

T-cell recognition of discrete regions of the thrombolytic drug streptokinase

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A B S T R A C T

Streptokinase (SK) is a bacterial protein used clinically as a thrombolytic agent in humans. Administration of SK causes a rapid increase in the frequency of anti-SK T cells and the titre of specific anti-SK antibodies that, on subsequent administration of SK, may neutralize the activity of the drug or elicit allergic-type reactions. By locating and modifying the immunogenic T-cell epitopes within the SK protein, it is possible that an agent with reduced immunogenicity but equal efficacy may be produced. We have investigated the T-cell epitopes within SK using nine non-overlapping, recombinant peptide fragments of SK. We investigated the proliferative T-cell response of peripheral blood mononuclear cells obtained from patients before and 6 days after administration of SK for myocardial infarction. We also examined the response of cultured anti-SK T-cell lines derived from patients 6 days after treatment with SK. Before administration of SK, peripheral blood mononuclear cells from six of nine patients showed a proliferative response to SK. The response was significantly higher 6 days after administration of SK ($P = 0.0004$). Cultured T-cell lines showed similar proliferative responses to clinical-grade SK and recombinant SK. Marked differences in T-cell responses were apparent in response to each recombinant SK fragment ($P = 0.04$). The mean proliferative response exceeded background to only two peptides, peptide 2 ($P = 0.04$) and peptide 3 ($P = 0.009$). Peptide 3, representing amino acids 100–150 of mature SK, was recognized preferentially in the majority of assays. Marked variation in the T-cell response to SK following treatment with this agent was observed between subjects. Despite these differences, peptides 2 and 3 induced T-cell proliferation at a level significantly above background in the majority of subjects. These epitopes may represent a region of enhanced immunogenicity within SK.

INTRODUCTION

Streptokinase (SK) is a protein, produced by beta-haemolytic streptococci [1], which initiates the conversion of plasminogen to plasmin and which is used clinically as a thrombolytic agent. The main clinical application of SK is in the treatment of acute myocardial infarction (AMI). SK has proven benefits in terms of both

mortality and morbidity when administered up to 12 h, and perhaps as late as 24 h, following AMI [2,3].

Approximately 60% of healthy adults have low levels of anti-SK antibodies, due to previous exposures to streptococcal antigens [4]. These antibodies are mostly IgG [5] produced following T-cell-dependent B-cell isotype switching and affinity maturation. T cells in the peripheral blood mononuclear cells (PBMC) of healthy

Key words: immunotherapy, peptide epitopes, streptokinase resistance, T cells.

Abbreviations: AMI, acute myocardial infarction; CI, confidence intervals; FCS, fetal-calf serum; EBV, Epstein-Barr virus; HS, human A⁺ve serum; PBMC, peripheral blood mononuclear cells; SK, streptokinase; rSK, recombinant SK.

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adults also proliferate in response to SK at low levels *in vitro* [6,7]. Following therapeutic administration of SK, high titres of anti-SK antibodies are produced, which, in a previous study [8], were shown to be detectable for up to 7.5 years after administration of the drug. We and others [7,8] have also shown that T-cell responsiveness to SK increases in the first few weeks after SK treatment but declines almost immediately to pre-treatment levels.

Although allergic reactions, including serum sickness and anaphylaxis following SK treatment, have been reported [9–12], the majority of allergic reactions are mild and not life threatening [3]. Of greater concern is the potential neutralization of SK by anti-SK antibodies; in particular, while anti-SK antibody levels remain elevated, there is the potential for the efficacy of subsequent treatment to be significantly impaired. Thus re-administration of SK is not recommended. This is of increasing clinical importance given the growing percentage of patients brought to hospital with AMI who have received treatment with SK previously.

Identification and removal of the immunogenic regions of the molecule may allow SK to be used safely and effectively more than once in the same patient. Mapping of the B cell epitopes of SK with murine anti-SK monoclonal antibodies has highlighted several distinct immunogenic regions located throughout the mature SK molecule [13–15]. The T-cell epitopes of SK are, however, only characterized to a low resolution. In a study by Bruserud et al. [16], each one of four SK fragments of various lengths was found to induce the proliferation of anti-SK T cells cultured from one healthy individual.

In an attempt to clarify the immunogenic epitopes of SK, we have produced a series of non-overlapping peptide fragments spanning the entire length of the SK molecule and tested the proliferative responsiveness of T cells, cultured from the peripheral blood of SK-treated patients, to each peptide. In the present work we present data which outline the complexities of determining the T-cell epitopes of SK.

