

Use of streptokinase as a recall antigen for lymphocyte proliferation testing in vitro

Lisa H. Youkeles, MD, Mohammed Y. Soliman, MD, and
David L. Rosenstreich, MD Bronx, N.Y.

The use of streptokinase (SK) as a recall antigen (Ag) for stimulation of human lymphocyte proliferation in vitro was evaluated. SK is a potent stimulant, inducing a greater response than three other Ags, tetanus toxoid, Candida albicans extract, or streptolysin. The optimum stimulatory concentration is 1000 U/ml, and peak stimulation occurs between days 6 and 8. Human cord-blood lymphocytes do not respond to SK, suggesting that it is an Ag and not a mitogen. The response rate in an adult population was 82%, higher than the rate of two other Ags tested (tetanus toxoid or Candida). These findings demonstrate the ability of SK to act as a potent recall Ag for human lymphocyte proliferation in vitro. (J ALLERGY CLIN IMMUNOL 1991;88:166-71.)

Key words: Streptokinase, lymphocyte proliferation, cell-mediated immunity, tetanus toxoid, antigens, mitogens, cord blood, lymphocytes

The in vitro proliferation of lymphocytes induced by recall Ags remains a useful technique for evaluating cell-mediated immunity.¹ Since lymphocyte responsiveness requires prior exposure to a given Ag, commonly used recall Ags for population studies have included material derived from ubiquitous organisms such as *Candida albicans* and *Trichophyton*, or proteins such as TT to which individuals are immunized. In the past, a very useful recall Ag was a mixture of SK/SD, which was sold as a therapeutic agent but widely used for in vitro studies. Analysis of in vitro lymphocyte proliferation with a partially purified 30 kd component of SK/SD demonstrated a response rate in humans of 50%.²⁻⁴ However, SK/SD was withdrawn from the market in the mid 1970s. Therefore, it was no longer available for in vitro testing.

From the Departments of Medicine and Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, N. Y. Supported by National Institutes of Health Grant AI-29871-01, National Institute of General Medical Sciences Grant T32 GM-7288, Albert Einstein College of Medicine Core Grant P30-CA-13330, and funds from the Saul and Lola Kramer Foundation, the G. Harold and Leila Y. Mathers Foundation, and the Irvington House Foundation.

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Reprint requests: David L. Rosenstreich, MD, Depts. of Medicine and Microbiology and Immunology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461.

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Abbreviations used

SK/SD:	Streptokinase/streptodornase
PBS:	Phosphate-buffered saline
PHA:	Phytohaemagglutinin
Con A:	Concanavalin A
TT:	Tetanus toxoid
MNC:	Mononuclear cell
Ag:	Antigen

SK is an enzyme produced by group C β -hemolytic *Streptococci* that has a molecular weight of 46 kd. Since the early 1980s, SK has been available for antithrombotic therapy of acute myocardial infarction.⁵⁻⁷ Several studies have evaluated its allergenic potential and in vivo immunogenicity.⁸⁻¹⁰ In a study of 292 individuals, it was found to induce a delayed-type skin reaction in only 11%.¹¹ However, its use as a recall Ag for stimulation of human lymphocytes in vitro has not been fully evaluated. We have therefore analyzed the ability of commercially available SK to stimulate in vitro human peripheral blood MNC proliferation.

MATERIAL AND METHODS

Patients

Patients were chosen from the population of an adult allergy clinic as well as a group of students, house officers, and laboratory and clinic personnel. Ages ranged between 19 to 69 years. Patients had no prior allergic immunotherapy and were not receiving oral corticosteroids or other im-

