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Antibody Responses of Monkeys and Rabbits to Streptokinase and Staphylokinase

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Introduction

In our previous paper[8] it was reported that no significant rise of antibody to Streptokinase (STK) and Staphylokinase (SAK) could be observed in the sera of rabbits and dogs immunized with these extracellular bacterial substances or with live streptococci and staphylococci, although in the sera of patients with streptococcal infections or patients treated with STK-preparations, a considerable rise of the antibody titers to STK was observed.

From the reports of numerous authors[2~4,7,10~12,14,16,21~22] who demonstrated an incease of anti-STK-titers in the sera of patients with streptococcal infections, the antigenicity of STK seems to be generally accepted. However, clear positive results of immunization of experimental animals with STK and SAK were not reported until Dillon and Wannamaker's paper[6] was published in 1965, except for a few suggestive data reported by several authors 10 years ago. Van Deventer[19] failed to demonstrate a significant rise of the anti-STK titer in the sera of rabbits immunzed with the ethanol precipitate of the culture filtrate of Group A, B and C streptococci. Aoi[1] immunized rabbits with staphylococcal culture filtrate and observed the inhibition by the globulin fraction of immune rabbit-sera to the fibrinolytic activity of SAK by the use of the stained fibrin plate method. Neter[15] reported that the commercial anti-staphylotoxin horse sera inhibit the fibrinolytic activity of a culture of staphylococci cultivated on a fibrin-contained agar plate. These results show the existence of antigenicity of SAK, although quantitative determinations of the antibody titer have never been made.

In the course of our present experiments Dillon and Wannamaker's paper[6] was published, reporting the dissimilarity of two kinds of STK produced by Group A and C streptococci as a result of an immuno-electrophoretic examination and the cross-neutralizing test of antisera of rabbits immunized with each kind of STK in conjunction with complete Freund's adjuvant. From the results of their experiments the antigenicity of STK to rabbits was clearly demonstrated.

Therefore, the negative results of anti-STK production in the sera of rabbits in contrast with the case of the experiments with human volunteers, previously reported by the present authors, might be explained by the weakness of antibody response of rabbit to STK. From the point of view, mentioned above, an attempt to use monkeys instead of rabbits was made in this experiment.

The present paper deals with the difference between antibody response of monkeys to STK and SAK and that of rabbits to these substances.

Materials and Methods

1) *Experimental animals*: Albino rabbits weighing 2.0～2.5 kg obtained from a local dealer and Rhesus macaca weighing 1.7～2.3 kg from the Inuyama Monkey Center, were used.

2) *Antigens*: (1) Streptokinase; Varidase (Lederle) (100,000 Christensen units of streptokinase and 25,000 Kunitz units of streptodornase per vial) was used. Some of the samples which were used in the initial experiment were contaminated with a small amount of streptolysin-O. (2) Staphylokinase; Staphylokinase was prepared in our laboratory with the culture filtrate of staphylococcus aureus (Terashima). The cocci were inoculated in the nutrient broth (Difco) containing yeast extract (0.3%), and a shaking culture was made at 37°C for 6 hours. The cocci were removed by centrifugation at 7,000 r.p.m. for 30 min. The supernatant was used as the original material. Three kinds of staphylokinase samples were prepared by different concentrating methods. Each method of concentration in detail was described in a separate paper[19] of the same series.

SAK-I (Crude staphylokinase preparation): The preparation was obtained from the staphylococcal culture filtrate by the fractionation with acid-ethanol at a low temperature.

SAK-II (Concentrated staphylokinase preparation): The preparation was obtained by the following three steps; 1) precipitation with zinc chloride, 2) elution with sodium phosphate solution from the zinc-SAK precipitate, and 3) further fractionation with ammonium sulfate.

SAK-III (Purified staphylokinase preparation): The preparation was purified from the SAK-II solution by the CM-cellulose collumn chromatographic method containing the NaCl-gradient elution. The specific activity of the lyophilized sample was 3,125 units per mg protein.

3) *Immunization*: Lyophilized STK (Varidase-Lederle) or lyophilized SAK was dissolved in a saline solution. The injections of 2～4% solution of these materials were given to rabbits or monkeys intraperitoneally and/or subcutaneouly at the footpads. Some of these primarily injected rabbits were given additional intraperitoneal and subcutaneous injections of the same preparations after a long period of recess as a booster immunization. In some cases, samples were injected with complete Freund's adjuvant subcutaneously at the footpads of these animals.

