havana, Cuba) contains a recombinant SKC-2 obtained after the expression of the skc-2 gene in *E. coli* (22). A study has been recently reported comparing Heberkinase and natural SK (Streptase, Hoechts, Germany) with respect to their immunogenicity in a randomized trial including acute myocardial infarction patients (24). Both, recombinant and natural SKs, induced similar Ab levels, as measured by enzyme-linked immunoassay and neutralizing activity assay. These results suggest that the difference in five amino acids between both molecules does not result in changes in immunogenicity.

In a previous study, we mapped the antigenic regions (linear epitopes) located on SKC-2, using cellulose-bound peptide scans and human total sera from patients treated with Heberkinase. As a result we obtained that C-terminal region 380–409, among other fragments, is notably immunodominant (25). We have also reported that around 30% from 1008 normal blood donors recognized a synthetic peptide resembling amino acids 373–414 from SKC-2 C-terminal region (26).

Previous results suggest that the C-terminal region of SK might play an important role in Plg activation. C-terminal deletions of 40 (27) and 41 residues (28) have no effect on Plg activation activity. However, SK truncated with 46 amino acids has about one-forth of the specific activity (28).

Results on the immunodominance of SK C-terminus (15, 25, 26) and evidence on the role of this region in Plg activation (27, 28) led us to: (a) the study of the reactivity of SKC-2(373–414) peptide with sera from acute myocardial infarction patients before and after Heberkinase therapy; and (b) the genetic construction and immunological characterization of an SKC-2 mutant protein (mut-C42), lacking the same 42 C-terminal residues.

MATERIALS AND METHODS

Titration of human sera by anti-SKC-2 ELISA. Human total serum from 64 Cuban patients with myocardial infarction was collected before and ten days after Heberkinase (Heber Biotec S.A., Havana, Cuba) therapy. All patients received intravenous SKC-2 (1.5 million units over 1 h) to treat the acute myocardial infarction. No patient had previously been treated with SK. Polyvinyl plates (Costar, Cambridge, Massachusetts) were coated with 10μg/ml SKC-2 in coating buffer (0.1 M Na_2CO_3, 0.1 M $NaHCO_3$, pH 9.6), and incubated overnight at 4°C. Then, plates were washed three times with 0.05% Tween 20 in phosphate buffered saline (PBS-Tween). One hundred μl of serial dilutions of each human serum (1:5–1:2621440, two-fold dilutions in PBS-Tween containing 3% skim milk) were added. After incubation for 1 h at 37°C, the binding of human anti-SKC-2 Abs was measured using a horseradish peroxidase-conjugated anti-human Ab (Sigma). The reaction was developed using 100 μl per well of 1 mg/ml *o*-phenylenediamine (Sigma), 0.03% H_2O_2 in substrate buffer (0.1 M citric acid, 0.2 M Na_2HPO_4, pH 5.0). After 30 min, the reaction was stopped with 50 μl of 4 M H_2SO_4. Results were measured on a Multiskan system (Titertek, Helsinki, Finland) at 492 nm. The anti-SKC-2 Ab titer was determined as the maximal dilution in which positive signal was obtained. Positive signal was considered when the value was at least two-fold the background.

Anti-SKC-2(373–414) peptide ELISA. A peptide corresponding to the sequence 373–414 of SKC-2 was synthesized. Plates were coated with 1 μg/ml SKC-2(373–414) peptide, and incubated overnight at 4°C. After washing three times with PBS-Tween, plates were blocked using 2% bovine serum albumin (BSA) (Sigma), and 100 μl of 1:50 dilution of each human serum were added. The binding of human Abs to SKC-2(373–414) peptide was measured using a horseradish peroxidase-conjugated anti-human Ab (Sigma). The reaction was developed and stopped as described above. Each sample was tested by duplicated. Positiveness was considered when sample was at least two-fold the background.

SKC-2(373–414) direct binding assay. Experimental conditions were determined by titration of samples against native SKC-2 and SKC-2(373–414) peptide in order to select those dilution conditions (dln.1 for SKC-2(373–414) and dln.2 for SKC-2) in which there is not excess of Ab directed to each molecule.

