Comparative Studies of Non–Hemoglobin Proteins of Adult and Newborn Red Blood Cell Lysates

KAORU SAGISAKA, MINEO IWASA, HIROKO YAMASHITA and SADAO YAMADA

Department of Legal Medicine, Gifu University School of Medicine, Gifu 500

SAGISAKA, K., IWASA, M., YAMASHITA, H. and YAMADA, S. Comparative Studies of Non-Hemoglobin Proteins of Adult and Newborn Red Blood Cell Lysates. Tohoku J. exp. Med., 1977, 123(3), 287–294—It has been known that hemolysate is composed of not only hemoglobin but also non-hemoglobin proteins (NHP). NHP isolated effectively from hemolysate of adult and newborn by CM-Sephadex chromatography was investigated by polyacrylamide disc or slab electrophoresis. It was demonstrated on slab electrophoresis that adult NHP is composed of 4 zones (approximately 14 bands) with different mobilities and that adult NHP shows a considerably different pattern from cord NHP. The difference was observed also on the electrophoresis stained for esterase or lactate dehydrogenase. Rabbit antiserum to adult NHP was adsorbed with cord NHP and specific antiserum for adult NHP was prepared. However, antiserum reacting with only cord NHP was not obtained from anti-cord NHP serum. These results suggested that adult specific components with passable antigenicity were present in adult NHP and that cord NHP had similar antigenicity as adult NHP. non-hemoglobin protein; adult specificity

Recent progress in separating techniques such as electrophoresis and column chromatography demonstrates that hemolysate is composed of many constituents. Stern et al. (1945) pointed out by Tiselius electrophoresis that hemolysate is composed of not only hemoglobin but also many non-hemoglobin proteins (NHP). Haut et al. (1962) reported that about 6% of hemolysate was estimated to be NHP. Moreover, Haut et al. (1964) revealed subsequently that some NHP zones on the starch gel electrophoresis had esterase, lactate dehydrogenase or glucose-6-phosphate dehydrogenase activity. Recently the significance of those constituents has been biochemically and genetically elucidated. In this paper, polyacrylamide disc or slab electrophoresis, of which resolution was much higher than other electrophoresis methods, was adopted to analyze NHP of adult and newborn, and adult specific components in NHP were immunologically investigated.

MATERIALS AND METHODS

Preparation of hemolysate. Blood samples from healthy adults and cord were washed five times with five-fold volumes of saline. Hemolysate was prepared by lysing the cells.
with an equal volume of distilled water and one-half volume of toluene. After shaking well, the mixture was centrifuged at 10,000 rpm for 20 min and cell debris was discarded. The hemolysate was dialysed against tap water and then against 0.01 M phosphate buffer, pH 5.9.

Preparation of NHP. CM-Sephadex (Farmacia Fine Chemicals, Sweden) was equilibrated with 0.01 M phosphate buffer, pH 5.9. The hemolysate was applied onto a CM-Sephadex column and eluted with the equilibrating buffer and with 0.1 M phosphate buffer, pH 7.4, containing 0.1 M NaCl. NHP was concentrated with ultrafiltration (Toyokagaku Co., type UK-10, cut off M.W. 10,000).

Polyacrylamide disc and slab electrophoresis. Seven percent polyacrylamide running gel of pH 8.9 and spacer gel of pH 6.7 as described by Davis (1964) were used. For slab electrophoresis, 7% gel of 2 mm in thickness and the same buffers as used in disc electrophoresis were employed.

Preparation of carbonic anhydrase (CA). CA was separated from adult hemolysate with the chloroform-ethanol extraction method described by Huennekens et al. (1957).

Antisera to adult NHP (NHPA) and cord NHP (NHPC). Rabbits were intramuscularly immunized with 1 ml of 2% NHP and an equal volume of Freund's complete adjuvant. After 5 to 7 weekly immunizations the rabbits were bled.

Adsorption of anti-NHP sera. Antiserum to NHPA or NHPC was adsorbed with NHPC or NHPA with various ratios in volume at 4°C for 3 hr. After centrifugation at 10,000 rpm for 30 min, the supernatant was removed.

Immunoelectrophoresis and immunoelectrosyneresis. One percent agarose plate and veronal buffer containing calcium lactate described by Hirschfield (1960) were used. For electrosyneresis, wells of 0.2 cm in diameter and 8 μl in capacity were cut at an interval of 0.5 cm in parallel. Both the electrophoresis were conducted at 10 V per cm for 30 to 45 min.

Esterase and lactate dehydrogenase (LDH). The enzyme activities of the acrylamide gels were detected with the following substrates. For esterase, the gels were incubated in 25 ml of 0.1 M phosphate buffer pH 7.3 containing 5 mg alpha-naphtyl acetate and 25 mg Fast blue RR salt at 37°C for 1 hr. For LDH, the gels were stained in the dark at 37°C for 1 hr in 50 ml of 0.1 M tris-HCl buffer, pH 8.0, containing 0.25 ml of 10% lactic acid, 5 mg NAD, 5 mg MTT tetrazolium and 5 mg phenazine methosulphate as described by Giblett (1969).

