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Mapping of the Antigenic Regions of Streptokinase in Humans after Streptokinase Therapy

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Streptokinase (SK) is efficaciously used as a thrombolytic drug for the treatment of myocardial infarction. Being a bacterial protein, SK is immunogenic in humans. Therefore, resulting from SK therapy, patients become immunized and anti-SK antibody (Ab) titers rise post-treatment. High Ab titers might provoke severe immune reactions during SK therapy and neutralize SK activity, preventing effective thrombolysis. Spot synthesis combined with peptide library techniques is a useful tool for studying protein-peptide interactions on continuous cellulose membranes. Here, we report on the mapping of antigenic regions of SK using a spot-synthesized peptide library and human total sera from patients receiving SK therapy. All tested samples have high anti-SK Ab titers and most of them show significant SK neutralizing capacity. Individual variations in peptide recognition were detected. However, patients treated with SK tend, in general, to show a common regional binding pattern, including residues 1-20, 130-149, 170-189, and 390-399. This is the first study reporting the probing of a cellulose-bound set of peptides with total human sera.

Streptokinase (SK) is a secretory protein of 414 residues produced by selected species of the genus *Streptococcus* that is used often as a thrombolytic drug for the treatment of several circulatory disorders, including myocardial infarction (1), wherein it has been demonstrated to be virtually as efficacious as its more expensive alternatives, namely urokinase (UK) and tissue-type plasminogen activator (TPA) (1).

Because SK is a bacterial protein, it is immunogenic in humans. Antibodies (Abs) to SK are found in most individuals as a result of recurrent streptococcal infection (2). Patients are also immunized with SK as result of thrombolytic therapy and Ab titers to SK rise post-treatment (3-8). High Ab titers may neutralize SK activity preventing effective thrombolysis. Furthermore, high Ab titers may provoke severe immune reactions during SK therapy. Indeed, allergic reactions are a common side effect, noted in up to 15% of patients (9). In addition to humoral immune responses to SK, strong cellular immune response has also been documented (10-13).

Despite this rich clinical information on the immunogenicity of SK, little is known about the structural basis for its antigenicity. It is known that certain regions of the molecule are more immunogenic than others (12, 14, 15), but nor have there been studies of the molecular mechanisms responsible for antibody-mediated neutralization of SK activity. There are several reports indicating the presence of neutralizing Abs in the sera of patients before and after SK therapy, but there are no studies regarding the antigenic regions to which these neutralizing Abs are directed. This type of studies could reveal structural regions, which are important for SK antigenicity in humans. This information could be used to genetically modify the immunoreactive regions of SK so as to generate a fully functional molecule, which does not produce immune reactions.

The patchy distribution of antigenic epitopes in SK was suggested by Redd et al. (14). For example, anti-SK Abs isolated from blood bank donors by elution from an SK agarose column show considerable variation in reactivity with monoclonal antibody-defined epitopes on recombinant SK fragments (14). Most strikingly, the amino terminal portions (residues 1-13)

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Abbreviations used: SK, streptokinase; UK, urokinase; TPA, tissue-type plasminogen activator, Plg, plasminogen; NAT, neutralizing activity titer; DIC, N,N′-diisopropylcarbodiimide; NMI, N-methylimidazole; DMF, N,N-dimethylformamide; TBS, Tris-buffered saline; BCIP, 5-Bromo 4-chloro 3-Indolyl Phosphate; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide.

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react with Abs in 80% of plasma samples, whereas segment 14-127 is devoid of reaction with all of them (14). The remaining segments (120-352 and 353-414) show moderate reactivity, with about 20-35% of plasma samples containing Abs that bind to these portions (14).

Parhami-Seren et al. (15) reported that certain regions of SK appear to be more antigenic or immunodominant. They have used murine anti-SK monoclonal Abs and recombinant SK-truncated fragments as a molecular probe to analyze human polyclonal Ab responses to SK in normal individuals and in patients before and after SK treatment. The data from this study suggests several conclusions. First, there are significant individual variations in the amount of anti-SK Abs before and after SK therapy. Second, patients who are treated with SK, tend in general, to show the same regional binding pattern after therapy as they did prior to treatment with SK, though the amount of specific anti-SK Abs increases an average of seven-fold. Third, the anti-SK Abs in normal subjects and patients are directed against three major antigenic regions in SK. These three regions comprise epitopes constructed by amino acids 1-253 and 120-352 (containing two distinct, non-overlapping epitopes. Bruserud et al. (12) have also identified a major T-cell epitope in the region of SK constructed by amino acid residues 238-246. Two other epitopes in SK, constructed by amino acids 1-13 and 353-414, were not antigenic in all humans tested by Parhami-Seren et al. (15). Human Abs from most subjects showed minimal or no binding to the region of SK constructed by amino acids 14-127 (15).