Plates were divided in two sections and coated with 10 μg/ml SKC-2 and 1 μg/ml SKC-2(373–414) peptide, respectively. After washing three times with PBS-Tween, plates were blocked with 2% BSA. One hundred μl of human sera collected from 21 patients ten days after Heberkinase therapy were added at previously determined optimal dilutions. After incubation for 1 h at 37°C, the binding of human anti-SKC-2 Abs to molecules on solid phase was measured using a horseradish peroxidase-conjugated anti-human Ab (Sigma). The reactions were developed and stopped as described above. Each sample was tested by duplicated. Percent direct binding of human anti-SKC-2 Abs to SKC-2(373–414) peptide was determined from the formula

$$100 \times \frac{(\text{Absorbance binding to SKC-2(373–414)}) \times \text{dln.1}}{(\text{Absorbance binding to SKC-2}) \times \text{dln.2}}.$$

Cloning of SK-truncated fragment. Truncated portion of the skc-2 gene (1245 nucleotides) was generated from the plasmid pEKG-3 (22) using polymerase chain reaction (PCR) employing the upstream specific primer (5′-GGAATTCATGATTGCTGGACCTGAGTGGC-3′) and specific internal primer that hybridize to the nucleotides 1092–1119 (5′-CGATATCTCGCTTGCCCATATAAACGG-3′). The amplified DNA sequence corresponding to truncated skc-2 fragment (nucleotides 1–1119) was digested with *Eco*RI and *Eco*RV and inserted into the expression vector pTrp (29) digested *Eco*RI and *Eco*RV, containing a 6xHis-affinity tag the very strong and regulable tryptophan promoter. The resulting plasmid was designated as pMC8.

Growth conditions and induction of tryptophan promoter. The recombinant protein mut-C42 was studied using 5L fermentors (Marubishi B.E, Japan). Induction of the tryptophan promoter was carried out in *E. coli* K-12 strain W3110 (F^-, mcrA, mcrB in (rrnD-rrnE)) as previously described (29) in a medium M9 containing, 1% triptone, 0.5% yeast extract, 2% glycerol, 100 μg/ml ampicillin, pH 7.0. Bacteria were grown at 37°C and 200 rpm. mut-C42 maximal expression level was obtained after 24 h culture.

Purification of mut-C42. Cells were harvested by centrifugation and disrupted by French Press. The supernatant was two fold diluted in 20 mM Tris-HCl, 500 mM NaCl, 40 mM imidazole, pH 7.0 and then chromatographed at a rate of 2 ml/min through a 10 ml IDA-Sepharose column (Pharmacia, Sweden) charged with Cu^{2+}, equilibrated with the same buffer containing 20 mM Imidazole (30). Elution was done with 20 mM Tris-HCl, 250 mM NaCl, 200 mM imidazole, pH 7.0. This protein eluate was passed at a rate of 4 ml/min over a 130 ml Sephadex G-25 column (Pharmacia, Sweden) previously equilibrated with 20 mM Tris-HCl, pH 8.0. The protein eluate from the filtration column was chromatographed at a rate of 3 ml/min through a 1 ml Resource Q column (Pharmacia, Sweden)

equilibrated with 20 mM Tris-HCl, pH 8.0. After the column was washed with the equilibration buffer, the elution was done by a NaCl gradient from 0 to 0.1 M in 5 ml, then from 0.1 M to 0.27 M in 35 ml, and finally from 0.27 M to 0.1 M in 5 ml. mut-C42 was collected between 0.1–0.15 M NaCl.

SDS-PAGE and Western blot analysis. SDS-PAGE and Western blot analysis were carried out as previously described (31, 32). For Western blot analysis, purified materials of SKC-2 and mut-C42 were tested using a rabbit anti-SK polyclonal antiserum (CIGB, Cuba).

