Enzymatic Study to Characterize the Slow Reacting Substance of Anaphylaxis (SRS-A) and Leukotrienes

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Abstract—Slow reacting substance of anaphylaxis (SRS-A) has been shown to be one of the major mediators in hypersensitive reactions and to be composed of leukotriene (LT) C₄, LTD₄ and LTE₄. In the present study, we examined the properties of SRS-A released from sensitized guinea pig lungs by antigen and SRS released from rat peritoneal exudate cells and from human leucocytes by ionophore A23187 (0.5 and 0.2 μg/ml, respectively). By the incubation with SRS-A, SRS and LTs with arylsulfatase (type V) in pH 5.7 buffered solution at 37°C for 30 min, SRS-A and LTD₄ were greatly inactivated and rat SRS was slightly inactivated, but human SRS and LTC₄ were not inactivated at all. The same results were obtained when aminopeptidase was used in place of arylsulfatase. Moreover, when SRS-A, LTC₄ and LTD₄ were incubated with 0.02 mg/ml of γ-glutamyltranspeptidase (γ-GTP) pH 8.0 buffered solution at 37°C for 30 min, the activities of SRS-A and LTD₄ were slightly decreased, but those of SRS and LTC₄ were obviously potentiated. On the other hand, incubation with a large amount of γ-GTP (0.2 mg/ml) a dose at which this enzyme preparation showed clear aminopeptidase activity, SRS-A, SRS, LTC₄ and LTD₄ were obviously inactivated. In addition, we found a peak of LTD₄ in guinea pig SRS-A, that of LTC₄ in human SRS, and that of LTC₄ in rat SRS on high performance liquid chromatograms. From these results, we demonstrated that guinea pig lung SRS-A is mainly composed of LTD₄, human leukocyte SRS is mainly LTC₄, and rat peritoneal SRS is composed of both LTC₄ and LTD₄. The inactivation of LTD₄ and SRS-A by arylsulfatase may be due to aminopeptidase contamination in the enzyme preparation.

Slow reacting substance of anaphylaxis (SRS-A), one of the major mediators in hypersensitive reactions (1), has been proposed to consist of leukotriene (LT) C₄, LTD₄ and LTE₄ which are biosynthesized from arachidonic acid (AA) by lipoxygenase pathways (2). SRS-A or SRS has been found to be released from many types of tissues and cells in human, guinea pig and other animal lungs (3–5), human leukocytes (6, 7), rat peritoneal cells (4), cat paws (8), and leukemia basophils of rats and humans (9, 10) with immunological stimulations or ionophore A23187.

In the present study, we compare the characteristics of SRS-A released from sensitized guinea pig lungs, SRS from rat peritoneal exudate cells (PEC) and human leukocytes with that of LTC₄ and LTD₄ using converting enzymes of LTs such as arylsulfatase, aminopeptidase and γ-glutamyltranspeptidase (γ-GTP).

Materials and Methods
Preparation of SRS-A and SRS
a) Guinea pig lungs: Male Hartley guinea pigs weighing about 300 g were sensitized with 100 mg of bovine serum albumin (BSA) as described by Michell and Denborough (11). After 20 to 30 days, the lungs of sensitized animals were removed and finely chopped. The lung fragments suspended in 1.0 ml of the buffered solution containing 8 g/l NaCl, 0.2 g/l CaCl₂, 0.2 g/l KCl, 0.2 g/l
MgCl₂·6 H₂O, 1.0 g/l NaHCO₃, 1.0 g/l glucose and 5 units/ml heparin was incubated at 37°C for 10 min, and then challenged by 10 mg/ml BSA as an antigen, followed by incubation for a further 10 min. The reaction mixture solution was kept in ice and was strained through muslin to remove the lung fragments. The filtrate containing crude SRS-A was kept at −20°C.

b) Rat PEC: Male Sprague-Dawley rats weighing about 250 g were sacrificed by a blow on the head and exsanguination. Rat PEC suspension was prepared as described by Nakazawa et al. (12) using the buffered solution and incubated at 37°C for 10 min; then 0.5 μg/ml of ionophore A23178 (Calbiochem, La Jolla, U.S.A.) was added, and incubation was continued for a further 10 min. The reaction mixture was kept in ice and centrifuged at 180 × g for 10 min to remove the sediment. The supernatant containing crude SRS was stocked at −20°C.