MATERIALS AND METHODS

Isolation of streptococcal genomic DNA

Genomic DNA was isolated from *Streptococcus equisimilis* H46A ATCC number 12449 (a gift from Professor T. Mitchell, University of Glasgow, U.K.) as described by Caparon and Scott [17]. The genomic DNA was used as a template in PCR and the PCR product was cloned into pMal c-2 (New England Biolabs, Hitchin, Herts., U.K.) for sequencing and as a source of template DNA in subsequent PCR reactions to generate nine SK minigenes.

PCR oligonucleotide primer design

Primers were designed based on the published sequence

of the *S. equisimilis* H46A SK coding sequence [18]. Coding strand primers included a *Bam*HI site at the 5' end, and non-coding primers included a *Pst*I site at their 5' end (Table 1). Primers were synthesized by the Protein and Nucleic Acids Chemistry Laboratory (University of Leicester, Leicester, U.K.).

Cloning and recombinant protein production

PCR reactions were conducted using ULTma proof-reading thermostable polymerase (Perkin-Elmer, Warrington, Cheshire, U.K.). Purified PCR products were recovered from 1.5% (w/v) agarose gels using a Sephaglas Bandprep kit (Pharmacia Biotech, Milton Keynes, Herts., U.K.) and were ligated into pMal c-2 vector DNA. Ligated constructs were transformed into competent *Escherichia coli* TB1 (New England Biolabs) and sequenced (Protein and Nucleic Acids Chemistry Laboratory, University of Leicester, U.K.). Consensus sequence data for each construct are presented in Figure 1. The sequences encoding mature recombinant SK and the nine SK fragment peptides were expressed fused to maltose binding protein in protease-deficient *E. coli* PR745. Fusion proteins were recovered from bacterial lysates using amylose resin (New England Biolabs). Total protein concentration for each fusion protein was determined using a micro-bicinchoninic acid ('BCA') protein assay (Pierce and Warringer, Cheshire, U.K.). The proportion of SK protein or SK peptide was calculated based upon the predicted molecular masses of the fusion peptide and SK component.

Culture medium and antigens

PBMC were cultured in RPMI 1640 supplemented with 2 mM Glutamax (Gibco, Paisley, Scotland, U.K.) and either 10% (v/v) fetal-calf serum (FCS) or 5% (v/v) pooled human A⁺ve serum (National Blood Service, Sheffield, U.K.). Proliferation assays were performed in AIM V serum-free medium (Gibco) supplemented with 2 mM Glutamax. SK for clinical use (Kabikinase; Pharmacia Biotech, Herts., U.K.) was diluted with sterile water to 1 mg/ml. Recombinant full-length SK (rSK), SK peptides 1–9 and a preparation of protein from induced bacteria harbouring the pMal c-2 vector alone, were each diluted to 1 mg/ml of recombinant protein, calculated as a percentage of the total fusion protein in PBS, and used at equal concentrations.

Blood samples

Blood was taken from patients who had each received a single dose of 1.5×10^6 units of SK as part of standard treatment of AMI at Leicester Royal Infirmary. All blood donors gave informed consent for participation in this study, which was approved by the local Ethical Research Committee. Samples were collected from nine patients (patients 1–9) immediately before the administration of

Table 1 Oligonucleotide primer pairs used to generate a copy of the SK coding sequence and nine SK minigenes

Sequences which did not anneal to the template are underlined. Restriction sites are shown in italics (*Bam*HI for coding primers and *Pst*I for non-coding primers). In each pair, the coding primer is shown first.