Determination of mut-C42 amidolytic activity. The amidolytic activity of SK was determined by the chromogenic substrate (S-2251) assay (33).

mut-C42 direct binding assay. Plates were divided in two sections and coated with 10 μg/ml of full length SKC-2 and deletion mutant mut-C42, respectively. Then, plates were washed three times with PBS-Tween. One hundred μl of human sera collected from eight patients ten days after Heberkinase therapy were added at a previously determined optimal dilution. Samples were diluted according to the predetermined anti-SKC-2 Ab titers. For sera with 5×10^5, 10^5 and 5×10^4 Ab titers, dilutions were of $1{:}3.2 \times 10^4$, $1{:}1.6 \times 10^4$ and $1{:}2 \times 10^3$, respectively. After incubation for 1 h at 37°C, the binding of human anti-SKC-2 Abs to molecules on solid phase was measured using a horseradish peroxidase-conjugated anti-human Ab (Sigma). The reaction was developed and stopped as described above. Each sample was tested by duplicated. Percent direct binding of human anti-SKC-2 Abs to deletion mutant mut-C42 was determined from the formula

$$100 \times (\text{Absorbance binding to mut-C42})/(\text{Absorbance binding to SKC-2}).$$

Competition assay. Plates were coated with 5 μg/ml of goat anti-human Abs in coating buffer. After washing three times with PBS-Tween, plates were blocked using 2% BSA (Sigma). One hundred μl of human sera collected from eight patients ten days after Heberkinase therapy were added at a previously determined optimal dilution. Samples were diluted according to the predetermined anti-SKC-2 Ab titers. For sera with 5×10^5, 10^5 and 5×10^4 Ab titers, dilutions were of $1{:}10^4$, $1{:}5 \times 10^3$ and $1{:}10^3$, respectively. This way, human anti-SKC-2 Abs were immobilized on the coated plates. After washing, 100 μl of a solution of 1 μg/ml of biotinylated SKC-2 mixed with different concentrations of non-labeled full length SKC-2 or deletion mutant mut-C42 (4–0.25 μg/ml, two-fold dilutions) were added. The binding of biotinylated SKC-2 to human anti-SKC-2 Abs, after competition with non-labeled molecules, was measured using a horseradish peroxidase-conjugated streptavidin. The reaction was developed and stopped as described above. The effective dose 50% (ED50) values for mutant and native proteins were determined from plots of absorbance versus concentration of non-labeled molecules using a Probit transformation.

SKC-2- and mut-C42-neutralizing activity in patient sera. The chromogenic substrate (S-2251) reaction was performed in polyvinyl plates (Costar, Cambridge, Massachusetts, U.S.A.). Serial dilutions of SKC-2 and mut-C42 (128–2 IU, two-fold dilutions in 20 mM Tris-HCl pH 8, 0.5 M NaCl) were prepared in a volume of 25 μl. Curves were mixed with 25 μl of 1:10 dilutions of each patient serum, and a negative control consisting of a human serum having low anti-SKC-2 Ab titer and preabsorbed with SKC-2. Fifty μl of 25 μg/ml human Plg were added and allowed to mix for 10 min at room temperature. The reaction was developed by addition of 50 μl of chromogenic substrate S-2251 (Chromogenix, Antwerp, Belgium). After incubation for 30 min, the reaction was stopped with 25 μl of 20% acetic acid. Results were measured on a Multiskan system (Titertek, Helsinki, Finland) at 405 nm. The activity of SKC-2 required to obtain an absorbance of 0.7 was determined from plots of absorbance versus activity. The neutralizing activity titer (NAT) was determined as the difference between the tested serum and the negative control and was expressed as micrograms of protein neutralized per milliliter of tested serum.

TABLE 1

Percentage Ab Binding of Patient Sera to Peptide SKC-2(373–414)

Patient	% Direct binding
01	2.67
11	0.14
12	2.91
13	1.39
20	0.71
24	0.27
28	2.69
30	1.37
31	0.63
32	0.33
33	9.54
34	2.65
41	0.63
45	10.68
50	0.74
52	2.40
53	0.29
54	10.00
55	3.22
66	2.89
67	5.99
Mean	2.96
St. dev.	3.30

Note. Percentage direct binding to SKC-2(373–414) was deduced from the formula

$$100 \times \frac{(\text{Absorbance binding to SKC-2(373–414)}) \times \text{dln1}}{(\text{Absorbance binding to SKC-2}) \times \text{dln2}}.$$

where dln1 is serum dilution in which there is no excess of Abs against SKC-2(373–414) peptide on solid phase, and dln2 is serum dilution in which there is no excess of Abs against SKC-2 on solid phase.