4) *Determination of antibody*: (1) Antistreptokinase (Anti-STK); Anti-STK was measured according to the method described in the previous paper[8]. In this experiment commercial bovine fibrinogen (N.B.C.) and thrombin (Mochida Pharmaceutical Co.) were employed. (2) Antistaphylokinase (Anti-SAK); The method of assay was almost the same as described in the previous paper[8], except that the following minor modification was made: the 20 fold diluted immune serum to be tested was heated at 65°C for 30 min. before the original procedure of assay was performed. (3) Antistreptodornase (Anti-DNase); McCarty's method[13] was employed in this experiment. 0.25 mℓ of DNase (10～15 units/mℓ) was added to each 0.25 mℓ of serially diluted serum. The mixture was incubated at 37°C for 30 min. Then, 0.5 mℓ of 0.1% DNA-Na (Calf thymus, Sigma) was added to each tube. After 30 min. incubation at 37°C, 0.1 mℓ of ethanol was added to

each tube and the tubes were shaken gently. The anti-DNase unit was defined as the highest dilution of the serum in which the DNase activity was inhibited and the floating fibrous precipitate of DNA was formed. (4) Antistreptolysin-0 (Anti-SLO); Rantz and Randall's method[18] was employed. (5) Antistaphylocoagulase (Anti-CAG); The method described by Rammelkamp[17] was employed. Twenty-fold diluted sera were incubated by heating at 65°C for 20 min. and subsequently by refrigerating at 4°C overnight. One half mℓ of staphylocoagulase solution (containing 1 Rammelkamp unit) was added to each 1.0 mℓ of the serially diluted sera and the mixture was incubated at 37°C for 90 min. Then 0.5 mℓ of 4 fold diluted rabbit citrated-plasma was added to each tube and these were incubated at 37°C for 180 min. The highest dilution of the sera in which the inhibition of clot-formation was observed was taken as the unit of anti-CAG. (6) Antistaphylolysin; The method was as described in our previous paper[8]. (7) Agar gel precipitation reaction (Ouchterlony's double diffusion method); Twenty mℓ of 1% J-Agar (Sanko Pure Chemicals) containing 0.001% merthiolate were used for the agar plates. The diameter of the basin was 7 mm and the distance from the perimeter of the basin to that of the adjacent one was 15 mm. The incubations were made at 25°C for a week, and daily inspections of the precipitin bands were made during that period.

5) *Antisera*: Specimens of the blood were taken from the marginal auricular vein of immunized rabbits and from the femoral artery of immunized monkeys. The sera were stored in a refrigerator at −20°C.

Results

A) I. *Antibody formation against streptokinase.*

The experiment of immunization consists of the following four parts:

(1) Monkeys received only the primary injection of Varidase without adjuvant.

(2) Rabbits received only the primary injection of Varidase without adjuvant.

(3) Rabbits received booster injection of Varidase after a long period of recess, following the primary injection.

(4) Rabbits received only the primary injection of Varidase with complete Freund's adjuvant.

The results were summarized in Table 1. A rise in anti-STK titer was observed in the sera of both monkeys immunized with Varidase without complete Freund's adjuvant (Experiment 1). However, it is to be noted that Monkey-2 had considerably high titers of anti-STK and had some other streptococcal antibodies even before the injection of Varidase. Anti-SLO, antibody against streptolysin-O, increased also in the sera of these monkeys in the course of the immunization. The results of agar-gel-diffusion precipitin reaction were as follows: The preimmune serum of Monkey-4 did not show any precipitin band with Varidase, whereas more than one precipitin band was observed between the immune sera and Varidase preparation and that of the precipitin bands became more distinct with the progress of immunization. The parallelism of the results obtained with agr-gel-diffusion precipitin reaction to those found with Varidase-inhibitory reaction was noted in all of these specimens (Fig. 1).

No increase of anti-STK was observed in the sera of six rabbits immunized with

Varidase intraperitoneally and/or subcutaneously at the footpad without an addition of adjuvant (Experiment 2).

Varidase without adjuvant was injected to these rabbits as a booster immunization about 80 days after the initial administration of the primary injection of the same antigen (Experiment 3). The rise in both anti-STK-and anti-DNase-titer was observed 10 days after the booster injection. The parallelism of the results of gel-diffusion precipitin reaction to those of the Varidase-inhibitory reaction was noticed also in this case of the experiment (Fig. 2).

