Cytoflowmetric Analysis of Binding Pattern and A New Binding Kinetic Constant for an Enterovirus and Receptor

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Summary: A bovine enterovirus (MZ-468) was adsorbed to Sarcoma-180 cells at 4°C. The cells were reacted with FITC-labeled IgG antibody for the virus and were studied with a laser fluorescence cytoflowmeter. From the histograms, binding-saturation of receptors was observed and the dissociation constant (K_d) was estimated. A new binding rate constant was deduced from the change of the ratio of virus-adsorbed cells against time and virus concentration. The constant has a dimensions of ($\text{cm}^3/\text{min} \cdot \text{receptor-site}$) and probably represents the receptor class on a cell with the highest rate constant.

Key words: virus receptor—binding kinetic constant—dissociation constant—binding saturation—enterovirus

Introduction

The host range of a virus depends on the existence of the specific receptors on the cell membrane (McLaren et al. 1959, Crowell and Landau, 1983). The specificity depends on the affinity of a virus for the receptor at an early stage of infection. Most of the research on binding patterns and kinetic constants of a virus and its receptors have utilized radioisotope-labeling of virus particles (Schlegel et al. 1982; Huggins et al. 1983) by determining radioactivity of labeled virus. The virus-labeling method for studying virus receptors gives the amount of bound-virus to an average cell but not the amount bound to individual cells, from the virus-side. Cytoflowmetry was employed in this study to give the amount of virus bound to individual cells and the amount of virus-bound cells at the same time (Shapiro and Volsky, 1983). By using the cytoflowmeter, a unique analysis of the virus receptor can be performed without radioisotope from cell-side.

Methods

Cell and Virus

Sarcoma-180 cells were spinner-cultured in Eagle's Essential Medium (MEM, GIBCO) containing 10 % calf serum. A bovine enterovirus (MZ-468) was isolated (Shingu, 1976) and propagated in monolayered HeLa cells at a multiplicity of infection (m.o.i.) of 1.0. The cells were frozen and thawed twice and the cellular debris was removed by centrifugation at $10,000 \times g$ for 20 min. The supernatant was ultracentrifuged twice at $1.4 \times 10^5 g$ for 1 hr with a Hitachi RP-50. The pellet was resuspended in a CsCl-saline solution (refractive index, 1.367) and was ultracentrifuged in the angle rotor (Hitachi-RP-50) at 40,000 rpm for 18 hrs. Peak fractions monitored at 260 nm by the spectrophotometer (Hitachi-220) were collected and dialized twice against one 1 of 0.02 M HEPES buffer (pH 7.0) for 24 hrs (OD 280/OD 260; 1.47). The virus titration was performed on monolayers of HeLa cells.

Immunization and purification of IgG

A rabbit was given a subcutaneous injection of 1 ml of the virus purified with CsCl (infectious titer; 2×10^8 pfu/ml) containing 20 % complete Freund's adjuvant (Difco Labo.). The same virus solution without the adjuvant was injected intravenously three times at three day intervals. After a three week interval without an injection, a single injection was administered. Twenty ml of the blood were collected and centrifuged at 2500 rpm for 10 min at room temperature. Ten ml of the serum were separated and heat-inactivated at 56°C for 30 min. The serum was dialyzed against one l of 0.05 M Tris-HCl buffer (pH 8.0) and was applied on 1.6 g (column; 1×6 cm) of protein-A-sepharose CL-4B (Pharmacia fine chemicals) which had been equilibrated with the same buffer. Ten ml of the IgG fraction were obtained by eluting at room temperature with 0.05 M acetic buffer (pH 4.3) monitoring at 280 nm with the spectrophotometer (Hitachi-220) (Goding, 1976). The IgG was confirmed by the Ouchterlony's immunodiffusion method.