Statistical analysis. The Student's *t* test for paired values was used to evaluate the statistical significance of differences between SKC-2 and mut-C42 results in direct binding, competition and neutralizing activity assays.

RESULTS

Human total sera collected from 64 patients in different hospitals in Havana, Cuba, before and ten days after Heberkinase therapy were tested in an anti-SKC-2 ELISA. Samples before therapy showed anti-SKC-2 Ab titers between 1:10 and $1{:}10^4$, while after therapy Ab titer range increased to $1{:}10^3$–$1{:}5 \times 10^5$. These samples were assayed in an anti-SKC-2(373–414) peptide ELISA in order to asses the increased recognition rate for SKC-2 C-terminus in patient sera after therapy with respect to samples collected before treatment. Before therapy, 39% of patients recognized SKC-2(373–414) peptide, and the recognition increased to 64% after therapy. This increase was not only due to a larger number of positive samples, but also to higher intensity of these positive signals.

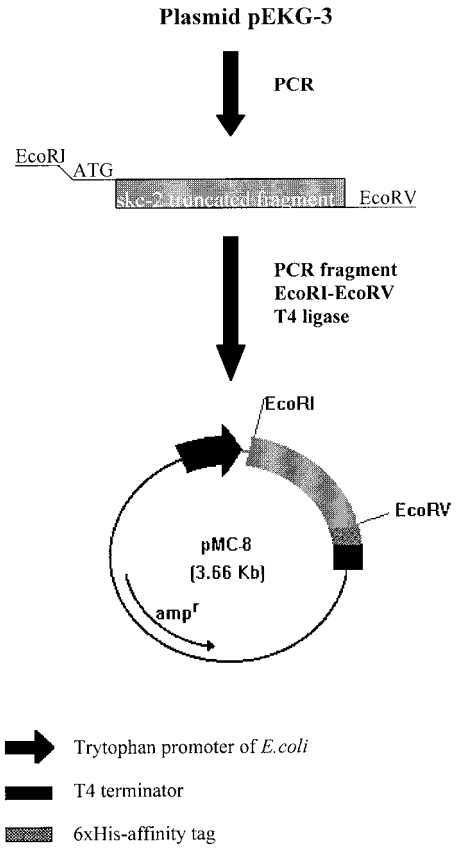

FIG. 1. Construction of expression plasmid with truncated fragment of the skc-2 gene (pMC8). The PCR-amplified fragment was digested with E*coR*I and EcoRV and ligated with the vector pTrp previously digested with *EcoR*I and *EcoR*V.

In order to asses the proportion of this anti-SKC-2(373–414) recognition with respect to the total anti-SKC-2 Ab response, we performed a direct binding assay with patient sera obtained after Heberkinase therapy. Percent Ab binding to SKC-2(373–414) ranged between 0.14 and 10.68% with respect to anti-SKC-2 Ab recognition (Table 1). The mean value from 21 samples was 2.96% (St. dev. = 3.30).

The truncated fragment of the skc-2 gene was generated by PCR from the plasmid pEKG-3 (22). The 5′ amplification primer introduced an ATG codon for translation initiation in *E. coli*. The amplified fragment was inserted into the expression vector pTrp (29) containing a 6xHis-affinity tag at the 3′ end (Fig. 1) that provides a simple method for rapid protein purification (30). Three clones were checked by DNA sequencing to exclude PCR mutations. No differences were found between their nucleotide sequences, which matched that of the skc-2 gene. The pMC8 expression vector, containing the truncated portion of skc-2 with a 6xHis-affinity tag at the C-terminus was introduced into the *E. coli* K-12 strain W3110 for expression. The tryptophan promoter was induced as described under Materials and Methods, and maximal protein expression (mut-C42) was obtained after 24 h. mut-C42 was found in the soluble fraction of the cell cytosol and constituted 10% of total cell protein. mut-C42 was purified by Cu^{2+}-IDA chromatography, gel filtration and ion exchange steps that yielded a pure product (95% as assessed by SDS-PAGE and Western blot) with high recovery (Fig. 2). This material was sterilized by filtration through a 0.22-μm Millipore filter. The specific activity of mut-C42 was determined by the chromogenic substrate assay (33) and was similar to that of the native protein SKC-2 (22).