Another group of rabbits were immunized with Varidase in conjunction with complete Freund's adjuvant subcutaneously at the footpad (Experiment 4). A marked rise of antibody titers was observed in the sera of the immunized rabbits about 20 days after the antigen injection. The picture of the agar-gel-diffusion precipitation was illustrated in Fig. 3.

Table 1 *Anti-STK production in rabbits and monkeys immunized with Varidase*

	Expt. animals	Method of immunization			Titers of anti-STK		Agar gel diffusion Precipitin reaction	
		Route	Total units of Varidase injected***	Complete Freund's adjuv.	Preimmune	Immune	Preimmune	Immune
Exp. 1	Monkey- 2	intraperitoneal and footpad	100,000 u.	−	640	4000$^{*}_{**}$	+	##
	Monkey- 4			−	20	640	−	#
Exp. 2	Rabbit- 7	intraperitoneal and footpad	100,000 u.	−	20	20	−	−
	Rabbit- 8			−	20	20	−	−
	Rabbit- 9	footpad	100,000 u.	−	20	20	−	−
	Rabbit-10			−	20	20	−	−
	Rabbit-11	intraperitoneal	100,000 u.	−	20	20	−	−
	Rabbit-12			−	20	20	−	−
Exp. 3	Rabbit- 7*	intraperitoneal and footpad	100,000 u.	−	20**	80	−	#
	Rabbit- 8*			−	20**	80	−	#
	Rabbit- 9*	intraperitoneal and footpad	100,000 u.	−	20**	80	−	#
	Rabbit-10*			−	20**	320	−	#
	Rabbit-11*	intraperitoneal and footpad	100,000 u.	−	20**	20	−	+
	Rabbit-12*			−	20**	20	−	+
Exp. 4	Rabbit- 5	footpad	100,000 u.	+	20	160	−	#
	Rabbit- 6			+	20	320	−	##

* The booster immunization was carried out with the same rabbits used in the Experiment 2.

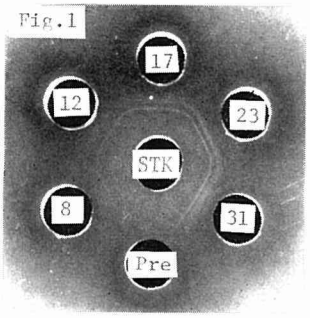
** Antibody titer of the serum taken immediately before the booster immunization.

*** The total units of Varidase was divided into three parts and each part was injected in every two days.

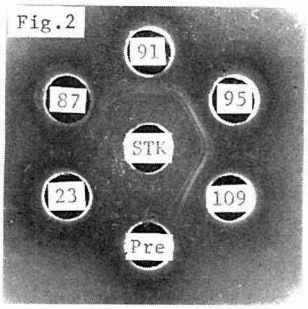
$^{*}_{**}$ The figure indicates the highest titer of anti-STK obtained in the course of immunization. Complete Freund's adjuvant +, and − indicate "with" and "without" respectively.

In the experiment of agar gel precipitin reaction the intensity of precipitin band appeared was indicated by the following symbols: ## clear band, # moderately clear band, + faint band, − no precipitin band appeared.

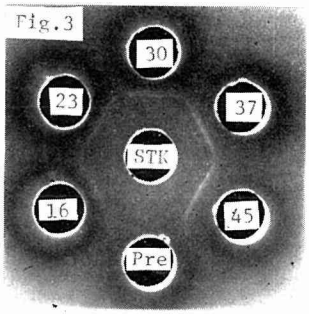
Agar-gel diffusion precipitin reaction of the sera of immunized animals to the proper antigen used for the immunization.



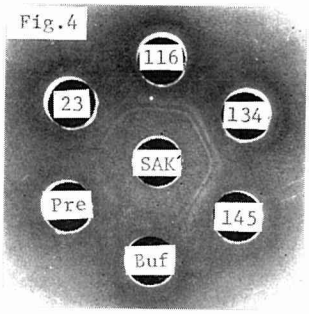
Monkey-4



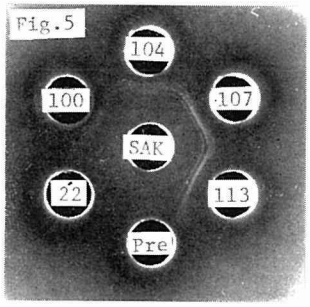
Rabbit-10



Rabbit-6



Rabbit-14



Rabbit-18

Central well: antigen, STK or SAK preparation.
Peripheral well: immune serum.
The figure expresses the number of days after immunization.
Pre: before the immunization.
Buf: buffer control.

