A New Individual Variation of Saliva Detected with Rabbit Anti-Human Saliva Serum

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IWASA, M., OHYA, I., YOKOI, T. and SAGISAKA, K. *A New Individual Variation of Saliva Detected with Rabbit Anti-Human Saliva Serum.* Tohoku J. exp. Med., 1985, **145**(3), 283-290 — A rabbit anti-saliva hyperimmune serum was raised against pooled human saliva. After absorption with human serum and semen, the immune serum formed two or three distinct precipitation lines with human saliva by the Ouchterlony method. Based on the presence or absence of a precipitation line, close to the antigen well designated as PI, all human saliva were divided into two groups; PI⁺ and PI⁻. The incidence of PI⁺ saliva was 114 out of 154 samples (74%). By immunoelectrophoresis with anti-saliva serum pre-absorbed with PI⁻ saliva, PI⁺ saliva formed a precipitation line in cathodal area. No amylase activity was found in this precipitation line but it was stained with the Schiff's reagent. PI protein was isolated by Sephadex G-100 and ion-exchange chromatography. Isoelectric focusing of the PI protein in polyacrylamide slab gel containing urea revealed that the protein differed from any known parotid salivary proteins such as Pr, Db, Pa and Pm. ——— saliva; anti-saliva; individual variation

In forensic science, identification of saliva stains has been performed by enzymatic and serological methods, i.e., amylase (Nelson and Kirk 1963; Willott 1974; Whitehead and Kipps 1975) and antiserum to saliva (Baxter and Ree 1975; Eckersall et al. 1981). Recently a number of genetic markers in saliva were reported which could be classified into two categories; the former was known as isozyme polymorphisms such as amylase (Ward et al. 1971), acid phosphatase (Tan and Ashton 1976a), glucose-6-phosphate dehydrogenase (Tan and Ashton 1976b), esterase (Tan 1976), etc. and the latter was protein polymorphisms of parotid saliva; Pb (Azen 1972), Pr (Azen and Oppenheim 1973), Db (Azen and Denniston 1974), Pa (Friedman et al. 1975), Pm (Ikemoto et al. 1977), etc. In this paper an individual variation of saliva detected with rabbit anti-human saliva serum was described.

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MATERIALS AND METHODS

Human saliva, serum and seminal plasma

Saliva samples were collected from 154 healthy individuals and were frozen at -20°C . Samples were fused at 4°C and centrifuged at 3,000 rpm for 30 min. Supernatants free of mucin were separated and stored at -20°C until use. Serum from five individuals was pooled. Semen from five persons was centrifuged at 3,000 rpm for 20 min, and the supernatant was pooled.

Rabbit anti-saliva serum

Three rabbits were intramuscularly injected with 1 ml of pooled saliva (protein concentration 0.8 mg/ml) once a week for five weeks. For the initial two injections, emulsion of the immunogen with the same volume of Freund's complete adjuvant was used. Ten days after the last immunization, the rabbits were bled. Anti-saliva serum was absorbed with human serum and semen in a test tube at the appropriate ratio. The mixture was allowed to stand at 4°C for 12 hr, followed by centrifugation at 10,000 rpm for 30 min.

Immunodiffusion, immunoelectrophoresis and isoelectric focusing

Immunodiffusion and immunoelectrophoresis were performed using 1% agarose (Litex, Glostrup, Denmark) and veronal buffer described by Hirshfeld (1965). Amylase activity of precipitation lines on the agarose gel was detected by the starch-iodine reaction, and carbohydrate was stained with the Schiff's reagent. Isoelectric focusing of saliva was performed in 7% polyacrylamide slab gel containing 5 M urea as described by Azen and Denniston (1981).

Extracts of salivary glands

Submaxillary, parotid and sublingual glands were homogenized with 10-fold volumes of saline. The homogenates were centrifuged at 10,000 rpm for 30 min and the supernatants were separated.