The skc-2 gene was cloned from *Streptococcus equisimilis* group C (ATCC 9542) (16). The nucleotide sequence differs from previously reported genes and contains five differences at the amino acid level with respect to the reported SKC protein (17): residues Ser-71, Asn-210, Arg-244, Arg-253 and Asp-303, in SKC are changed to Pro, Thr, His, Glu and Asn respectively in SKC-2 (16).

The recent publication of X-ray crystallographic data of SK-plasmin catalytic complex (18) permits the analysis of SK antigenic regions elucidated by complementary approaches (14,15). Spot synthesis (19) in combination with peptide library techniques serve as useful tools to study protein-peptide interactions on continuous cellulose membranes (20-25). Here we report on the mapping of antigenic regions located on SKC-2 using cellulose-bound peptide scans and human total sera from patients treated with SKC-2. As a result we obtained several regions with different degree of immunological dominance.

Spot synthesized membranes have been probed using exclusively mAbs. Therefore a novel result from this study is the screening of spot synthesized peptide libraries using human total sera from patients treated with SKC-2. This study, together with other complementary experiments, sets the bases for the construction of active mutant SKC-2 proteins with immunogenicity.

MATERIALS AND METHODS

Titration of human sera by anti-SK ELISA. Human total serum from 28 Cuban patients with myocardial infarction was collected ten days after SKC-2 (Heberkinase, Heber Biotec SA, Havana, Cuba) therapy. Heberkinase contains a recombinant SK obtained after the expression of the skc-2 gene in *E. coli* (16). All patients received intravenous Heberkinase (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, U.S.A.) were coated with 10 μg/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 PBS (PBS-Tween). One hundred μl of serial dilutions of each human serum (1:1 000–1:2 048 000, two-fold dilutions in PBS-Tween containing 3% skim milk) were added. After incubation for 1 h at 37°C, the binding of human Abs to SKC-2 was measured using a horseradish peroxidase-conjugated anti-human Ab (Sigma) at 1:10000 dilution. 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.

SKC-2-neutralizing activity in patient sera. The chromogenic substrate (S-2251) reaction was carried out in polyvinyl plates (Costar, Cambridge, Massachusetts, U.S.A.). Serial dilutions of SKC-2 (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. SKC-2 dilutions were mixed with 25 μl of 1:10 dilution of each human 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 plasminogen (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 negative control value and was expressed as micrograms of SKC-2 neutralized per milliliter of tested serum.

Synthesis of overlapping 20-mer peptides corresponding to the amino acid sequence of SKC-2 on cellulose support. To identify the regions of SK involved in Abs-SK binding, the peptide spot synthesis approach as previously described by Frank (19), was used. Briefly, the derivation of five Whatman 540 papers was carried out sterifying the first anchor component, Fmoc-β-Ala-OH, using N,N′-diisopropylcarbodiimide (DIC) and N-methylimidazole (NMI) in dry N,N-dimethylformamide (DMF). The spot array on the membranes was defined anchoring Fmoc-β-Ala-OH on the previously marked positions according to the required number of 20-mer peptides. For the assembly of the overlapping the SKC-2 sequence peptides, the standard Fmoc-/tBu chemistry was used. After the final cycle, all peptides were N-terminally acetylated.