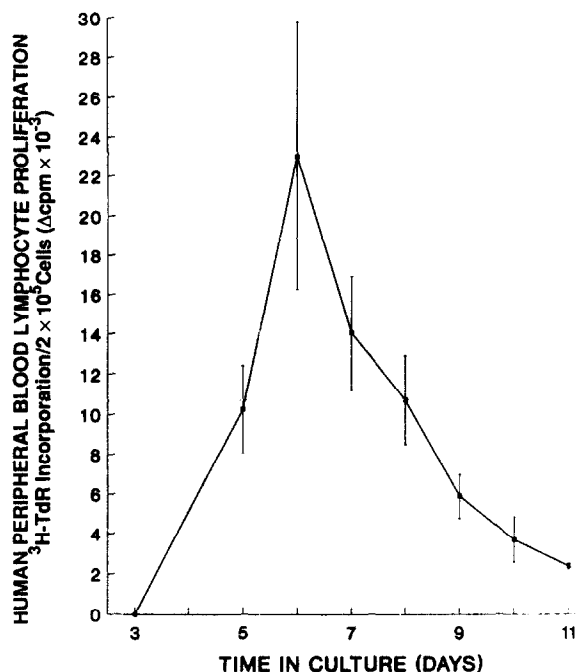


FIG. 1. Time course of human lymphocyte response to SK in vitro. Human peripheral blood MNCs were stimulated in culture with 1000 IU/ml of SK. Cells were pulsed and harvested on days 3 through 11. Results represent the arithmetic means $\pm$ SEM of data from 12 different donors.

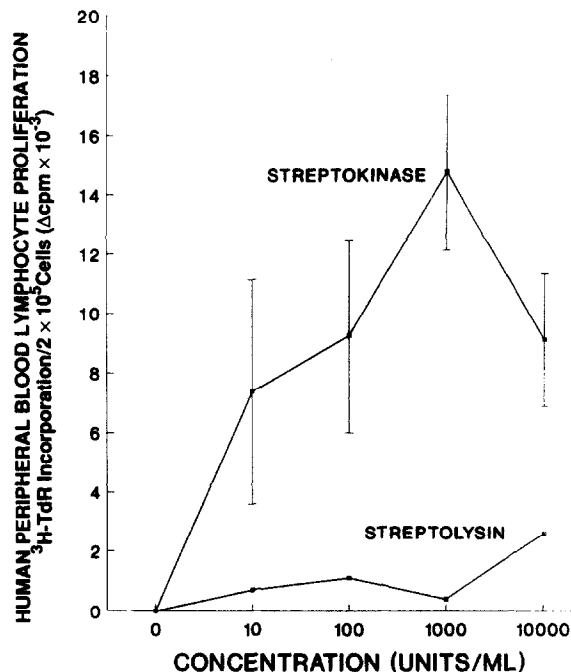


FIG. 2. Dose dependency of human lymphocyte responsiveness to Streptococcal products in vitro. Peripheral blood MNCs were cultured in the presence of SK and streptolysin at various concentrations. Results represent the arithmetic means $\pm$ SEM of 39 individuals.

munosuppressive drugs. The study procedure was approved by the Albert Einstein College of Medicine Institutional Review Board, and informed consent was obtained from all subjects.

Preparation of Ags and mitogens

SK was obtained from Hoechst-Roussel Pharmaceuticals, Inc., Somerville, N.J., containing 750,000 IU per vial. A stock solution was prepared by diluting with tissue-culture medium (RPMI 1640) to a final concentration of 100,000 IU/ml. This solution was then filter sterilized (0.22 $\mu\text{mol/L}$, Millex-Gv, Millipore Corp., Bedford, Mass.) and stored in small aliquots (100 μl) at 4° C. TT (Lederle Laboratories, Pearl River, N.Y.) was dialyzed against PBS overnight, and the protein concentration was measured by a dye-binding assay (Bio-Rad Laboratories, Richmond, Calif.). *Candida albicans* and *Trichophyton* Ags (Hollister-Stier Laboratories, Spokane, Wash.) were prepared in the same manner. Streptolysin O (Difco Laboratories, Detroit, Mich.) was prepared by reconstituting the powder in 10 ml of RPMI 1640. The mitogens, Con A (Sigma Chemical Co., St. Louis, Mo.) and PHA (Wellcome Laboratories, Beckenham, England), were reconstituted in PBS and filter sterilized.