Oligonucleotide primer pairs	PCR product (SK coding sequence)	Calculated mass (kDa) of the rSK portion of the fusion protein (peptide number)
<u>CCC</u> <i>GGATCC</i> ATGAAAATTACTTATCTTTGGG	Full-length SK (1–1323)	50
<u>CCC</u> <i>CTGCAGT</i> CATTATTTGTCGTTAGGGTATCAG		
<u>CCC</u> <i>GGATCC</i> ATGAAAATTACTTATCTTTGGG	Minigene 1 (1–150)	6.1 (1)
<u>CCC</u> <i>CTGCAGT</i> TAACCAAGCTGACTACTAA		
<u>CCC</u> <i>GGATCC</i> ACTGTTGAGGGGACGAATC	Minigene 2 (151–300)	5.1 (2)
<u>CCC</u> <i>CTGCAGT</i> TAAAGTTTATGTGACATCGCG		
<u>CCC</u> <i>GGATCC</i> GAGAAAGCTGACTACTAA	Minigene 3 (301–450)	6.2 (3)
<u>CCC</u> <i>CTGCAGT</i> TACGAACCATCTTTGTGACGAA		
<u>CCC</u> <i>GGATCC</i> GTAACCTTGCCGACCAACC	Minigene 4 (451–600)	6.4 (4)
<u>CCC</u> <i>CTGCAGT</i> TAATCGTCATCAGGGTTAAGG		
<u>CCC</u> <i>GGATCC</i> TCCAGACAGGTCTCAAGA	Minigene 5 (601–750)	6.2 (5)
<u>CCC</u> <i>CTGCAGT</i> TAGACGATTGAGGATCAGCTT		
<u>CCC</u> <i>GGATCC</i> CACTCATGACAATGACATTT	Minigene 6 (751–900)	6.7 (6)
<u>CCC</u> <i>CTGCAGT</i> TAATTTCTCAGAGATCAGGT		
<u>CCC</u> <i>GGATCC</i> TACGTCCTAAAAAGGCGA	Minigene 7 (901–1050)	6.6 (7)
<u>CCC</u> <i>CTGCAGT</i> ATCTGAAGTCTAAGTTACGTT		
<u>CCC</u> <i>GGATCC</i> GATTATACGATCCTCGTGA	Minigene 8 (1051–1200)	6.5 (8)
<u>CCC</u> <i>CTGCAGT</i> ATTCCGGTCGCTGCCATAT		
<u>CCC</u> <i>GGATCC</i> GAGAGAATGCTAGCTATCA	Minigene 9 (1201–1323)	5.4 (9)
<u>CCC</u> <i>CTGCAGT</i> CATTATTTGTCGTTAGGGTATCAG		

SK and at 6 days after treatment to investigate the proliferative responsiveness of PBMC before and after treatment. For seven additional patients (patients 10–16), blood was collected 6 days after the administration of SK and used for the assessment of T-cell responsiveness to Kabikinase, rSK and SK peptides.

Four SK-specific T-cell lines were established (patients 10, 11, 12 and 13). SK-specific T-cell lines were maintained with irradiated autologous B cells (7500 rad) transformed with the Epstein–Barr virus (EBV) (patients 10, 11 and 12) or fresh irradiated PBMC (2500 rad) (patient 13). Three additional T-cell proliferation assays were performed using freshly isolated PBMC (patients 14, 15 and 16). All cultures were performed at 37 °C in a 100% humidified, 5% CO₂ atmosphere.

Isolation of PBMC

Whole blood was collected into preservative-free heparin (10 units/ml) and diluted 1:1 with RPMI 1640 containing FCS. Diluted blood was layered on to Lymphoprep (Nycomed U.K. Ltd., Birmingham, U.K.) and centrifuged at 800 g for 30 min at 20 °C. PBMC were aspirated from the interface, washed twice in the required medium and adjusted to 1 × 10⁶/ml. Cells were used either directly in PBMC proliferation assays or to establish T-cell lines and EBV–B cell lines.

T-cell lines

PBMC were cultured at 1.25 × 10⁶/ml in RPMI containing HS with 10 µg/ml Kabikinase and 20 units/ml interleukin-2 (Aldelesleukin; Eurocetes U.K. Ltd., Harefield, Middx., U.K.). Cells were cultured for 2 weeks before use in proliferation assays.

EBV–B cell lines

EBV-secreting B958 cells were kindly donated by Professor A. Rickinson (Department of Cancer Studies, University of Birmingham, U.K.). PBMC were adjusted to 1 × 10⁶/ml in fresh RPMI containing FCS. Filtered supernatant from B958 cells, which had been cultured for 7 days, was incubated with 1 × 10⁶ PBMC for 1 h at 37 °C with occasional shaking. Cells were centrifuged and resuspended in 2 ml of RPMI containing FCS, supplemented with a final concentration of 0.2 µg/ml cyclosporin A. Cells were cultured for 5 days, then subcultured as required.