c) Human peripheral leucocytes: Ten ml of citrated blood from healthy volunteers was mixed with 2.0 ml of 4% dextran (M.W. = 180000, Nakarai, Kyoto, Japan) dissolved in 0.9% sodium chloride. The mixture was allowed to stand for 1 hr at room temperature, and its upper phase was washed 2 or 3 times with the buffered solution at 4°C. Leucocytes (neutrophil:lymphocyte:eosinophil:monocyte:basophil = 82:10:4:3:0.8) suspension was reacted with 0.2 μg/ml of ionophore A23187, and the supernatant containing crude SRS was prepared as described in b).

Inactivation by arylsulfatase and aminopeptidase
An aliquot of SRS-A or SRS and 1.0 ng/ml of LTC₄ or LTD₄ was incubated with and without 4 units/ml of arylsulfatase type V (Limpet, Sigma, St. Louis, U.S.A.) and 0.5 units/ml of leucine aminopeptidase (porcine kidney microsomes type VI-S, Sigma) in the pH 5.7 buffered solution at 37°C for 30 min. Then the reaction mixture was kept in ice and bioassayed.

Conversion by γ-GTP
An aliquot of SRS-A or SRS and 1 ng/ml of LTC₄ or LTD₄ in the buffered solution (pH 8.0) containing 10 mM of MgCl₂ was incubated with and without 0.02 or 0.2 mg/ml of γ-GTP (γ-glutamyltransferase from beef kidney, Seikagaku Kogyo, Tokyo, Japan) at 37°C for 30 min. The mixture was kept in ice and bioassayed.

Bioassay
Bioassay of SRS-A, SRS and LTs was performed on the isolated guinea pig ileum which was mounted at a load of 1.0 g in a 5 ml organ bath filled with the Tyrode's solution and bubbled with air. The activity of SRS-A and SRS was assayed in the presence of 10⁻⁷ g/ml of atropine sulfate (Wako, Osaka, Japan) and 10⁻⁶ g/ml of mepyramine hydrochloride (Sigma). Activities of LTs were assayed in the absence of blockers. The contractile activities of SRS-A, SRS and LTs incubated with the enzyme solutions or various pH solutions were expressed as a percentage of the contraction by the respective non-incubated preparation.

Activity of aminopeptidase
7-L-leucyl-4-methyl-cumarynylamide hydrochloride (Sigma) was used as a substrate to determine aminopeptidase activity in the arylsulfatase and γ-GTP preparation. One ng/ml of the substrate was incubated with the aminopeptidase or arylsulfatase preparation in the pH 5.7 or pH 8.0 buffered solution at 37°C for 30 min. The mixture was kept in ice, and the fluorescence intensity was measured using an excitation wave length of 380 nm and an emission wave length of 440 nm by a fluorescence spectrophotometer (650–10S, Hitachi, Tokyo, Japan).

High-performance liquid chromatography (HPLC)
The filtrate from guinea pig lung fragments and the supernatant from human leucocytes and rat PEC were each added to 4 volumes of ethanol. LTC₄ and LTD₄ were extracted with the procedure described by Koshihara and Isono (13). The fractions eluted with methanol from a Sep-Pak C18 column (Waters) were analyzed by HPLC using methanol : water : acetic acid (60:30:0.05), adjusted pH 5.1 with ammonium hydroxide, as a solvent, with a flow rate of 1.0 ml/min, (column, YMC-PACK A303 ODS; size, 4.6×250 mm; TRI ROTAR-VI and UV/DECT-100-VI, Nipponbunko, Tokyo, Japan).

Results
Release of SRS-A and SRS: The filtrate
prepared from guinea pig lung fragments and the supernatant prepared from rat PEC and human leucocytes stimulated with ionophore A23187 caused a potent dose-dependent contraction of isolated guinea pig ileum (Fig. 1). Similarly, LTC₄ and LTD₄ contracted the ileum dose-dependently. The activity of LTD₄ was about 10 times as potent as that of LTC₄, and it was about 100 times as potent as that of histamine (Fig. 2).