Lymphocyte proliferation

Peripheral blood, 30 to 35 ml from each subject, was drawn into a lightly heparinized syringe and mixed thoroughly. The blood cells were washed three times with PBS,

with the last PBS wash containing 0.03 mol/L of ethylenediaminetetraacetic acid. The prewashing procedure was developed for other studies to remove platelet contamination. However, it was found to improve the reliability and the magnitude of in vitro proliferative responses, and we therefore used this method in these studies as well. The blood cell suspension was overlaid on a Hypaque-Ficoll density gradient (Ficoll-Paque, Pharmacia Fine Chemicals, Piscataway, N.J.) and centrifuged at 550 g at room temperature for 35 minutes. The interface containing the MNCs was aspirated, and the cells were washed twice with PBS. Cells were resuspended at a concentration of $2 \times 10^6/\text{ml}$ in RPMI 1640 tissue-culture medium containing 10% heat-inactivated human serum, gentamicin (50 $\mu\text{g}/\text{ml}$), and L-glutamine (2 mmol/L). The cells were dispensed into 96-well plastic plates (Linbro, Flow Laboratories Inc., McLean, Va.) at a concentration of 2×10^5 cells per well in a volume of 0.1 ml.

Several experiments were done on human cord blood; 15 to 20 ml of blood was collected from the umbilical veins of normal, full-term, vaginal delivery placentae. Cord-blood MNCs were prepared and cultured in a similar manner as the peripheral blood cells.

Ags and mitogens were diluted into RPMI 1640 at various concentrations immediately before each experiment and were tested in triplicate. PHA and Con A were added in a 1 $\mu\text{g}/\text{ml}$ final concentration per well, and SK in 10, 100, 1000, and 10,000 IU/ml of final concentration. Plates were incubated at 37° C in a humidified atmosphere containing