RESULTS

Preparation of NHP. As shown in Fig. 1, three fractions were eluted with two buffers. The first fraction with faint yellow was proved to contain no hemoglobin (Hb) by staining with benzidine. The second fraction had a light color of Hb, whereas thick Hb was eluted with the second buffer. The similar eluting pattern was observed on the chromatography of NHPC.

Electrophoretic analyses of NHP. Disc electrophoresis revealed numerous NHP in the first fraction (tube No. 4). The second fraction (No. 13) consisted of Hb and NHP; the former corresponded to major component of Hb, HbA0, and the latter was estimated to be isozyme of CA, CAB, from their electrophoretic mobilities. On the other hand, the last fractions (Nos. 57 and 59) contained major (HbA0) and minor (HbA2) components of Hb but not any NHP (Fig. 2).

Slab electrophoretic patterns of pooled NHPA and NHPC are shown in Fig. 3.
Fig. 1. Chromatography of adult hemolysate on a CM-Sephadex column.

Twenty ml of adult hemolysate was applied on a column (2.5 x 25 cm) and eluted stepwise with 80 ml of 0.01 M phosphate buffer, pH 5.9, and with 100 ml of 0.1 M phosphate buffer, pH 7.4, containing 0.1 M NaCl. Optical densities of each fraction (2.5 ml) were measured at 280 (---) and 415 (----) nm. The scale of the tube numbers from No. 47 to 67 is reduced to 1/50.

Fig. 2. A polyacrylamide disc electrophoretic pattern of adult hemolysate fractionated with CM-Sephadex chromatography.

The numerals show the tube numbers of CM-Sephadex chromatography (Fig. 1). Tube No. 16 consisted of numerous NHPs and no Hb. A small amount of Hb was contained in tubes No. 25 and 27 and it corresponded to major component of Hb, HbA0. Tube No. 59 composed of HbA4 and minor component of Hb, HbA2, and no NHP.

Electrophoretic bands of NHPA could be separated into four zones (A, B, C and D). A zone was composed of two bands, and the thicker band was identified as CAB by comparison with the pattern of CA. There were three bands in B zone which were not differentiated on the photograph. C zone consisted of three definite bands, and D zone of several indistinct bands. One band of B zone was estimated to be an isozyme of CA, CAA. NHPC had a considerably different pattern from NHPA at the following several points: A zone and the latest moving band of C zone were
Fig. 3. Polyacrylamide slab electrophoresis of NHPA, NHPC and CA (above) and its schema (below).

NHPA consisted of 4 zones (A, B, C, and D) of bands. A zone, which was estimated to be CA isozymes (CAA and CAB) from the electrophoretic pattern of CA, was obscure in the track of NHPC. In C zone, the thick band moving lastly in NHPA was not apparent in NHPC. On the other hand, the band moving fastestly in this zone was thicker in NHPC than in NHPA, and NHPC contained several bands at D zone which were more definite than in NHPA.

very faint, whereas at D zone, two to three bands, which were thicker than those of NHPA, were observed. At B and C zones except the above-mentioned bands, the similar pattern to NHPA was noted. LDH staining of the gels demonstrated that NHPA and NHPC had four and five zones, respectively. Each of LDH–2 and –3, which had thicker intensity than the others, consisted of two bands. The intensities of LDH–3 and –4 in NHPC were extremely low as compared with those in NHPA (Fig. 4).

On the immunoelectrophoresis, antisera to NHPA and NHPC exhibited different patterns; anti-NHPA serum produced two potent lines against NHPA at the origin and a long line extending to cathodal side against NHPC. At the anodal area, the antiserum produced several faint lines against NHPA, but those were not apparent against NHPC. Anti-NHPC serum produced somewhat fewer lines against NHPA than anti-NHPA did (Fig. 5). Esterase staining showed that anti-NHPA
Fig. 4. Polyacrylamide disc electrophoresis of NHPA and NHPC. Track A, NHPA; Track C, NHPC.
Four LDH zones were observed at NHPA. The LDH-2 and -3 zones composed of two bands, respectively. The intensities of LDH-3 and -4 zones of NHPC were extremely lower than those of NHPA. The latestly moving band, LDH-5, was observed in only NHPC.

Fig. 5. Immunoelectrophoretic patterns of NHPA and NHPC against anti-NHPA and anti-NHPC.
Well 1, NHPA; Well 2, NHPC; Trough A, anti-NHPA; Trough C, anti-NHPC (details in the text).