A direct binding assay was performed in order to compare the new mut-C42 mutant with native SKC-2 protein regarding their capacity for binding human anti-SKC-2 Abs present in sera from patients after SKC-2 therapy. All eight tested sera showed a similar binding pattern (Fig. 3). As it was expected, binding of human anti-SKC-2 Abs to mut-C42 was 50.73% (St. dev. = 3.82) of their binding to native SKC-2 ($P = 1.52 \times 10^{-9}$).

Similar results were obtained from the same eight samples using a competition assay in which both, native and mutant proteins competed with a biotinylated SKC-2 for binding human anti-SKC-2 Abs. Results are shown in Fig. 4. ED50 values for mut-C42 were in all cases higher than those for SKC-2. Mean values for SKC-2 and mut-C42 were 0.26 μg/ml (St. dev. = 0.10) and 0.41 μg/ml (St. dev. = 0.18), respectively ($P = 0.004$). We expressed mut-C42 ED50 values in terms

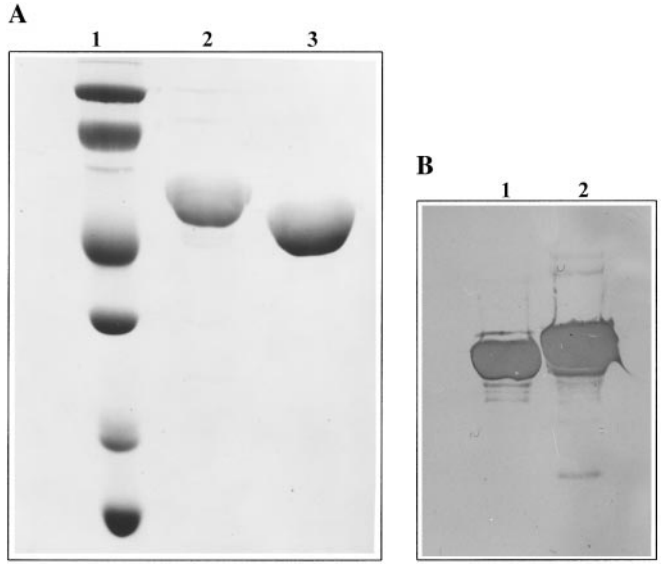

FIG. 2. Analysis of purified SKC-2 and mut-C42 proteins. Commasie blue-stained SDS–15% polyacrylamide gel. Lane 1: Low molecular weight marker (Amerscham Pharmacia Biotech), phosphorylase: 94,000 Da, albumin: 67,000 Da, ovalbumin: 43,000 Da, carbonic anhydrase: 30,000 Da, trypsin inhibitor: 20,100 Da, α-Lactalbumin: 14,400 Da. Lane 2: SKC-2 (3 μg). Lane 3: mut-C42 (3 μg). (B) Western blot analysis of purified material developed with an anti-SK polyclonal antiserum. Lane 1: mut-C42 (2 μg). Lane 2: SKC-2 (2 μg).

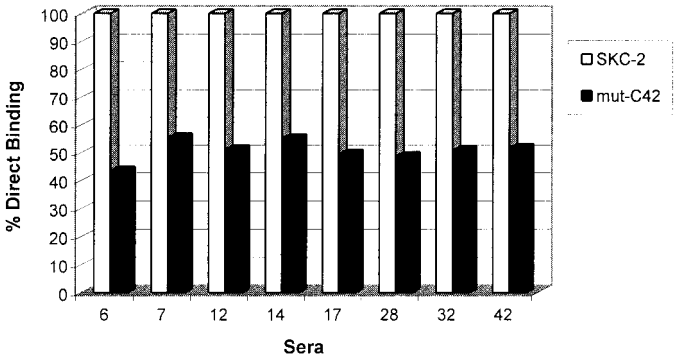

FIG. 3. Percentage Ab binding of patient sera to mut-C42. Anti-SKC-2 Abs from patient sera reacted with SKC-2 and mut-C42 on solid phase. Percentage direct binding of human anti-SKC-2 Abs to mutant protein was determined from the formula

$$100 \times (\text{Absorbance binding to mut-C42}) / (\text{Absorbance binding to SKC-2}).$$