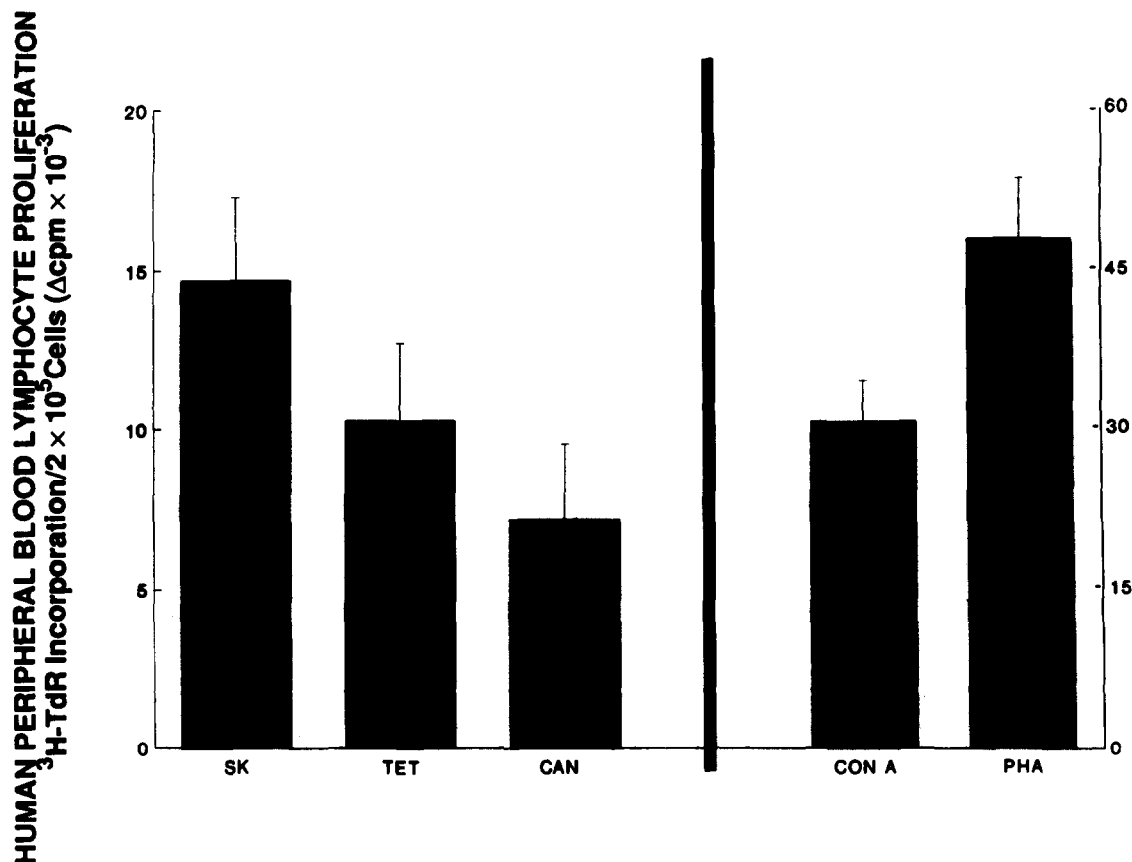


FIG. 3. Mean maximal stimulation response of peripheral blood lymphocytes to Ags and mitogens. Peripheral blood MNCs were cultured for 144 hours with multiple concentrations of SK, TT (*Tet*), *Candida* (*Can*), or with Con A or PHA for 72 hours, and the maximum response for each stimulant was determined. Results represent the arithmetic means $\pm$ SEM of 27 to 43 patients. Mean baseline is 700 cpm.

7% CO₂. Except where this is indicated, cultures were harvested after 144 hours. The cells were pulsed with 0.5 μ Ci per well of tritiated thymidine (methyl-³H-TdR, New England Nuclear, Boston, Mass.) and harvested after 4 hours of incubation at 37° C onto glass-fiber filter paper (M. A. Bioproducts, Walkersville, Md.) with a semiautomated cell harvester (Mash II, Microbiological Associates, Bethesda, Md.). The filters were either air-dried overnight or dried for 1 hour at 150° F, placed in a scintillation cocktail (ACS II, Amersham, Arlington Heights, Ill.) and then counted in a β -scintillation spectrometer (Beckman Instruments Inc., Fullerton, Calif.).

The arithmetic means of triplicate wells were calculated, and the results were expressed as the Δ cpm (Δ cpm = cpm Ag - cpm media).

RESULTS

Time course of human lymphocyte response to SK in vitro

Human peripheral blood MNCs were cultured with 1000 U/ml of SK and harvested daily on days 3 to 11. Lymphocyte proliferation was first detectable after 5 days in culture, and maximum stimulation occurred

on day 6 (Fig. 1). The response decreased thereafter but was still detectable as late as day 11.

Comparative dose dependency of SK and streptolysin

Lymphocyte proliferation induced by four concentrations of SK (10 to 10,000 IU/ml) was determined after 6 days in culture. Significant stimulation was observed at an SK concentration as low as 10 IU/ml and was maximal at 1000 IU/ml (Fig. 2). Streptolysin was also tested since it has been reported to stimulate human lymphocyte proliferation in vitro.^{12, 13} However, in our study, streptolysin was a very weak stimulant with essentially no stimulation at concentrations from 10 to 1000 IU/ml and only a minimal response at a concentration of 10,000 IU/ml.

Comparative response of peripheral blood lymphocytes to SK and other stimulants

To determine the comparative potency of SK, lymphocytes from a large number of individuals were stimulated in culture with multiple concentrations of