Fig. 1 Monkey- 4
Fig. 2 Rabbit -10
Fig. 3 Rabbit - 6
Fig. 4 Rabbit -14
Fig. 5 Rabbit -18

B) II. *Antibody formation against staphylokinase.*

The experiment of immunization consists of the following four parts:

(1) Monkeys received only a primary injection of SAK-preparation without adjuvant.

(2) Monkeys received a booster injection of SAK-preparation with adjuvant in addition to the primary injection of the same antigen.

(3) Rabbits received a booster injection of SAK-preparation without adjuvant in addition to the primary injection of the same antigen without adjuvant after a long period of recess.

(4) Rabbits received only a primary injection of SAK-preparation with complete Freund's adjuvant.

The results of the above four kinds of experiment in immunization are summarized in Table 2. The marked rise of anti-SAK titer was observed in the sera of Monkey-1 immunized with SAK-III preparation intraperitoneally and subcutaneously at the footpad, whereas in the case of Monkey-3 no marked rise of the antibody titer was measured even though the same immunizing procedure was given to this animal (Experiment 5). After the booster injection of SAK-III with complete Freund's adjuvant, however, a marked rise of the anti-SAK- and anti-CAG-titers was observed in the sera of both of the immunized monkeys (Experiment 6).

Table 2 *Anti-SAK production in rabbits and monkeys immunized with SAK preparations*

	Expt. animals	Method of immunization			Titers of anti-SAK		Agar gel diffusion precipitin reaction	
		Route	Total units of SAK-prepar. injected	Complete Freund's adjuv.	Preimmune	Immune	Preimmune	Immune
Exp. 5	Monkey-1	intraperitoneal and footpad	SAK-I 14,000 u.***	−	160	640$^{*}_{**}$	+	++
	Monkey-3			−	80	160	+	+
Exp. 6	Monkey-1*	footpad	SAK-II 4,270 u.	+	80	640	+	++
	Monkey-3*			+	80	640	+	++
Exp. 7	Rabbit-18	footpad	SAK-III 5,300 u.$^{**}_{*}$	−	20**	20	−	−
	Rabbit-18*	footpad	SAK-III 3,400 u.$^{**}_{*}$	−	20**	640	−	++
Exp. 8	Rabbit-14	footpad	SAK-III 8,160 u.$^{**}_{**}$	+	20	160	−	+
	Rabbit-16			+	20	80	−	+

* The booster immunization was carried out.
** Antibody titer of the serum taken immediately before the booster immunization.
*** The total units of SAK-I was divided into five parts and each part was injected in every two days.
$^{*}_{**}$ The figure indicates the highest titer of anti-SAK obtained in the course of immunization.
$^{**}_{*}$ 5,300 units of SAK-III was divided into three parts, and 3,400 units of SAK-III was divided into two parts. Each part of the sample was injected in every two days.
$^{**}_{**}$ 8,600 units of SAK-III was divided into two parts and each part was injected with the interval of 6 days.

Although no rise in anti-SAK titer could be detected in the sera of primary immunized rabbit (Rabbit-18), considerably high titers of anti-SAK was observed in the sera of the same rabbit 10 days after the booster immunization (Experiment 7). When the complete Freund's adjuvant with SAK-III preparation was used for the immunization of rabbits (Rabbit-14 and 16), a marked rise of anti-SAK titer was observed in the sera of the rabbits about 20 days after primary injection of the antigen (Experiment 8). The results of agar-gel-diffusion precipitin reaction between these immune sera and SAK-III preparation are illustrated in Fig. 4, 5. The density of the precipitin band appearing between the antigen and the immune serum was comparable with the titer of anti-SAK in the same immune serum, which was estimated by the SAK-inhibiting reaction.