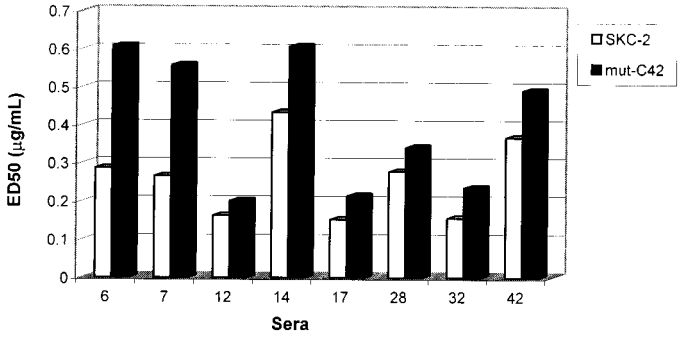

FIG. 4. Competition assay of native SKC-2 and mutant mut-C42 proteins using anti-SKC-2 Abs from patient sera. SKC-2 and mut-C42 competed with a biotinylated SKC-2 for binding human anti-SKC-2 Abs immovilized on coated plates. ED50 values were determined from plots of absorbance versus concentration of nonlabeled molecules, using a Probit transformation.

of percent with respect to SKC-2. Binding of mut-C42 to human anti-SKC-2 Abs was 67.57% (St. dev. = 13.47) of reactivity of native SKC-2 ($P = 0.0001$).

The neutralizing activity titers (NAT) against mut-C42 mutant and native SKC-2 proteins were determined for 15 patients, ten days after Heberkinase therapy. SKC-2-NAT values ranged between 2.59 and 428.27 μg of SKC-2 neutralized per mL of tested serum, while mut-C42-NAT ranged between 0 and 277.24 μg of mut-C42 neutralized per ml of tested serum (Fig. 5). For most of the individuals mut-C42-NAT decreased with respect to SKC-2-NAT, ranging from 30 to 91% of the native protein value ($P = 0.0013$).

DISCUSSION

Although immunogenicity has not constituted an insurmountable obstacle for the use of streptokinase, it clearly hampers its unrestricted use and precludes its repeated administration.

The present study is based on evidences about the immunological significance of the C-terminal region of SK, obtained from previous studies using soluble recombinant SK fragments (15), a spot-synthesized SKC-2 peptide library (25) and a synthetic peptide resembling SKC-2 C-terminal region, used for testing sera from normal blood donors by Ultra-Micro-ELISA (26) as well as previous reports on the SK C-terminus role in Plg activation (27, 28).

We studied the immunoreactivity of a synthetic 42-residue peptideresembling amino acids 373–414 of SKC-2 C-terminus using a panel of sera collected from patients before and ten days after Heberkinase therapy. SKC-2(373–414) peptide was recognized by 39% of patients before therapy. The presence of anti-SK Ab titers in individuals not yet exposed to SK therapy is in agreement with previous reports (2, 26). This response is a consequence of common streptococcal infections occurring among general population. Unless such infections occurred short before SK therapy, anti-SK Ab titers are not very high. Therefore, the recognition of the peptide by 39% of individuals confirm a significant immunodominance for the studied region. As it was expected, recognition increased to 64% after therapy due to the booster effect associated to the administered SKC-2 dose. This is also in agreement with previous studies reporting increased anti-SK Ab levels in patients receiving SK therapy (5, 15). Post-therapy anti SKC-2(373–414) recognition was about 3%, as a mean value, with respect to total anti SKC-2 Ab response. Although individual variations in percent binding to SKC-2(373–414) were observed, there is an evident direction in immune response towards SKC-terminal region. Particular differences may be a consequence of individual variation in immune response to SKC-2. We decided to construct an SKC-2 deletion mutant lacking