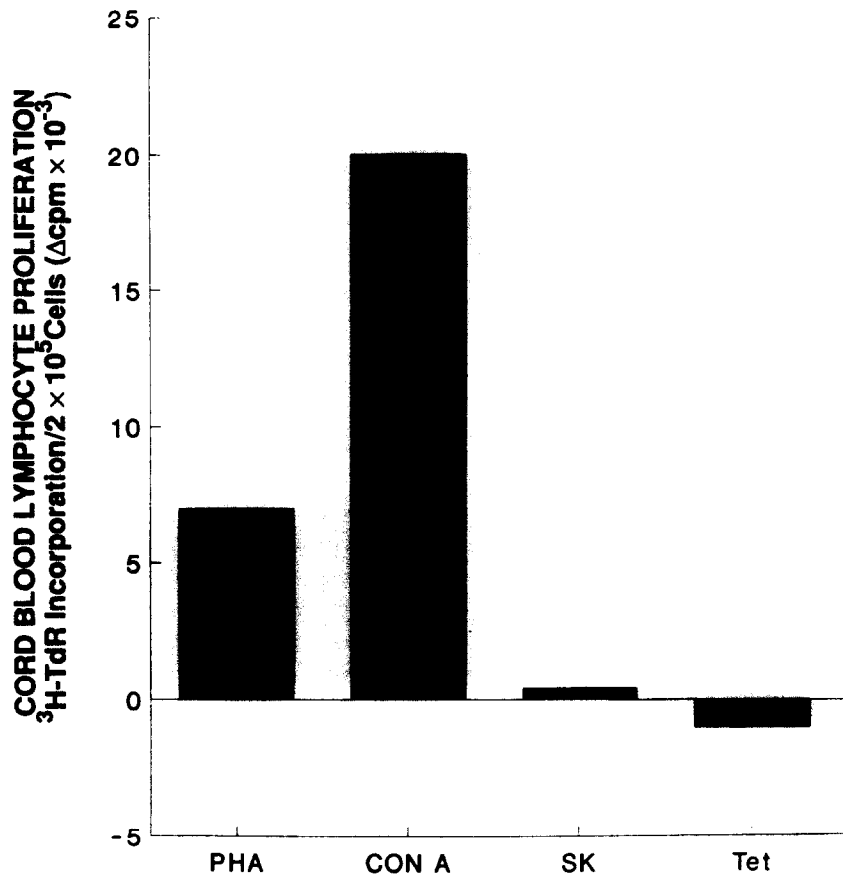


FIG. 4. Cord-blood lymphocyte response to Ags and mitogens. Human cord-blood MNCs were cultured for 144 hours with several concentrations of SK, TT (*Tet*) Ags, or with the mitogens, Con A or PHA, for 72 hours, and pulsed and harvested in the same manner as the peripheral blood lymphocytes. The maximum response to each stimulant was recorded. Results represent the arithmetic means $\pm$ SEM of two experiments. Mean baseline is 700 cpm.

this Ag as well as two other Ags, TT and *Candida albicans* extract. Proliferative responses were determined, and the maximum response at any concentration was used for calculations.

SK was a more potent stimulant than either of the two other Ags, producing a mean maximal response (Δcpm) of 14,729 cpm, in comparison to a Δcpm of 10,263 for tetanus toxoid and a Δcpm of 7164 for *Candida* (Fig. 3). The mean SK response was approximately 48% of the mean response to the T cell mitogen, Con A, and 31% of the mean PHA response.

Umbilical cord-blood lymphocyte response to SK

Human umbilical cord-blood lymphocytes were cultured with SK to determine if it was acting as a recall Ag or as a nonspecific mitogen. The cord-blood lymphocytes were cultured for 72 hours with the mitogens, PHA and Con A, or for 144 hours with two dilutions of TT (1 and 10 $\mu\text{g}/\text{ml}$) or four, tenfold dilutions of SK (10 to 10,000 IU/ml). The cord-blood

lymphocytes did not exhibit any significant proliferation in response to SK or to TT (Fig. 4). In contrast, these cells responded well to both T cell mitogens.

Response rate of an adult population to SK

The frequency of in vitro responsiveness to SK was determined in a population of 39 adults (aged 19 to 69 years) who were without known immunodeficiency disease and not receiving any immunosuppressive drugs. A positive response was considered to be >2 SD over the baseline response (approximately a three-fold stimulation); 82% of this population responded to SK (Fig. 5). In contrast, the frequency of responsiveness to TT and *Candida* Ags was lower (58% and 65%, respectively). The SK response rate was almost as high as that of the two mitogens, Con A and PHA (86% and 93%, respectively). Among the seven SK nonresponders, three did not respond to any Ag or mitogen, suggesting that they were anergic. The response rate among immunologically intact adults to SK may therefore be as high as 89% (32/36).