Proliferation assays using PBMC

Antigens were prepared in AIM V medium at final concentrations of 1, 3, 10 and 30 µg/ml per well, with 1 × 10⁵ cells/well in a 96-well U-bottomed plate and cultured for 5 days. At 16 h before the end of the culture period, each well was pulsed with 37 kBq of [³H]thy-

1	ATGAAAAATTACTTATCTTTTGGGATGTTTGCAC TGCTGTTTGCAC TAA CATT TGGAACAGTCA	64	SK
	ATGAAAAATTACTTATCTTTTGGGATGTTTGCAC		SK peptide 1
65	ATTCTGTCCAAGCTATTGCTGGACCTGAGTGGCTGCTAGACCGTCCATCTGTCAACAACAGCCA	128	SK
	ATTCTGTCCAAGCTATTGCTGGACCTGAGTGGCTGCTAGACCGTCCATCTGTCAACAACAGCCA		SK peptide 1
129	ATTAGTTGTTAGCGTTGCTGCTAGTCTGTTGAGGGGACGAATCAAGACATTAGTCTTAAATTTTTT	192	SK
	ATTAGTTGTTAGCGTTGCTGGT		SK peptide 1
	ACTGTTGAGGGGACGAATCAAGACATTAGTCTTAAATTTTTT		SK peptide 2
193	GAAATCGATCTAACATCACGACCTGCTCATGGAGGAAAGACAGAGCAAGGCTTAAGTCCAAAAT	256	SK
	GAAATCGATCTAACATCACGACCTGCTCATGGAGGAAAGACAGAGCAAGGCTTAAGTCCAAAAT		SK peptide 2
257	CAAAACCATTGCTACTGATAGTGGCGCGATGTCACATAAACTTGAGAAAGCTGACTTACTAAA	320	SK
	CAAAACCATTGCTACTGATAGTGGCGCGATGTCACATAAACTTG		SK peptide 2
	AGAAAGCTGACTTACTAAA		SK peptide 3
321	GGCTATTCAAGAACAATTGATCGCTAACGTCACAGTAACGACGACTACTTTGAGGTCATTGAT	384	SK
	GGCTATTCAAGAACAATTGATCGCTAACGTCACAGTAACGACGACTACTTTGAGGTCATTGAT		SK peptide 3
385	TTTGCAAGCGATGCAACCAATTACTGATCGAAACGGCAAGGTCTACTTTGCTGACAAAGATGGTT	448	SK
	TTTGCAAGCGATGCAACCAATTACTGATCGAAACGGCAAGGTCTACTTTGCTGACAAAGATGGTT		SK peptide 3
449	CGGTAACCTTGCCGACCCAACTGTCCAAGAATTTTTGCTAAGCGGACATGTGCGCGTTAGACC	512	SK
	CG		SK peptide 3
	GTAACCTTGCCGACCCAACTGTCCAAGAATTTTTGCTAAGCGGACATGTGCGCGTTAGACC		SK peptide 4
513	ATATAAGAAAAACCAATACAAAACCAAGCGAAATCTGTTGATGTGGAATATACGTACAGTTT	576	SK
	ATATAAGAAAAACCAATACAAAACCAAGCGAAATCTGTTGATGTGGAATATACGTACAGTTT		SK peptide 4
577	ACTCCCTTAAACCTGATGACGATTTTCAAGACAGGTCTCAAAGATACTAAGCTATTGAAAACAC	640	SK
	ACTCCCTTAAACCTGATGACGAT		SK peptide 4
	TTTCAAGACAGGTCTCAAAGATACTAAGCTATTGAAAACAC		SK peptide 5
641	TAGCTATCGGTGACACCATCACATCTCAAGAATTACTAGCTCAAGCACAAAGCATTTTAAACAA	704	SK
	TAGCTATCGGTGACACCATCACATCTCAAGAATTACTAGCTCAAGCACAAAGCATTTTAAACAA		SK peptide 5
705	AAACCACCCAGGCTATACGATTTATGAACGTGACTCCTCAATCGTCACTCATGACAATGACATT	768	SK
	AAACCACCCAGGCTATACGATTTATGAACGTGACTCCTCAATCGTC		SK peptide 5
	ACTCATGACAATGACATT		SK peptide 6
769	TTCCGTACGATTTTACCAATGGATCAAGAGTTTACTTACCGTGTTAAAAATCGGGAACAAGCTT	832	SK
	TTCCGTACGATTTTACCAATGGATCAAGAGTTTACTTACCGTGTTAAAAATCGGGAACAAGCTT		SK peptide 6
833	ATAGGATCAATAAAAAATCTGGTCTGAATGAAGAAATAAACAACTGACCTGATCTCTGAGAA	896	SK
	ATAGGATCAATAAAAAATCTGGTCTGAATGAAGAAATAAACAACTGACCTGATCTCTGAGAA		SK peptide 6
897	ATATTACGTCCTTAAAAAAGGGGAAAAGCCGTATGATCCCTTTGATCGCAGTCACCTGAAACTG	960	SK
	ATATTA		SK peptide 6
	CGTCCTTAAAAAAGGGGAAAAGCCGTATGATCCCTTTGATCGCAGTCACCTGAAACTG		SK peptide 7
961	TTCACCATCAAATACGTTGATGTCGATACCAACGAATTGCTAAAAAGTGAGCAGCTCTTAACAG	1024	SK
	TTCACCATCAAATACGTTGATGTCGATACCAACGAATTGCTAAAAAGTGAGCAGCTCTTAACAG		SK peptide 7
1025	CTAGCGAACGTAACCTTAGACTTCAGAGATTTATACGATCCTCGTGATAAGGCTAAACTACTCTA	1088	SK
	CTAGCGAACGTAACCTTAGACTTCAGA		SK peptide 7
	GATTTATACGATCCTCGTGATAAGGCTAAACTACTCTA		SK peptide 8
1089	CAACAATCTCGATGCTTTTGGTATTATGGACTATACCTTAACTGGAAAAGTAGAGGATAATCAC	1152	SK
	CAACAATCTCGATGCTTTTGGTATTATGGACTATACCTTAACTGGAAAAGTAGAGGATAATCAC		SK peptide 8
1153	GATGACACCAACCGTATCATAACCGTTTATATGGGCAAGCGACCCGGAAGGAGAGAATGCTAGCT	1216	SK
	GATGACACCAACCGTATCATAACCGTTTATATGGGCAAGCGACCCGGA		SK peptide 8
	GGAGAGAATGCTAGCT		SK peptide 9
1217	ATCATTTAGCCTATGATAAAGATCGTTATACCGAAGAAGAACGAGAAGTTTACAGCTACCTGCG	1280	SK
	ATCATTTAGCCTATGATAAAGATCGTTATACCGAAGAAGAACGAGAAGTTTACAGCTACCTGCG		SK peptide 9
1281	TTATACAGGGACACCTATACCTGATAACCCCTAACGACAAATAA	1323	SK
	TTATACAGGGACACCTATACCTGATAACCCCTAACGACAAATAA		SK peptide 9