Figure 3 shows the antagonistic effects of FPL-55712 on the bioactive substances in the contractions of the guinea pig ileum. The contractile responses by the filtrate from guinea pig lungs, the supernatant from rat PEC and human leucocytes, LTC₄ and LTD₄ were markedly inhibited by 10⁻⁵ or 10⁻⁶ M of FPL-55712.

Stability to acid and base: Figure 4 shows the stability of SRS-A, rat SRS, human SRS, LTC₄ and LTD₄ in the buffered solutions of acidic and alkaline pH. SRS-A and LTD₄ were incubated for 15 min at pH 3.0, 6.8 and 11.0, respectively. The bioactivity of SRS-A and LTD₄ decreased to 56% and 37% at pH 3.0, and it decreased to 66% and 68% at pH 11.0, respectively. These substances were more stable at the alkaline pH than at the acidic pH. When human SRS or rat SRS and LTC₄ were incubated for the longer period of 120 min at 37°C under the same condition of pH as described above, these substances were found be considerably stable at the alkaline pH and markedly inactivated at the acidic pH.

Inactivation by arylsulfatase and amino-
peptidase: Figure 5 shows the inactivation of guinea pig SRS-A, rat SRS, human SRS, LTC₄ and LTD₄ by arylsulfatase and aminopeptidase. Although SRS-A and LTD₄ was markedly inactivated by the enzyme to 20% (P<0.01) and to 49% (P<0.01), respectively, the activity of rat SRS was slightly decreased. On the other hand, human SRS and LTC₄ was not inactivated at all. The similar results were obtained from the experiments using aminopeptidase.

Conversion by γ-GTP: Figure 6 shows changes in the activities of guinea pig SRS-A, rat SRS, human SRS, LTC₄ and LTD₄ incubated with 0.02 or 0.2 mg/ml of γ-GTP. When SRS-A, SRS and LTs were incubated with 0.02 mg/ml of γ-GTP, the bioactivities of LTC₄, human SRS and rat SRS increased 1.5 (P<0.01), 1.3 (P<0.05) and 1.09 times, respectively, while the activities of SRS-A and LTD₄ (P<0.05) were decreased. On the other hand, when SRS-A, SRS and LTs were
Fig. 4. Decrease in the activity of guinea pig SRS-A (1), rat SRS (2), human SRS (3), LTD₄ (4) and LTC₄ (5) in the buffered solution of each pH. SRS-A and LTD₄ were incubated for 15 min, while human SRS, rat SRS and LTC₄ were incubated for 120 min. Each value indicates the mean of 3 or 4 experiments. Vertical bars show the S.E. of the mean.

Fig. 5. Inactivation of guinea pig SRS-A, rat SRS, human SRS, LTC₄ and LTD₄ by 4 units/ml of arylsulfatase type V (upper panel) and 0.5 units/ml of aminopeptidase (lower panel). Each value indicates the mean of 4 or 6 experiments. Vertical bars show the S.E. of the mean. ** and ***: significantly different from the value without the enzyme (−) at P<0.01 and P<0.001, respectively. (+) incubated with arylsulfatase in the pH 5.7 buffered solution at 37°C for 30 min. (−) incubated without the enzyme.

incubated with 0.2 mg/ml of γ-GTP, which is contaminated with aminopeptidase activity, these bioactive substances were inactivated by 50% or more (P<0.01 or P<0.05).

Separation by HPLC: Figure 7 shows chromatographic differences of guinea pig SRS-A, rat SRS and human SRS. There is mainly a peak of LTD₄ on the chromatogram of SRS-A. Conversely, a peak of LTC₄ is mainly found in human SRS. There are two peaks corresponding to LTC₄ and LTD₄ in rat SRS.