Isolation of salivary protein

Isolation of salivary protein was performed by the method of Masson et al. (1965) with slight modifications. Pooled saliva was dialyzed against 0.05 M phosphate buffer pH 8.0 and applied onto a Sephadex G-100 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) column which was equilibrated with the same buffer. Protein concentration of the effluents was examined with absorbance at 280 nm and saliva specific antigen of each fraction was monitored by the Ouchterlony method with absorbed anti-saliva serum. The fractions containing the antigen were concentrated by ultrafiltration (Toyo Kagaku, cut off mol. wt. 10,000). After dialysing against 0.05 M phosphate buffer pH 8.0 the fractions were applied onto a DE 32 (Whatman Inc., Clifton, New Jersey, USA) column which was equilibrated with the same buffer. Elution was carried out stepwise with the starting buffer, 0.15 M phosphate buffer pH 6.0 and 0.15 M phosphate buffer pH 6.0 containing 2.0 M NaCl. The antigen rich fractions were pooled and dialyzed against 0.005 M phosphate buffer pH 5.0. The fraction was applied onto a CM 32 (Whatman Inc., Clifton, New Jersey, USA) column which was equilibrated with the same buffer. Elution was carried out stepwise with the starting buffer, 0.3 M phosphate buffer pH 5.0 and 0.3 M phosphate buffer pH 7.5. The final preparation was dialyzed against distilled water and lyophilized.

RESULTS

Absorption of crude anti-saliva serum with serum and semen

Crude anti-saliva serum formed clear precipitation lines against saliva, serum

Fig. 1. Ouchterlony test of 12 saliva samples with specific anti-saliva serum. Wells 1-18, saliva samples; Well A, anti-saliva serum absorbed with serum and semen. Two to three precipitation lines were developed but Nos. 2, 7, 13, 14 and 15 failed to develop a precipitation line near the antigen well.

and seminal plasma by the Ouchterlony method. After absorption of crude anti-saliva serum with human serum (ratio 1 : 0.05) and semen (ratio 1 : 0.3), an antiserum specific for saliva was obtained, which formed two to three precipitation lines against saliva only. However, some samples failed to develop a precipitation line near the antigen well (Fig. 1).

Absorption of antiserum specific for saliva

Saliva specific antiserum was further absorbed with saliva lacking of a precipitation line near the antigen well at a ratio of 1 : 0.3. Absorbed antiserum could divide saliva samples into two groups (Pl^+ and Pl^-) depending on the precipitation line (Fig. 2). Incidence of Pl^+ and Pl^- types among 154 subjects is

Fig. 2. Immunodiffusion test of 20 saliva samples with antiserum specific for Pl protein. Wells 1-20, saliva samples; Trough, antiserum specific for Pl protein. Nos. 5, 7, 8, 9, 10, 12, 13, 14, 15, 16 and 20 were grouped as Pl^+ type, and Nos. 1, 2, 3, 4, 6, 11, 17, 18 and 19 were grouped as Pl^- type.

TABLE 1. Incidence of Pl^+ and Pl^- type in saliva

	Number	Percent
Pl^+	114	74
Pl^-	40	26
Total	154	100

shown in Table 1.

Immunoelectrophoresis and immunodiffusion using absorbed anti-saliva serum

Saliva of PI^+ and PI^- types were examined by immunoelectrophoresis using anti-saliva serum reacting with only PI^+ saliva. As shown in Fig. 3, PI^+ saliva

Fig. 3. An immunoelectrophoretic pattern of PI^+ and PI^- type saliva with anti-saliva serum. Well 1, PI^- saliva; Well 2, PI^+ saliva; Trough A, crude anti-saliva serum; Trough B, specific anti-saliva serum; Trough C, antiserum specific for PI protein. PI^+ type saliva developed a precipitation line at cathodal area (indicated by an arrow).

Fig. 4. A histogram of area of precipitation rings developed with antiserum specific for PI protein. Immunodiffusion test was performed with agarose gel plates containing 10% the antiserum. Three μ l of PI^+ type saliva from 63 subjects was applied to the well. After standing at room temperature for 24 hr, precipitation rings were measured.

developed a precipitation line at cathodal area. This line had no amylase activity but stained with the Schiff's reagent. At immunoelectrophoresis of extracts from salivary glands, extracts of submaxillary and parotid glands gave a precipitation line near the origin. To examine the concentration of the protein reacting with the antiserum, relative quantitation on 63 subjects was performed by a single radial immunodiffusion technique with the antiserum. A histogram of the area of each precipitation ring is shown in Fig. 4.

