CHEMISTRY LETTERS, pp. 1467-1470, 1990.

Competitive Homogeneous Chemiluminescent Immunoassay Using Antigen-modified Sheep Red Blood Cells

Yoshiro TATSU and Susumu YOSHIKAWA
Government Industrial Research Institute, Osaka
Midorigaoka, Ikeda, Osaka 563

A novel method for immunoassay is reported. A competitive assay for dinitrophenylated bovine gamma globulin (DNP-GGB) was performed using anti-DNP serum and DNP-sheep red blood cells (DNP-SRBC). After complement-mediated hemolytic reaction, the chemiluminescent measurements were performed without any separation procedure. The sensitivity was 0.1 μg / ml of DNP-BGG.

The chemiluminescent reaction of luminol catalyzed by heme iron has long been used for sensitive blood-stain judgments. The immunoassay using luminol has been carried out with luminol-antibody conjugate[1] or peroxidase-antibody conjugate.[2] However, these methods require the separation of the antigen-antibody (Ag-Ab) complex from the free conjugates. There are several reports of a homogeneous immunoassay using chemiluminescent energy transfer[3] or electrochemical luminescence[4], where no separation process is needed. Here we report a novel type of homogeneous immunoassay based on the fact that red blood cells without hemolysis have virtually no catalytic effect on the luminol reaction.

Figure 1 represents the principle of this method. Sheep red blood cells are modified with dinitrophenyl group as an antigen. Formation of the Ab-Ag complex on the cell membrane activates the complement system and eventually causes the hemolysis of the cell. Hemoglobin is an effective catalyst for the chemiluminescent reaction of luminol.[5] Its concentration within the cell is 0.3 kg / dm^3.[6] Hemoglobin released from the cells by hemolysis catalyzes the chemiluminescent reaction, which can be detected photometrically.

Antigen modification of sheep red blood cells (SRBC) was performed

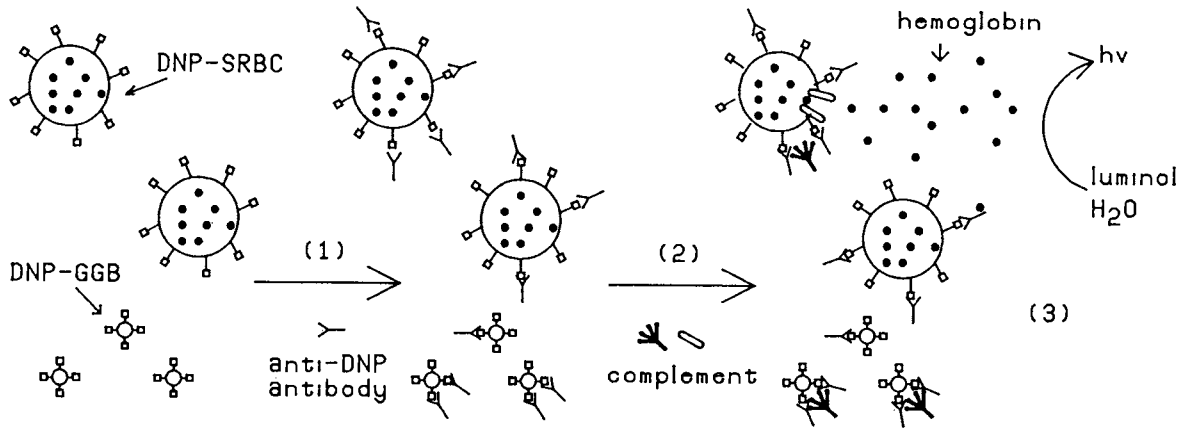

Fig. 1. The principle of the immunoassay. DNP-SRBC and DNP-GGB were competitively reacted with anti-DNP antibodies (1). Complement bound with antigen-antibody complex (2) and made the channels on the cell membrane (3). Hemoglobins released from the cell were detected by the luminol reaction.

according to Bullock's method.[7] SRBC from Cappel was washed with 0.01 M EDTA buffered saline solution (pH8.6) and suspended in the same buffer at 1×10^9 cells / ml. The suspension (5 ml) was mixed with 10 ml of the same buffer containing 150 µl of 2% dinitro-fluorobenzene in acetone and incubated at 37 °C for 10 min. After the incubation, the cells were centrifuged and washed several times with gelatin veronal buffered saline solution (pH 7.2, GVB^{++}). Lyophilized guinea-pig complement from Handai-Biken was reconstituted with Green's solution and was incubated with SRBC at 4 °C for 30 min to remove any anti-sheep antibodies. DNP-GGB was synthesized according to Eisen's method.[8] Rabbit anti-DNP serum was obtained from a male white rabbit immunized with DNP-GGB. The serum was heated at 56 °C for 30 min.