Discussion
The filtrate from immunologically challenged lungs of guinea pig sensitized to BSA, the supernatant from human leucocytes or rat PEC treated with ionophore A23187 produced a large quantity of substances that contracted the isolated guinea pig ileum in the presence of atropine and mepyramine (Fig. 1). The contraction was markedly inhibited by the selective anti-SRS-A agent FPL-55712 (Fig. 3). These bioactive substances were more stable at an alkaline pH than at an acidic pH. LTC₄ and LTD₄ showed the same properties (Fig. 4). Thus we demonstrated that SRS-A and SRS is released from sensitized guinea pig lungs, rat PEC and human leucocytes.

LTC₄ and/or LTD₄ have been identified in SRS-A or SRS released from various sources. Morris et al. (5) demonstrated that SRS-A released from sensitized guinea pig lungs by the addition of antigen was identified as LTD₄ mainly. Bach et al. (14, 15) reported that SRS produced by rat peritoneal mononuclear cells with ionophore A23187 consists of two main
components, LTC₄ and LTD₄. Moreover, when human leucocytes were incubated with LTA₄, the main product with SRS activity is LTC₄ (6). In our HPLC experiments, LTD₄ was identified in guinea pig SRS-A, LTC₄ was in human SRS, and both LTC₄ and LTD₄ were in SRS from rat PEC (Fig. 7). Furthermore, from the experiments on the inactivation by arylsulfatase and aminopeptidase, the susceptibility to γ-GTP, and the stability at the various pH's, our presented results suggest that guinea pig SRS-A is mainly composed of LTD₄, human SRS is mainly LTC₄, and rat SRS consists of LTC₄ and LTD₄.

A variety of arylsulfatases have been known to inactivate the spasmogenic activity of SRS-A (16–18). Consequently, it is suggested that arylsulfatase may be responsible for the inactivation of SRS-A in vivo following antigen-antibody reaction (17). Moreover, the susceptibility to arylsulfatase was used as an important criterion in the positive identification of SRS-A until recently. However, it has been shown that while the various forms of SRS-A or SRS contain sulfur, the sulfur is bound in a thioether rather than a sulfate ester linkage (4, 9, 19). Therefore, it has been suspected that the inactivation of SRS-A by the arylsulfatase preparation is due to a contaminating protease rather than to the arylsulfatase itself. This enzyme preparation cleaves the peptide bond in highly bioactive LTD₄ (SRS-cystinyl-glycyl form) to form much less bioactive LTE₄ (SRS-cystinyl form) (8, 20, 21). In our experiments, limpet arylsulfatase type V which was contaminated by aminopeptidase activity markedly inactivated LTD₄ and
inactivated guinea pig SRS-A and rat SRS to a lesser extent, but did not inactivate LTC₄ and human SRS at all (Fig. 5). The same results were obtained from the susceptibility to leucine aminopeptidase. From these results, we suggest that the inactivation of LTD₄ and guinea pig SRS-A by the arylsulfatase preparation is due to aminopeptidase contamination in the enzyme preparation rather than the arylsulfatase itself.

γ-GTP, which localizes mainly on the outer surface of the membrane of certain cells, converts LTC₄ to LTD₄ by the removal of a glutamyl residue (9). This transformation increases the biological activity on the guinea pig ileum (8, 9). In this study, the activity of LTD₄ was about 10 times more potent than that of LTC₄ in the isolated guinea pig ileum (Fig. 2). Although 0.02 mg/ml of γ-GTP potentiated the bioactivity of LTC₄ and human SRS to 1.3 and 1.5 times, respectively, and slightly potentiated that of rat SRS, that of guinea pig SRS-A and LTD₄ did not increase by the enzyme. Thus, we think that LTC₄ contained in human and rat SRS was converted to highly bioactive LTD₄ by γ-GTP. On the other hand, 0.2 mg/ml of γ-GTP containing aminopeptidase activity clearly inactivated guinea pig SRS-A, human SRS, rat SRS and LTs (Fig. 6). These results suggest that LTC₄ and SRS is converted to LTD₄ by the removal of a γ-glutamyl residue, then, converted to less bioactive LTE₄ by aminopeptidase in the γ-GTP preparation, while guinea pig SRS-A and LTD₄ is directly converted to less bioactive LTE₄ by the aminopeptidase contaminating the γ-GTP preparation.

References
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