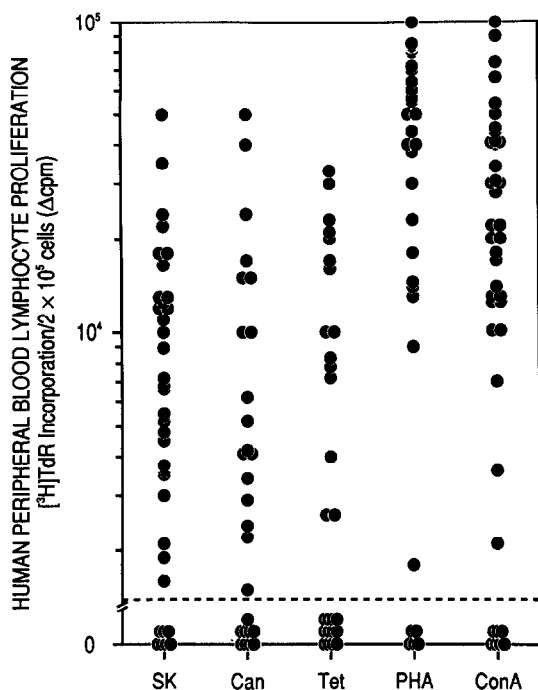


FIG. 5. Response rates to Ags and mitogens in an adult population. Peripheral blood MNCs from 27 to 43 individuals were cultured in the presence of multiple concentrations of SK, *Candida* (Can), TT (Tet), PHA, or Con A, and the maximum response to each was determined. Results represent the maximum response of each patient to each Ag. The dashed line (---) represents 2 SD over baseline, and responses greater than this were considered to be positive.

DISCUSSION

A potent and reliable recall Ag for in vitro lymphocyte transformation would be useful for evaluating cell-mediated immunity.¹ Previously, SK was combined with SD (SK/SD) and used in standard evaluations for cell-mediated immunity by delayed cutaneous hypersensitivity testing, as well as for in vitro studies.^{14, 15} SK/SD, which is a potent recall Ag in most adults, has not been freely available for at least 10 years, making a good substitute necessary. The purpose of this study was to investigate the use of the more recently available product, SK, in comparison to more common in vitro Ags, such as TT, *Candida* Ag, and streptolysin. Our results indicate that SK is a potent recall Ag in vitro with a response rate of at least 82% in a healthy adult population. This finding was significantly greater than the response rates of TT or *Candida* Ag, which were only 58% and 65%, respectively. These findings suggest that SK should be as useful as an in vitro recall Ag as was the previously available product, SK/SD.

The failure of SK to stimulate cord-blood lympho-

cytes supports the conclusion that it is acting as a recall Ag and not as a nonspecific mitogen. Similar findings have also been recently reported by Pabst et al.¹⁶ who noted low to absent responsiveness to SK and purified protein derivative in cord-blood cell preparations. They also noted a gradual increase in lymphocyte SK responses as infants aged, supporting the conclusion that SK is acting as a recall Ag.

Haverly et al.¹¹ previously reported that SK/SD induced a positive delayed hypersensitivity skin reaction in 21% of their patients, whereas SK alone induced a positive reaction in only 11%, a response rate significantly lower than the 82% positive in vitro response rate we found in our study population. An apparent discordance may therefore exist between SK responsiveness in vitro and in vivo, the reasons for which are not clear. One contributing factor is that the two study populations were not comparable. In the current study, all subjects were either normal or ambulatory outpatients with allergies or asthma, whereas subjects in the study of Haverly et al.¹¹ were all hospitalized and were selected because of a clinical suspicion of a diminished cell-mediated immunity. In fact, in their population, 40/292 (14%) were found to be completely anergic. Another possible factor is that the sensitivity of the in vitro assay is much greater than delayed hypersensitivity skin testing. However, preliminary results in our laboratory with a small number of volunteers indicate that the material we have used in vitro induces large delayed-type skin reactions. However, we have not yet tested a large enough population to compare our results to results of Haverly et al.¹¹

Alternatively, this dissociation between in vitro and in vivo responsiveness may be real, perhaps because lymphocytes that proliferate in vitro in response to SK are not major participants in the in vivo skin reaction. The finding of Tomar et al.³ that the major antigenic protein derived from SK/SD was 30 kd in size, whereas SK is 46 kd, suggests that other streptococcal Ags may be involved in human cell-mediated immunity and are responsible for delayed-testing hypersensitivity responses in vivo.