FITC-labeling of IgG

Ten ml of the IgG fraction were dialyzed against 0.05 M carbonate and bicarbonate buffer (CBB, pH 9.2) for 4 hrs at 4°C. The fraction was dialyzed again against 0.6 l of 0.05 M CBB containing 100 µg/ml of fluorescein isothiocyanate (FITC, Dotite) for 17 hrs at 4°C. The reaction was stopped by dialysis against 0.02 M of phosphate buffer (pH 6.6) and was applied on the DEAE-cellulose column (1×10 cm) which had been equilibrated with the same buffer. The conjugate was fractionated by 0.0175 M PBS containing NaCl (from 0 to 1 M) (Goding, 1976). The fractions with F/P ratios of 1.5-2.0 were collected (Brighton and Jhonson, 1971). The neutralizing titer of the conjugate was 1:180, as assessed by the 50 % plaque re-

duction method.

Absorption of FITC-labeled IgG by cells

HeLa cells that were monolayer-cultured in two bottles (10×15 cm) were detached physically with a rubber policeman and suspended in saline. The suspension was centrifuged at 1,000 rpm for 6 min and the cell pellet was obtained. The pellet was resuspended in the solution of FITC-labeled IgG and incubated for 6 hrs at room temperature. This procedure was repeated once.

Adsorption of virus and cytoflowmetry

The following experiments were executed at 4°C except the measurement with the cytoflowmeter.

(1) Twelve 2 ml samples of the cell suspension (Sarcoma-180 cells 5×10^3 cells/ml) were centrifuged at 1000 rpm for 6 min. One-half ml of 1/2 diluted virus (original; 2×10^9 pfu/ml) in 0.02 M HEPES buffer (pH 7.0, Dotite) was added to eleven samples and the same buffer was added to the other sample without virus for the control. After 2 hrs adsorption all the samples were washed twice with 3 ml saline. Forty µl of FITC-labeled IgG (0.2 mg/ml protein by O.D.) were added to the cell pellets and they were incubated for 30 min and then washed with saline. The cells were resuspended in 0.02 M HEPES buffer and measured by the cytoflowmeter (Orthospectrum III) with a green filter, gain 430 (5).

(2) Five ml of the cells (5×10^3 cells/ml) were washed twice in 0.02 M HEPES buffer and the virus (infectious titer; 2×10^9 pfu/ml) in the same buffer was added to the pellets. At 0, 0.5, 1, 2, 4, 10, 16 min, 0.5 ml of the cell suspension was extracted and washed twice in 1.5 ml of the buffer. The samples were then reacted with the FITC-labeled IgG, as above, and measured by the cytoflowmeter, under the same conditions as above.

(3) Two ml of the cells (5×10^3 cells/ml) in 20 mM HEPES buffer were pipetted into 8 conical test tubes and washed twice in the buffer. One-half ml aliquots of the various concentrations of virus in the same buffer (0, 0.2, 0.4, 1, 2, 4, 7, 20×10^8 pfu/ml) were added to the cell pellets and the preparations were incubated for 30 min. After washing, the cells were reacted with FITC-labeled IgG and measured by the cytoflowmeter, as described above.

Results

Study of virus binding pattern

To study the binding pattern of an enterovirus (MZ-468) to Sarcoma-180 cells, the cells were adsorbed to the virus at 4°C for two hours and were measured by the laser fluorescence cytoflowmeter. In the histograms in Fig. 1, the ordinate represents the relative number of cells on a linear scale and the abscissa represents

Fig. 1. Virus binding patterns to Sarcoma-180 cells determined by fluorescence cytoflowmetry.