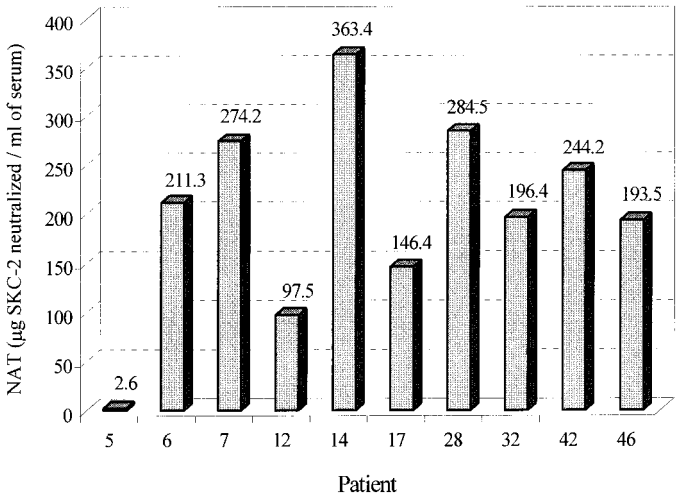

FIG. 1. Schematic representation of the reactivity of patient sera to a spot-synthesized peptide library spanning the whole sequence of SKC-2 protein. N_1: number of patients whose sera react with each individual spot. N_2: number of spots recognized by each patient serum.

Binding of anti-SK Abs to overlapping 20-mer peptides corresponding to the amino acid sequence of SKC-2. Cellulose sheets exhibiting the overlapping 20-mer synthetic peptides corresponding to SKC-2 (amino acids 1-414) (16) were soaked in ethanol to prevent possible hydrophobic interactions between the peptides. Ethanol was exchanged against Tris-buffered saline (TBS) (10 mM Tris, pH 7.6, 150 mM NaCl) by sequential washing, and nonspecific binding was blocked by incubating overnight in 10 ml of T-TBS blocking buffer (0.05% Tween 20 in TBS). The sheets were subsequently incubated for 3 h with serum samples obtained from ten patients 10 days after Heberkinase therapy, diluted in 10 ml of T-TBS blocking buffer. Serum 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 1:1000, 1:500 and 1:300, respectively. Cellulose sheets were washed three times with T-TBS. Then, an alkaline phosphatase-conjugated anti-human Ab (Sigma) was added at 1:2500 dilution in T-TBS blocking buffer for 1h. Sheets were washed three times with T-TBS. Detection of bound anti-SKC-2 Abs was achieved by incubating the sheets with 0.3 mg/ml 5-Bromo 4-chloro 3-Indolyl Phosphate (BCIP) (Sigma), 4.5 mg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma) in substrate buffer (100 mM Tris, pH 8.9, 100 mM NaCl, 2 mM $MgCl_2$). Positive spots develop a violet color. Washing with PBS stopped staining. Cellulose sheets carrying the peptides were finally regenerated for the next test as described (19).

RESULTS

In order to study the antigenic regions of SKC-2, a cellulose-bound set of overlapping 20-mer peptides (10 overlapping amino acids) spanning the primary sequence of SKC-2 was elaborated (Fig. 1). The cellulose sheet was probed with human sera collected from ten patients in different hospitals at Havana, Cuba, 10 days after SK (Heberkinase) therapy. These ten human sera were selected from a group of 28 patients, which were previously tested in an anti-SKC-2 ELISA. The result of titration of the patients' sera is shown in Table I. We selected those samples showing anti-SKC-2 Ab titers of at least $1:5\times10^4$ in order to work at high dilutions (1:300–1:1000), thus avoiding elevated background levels.

Selected sera were subjected to a neutralization assay in order to determine their neutralizing activity titer (NAT). Abs from nine out of ten patients inhibited the formation of SKC-2-Plg activator complex in vitro (Fig. 2). Neutralizing activity titers ranged between 97.5–363.4 μg of SK neutralized per ml of tested serum. Despite the high anti-SKC-2 Ab titer presented by serum from patient 5 (Table I), it showed an insignificant value of NAT (Fig. 2). On the other hand, patient 14, having the highest anti-SKC-2 Ab titer (Table I), showed the most elevated value of NAT (Fig. 2).

Several distinct binding areas were observed for the ten tested sera (Fig. 1). However, there are in the SKC-2 molecule binding sequences that are common for most of the patients. Eight out of ten sera recognized spot 14 comprising amino acids 130-149. Seventy percent of patients recognized spot 18 comprising residues 170-189. Six out of ten samples bound at spot 1 comprising amino acids 1-20 of the SKC-2 N-terminal region. Other six

TABLE I

Anti-SKC-2 Ab Titers of Patient Sera[a]