The assay was carried out as follows. DNP-SRBC (0.1 ml) was mixed with 0.05 ml of DNP-GGB solution and 0.05 ml of the diluted anti-DNP serum, and the mixture was incubated at 37 °C for 30 min and cooled at 4 °C for 30 min. 260-fold diluted complement solution (1.3 ml), which contains 0.68 CH50 units / ml, was added and incubated at 37 °C for 1 h. The total sample volume at this point was 1.5 ml. After hemolytic reaction, GVB (1.5 ml) was added. Chemiluminescent measurements were made by adding 50 µl each of 1 mM luminol solution and 5 mM H_2O_2 solution to 75 µl of the cell suspension. The final sample volume was 175 µl. All measurements were performed by the photon counting method with Hamamatsu R-2757 photo-multiplier tube operating at 2.0 kV and Hamamatsu C2550 signal processor. The photon counting was carried out for 205 s with gate-time of 50 ms.

Figure 2 shows the time response of the chemiluminescent intensity

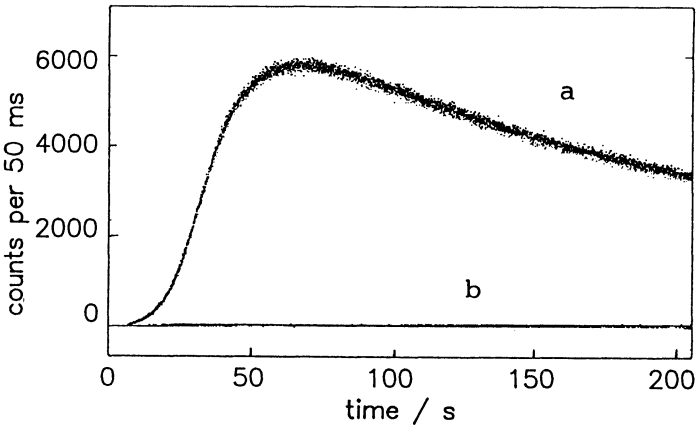

Fig. 2. Time course of chemiluminescent intensity during the reaction of 50 µl of 1 mM luminol and 50 µl of 5 mM H_2O_2 with 75 µl of sheep red blood cells suspension.
a) 100% hemolysed cells.
b) non-hemolysed cells.

both for the 100% hemolysed and the non-hemolysed cells. It is clear that SRBC without hemolysis exhibited almost no chemiluminescence. The total count was 1.339×10^5 in 205 s, whereas for the hemolysed cells, the count was 1.641×10^7 over the same period. This large difference makes the assay possible without separating the hemolysed and non-hemolysed cells. For our equipment, the background was 6000 ± 100 counts in 205 s. Non-specific chemiluminescence of ca. 1.279×10^5 counts was observed for non-hemolysed cells, which may be attributed to the presence of a catalyst or the leaked hemoglobin caused by some damage to the cells during the modification. The immunological signal, i.e., the formation of Ab-Ag complex is amplified by the hemolysis as a large amount of hemoglobin is released. Proteins contained in red blood cells are mainly hemoglobin.[6] Even if the serum sample to be measured contains the peroxidase-like enzymes or hemolysed hemoglobins, this amplification enables the detection of the specific immunoagents.

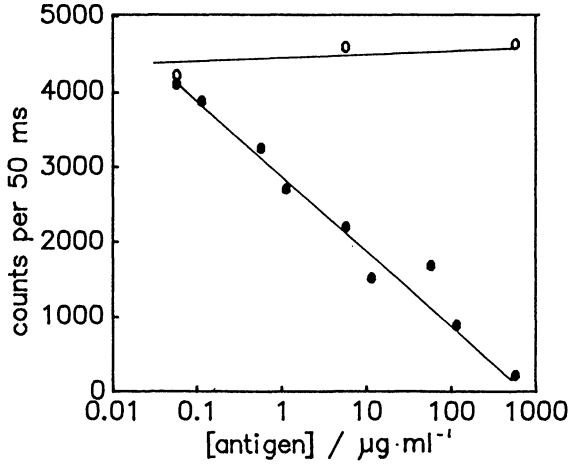

Fig. 3. Chemiluminometric response curve of DNP-GGB (●) and GGB (o). The maximum count per 50 ms interval was plotted against the concentration.

