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One Point Method for the Assay of the Alternative Complement Pathway Hemolysis in Rats

Ayako UEKI and Yoshio MOCHIZUKI

Department of Hygiene, Kawasaki Medical School, Kurashiki 701-01, Japan

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ABSTRACT. One point method to assay the functional activity of alternative complement pathway (AP50) was performed using rats sera. As the titration of CH50 or AP50 to see the activity of complement system with ordinary method needs at least 0.1 ml of serum, experiments with small animals find difficulties to assay CH50 and AP50 at the same time. The method in this report is a modification of one point method for CH50, and instrumental in the case of experiments with small animals.

Key words : Complement — Alternative — Rats

In the case of xenogenic organ transplantation, tissue rejection is a complex cascade of events that may take weeks or months depending on the type of tissue and the immune status of the recipient for the tissue. One functional component among the total immune response is known to be brought into operation immediately and has no need for prior sensitization or contact with the transplanted tissue. This is the alternative pathway of complement where materials by virtue of hindrance to the control inhibitors C3b or β1H cause the alternative pathway amplification resulting in C3-9 activation. If the material resides on a cell surface, lysis of the cell can occur because of the formation of complement-dependent transmembrane channels.

Edwards[1] studied the activation of the alternative complement pathway by cells of different species using sera of other species as complement sources. According to the experiments, dog, horse, guinea pig, or rabbit red blood cells were useful to see alternative pathway hemolysis using rat serum as complement sources.

The schedules to estimate alternative complement pathway activity were made by Brai and Osler[2] as CVFHA50 (50% lytic U/ml by alternative pathway using Cobra Venom Factor) and by Platts-Mills and Ishizaka[3] as AP50 (50% lytic U/ml by alternative pathway using rabbit red blood cells). AP50 has often been used because of its reproducibility and technical simplicity.

It is known that AP50 or CH50 are correlated with the concentration of serum or complement components in samples. Inai[4] modified the method by Mayer[5] to assay CH50, and scheduled one point method in stead of trying several points of serum dilution. The formula for one point method is as follows.

$$\text{Lytic Unit} = \frac{1000\mu l}{x\mu l} \times \frac{1}{2.5} \times \left(\frac{y}{1-y}\right)^{0.2}$$

x ; serum volume y ; hemolysis

植木絢子，望月義夫

In this report the authors intended to arrange one point method to assay AP50 using rats sera, because it is very instrumental if AP50 of experimental animals can be estimated with small amount of sera.

MATERIALS AND METHODS

Buffer : Veronal buffer stock solution (×5), pH 7.3 and 0.1M ethyleneglycol-bis-(β-aminoethyl ether) N,N'- tetraacetic acid (EGTA), 0.02M $MgCl_2$ stock solutions were made previously. EGTA·Mg·GGVB was used through the experiment and prepared as follows.

Veronal buffer stock solution (×5)	50 ml
0.1M EGTA, 0.02M $MgCl_2$ stock solution	50 ml
glucose	12.5 g
gelatin	0.5 g

make total volume 500 ml with distilled water.

Guinea pig red blood cells : Guinea pig red blood cells suspended in Alsever's solution were purchased from MBL (Nagoya, Japan), washed and suspended in EGTA·Mg·GGVB. Cell count was adjusted to 5×10^8 cells/ml, and optical density in 100% hemolysis of 5×10^8 cells/ml was 0.666 at 542 nm.

Assay of AP50 : Procedures for the assay of AP50 were shown in Fig. 1. As Mg^{++} ions are unevitable for the activation of alternative complement pathway, EGTA is used to chelate not Mg^{++} but Ca^{++} ions required in the classical pathway of complement.

Fig. 1 Assay of alternative pathway hemolysis (AP50)

guinea pig red blood cells 0.2 ml (2.5×10^7 cells)
+
diluted serum 0.8 ml
↓
incubated at 37°C, 60 min
↓
add EGTA·Mg·GGVB 2.0ml
↓
thoroughly mixed and centrifuged at 2,000rpm, 10 min
↓
OD_{415}

RESULTS

The results of assay were demonstrated in Table 1. Mean value of AP50 with 5 rats sera was 42.2±4.8 (s.d.) U/ml.

DISCUSSION

The functional activity of the alternative complement pathway in rat serum was measured by Edwards[1] or Coonrod and Jenkins[6] with rabbit red blood cells using multipoint method. There are some differences in reported AP50 values among authors each other. Yukiyama reported the results of AP50 assay with guinea pig red blood cells and rats sera as a source of complement components[7]. In this report the authors demonstrated the results of AP50 assay

TABLE 1. One example of the assay of AP50 in rats sera using one point method.

Sample	1	2	3	4	5	CB	CB	100%	100%
EGTA·Mg·GGVB (ml)	0.79	0.79	0.79	0.79	0.79	0.8	0.8	—	—
serum (μl)	10	10	10	10	10	—	—	—	—
distilled water (ml)	—	—	—	—	—	—	—	0.8	0.8
E (2.5×10^7)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
	incubation at 37°C, 60 min								
	add 2.0 ml of EGTA·Mg·GGVB								
	centrifugation								
	estimation of optical density at 415 nm								
OD 415	0.428	0.355	0.410	0.651	0.528	0.014	0.017	0.832	0.855
OD'*	0.412	0.339	0.394	0.635	0.512			0.816	0.839
y **	0.498	0.409	0.476	0.767	0.618				
1-y	0.502	0.591	0.524	0.233	0.382				
$\frac{y}{1-y}$	0.992	0.692	0.908	3.292	1.618				
$\left(\frac{y}{1-y}\right)^{0.2}$	0.998	0.929	0.981	1.269	1.101				
AP50 (U)	39.92	37.16	39.24	50.76	44.04				

* OD' = OD 415 (sample) − OD 415 (CB)

** $y = \frac{\text{OD 415 (sample)}}{\text{OD 415 (100\% hemolysis)}}$

using guinea pig red blood cells with one point method, and AP50 titer was relatively higher than that of Yukiyama.

The advantage of one point method is that the measurement of functional activity of complement is capable with small amount of serum, especially in the case of experiments with small animals. It is important to perform several test assay with various volume of serum, and to decide optimum conditions for each experiment previously.

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