Patient	Anti-SKC-2 titer
1	$1:1\times10^4$
3	$1:1\times10^4$
5	$1:5\times10^4$
6	$1:1\times10^5$
7	$1:1\times10^5$
8	$1:1\times10^4$
9	$1:1\times10^4$
10	$1:1\times10^4$
11	$1:5\times10^4$
12	$1:5\times10^4$
14	$1:5\times10^5$
15	$1:5\times10^4$
17	$1:1\times10^5$
18	$1:1\times10^4$
20	$1:1\times10^4$
21	$1:1\times10^4$
24	$1:5\times10^4$
25	$1:1\times10^4$
26	$1:1\times10^4$
28	$1:1\times10^5$
30	$1:5\times10^4$
31	$1:1\times10^4$
32	$1:1\times10^5$
42	$1:1\times10^5$
45	$1:1\times10^4$
46	$1:5\times10^4$
47	$1:1\times10^4$
48	$1:5\times10^4$

[a] The anti-SKC-2 Ab titer is represented as the maximal dilution in which positive signal was obtained. Positive signal was considered when the value was at least two-fold the background.

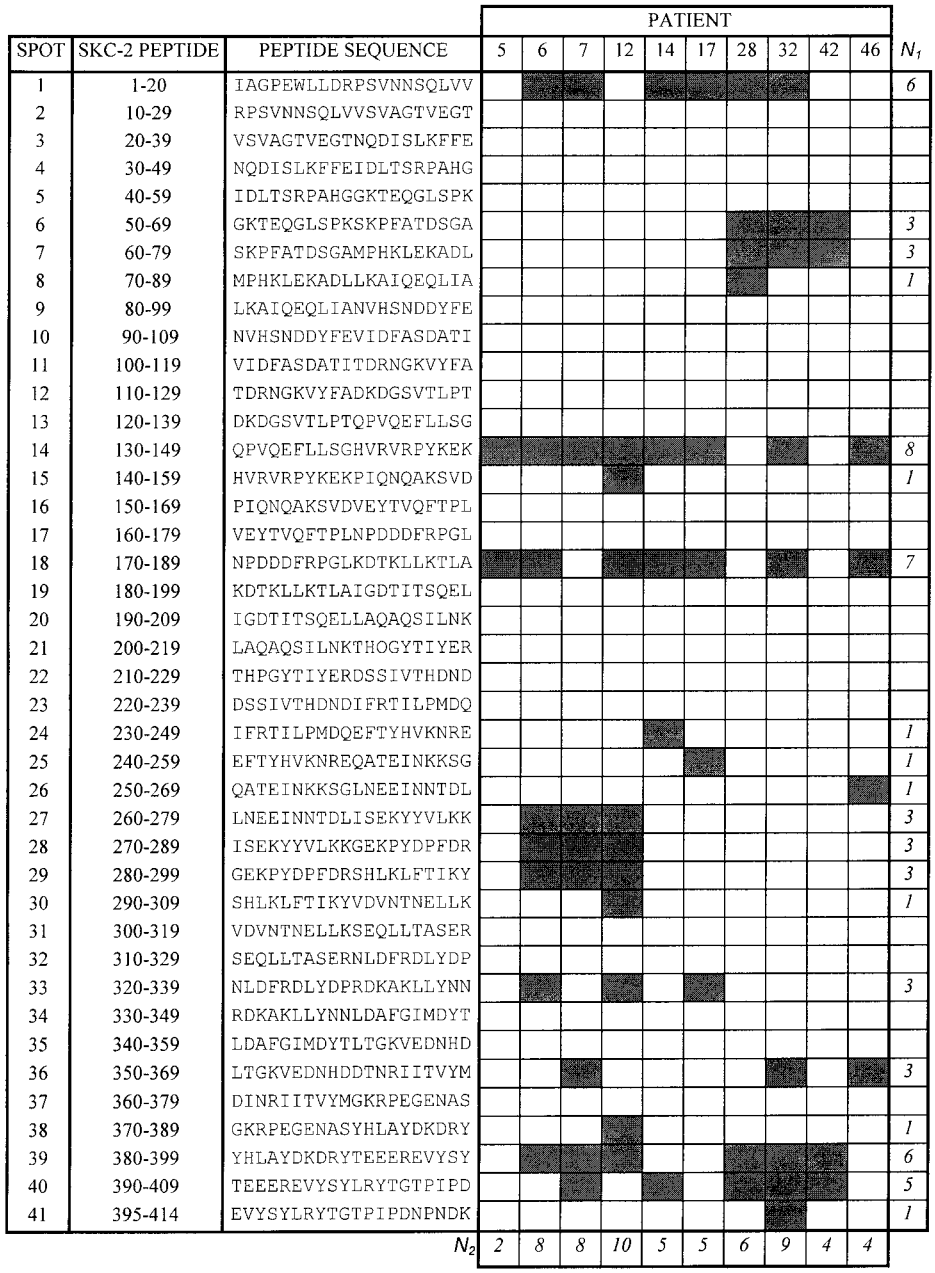

FIG. 2. Neutralizing activity titer (NAT) of patient sera against SKC-2 protein. Each bar indicated the NAT obtained from one patient 10 days after SKC-2 therapy.

patients recognized spot 39 comprising residues 380-399. Fifty percent of tested sera recognized spot 40 comprising amino acids 390-409 within the C-terminal region.

The simultaneous recognition of the spots 6 and 7 indicated the presence of a continuous epitope comprised between residues 60-69 (SKPFATDSGA). Likewise, for spots 39 and 40, the existence of a continuous epitope comprising residues 390-399 (TEEEREVYSY) was delineated. The recognition of spots 27, 28 and 29 indicated the presence of one or more continuous epitopes comprised between residues 270-289 (ISEKYYVLKKGEKPYDPFDR). Spots showing isolated positive signals, without recognition of adjacent positions, suggested the existence of continuous epitopes including more than ten amino acids (Fig. 1).

DISCUSSION

Being a bacterial protein, SK is immunogenic in human (2). Therefore, resulting from SK therapy, pa-

tients become immunized and anti-SK Ab titers rise post-treatment (3-8). High Ab titers might provoke severe immune reactions during SK therapy (9), and neutralize SK activity preventing effective thrombolysis (2). Although other thrombolytic agents like UK and TPA are available (1), they are significantly more expensive than SK. In this regard the study of the immunodominant regions of SK becomes an important aspect for the improvement of this thrombolytic agent.