The determination of whether distinct proteins mediate delayed hypersensitivity and lymphocyte proliferation will require additional experimentation, including a larger population study with simultaneous measurement of in vivo and in vitro responsiveness to SK. However, whether or not SK itself is found to be a good Ag for in vivo use, the findings of this study indicate that it is a useful and reliable recall Ag for in vitro lymphocyte proliferation testing.

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REFERENCES

1. WHO report. Use and abuse of laboratory tests in clinical immunology: critical considerations of eight widely used diagnostic procedures. *Clin Exp Immunol* 1981;46:662-74.
2. Tomar RH, Taylor FB, Green GR. Delayed hypersensitivity to SK-SD: in vitro lymphocyte study with an active component. *J Immunol* 1972;108:231-5.
3. Tomar RH. Delayed skin reactor from streptokinase-streptodornase: stability studies and amino acid analysis. *Int Arch Allergy Appl Immunol* 1976;50:220-4.
4. Tomar RH, Knight R. Cellular requirements for sensitive lymphocytes to proliferate in vitro to the purified streptococcal antigen. *Fed Proc* 1976;35:511.
5. Weinstein J. The international registry to support approval of intracoronary streptokinase thrombolysis in the treatment of myocardial infarction. Assessment of safety and efficacy. *Circulation* 1983;68:161-6.
6. Cowley MJ, Hastillo A, Vetrovec GW, Hess ML. Effects of intracoronary streptokinase in acute myocardial infarction. *Am Heart J* 1982;102:1149-58.
7. Mason DT. International experience with percutaneous transluminal coronary recanalization by streptokinase-thrombolysis reperfusion in acute myocardial infarction: new, safe, landmark therapeutic approach salvaging ischemic muscle and improving ventricular function. *Am Heart J* 1981;102:1126-33.
8. McGrath KG, Zeffren B, Alexander J, Kaplan K, Patterson R. Allergic reactions to streptokinase consistent with anaphylactic or antigen-antibody complex-mediated damage. *J ALLERGY CLIN IMMUNOL* 1985;76:453-7.
9. Totty WG, Romano T, Benian GM, Gilula L, Sherman LA. Serum sickness following streptokinase therapy. *Am J Radiol* 1982;138:143-4.
10. Weatherbee TC, Esterbrooks DJ, Katz DA, Aronow WS, Kenik JG, Mohiuddin SM. Serum sickness following selective intracoronary streptokinase. *Curr Ther Res* 1984;35:433-8.
11. Haverly RW, Mansfield LE, Ting S. A comparison between streptokinase/streptodornase and streptokinase on delayed hypersensitivity skin testing. *J ALLERGY CLIN IMMUNOL* 1984;74:205-7.
12. Oppenheim JJ, Blaese RM, Waldmann TA. Defective lymphocyte transformation and delayed hypersensitivity in Wiskott-Aldrich syndrome. *J Immunol* 1970;104:835-44.
13. Francis TC, Oppenheim JJ. Impaired lymphocyte stimulation by some streptococcal antigens in patients with recurrent aphthous stomatitis and rheumatic heart disease. *Clin Exp Immunol* 1970;6:573-86.
14. Kniker WT, Anderson CT, Roumiantzeff F. The Multi-Test system: a standardized approach to evaluation of delayed hypersensitivity and cell-mediated immunity. *Ann Allergy* 1979;43:73-7.
15. Ponte CD. Delayed cutaneous hypersensitivity testing. *South Med J* 1982;75:1076-9.
16. Pabst HF, Godel JC, Spady DW, McKechnie J, Grace M. Prospective trial of timing of *Bacillus Calmette-Guérin* vaccination in Canadian Cree infants. *Am Rev Resp Dis* 1989;140:1007-1001.