Measurement of Donath-Landsteiner Antibody-producing Cells in Idiopathic Nonsyphilitic Paroxysmal Cold Hemoglobinuria (PCH) in Children

By Yukioki Miyagawa, Sachihiro Yamada, Atsushi Komiyama, and Taro Akabane

A 2-yr-old girl with a nonsyphilitic type of paroxysmal cold hemoglobinuria (PCH) is reported. We applied the plaque-forming cell (PFC) assay to the measurement of Donath-Landsteiner (DL) antibody-producing cells in the patient. Some of the circulating B lymphocytes formed plaques in our assay system. Initially the number of PFC was 1442 ± 225/10⁶ lymphocytes and DL titer was 1:16. Thereafter the PFC decreased in number as the DL titer decreased. Eight weeks later circulating PFC were not found but the DL titer was still 1:1 positive. The PFC assay appears to be useful as a new method for obtaining rapid information as to the disease status of PCH.

PAROXYSMAL COLD HEMOGLOBINURIA (PCH) is relatively uncommon. In the earlier period of its history, PCH usually occurred as a chronic complication of syphilis, but in recent years it has occurred more often as a transient sequel to certain virus infections, including chickenpox, measles, and mumps.

In PCH a complement-dependent cold-acting autoantibody, Donath-Landsteiner (DL) antibody, produces intravascular hemolysis and hemoglobinuria after exposure to the cold. The DL antibody belongs to IgG, and its presence is essential for the diagnosis of the illness.

Recent advances in immunology have made it possible to measure antibody-producing human lymphocytes, although under limited conditions, by plaque-forming cell (PFC) assay. We applied the PFC assay to the measurement of DL antibody-producing cells in PCH. We report the result of PFC assay compared with DL titer in a child with nonsyphilitic PCH.

CASE REPORT

Patient Y.K., a 2-yr-old girl, was admitted with an 8-day history of progressive darkening of skin and urine. Two weeks previously she had had nonproductive cough and fever and been treated with aminopyrin. There was no history of exposure to any hepatotoxins or of ingestion of any medication. No member of the family had any heritable hematologic disorder.

Physical examination showed no abnormalities except for jaundice. The initial laboratory values were as follows: hemoglobin 11.4 g/100ml, hematocrit 35.1%, platelet count 212,000/mm³, reticulocyte count 13%, white blood cell (WBC) count 10,100/mm³ with normal differential. No red blood cells (RBC) were seen in the urine. Both the reddish-colored serum and urine, examined by spectroscopy, contained oxyhemoglobin and methaemalbumin. The total bilirubin concentration was 3.0 mg/dl (2.3 mg/dl indirect). The lactate dehydrogenase (LDH) level was 700 MIU. SGOT and SGPT levels were 55 and 15 KU, respectively; IgG was 857, IgA 52, and IgM 72.

From the Department of Pediatrics, Faculty of Medicine, Shinshu University, Matsumoto, Japan. Submitted December 21, 1977; accepted February 21, 1978.
Address for reprint requests: Yukiaki Miyagawa, M.D., Dept. of Pediatrics, Faculty of Medicine, Shinshu University, Matsumoto 390, Japan.
© 1978 by Grune & Stratton, Inc. ISSN 0006-4971/78/5201-2003$01.00/0
mg/dl. Serum complement (β1C/β1A-globulin) was 140 mg/dl. The PPD skin test was positive. E-rosette forming cells were 47% and surface immunoglobulin-bearing cells 41% of circulating mononuclear cells. A Coombs’ test performed with a mixture of gamma and nongamma anti-globulin sera was negative at room temperature. Cold agglutinin titer, acid hemolysis (Ham) test, Heinz body preparation, and glucose-6-phosphate dehydrogenase level were either negative or within normal limits. The Wasserman and Kahn tests for syphilis were negative.

The patient’s hospital course was marked by a further increase in hemoglobin value and decrease in bilirubin level without any medication. The DL test was performed on 12th hospital day and the titer was 1:16 positive. There was no rise in titer to respiratory syncytial, influenza, mumps, rubella, adenovirus, or pleuropneumonialike organisms. The LDH was high in the first half of the hospital course; however, in the latter half it was within normal range. IgG levels decreased by about 200 mg/dl during the hospital course.

The patient was discharged on the 61st day of hospitalization. One month after discharge the hemoglobin value was normal and DL titer was 1:1, compared to the initial titer of 1:16. DL antibody was still 1:1 positive after 2 mo and was not demonstrable when examined after 12 mo. The patient has been doing well for more than 18 mo after discharge.

**MATERIALS AND METHODS**

The DL antibody production by stimulated PCH peripheral B lymphocytes was measured by direct PFC assay.

**Preparation of B lymphocytes.** Venous blood was drawn from the PCH patient and healthy volunteers in sterile disposable syringes with 10–20 units sodium heparin/ml blood. RBC were sedimented by gravity at 37°C. The plasma supernatant was obtained, and purification of mononuclear cells was done by using standard Ficoll-Hypaque density centrifugation.11 Procedures for the enrichment of B lymphocytes were essentially similar to the rosette sedimentation method of Wybran et al.12 Briefly, 6–8 × 10⁶ mononuclear cells were incubated with sheep RBC (SRBC) for 15 min at 37°C and centrifuged at 400 g for 30 min. The top of the cell fraction contained 92.7±1.5% C3 receptor-positive mononuclear cells. They were washed three times with Hanks’ balanced saline solution (HBSS) and used as B lymphocytes although 10%–30% of them were monocytes with peroxidase activity.