Mapping of antigenic determinants using overlapping synthetic peptides permits the elucidation of linear epitopes only, that is, with this method, we can not determine the location of topographic epitopes formed by two or more protein regions distant in sequence but close in three dimensional space. Certain stereochemical properties favor linear antigenicity, being the mobile, protruding highly exposed surface loops, the most capable of inducing peptide-crossreactive anti-protein Abs (26). Alternatively, Abs can be generated against unfolded or degraded protein molecules, allowing the recognition of linear peptides even if the corresponding region in the native 3D structure is cryptic or not accessible.

This is the first study reporting the probing of a cellulose-bound set of peptides with total human sera. In previous reports, probing was performed using monoclonal Abs (20-25). Using total serum has been limited by the occurrence of high background levels. In our experiments we used sera with high anti-SKC-2 Ab titers, which allowed to work at high dilutions and therefore, to reduce the background level. This is an important achievement because it made possible not only to map the recognition site of a particular Ab, but also to characterize the immune response against a given antigen.

From ten tested patients eight immunoreacted with peptide 130-149, seven bound to 170-189, six immunoreacted with 1-20 and 380-399, and five reacted with 390-409. Peptides 50-69, 60-79, 260-279, 270-289, 280-299, 320-339, and 350-369 were recognized by 30% of tested patients. Another seven peptides were recognized by only one patient. We observed different reactivity patterns among the tested patients. Individual variation in the recognition of different spots may be a consequence of individual variation in immune response to SKC-2, and is in agreement with previous reports using human sera (15). Because of the limited number of patients tested in our study, we were not able to correlate the pattern of peptide reactivity with the allergic responses of the patients.

Our results with the spot synthesized library are in agreement with previous reports. In 1995, Parhami-Seren et al. (15) found three major antigenic regions in SK mapping with anti-SK Ab from human sera. They comprise amino acids 1-253 and 120-352 (containing two distinct, non-overlapping epitopes). Bruserud et al. (12) have also identified a major T-cell epitope in the region of SK constructed by amino acid residues 238-246. Regions most frequently found in our experiments comprise amino acids 1-20 (spot 1), 130-149 (spot 14) and 170-189 (spot 18); being included within these ranges previously described. We have also found other sequences contained in these ranges, though appearing with lower frequency.