Fig. 6. Immunoelectrophoretic patterns of NHPA and NHPC against anti-NHPA and anti-NHPC stained for esterase.
Abbreviations were the same as those in Fig. 5.
Anti-NHPA serum produced three precipitation lines against NHPA and two lines against NHPC. On the other hand, anti-NHPC serum gave a weak line against NHPC and two weak lines against NHPA which were not apparent in the photograph.

serum produced three lines with the activity against NHPA and two lines against NHPC (Fig. 6). The line locating near the origin was observed only between anti-NHPA serum and NHPA. The line with LDH activity, which was not recognized before staining, was detected near the origin only between anti-NHPA serum and NHPA.
Fig. 7. Immunoelectrophoretic patterns of NHP against specific anti-NHPA serum. Well 1, NHPA; Well 2, NHPC; Well 3, CA; Trough A, specific anti-NHPA serum. Three precipitation lines were detected near the origin only in the reaction with NHPA.

Fig. 8. Immunoelectrosyneresis of NHP against specific anti-NHPA serum. A, specific anti-NHPA serum; 1, NHPA; 2, NHPC; 3, CA; 4, adult serum. A clear precipitation line was detected only with NHPA.

Preparation of specific anti-NHPA serum. Anti-NHPA and anti-NHPC sera were adsorbed with NHPC, NHPA and CA at various ratios in volume. When 16 parts of anti-NHPA serum (No. 1) was adsorbed with 6 parts of NHPC (12 mg/ml) and one part of CA (22 mg/ml), specific anti-NHPA serum was prepared, whereas NHPA removed all of the antibody in anti-NHPC serum. Fig. 7 shows that specific anti-NHPA serum produced three precipitation lines against only NHPA, one of which was located near the origin and the others rather at anodal region. The precipitation line at the origin had esterase activity, and a line of anodal area had slight enzymatic activity of LDH. The specificity of adsorbed antiserum was confirmed by immunoelectrosyneresis in which all NHP from 70 normal subjects gave positive reaction (Fig. 8).

DISCUSSION

To separate NHP from hemolysate, column chromatography using many ion-exchangers has been adopted (Haut et al. 1962, 1964; Rickli et al. 1964). In
the present experiment, CM-Sephadex with two phosphate buffers was proved to be useful for effective separation. Following the first peak of NHP, NHP containing a small amount of Hb which had the same electrophoretic mobility as that of HbA\textsubscript{0} were eluted from adult and cord hemolysates. It might indicate the heterogeneity of HbA\textsubscript{0} as demonstrated electrophoretically by Kuenzer (1959) or combining capacity of some NHP with HbA\textsubscript{0}. On the slab electrophoresis, clearly different pattern was observed between NHPA and NHPC. Haut et al. (1962) demonstrated that adult and cord hemolysates were separated into 4 bands on starch gel electrophoresis. The fastest moving band of NHP was proved to have CA activity. However, this investigation did not yet point out the difference in pattern between NHPA and NHPC. According to the advanced investigation by Haut et al. (1964), concentrated NHP which was separated by DEAE-cellulose showed eleven bands. On the other hand, Sawamura (1969) reported that NHP isolated by CM-cellulose chromatography contained 18 bands of which pattern differed slightly from that of cord NHP. LDH zymogram exhibited also the difference between NHPA and NHPC. Sawamura (1969) reported that LDH activity of NHPC was higher than in NHPA, although similar electrophoretic pattern was noted at the two NHPs. These discrepancies might depend on the procedure to prepare NHP or on the concentration of NHP tested. The results of immuno-electrophoresis supported the difference of constituents between NHPA and NHPC. Anti-NHPA serum produced more precipitation lines against NHPA than against NHPC.

By esterase staining, two or three lines were noted between antisera and NHPs. Moreover, the esterase active line at the origin was observed only between anti-NHPA and NHPA. It was confirmed by the reaction of specific anti-NHPA serum. Accordingly, one of the adult specific NHPs had esterase activity and migrated scarcely under the electrophoretic condition employed.

Specific anti-NHPA serum was prepared by adsorption with NHPC. The investigation of NHP extracted by the chloroform and ethanol method exhibited that activity of CA in cord blood was by far lower than that in adult blood (Stenvenson 1943; Jones and McCance 1949). Therefore, it was predicted that anti-NHPA serum adsorbed with NHPC retained the antibody to CA. However, specific anti-NHPA serum did not react with CA. Ohya (1970) reported that antiserum to NHPA prepared by DEAE-cellulose chromatography did not produce distinct precipitation line against NHPC. The results of the present experiment indicated some components with antigenicity in NHPA which were not yet developed in newborn red cell. Immuno-electrophoresis using specific anti-NHPA serum revealed that there were at least three components with adult specificity. The more detailed investigation is required to elucidate the significance or mechanism of development of adult specific components in NHP.

Acknowledgment

We wish to express our thanks to Dr. G. Hayasaki, Department of Obstetrics and
Gynecology, Gifu University School of Medicine, for providing cord blood.

References