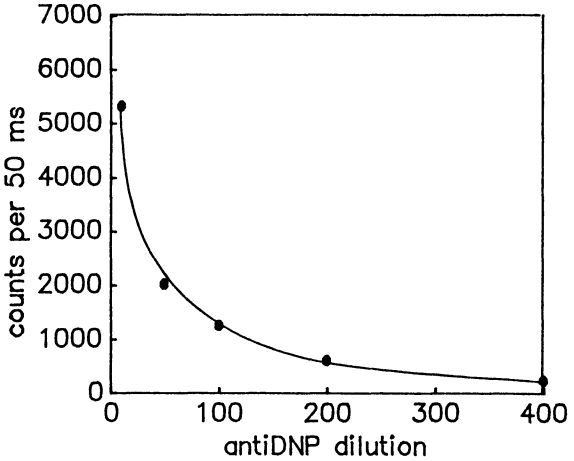

Fig. 4. Chemiluminometric response curve of anti-DNP serum. The maximum count per 50 ms was plotted against the dilution factor of the serum.

Figure 3 shows the response of sheep red blood cells, via chemiluminescence, to varying dilutions of antigen. As the DNP-GGB solution was diluted the chemiluminescence increased, while almost no change in the chemiluminescence was observed for GGB. This difference may be attributed to the antigenicity of the DNP group of DNP-GGB. In the presence of DNP-GGB, competitive binding of anti-DNP antibodies between DNP-GGB and DNP-SRBC occurs. The hemolysis is therefore inhibited and the chemiluminescence decreases. However, in the presence of GGB, anti-DNP antibodies can bind only with DNP-SRBC so that the DNP-SRBC is almost entirely hemolysed at any concentration of GGB. DNP-GGB was specifically detected at 0.1 μg / ml by this method.

Figure 4 shows the dependence of the chemiluminescence on the concentration of anti-DNP. The procedure was the same except that 0.1 ml of DNP-SRBC was incubated with 0.1 ml of the serum without DNP-GGB. Since the serum has no complement activity when heated at 56 °C, this dependence was attributed to the anti-DNP antibody in the serum. It is also possible to measure the level of antibody by this method.

This homogeneous assay is superior to the liposome immunoassay[9] with respect to the ease of the sample preparation and because of the stability of the cells. For other antigens, this assay can be easily made applicable by using the modification methods of the cells developed in the hemagglutination test or the plaque forming assay.

References

1) J. S. A. Simpson, A. K. Campbell, M. E. T. Ryall, and J. S. Woodhead, Nature, **279**, 646 (1979).
2) T. P. Whitehead, G. H. G. Thorpe, T. J. N. Carter, C. Groucutt, and L. J. Kricka, Nature, **305**, 158 (1983).
3) A. K. Cambell and A. Patel, Biochem. J., **216**, 185 (1983).
4) Y. Ikariyama, H. Kunoh, and M. Aizawa, Biochem. Biophys. Res. Commun., **128**, 987 (1985).
5) H. A. Neufeld, C. J. Conklin, and R. D. Tower, Anal. Biochem., **12**, 303 (1965).
6) G. Rajeshwar Rao, P. R. Narasinhalu, V. Krishna Redy, M. Ahmed Hussian, Indian Vet. J., **39**, 429 (1962).
7) Ward E. Bullock and Fred S. Kantor, J. Immunology, **94**, 317 (1965).
8) H. N. Eisen, S. Belman, and M. E. Carsten, J. Am. Chem. Soc., **75**, 4583 (1953).
9) S. C. Kinsky, J. A. Haxby, D. A. Zopf, C. R. Alving, and C. B. Kinsky, Biochemistry, **8**, 4149 (1969); K. Shiba, Y. Umezawa, T. Watanabe, S. Ogawa, and S. Fujiwara, Anal. Chem., **52**, 1610 (1980); M. Haga, H. Itagaki, S. Sugawara, and T. Okano, Biochem. Biophys. Res. Commun., **95**, 187 (1980); M. Umeda, Y. Ishimori, K. Yoshikawa, M. Yakada, and T. Yasuda, J. Immunol. Methods, **95**, 15 (1986).

(Received May 1, 1990)