Figure 1 Consensus sequence of SK cDNA and SK minigenes 1–9

Each clone was sequenced twice; vector sequences and restriction sites on cloning primers are not shown. Each minigene sequence is compared with the sequence from the pMal c-2 SK clone (bold type) that was identical to the published sequence [18].

midine (Amersham, Little Chalfont, Bucks., U.K.). Cells were harvested on to glass-fibre filters and radiation was monitored using a Wallac 1450 Microbeta scintillation counter. Data are presented as c.p.m. Unless otherwise stated, proliferative responsiveness to SK was considered significant if it was at least 2.5 times greater than proliferation to culture medium alone. All proliferation

assays were carried out in triplicate and data shown represent the means $\pm$ S.D.

Proliferation assays using T-cell lines

EBV-B cells were γ -irradiated (7500 rad) and EBV-B cells and T cells were resuspended separately in AIM V medium at 5×10^5 cells/ml. A T-cell/EBV-B-cell ratio of

1:1 was used in each assay, with final antigen concentrations of 0, 3 and 30 $\mu\text{g}/\text{ml}$. Cells were incubated for 56 h and pulsed with 37 kBq of [^3H]thymidine for the final 16 h of culture. Cells were harvested on to Wallac glass-fibre filters and radioactivity was counted as described above.

Statistical analysis

Differences between proliferative responses to 1 $\mu\text{g}/\text{ml}$ SK before and after treatment were analysed for PBMC isolated from patients 1–9 using the paired Student's *t* test. Differences in T-cell response among Kabikinase, rSK and the 9 SK peptides was assessed by analysis of variance (ANOVA) followed by multiple Student's *t* tests where appropriate. Statistical analyses were performed using the statistical package Minitab. The PBMC response to some SK peptides was not normally distributed and, thus, all PBMC proliferation data were log transformed before analysis. Comparisons with $P \leq 0.05$ were considered significant.