Two other epitopes in SK constructed by amino acids 1-13 and 353-414 were not antigenic in all humans tested by Parhami-Seren et al. (15). In contrast, we obtained regions comprising amino acids 1-20 (spot 1), 380-399 (spot 39) and 390-409 (spot 40), among the most frequently recognized. In the same previous report, human Abs from most subjects showed minimal or no binding to the region of SK constructed by amino acids 14-127 (15). Differing from these results, we have found slightly antigenic epitopes within this region, such as residues 50-89 (spots 6-8).

Differences observed between our results and previous reports might be due to several causes. First, differences existing between skc-2 and skc genes. It has been reported that this heterogeneity at the gene level may be the reason for previous findings of immunological and chemical differences between SKs (27-32). Second, we performed our study using small peptides (20 residues) bound to a cellulose membrane, while other researchers used much larger soluble recombinant fragments. Therefore they were able to detect conformational epitopes, which could not be detected by our technique. However, we could more accurately delineate different linear epitopes included within the same fragment, keeping in mind that recombinant fragments as well as peptides might improperly fold, hiding immunologically important epitopes and presenting other, which do not exist in the native molecule.

The crystallographic structure of SK complexed with the catalytic domain of human plasmin has been recently solved (18). 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 (1-15), the COOH-terminal 32 residues and the loop region 46-70 are disordered, suggesting that they are highly mobile and accessible even after the formation of the complex with μ plasmin. This is consistent with the observed antigenicity of spots 1, 6, 7, 39 and 40. The antigenic regions 130-149 (spot 14) and 260-299 (spots 27, 28 and 29) include highly accessible loops linking domains α with β and β with γ, respectively. The region 350-369 (spot 36) comprises the accessible beta hairpin $^{\gamma}\beta_3$ and $^{\gamma}\beta_4$. The peptide 320-339 (spot 33) maps a loop region of domain γ, which is buried upon formation of the complex and likely involved in activation. Never-

theless, this loop should be accessible in the uncomplexed SK.

Anti-SK Abs can neutralize SK in two different ways: affecting directly the binding of SK to Plg and/or in an indirect way affecting the binding of the Plg substrates to the activating complex. Abs recognizing peptide 320-329 (30% of the studied sera) should neutralize SK directly, because this region is involved in the interaction between domain γ and plasmin in the activating complex. Moreover, Wang et al. (18) have suggested this region to be directly implicated in a mechanism of binding activation of Plg.

Abs directed against the rest of the most frequent linear antigenic regions could neutralize SK by a more indirect mechanism. For instance, epitopes present in spot 1 and spots 6-7 seem to be accessible in the complex; hence the corresponding antibodies are not likely to inhibit the complex formation itself. However, considering the dimensions of the antigen-binding domain of these Abs it seems probable that they block the interaction with the substrate Plg by simple steric reasons. In fact, an interaction between the region 45-50 of SK and the Plg kringle 5 has been suggested previously (18,33).

Similarly, it is not excluded the possibility that antibodies recognizing other linear epitopes interfere sterically with the kringle domains of Plg, inhibiting the formation of the complex or affecting the binding of the substrate Plgs. Otherwise, as seen for serum No. 5, the recognition of certain linear epitopes does not lead to SK-neutralization at all. Apart of inducing neutralization, anti-SK Abs can mediate the allergic response observed in certain clinical cases. It is worth mentioning that peptide-crossreactive anti-protein antibodies represent only a fraction of the immune response. However, the study of the antigenicity corresponding to topographic epitopes should be done using SK mutants with amino acid changes in topographic zones of the protein surface.

The data from this study provided the following conclusions: (a) The spot-synthesis is a technically very easy and straightforward method, now having proven to be useful for epitopic mapping of proteins using patient total sera; (b) the possibility of accurately locate a given epitope being present in more than one overlapping peptide, extends the usefulness of the spot synthesis technique for mapping of antigenic regions; (c) although individual variations in peptide recognition were detected, patients treated with SKC-2 tend, in general, to show a common regional binding pattern including residues 1-20 (spot 1), 130-149 (spot 14), 170-189 (spot 18), and 390-399 (spots 39,40).

Antigenic regions of SKC-2 include linear epitopes, identified by our technique, as well as conformational epitopes, elucidated by complementary studies (12, 14,15).

The knowledge of the antigenic regions of SK can help in the design of mutant molecules with reduced antigenicity and perhaps reduced toxic effects.

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