Isolation of P_I protein and isoelectric focusing

P_I⁺ type saliva dialyzed against 0.05 M phosphate buffer pH 8.0 was fractionated in Sephadex G-100. Ouchterlony test on the two fractions (Frs. 1 and 2) separated with the gel filtration revealed that P_I protein resided only in Fr. 1 (Fig. 5). Then, Fr. 1 was separated in DE 32 with stepwise elution. Of three fractions separated, only the first fraction (Fr. 1-1) contained P_I protein (Fig. 6). Further fractionation of Fr. 1-1 in CM 32 column revealed that P_I protein was contained in only the second fractions (Fr. 1-1-2) (Fig. 7). The final preparation was dialyzed against distilled water and lyophilized. Isoelectric focusing pattern of P_I⁺, P_I⁻ salivas and purified P_I protein on 7% polyacrylamide gel containing carrier ampholite (pH 3.5-5.2) was shown in Fig. 8. P_I protein migrated to cathodal area as a broad band.

Fig. 5. Chromatography of saliva on Sephadex G-100 with 0.15 M phosphate buffer pH 7.0. Two ml of saliva (protein concentration 6 mg/ml) was applied onto a Sephadex G-100 column of 3 × 45 cm in size. Ouchterlony test of each fraction with antiserum specific for P_I protein showed that the fraction indicated by the bar contained P_I protein.

Fig. 6. Chromatography of the first fraction from Sephadex G-100 chromatography on DE 32. The first fraction dialyzed against 0.05 M phosphate buffer pH 8.0 was applied onto a DE 32 column. Elution was carried out stepwise with the starting buffer, 0.15 M phosphate buffer pH 6.0 and 0.15 M phosphate buffer pH 6.0 containing 2 M NaCl. The fraction indicated by the bar contained P1 protein.

Fig. 7. Chromatography of the first fraction from DE 32 chromatography on CM 32. The first fraction dialyzed against 0.005 M phosphate buffer pH 5.0 was applied onto a CM 32 column. Elution was carried out stepwise with the starting buffer, 0.3 M phosphate buffer pH 5.0 and 0.3 M phosphate buffer pH 7.5. The fraction indicated by the bar contained P1 protein.

DISCUSSION

Recent developments in analytical methods enable to elucidate many genetic markers in serum and saliva. It was generally known that quantitative difference in saliva constituents among individuals was more evident than that in serum

Fig. 8. Isoelectric focusing of PI^+ , PI^- type saliva and purified PI protein. Nos. 1, 2, 3 and 4, PI^+ type saliva; No. 5, purified PI protein; Nos. 6, 7, 8 and 9, PI^- type saliva. Purified PI protein migrated to cathodal area as a broad band.

proteins (Claman et al. 1967). The saliva protein discussed in this study was considered as one of saliva specific substances. As precipitin activity of specific antiserum for PI^+ saliva was abolished completely by absorption with PI^- saliva at higher ratios, the classification with the antiserum was attributed to quantitative difference. Masson et al. (1965) examined saliva proteins with ion-exchange chromatography and immunoelectrophoresis in which 9 of 15 salivary proteins were identified as saliva specific. By comparing the precipitation lines and chromatographical behaviours of PI protein and those described by Masson et al. (1965), PI protein was considered to correspond with a protein designated as β_2Sa by these authors.