Discussion

When monkeys were used as the experimental animals, antibodies against STK were formed after primary injection of STK without complete Freund's adjuvant, although negative results were previously obtained with rabbits immunized with STK in the same manner. The difference of the results obtained by the experiment with the two different animal species may be attributed to the difference of response of each animal species

to the antigenic substance. However, it should be noted that Monkey-2 had a considerably higher titer of anti-STK even in the preimmune serum, as shown in Table 1. It might be suggested that the monkey had a natural infection of streptococci. In such a case it should be considered that the natural infection could have been a primary immunization and the initial injection of antigen, given in our experiment, may have stimulated the antibody-forming cells actually in a booster immunization fashion. The unnecessity of the booster immunization for the anti-STK formation in the case of monkeys might be due to the existence of a common distribution of natural streptococcal infection among this animal species. From our experience with rabbits no case of natural infection of streptococci was ever found, and also in these experiments no rabbit had antibodies to any kind of streptococcal extracellular antigenic substance in the preimmune serum. Moreover, the above consideration may be more readily acceptable from the evidence that positive results of anti-STK formation were obtained even in the experiment with rabbit by the booster immunization without an addition of adjuvant, in spite of the fact that the result of the antibody formation in the same rabbit by the primary immunization of STK without adjuvant was entirely negative.

The antibody response of monkeys and rabbits to SAK was not too different from that to STK. The evidence that the preimmune sera of both of the two monkeys used for the experiment of immunization with SAK showed some titers of anti-SAK suggests that the natural staphylococcal infection is also widely distributed among this animal species.

Neither STK nor SAK has such potent antigenicity in rabbits and dogs as other streptococcal and staphylococcal extracellular antigenic substances, e.g. streptolysin and staphylolysin. The failure in demonstrating antigenicity of STK and SAK in these experimental animals in our previous study[8], may be mainly attributed to the inadequateness of the method of immunization. However, the incompleteness of purification of the antigenic substances should also be considered as a possible cause of the weakness of antigenicity. In our experiments repoted previously[8] one of the dogs was immunized with SAK preparation in almost the same manner as that adopted in the present experiment with rabbit. However, the anti-SAK could not have been detected in the sera of that dog. This might be due to the impurity of the antigen preparation injected. The sample of SAK used in the previous experiment was a crude preparation containing not only staphylolysin but also some inert substances which may have inhibited the antigenic potency of the active specific antigenic molecules.

Since almost any patient may well have experienced natural infection of streptococci, it can be considered that the treatment with Varidase would act as a booster immunization. Indeed a marked rise in anti-STK titer has been clearly demonstrated in almost all cases in which Varidase were administered parenterally, as reported in our previous paper[8].

The positive results in agar-gel diffusion precipitin reaction was obtained in these experiments and the parallelism of the results of the precipitin reaction to those of the kinase-inhibiting reaction was noticed in each case of the experiment. However, since the antigens used for the immunization were not completely purified specimens

and since other antigenic substances were contained in these samples, the possibility, that the precipitin bands appearing may not necessarily be those formed by a combination of kinase and anti-kinase, can not be excluded.

The studies of the physicochemical nature of the immunoglobulins produced by the immunization with STK and SAK will be reported later separately.

Summary

In our previous experiments, because of the weakness of antigenicity of streptokinase (STK) and staphylokinase (SAK), the antibody response of rabbits and dogs to these extracellular bacterial substances could not be demonstrated by the measurement of antibody titers in the sera of the immunized animals, although the antibody response of humans to STK was proved by demonstrating the rise of anti-STK titers in the sera of human volunteers treated therapeutically with a commercial STK-preparation (Varidase-Lederle).

In our present experiments, however, the antigenicity of these substances was clearly demonstrated by the used of monkeys as the experimental animals, in substitution for rabbits, or by the improvement of the immunizing method in rabbits.

In the experiments with monkeys anti-STK and anti-SAK were readily produced in the sera of the immunized animals by the primary injections of STK and SAK respectively without adjuvant. In the case of a primary immunization of rabbits, however, the addition of complete Freund's adjuvant to STK or SAK was necessary for the production of anti-STK or anti-SAK in the sera of the immunized animals. Considerablly high titers of anti-STK or anti-SAK were detected in the sera of rabbits, receiving a booster injection after a long lapse of time following the primary injection of the same antigens.

The difference between the antibody response of monkeys and that of rabbits to STK and SAK was discussed in connection with natural infection of streptococci and staphylococci among these animal species.

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