RESULTS

Peripheral T-cell responsiveness to SK increases in the first week after SK treatment

PBMC proliferation in response to Kabikinase, at a final concentration of 1 $\mu\text{g}/\text{ml}$, was determined for nine subjects immediately before and 6 days after the adminis-

tration of SK following AMI. Both the initial magnitude of the proliferative response and the change in response after treatment with SK varied among subjects. Before the administration of SK, PBMC isolated from six out of nine patients gave a modest but significant (i.e. a stimulation index of > 2.5) proliferative response to SK (Figure 2). After correction for the background response to culture medium alone, the response to SK was significantly greater after administration of SK (median 18830 c.p.m., range 6404–68463) compared with before (median 2222 c.p.m., range 0–16289) administration of SK ($P = 0.0004$). The PBMC response to SK before and after treatment showed no significant change in one patient (patient 9).

rSK is recognized by SK-specific T-cell lines and PBMC

Blood from seven donors (patients 10–16) was used to test lymphocyte responses to rSK (Figure 3), Kabikinase, the pMal c-2 maltose-binding portion of the fusion proteins, which was used as a negative control for stimulatory contaminants associated with the recombinant protein preparation (mal in Figure 3), each of the nine SK-fusion peptides, and the T cells and antigen-presenting cells with no antigen, i.e. true background (negative) control (0 in Figure 3).

Limited proliferation in response to the pMal c-2 maltose-binding portion of the fusion proteins was detected in each volunteer but this was the same as that seen with medium alone [95 % confidence intervals (CI)

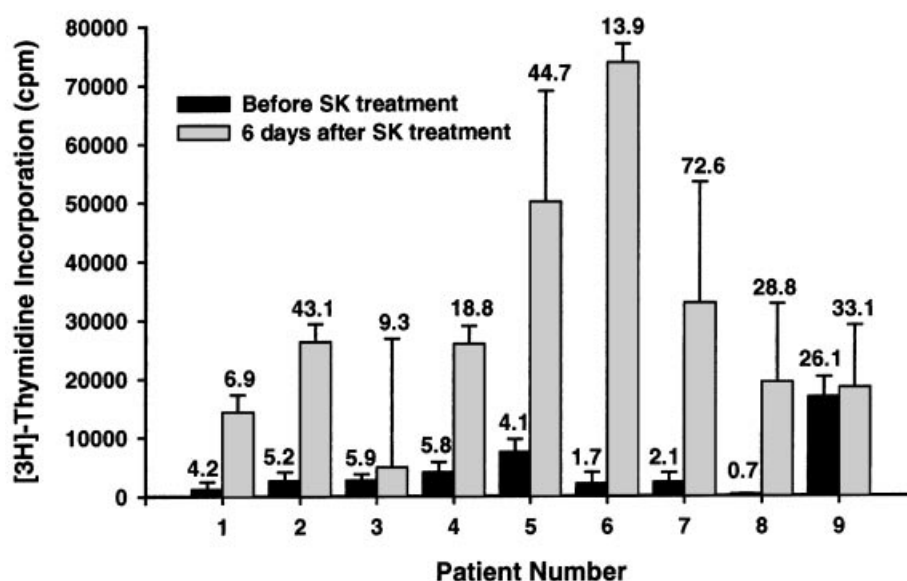


Figure 2 Proliferative response of PBMC to SK before and after treatment with SK

Proliferative response of PBMC to SK isolated before (black bars) and 6 days after (grey bars) treatment with SK for AMI. PBMC proliferation assays were cultured, in triplicate, for 5 days in AIM V serum-free medium with Kabikinase at a final concentration of 1 $\mu\text{g}/\text{ml}$. Data are presented as mean c.p.m. ($\pm$ S.D.). Values above the bars represent the stimulation indices for each assay.