Eckersall and Beeley (1980) reported that greater heterogeneity in saliva proteins could be demonstrated by two new methods, crossed immunoelectrophoresis and crossed immunoelectrofocusing. Azen and Denniston (1981) reported genetic polymorphisms of parotid proline-rich protein. They adopted isoelectric focusing using polyacrylamide slab gel containing carrier ampholite of pH range 3.5-5.2, by which genetic polymorphisms in parotid saliva such as Pr , Db and Pa were detected simultaneously. Purified PI protein migrated to cathodal area as a broad band under the condition. Accordingly, it seemed that PI protein was independent with parotid salivary proteins showing the polymorphisms. The problems that PI protein is a genetic marker or not and biochemical nature of PI protein are under investigation.

References

- 1) Azen, E.A. (1972) Genetic polymorphism of basic protein from parotid saliva. *Science*, **176**, 673-674.
- 2) Azen, E.A. & Denniston, C.L. (1974) Genetic polymorphism of human salivary proline-rich proteins: further genetic analysis. *Biochem. Genet.*, **12**, 109-120.
- 3) Azen, E.A. & Denniston, C. (1981) Genetic polymorphism of PIF (parotid isoelectric focusing variant) proteins with linkage to the PPP (parotid proline-rich protein) gene complex. *Biochem. Genet.*, **19**, 475-485.

- 4) Azen, E.A. & Oppenheim, F.G. (1973) Genetic polymorphism of proline-rich human salivary proteins. *Science*, **180**, 1067-1069.
  - 5) Baxter, S.T. & Ree, B. (1975) The identification of saliva in stains in forensic casework. *Med. Sci. Law*, **15**, 37-41.
  - 6) Claman, H.N., Merrill, D.A. & Hartley, T.F. (1967) Salivary immunoglobulines: Normal adult values and dissociation between serum and salivary levels. *J. Allergy*, **40**, 151-159.
  - 7) Eckersall, P.D. & Beeley, J.A. (1980) A comparison of crossed immunoelectrophoresis and crossed immunoelectrofocusing for the investigation of human salivary proteins. *Electrophoresis*, **1**, 62-67.
  - 8) Eckersall, P.D., Beeley, J.A., Dolton, P., Whitehead, P.H., Fletcher, S.M. & Eynon, K. (1981) The production and evaluation of an antiserum for the detection of human saliva. *J. Forens. Sci. Soc.*, **21**, 293-300.
  - 9) Friedman, R.D., Merritt, A.D. & Rivas, M.L. (1975) Genetic studies of human acidic salivary protein. *Amer. J. hum. Genet.*, **27**, 292-303.
  - 10) Hirshfeld, J. (1960) Immuno-electrophoresis procedure and application to the study of group specific variation in sera. *Science Tools*, **7**, 18-25.
  - 11) Ikemoto, S., Minaguchi, K., Suzuki, K. & Tomita, K. (1977) New genetic marker in human parotid saliva (Pm). *Science*, **197**, 378-379.
  - 12) Masson, P.L., Carbonaram, A.O. & Heremans, J.F. (1965) Studies on the proteins of human saliva. *Biochim. biophys. Acta*, **107**, 485-500.
  - 13) Nelson, D.F. & Kirk, P.L. (1963) The identification of saliva. *J. forens. Med.*, **10**, 14-21.
  - 14) Tan, S.G. (1976) Human saliva esterases: genetic studies. *Hum. Hered.*, **26**, 207-216.
  - 15) Tan, S.G. & Ashton, G.C. (1976a) Saliva acid phosphatases: genetic studies. *Hum. Hered.*, **26**, 81-89.
  - 16) Tan, S.G. & Ashton, G.C. (1976b) An autosomal glucose-6-phosphate dehydrogenase (hexose-6-phosphate dehydrogenase) polymorphism in human saliva. *Hum. Hered.*, **26**, 113-123.
  - 17) Ward, J.C., Merritt, A.D. & Bixler, D. (1971) Human salivary amylase: genetics of electrophoretic variants. *Amer. J. hum. Genet.*, **23**, 403-409.
  - 18) Whitehead, P.H. & Kipps, A.E. (1975) A test paper for detecting saliva stains. *J. forens. Sci. Soc.*, **15**, 39-42.
  - 19) Willott, G.M. (1974) An improved test for the detection of salivary amylase in stains. *J. forens. Sci. Soc.*, **14**, 341-344.
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