**Assay for PFC.** The number of antibody-forming cells per culture was determined by the Cunningham and Szenberg13 modification of Jerne plaque assay. Briefly, the patient’s and normal B lymphocyte suspensions in HBSS (4 × 10⁶ cells/ml), 20% patient RBC and type O RBC suspensions in HBSS, and 50%, fresh human AB serum were prepared and kept at 37°C. Two droplets of one of the B lymphocyte suspensions, one of the RBC suspensions, and fresh human AB serum were mixed together and poured into the PFC chamber. The PFC chamber was incubated at 37°C for 60 min, and then half of the mixture was chilled at 4°C for 30 min and incubated again at 37°C for 60 min. The other half was kept to incubate at 37°C for another 90 min. Part of the B lymphocytes were cultured in RPMI-1640 (Flow Laboratories, Rockville, Md.) with fetal calf serum (Flow Laboratories) at 37°C in an atmosphere of 5% CO₂ for 48 hr and served for the study.

An additional experiment was performed 12 mo after the patient’s discharge. The plaque formation by the patient’s and control B lymphocytes was measured as described above after incubation with the patient’s serum (obtained on the 12th hospital day and stored at −20°C) at 37°C for 60 min. The number of PFC was calculated from triplicate cultures.

**RESULTS**

On the 12th day of hospitalization the first PFC assay was performed. Table I shows the results of our experimental and control studies of PFC assay. The patient’s B lymphocytes formed 1442 ± 225 plaques/10⁶ cells with her RBC (A, MN, CDe, P1) and 1216 ± 212 with control RBC (O, M, CDe, P1) from an adult volunteer after incubation at 4°C followed by warm incubation at 37°C. When kept to incubate at 37°C, however, they did not form plaques.
The patient's B lymphocytes studies after the cultivation in vitro for 48 hr formed 1156 ± 188 plaques with her RBC under the bithermic condition as well. Control B lymphocytes did not form the plaque under either temperature condition studied. Neither the patient's B lymphocytes after recovery nor control B lymphocytes formed plaques after incubation with the patient's acute serum, which had been stored. Figure 1 shows one of the PFC and the plaque hole measuring 67 μm in diameter. Table 2 shows the PFC and DL titer during the hospitalization.

The PFC were measured on days 12, 34, 41, 47, 54, and 61 and compared with the DL titer. As the DL titer decreased, both the number of PFC and the size of plaques they formed decreased. On discharge PFC were not found, but

![Fig. 1. Appearance of plaque. Lymphocyte clearly visible in center of plaque.](image)
the DL titer was 1:1 positive. The DL titer was continuously 1:1 positive even 2 mo after the disappearance of PFC.

DISCUSSION

It is often difficult to determine the exact circumstances that lead to the production of the DL antibody in patients with no evidence of syphilis. A few cases have been associated with viral illness. In our patient the history suggested a possible antecedent upper respiratory tract infection despite the fact that study of the acute and convalescent sera by the currently available laboratory methods did not indicate any previous specific viral illness. An increase of the number of circulating B cells may be further evidence to suggest the antecedent infections.

DL antibody belongs to IgG and is bithermic in the sense that sensitization and complement fixation take place at low temperature and hemolysis occurs near body temperature. The PFC assay has recently been used for the measurement of antibody-producing cells in vitro. We expected that it would be possible to measure the DL antibody-producing cells in vitro by the PFC assay; this was indeed the case. Our study showed that some of the peripheral B lymphocytes from our patient were able to form plaques of hemolysis of both her own (A, MN, CDe, Pl) and control (O, M, CDe, Pl) RBC in vitro in the presence of fresh human AB serum containing complement. However, plaque formation occurred exclusively after cold incubation at 4°C followed by warm incubation at 37°C. No plaques were observed with incubation at 37°C only. Such thermodependency of the plaque formation indicates that the hemolysis is due to the DL antibody and complement. The direct correlation, although not exact, between number of PFC and DL titer is further evidence to support this.

The DL antibody, as previously shown, has blood group specificity in the P system. Unfortunately, however, we failed to examine the absence of hemolysis with rare p1, p2, or p RBC in our PFC assay system. Cultivation of the patient's B lymphocytes in vitro for 48 hr did not decrease the plaque formation; this is impossible in terms of hemolysis due to the DL antibody attached to the surface Fc receptors of mononuclear cells. As further evidence to rule out this possibility, the patient's B lymphocytes after recovery, as well as control B cells, did not form plaques after incubation with the patient's acute serum. Therefore we think that the PFC in our study are the cells that produced and secreted the DL antibody. Since DL antibody belongs, as mentioned above, to IgG, whose half-life in circulating blood is about 20 days, it seems reasonable that the disappearance of circulating PFC would precede that of DL antibody. There may be a possibility, however, that DL antibody production by cells in lymphoid tissue other than peripheral blood is responsible for the lack of exact correlation between PFC number and DL titer. Although further studies need to be done on this aspect, PFC assay appears useful as a new method for obtaining information rapidly as to the disease status of PCH.

ACKNOWLEDGMENT

The skilled technical assistance of Kyoko Hanazato and Mariko Higuchi is gratefully acknowledged.
REFERENCES

Measurement of Donath-Landsteiner antibody-producing cells in idiopathic nonsyphilitic paroxysmal cold hemoglobinuria (PCH) in children

Y Miyagawa, S Yamada, A Komiyama and T Akabane