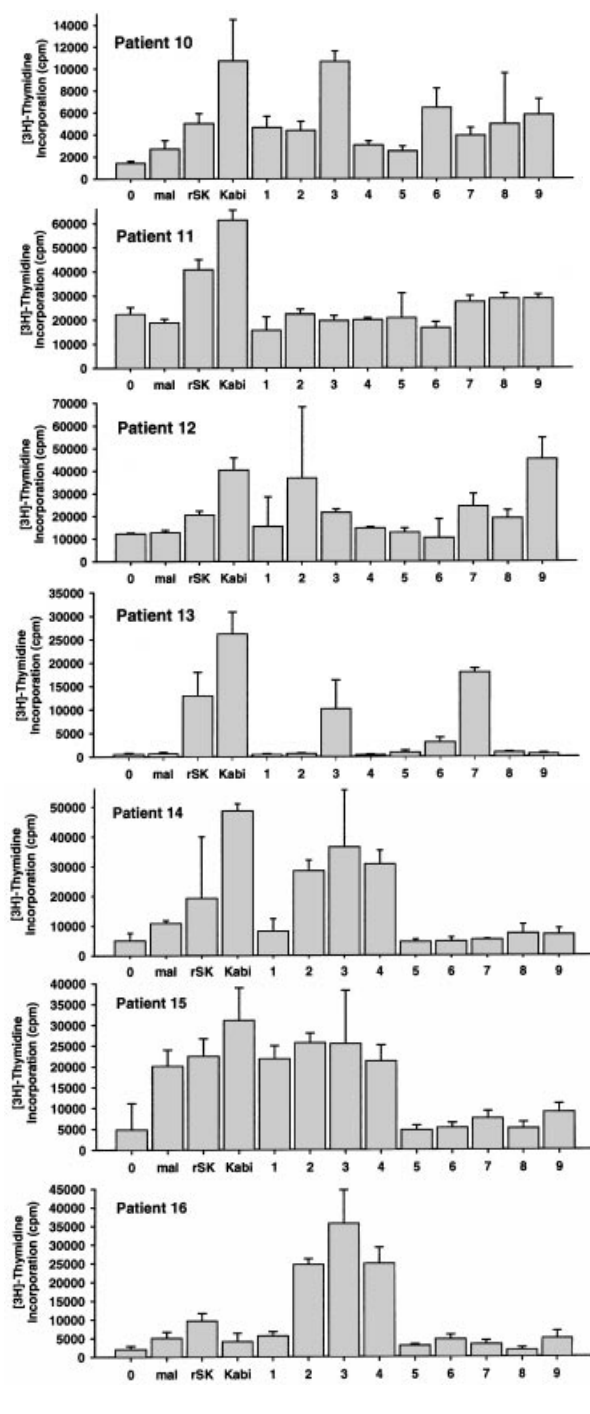


Figure 3 Proliferative response of SK-specific T-cell lines and PBMC to peptides 1–9 of SK

Upper four panels: proliferative response of SK-specific T-cell lines from patients with AMI 6 days after treatment with SK using EBV-B cells (patients 10, 11 and 12) or PBMC (patient 13) as antigen-presenting cells. Lower three panels: proliferative response of freshly isolated PBMC from patients 14, 15 and 16, 6 days after treatment with SK for AMI. All proteins were tested in triplicate at 3 µg/ml. Data are presented as mean c.p.m. ($\pm$ S.D.). 0, no antigen (negative control); mal, pMal c-2 maltose-binding portion of the fusion proteins; Kabi, Kabikinas.

for difference between means -13214 to 5864 c.p.m., $P = 0.41$]. For each subject, although the response varied greatly, the proliferative response to both Kabikinas and rSK was significantly greater than the response to the pMal c-2 maltose-binding portion of the fusion proteins (95% CI for difference between means -40066 to -2043 c.p.m., $P = 0.034$) and (95% CI for difference between means -20106 to -4051 c.p.m., $P = 0.042$) respectively (Figure 3). Although responses to Kabikinas were quantitatively greater than those to rSK, this did not reach statistical significance ($P = 0.17$).

Of the nine SK peptides, peptide 3 was preferentially recognized in the majority of assays, whereas proliferation in response to peptide 5 did not significantly exceed background proliferation in any assay. The mean proliferative response significantly exceeded that to culture medium alone for peptides 2 ($P = 0.04$) and 3 ($P = 0.009$) only. The mean proliferative response to all other peptides (peptide 1, $P = 0.44$; peptide 4, $P = 0.09$; peptide 5, $P = 0.99$; peptide 6, $P = 0.92$; peptide 7, $P = 0.25$; peptide 8, $P = 0.58$; peptide 9 $P = 0.52$) did not differ significantly from background.

T-cell responses differed among the nine SK peptide fragments ($P = 0.035$, ANOVA). When direct comparisons were made of the T-cell responses with randomly paired peptides, peptides 2 and 3 showed greater antigenicity than the remaining fragments. Peptide 2 was significantly more antigenic than both peptides 5 ($P = 0.04$) and 6 ($P = 0.04$), and peptide 3 was significantly more antigenic than peptides 1 ($P = 0.03$), 5 ($P = 0.008$), 6 ($P = 0.008$), and 8 ($P = 0.04$).