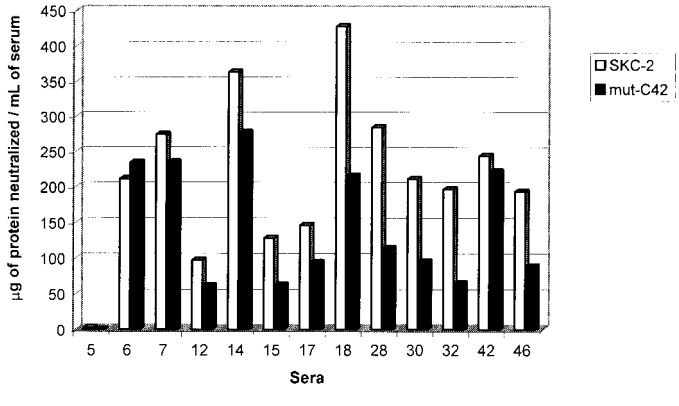

FIG. 5. Neutralizing activity titer (NAT) of patient sera against mut-C42 mutant and native SKC-2 proteins.

the 42 C-terminal residues. This mutant (mut-C42) was subjected to evaluation of its reactivity *in vitro* using human sera collected from patients ten days after SKC-2 therapy. All tested sera showed similar binding patterns in a direct binding assay comparing mut-C42 with native SKC-2. According to this approach, approximately 51% of human anti SKC-2 Abs bound to mut-C42. However, steric hindrance is a potential limitation for this assay, in addition to the fact that proteins fixed on solid phase may improperly fold, hiding immunologically important epitopes. Therefore we complemented these studies by performing a competition experiment in which both, native and mutant proteins reacted with human anti SKC-2 Abs in solution. This assay showed that binding of human anti-SKC-2 Abs to mut-C42 was approximately 68% of reactivity to native SKC-2. These results indicate that mut-C42 is significantly less recognized by pre-existing anti-SKC-2 Abs than the native SKC-2. Results from mut-C42 studies might seem not to be in total agreement with those from SKC-2(373–414) peptide. A possible cause could be that upon immobilization on solid phase, the peptide might adopt altered conformations resulting in loss of recognition by some samples. Besides, all these experiments are partial approaches to a real phenomenon that support the idea of reducing SK immunogenicity by means of genetically engineered variants.

The crystallographic structure of SK complexed with the catalytic domain of human micro-Pl has been recently solved (21). SK appears in the complex as three domains (α, β and γ), each domain being structurally independent and showing a similar folding. Inspection of the structure suggests that the determined antigenic peptides map protein regions with structural propensity for antigenicity. The NH_2-terminal region, the COOH-terminal and the loop region (amino acids 46–70) are disordered, suggesting that they are highly mobile and accessible even after the formation of the complex with micro-Pl. This is consistent with the observed antigenicity of the C-terminal region in SKC-2.

Anti-SK Abs not only mediate the allergic response observed in certain clinical cases (17–20), but also induce neutralization of SK activity (10–15). For most of tested sera, mut-C42-NAT significantly decreased with respect to SKC-2-NAT. The fact that mut-C42 was neutralized by anti-SKC-2 Abs present on patient sera in minor extension than native SKC-2 is encouraging for the achievement of a thrombolytic agent not only having less immunogenicity, but also reducing the possibilities of being neutralized.

In conclusion, present data indicate that the immunodominant C-terminal epitope of native SKC-2 can be eliminated without loss of specific activity. This study thus shows that it is possible to produce an engineered variant of SK lacking this immunodominant cluster while retaining intact specific activity, and being less reactive with preexisting Abs in patient sera. Similar results have been reached with recombinant staphylokinase (Sak), another Plg activator obtained from bacteria. Sak mutagenesis resulted in the variant Sak-START having intact specific activity and less immunogenicity (34). If mut-C42 shows to be less immunogenic in animals, it could constitute a preferred alternative to native SK for thrombolytic therapy in patients with thromboembolic diseases.

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