Assays with EBV-B cells as antigen presenting cells were found to be least informative, whereas assays with PBMC consistently resulted in low background proliferation and discrete recognition of certain regions of SK, namely those represented by peptides 2, 3 and 4.

DISCUSSION

This investigation represents, to our knowledge, the first published report of the recognition of peptide fragments of SK by T cells derived from patients following therapeutic administration of SK. Immediately before treatment with SK, lymphocytes isolated from six of nine patients were capable of responding significantly to SK; 6 days later, lymphocytes from all nine patients responded significantly to SK, with a ten-fold increase in proliferation in five cases. The degree of response was, however, highly variable between patients. As none of these patients had previously received SK therapeutically, these results probably reflect the varied immunological exposures of individuals to streptococcal antigens. Our data also show clearly that a single therapeutic dose of SK is sufficient in the majority of individuals to induce SK-specific T-cell clonal expansion, and that the responses of

these T cells are directed at immunologically distinct regions of SK in different individuals.

SK is a therapeutically effective thrombolytic therapy but its clinical application is limited by the potential consequences of T-cell-dependent production of anti-SK neutralizing antibodies. The T-cell response to SK increases in some individuals shortly after administration of SK [19]. Anti-SK T-cell responses may be initiated by epitopes within at least five regions of the SK molecule, at least in healthy volunteers [16]. Our data indicate that differential responses to SK peptides are most clearly observed in assays with a low background proliferation and high SK responsiveness. In assays with high background proliferation and low recognition of SK, minimal variation in responsiveness to different SK peptides was detected. Our data indicate preferential recognition by T cells of particular fragments of SK: peptide 2 and especially peptide 3.

A potential problem with our experimental design may lie in the use of pMal c-2 as a negative control. This protein represents the fusion peptide portion of each recombinant SK peptide and, as such, was used to monitor proliferation in response to this fusion peptide rather than the fused portion of SK. However, it does not control for proliferation in response to the neo-epitope created by the joining region between the pMal c-2 maltose-binding portion of the fusion protein and the recombinant SK protein. Theoretically, the differential responsiveness of lymphocytes to each SK peptide may reflect the unlikely event that an immune response was directed towards the unique fusion-protein-recombinant-protein joining region. Although we cannot fully discount this possibility, it is important to note that lymphocytes were shown to be responsive to Kabikinase, i.e. SK prepared for clinical administration, and were isolated from patients known to have a significantly expanded population of SK-specific T cells.

Within our small study population, marked inter-individual variation in T-cell response to each of the SK fragments was observed. Whereas, in some individuals, responses were observed to peptides near the C-terminus, peptides 2 and 3, situated towards the N-terminus of the SK sequence, elicited the greatest average response and were statistically most antigenic (Figure 3). Our data suggest that one region of SK may be more antigenic than others and that the T-cell response of different individuals may be directed towards epitopes in this region.

A situation has been reported in which a group of at least three nested T-cell epitopes were detected within a 30 amino acid fragment of the house-dust mite allergen, Der p1. The epitopes were recognized in association with several different HLA-DR and HLA-DP molecules [20]. Clusters of immunodominant epitopes have also been reported in the autoimmune antigen, myelin basic protein [21], and the measles virus fusion protein [22]. Such

clusters may be important if the SK molecule were to be altered to reduce its immunogenicity in population terms. It is plausible that the immunogenicity of SK may be reduced by substitution of a very small number of amino acids. Substitution of a single amino acid has been shown to ablate the immune response to a myelin basic protein peptide in a murine model of autoimmunity [23]. Amino acid alterations within discrete immunodominant regions may thus represent an important mechanism of antigenic escape by viral pathogens [24].

The clinical importance of our findings is unclear. Although non-immunogenic thrombolytic agents are available for use in man, SK remains the most widely used thrombolytic agent in the U.K. and in many other countries. Moreover, alternative agents are associated with a higher incidence of clinically significant haemorrhagic complications [25]. If SK is to remain in routine clinical use as a thrombolytic agent, further studies of the immunological epitopes of the SK protein and their manipulation may be justified.

In the present investigation we have shown that the administration of SK for the treatment of AMI results in a rapid and substantial increase in T-cell responsiveness to SK. We have additionally detected a putative region of enhanced antigenicity within SK. Further studies in larger numbers of patients will be required, to both confirm our observations and determine precisely the T-cell epitopes within this region. Our results suggest that it may be feasible to modulate non-human protein drugs to reduce their immunogenicity and allow them to be useful in the treatment of human disease.

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