Virus (MZ-468) at various concentrations was adsorbed to Sarcoma-180 cells at 4°C , reacted with FITC-labeled IgG and measured by the cytoflowmeter, as described in the methods. The vertical bars indicate the relative cell numbers on a linear scale and the abscissa is the relative intensity of fluorescence on a log scale. 1-control, 2, 3, 4, ..., 9, 10, 11 — $(1/2)^{10}$, $(1/2)^9$, $(1/2)^8$, ..., $(1/2)^2$, $(1/2)^1$, $(1/2)^0$ of 2×10^9 pfu/ml.

the relative intensity of fluorescence on a log scale. The fluorescence intensity should be related to the amount of the virus bound to cell membrane. Each panel in Fig. 1 corresponds to a specific virus concentration, ranging from $(1/2)^{10}$ to $(1/2)^0$ of 2×10^9 pfu/ml. Number 1 is a control without the virus. A typical normal distribution is found in panel 5 and 6. At concentrations greater than 2.5×10^8 pfu/ml (9 in Fig. 1), there was no further shift of the mode to the right.

Although the whole IgG fractions were used as the antibody, instead of Fab fragments, no significant aggregation of cells was observed. These experiments were performed under antibody excess.

Analysis of virus-binding equilibrium

Binding equilibration between the cells and viruses was achieved by adsorption at 4°C for more than two hours. The equilibrium can be expressed as follows.

The virus-concentrations were much greater than the cell-concentrations ($V \gg C$), thus V can be substituted with the initial virus concentration (V_0). The dissociation constant for the relationship can be expressed as follows.

$$K_d = [C] \cdot [V_0] / [C \cdot V] \quad \dots\dots\dots (2)$$

The cytoflowmeter gives us a negative ratio; r , the number of virus free-cells/ the number of total cells. The ratio is expressed as follows.

$$r = [C] / [C] + [C \cdot V] \quad \dots\dots\dots (3)$$

From equations (2) and (3),

$$1/r = 1 + (1/K_d) \cdot V_0$$

From the experiments on the virus binding patterns, previously described, $1/r$ was plotted against V_0 (Fig. 2). Two slopes are present. From the slopes, the K_d s

Fig. 2. Plot of $1/r$ against the initial virus concentration for the experiment in Fig. 1.

The negative ratios (r) were obtained from the cytoflowmeter and the values were derived from the equation 3 in the text. The data correspond to the results in Fig. 1.

were calculated as 2.2 pM and 0.1 nM, assuming 1 pfu equals 1000 virus particles, as estimated from optical density (Rueckert, 1976; Schwerdt and Schaffer, 1955).

Induction of an initial rate constant from receptor-side

Two series of experiments were performed to determine the binding rate constant by using a cytoflowmeter. The duration of adsorption was varied at a constant virus-concentration or virus-concentration was varied at a constant duration of adsorption. In both experiments the negative ratios (r) decreased with time or with virus-concentration, generating hyperbolic curves (Fig. 3, 4). The rate of the forward reaction in the above equilibrium (equation 1) can be measured by the change in concentration of virus-free cells with time, t , early in the experiment before the concentration of virus-bound cells become significant (Incardona, 1983).

The forward reaction is expressed as follows.

The decreasing concentration of virus-free cells can be expressed as follows.

$$dC/dt = -kc \cdot C \cdot V$$

$$V \gg C \dots\dots\dots V = V_0$$

$$dC/dt = -kc \cdot C \cdot V_0$$

The above equation can be solved,

$$\log C = -kc \cdot V_0 \cdot t + \text{constant}$$

$$t=0 \dots\dots C = C_0 \text{ (} C_0; \text{ initial cell-concentration)}$$

$$\therefore C/C_0 = \exp(-kc \cdot V_0 \cdot t)$$

C/C_0 is the negative ratio (r). This equation was optimized by including parameters, a and b , arising from the non-specific reaction of fluorochrome and the error of measurement. The final equation was expressed as:

$$r = (a - b) \exp(-kc \cdot V_0 \cdot t) + b \text{ (} kc, \text{ initial rate constant; } t, \text{ time; } a, b, \text{ parameters)}$$

The two series of experimental data were curve-fitted to the above equation. The parameter a was the negative ratio of the virus free sample. According to a computer program (the program was provided by Dr. K. Harada of the Computer Center of Kurume Univ.), kc and b were calculated by inputting r , a , and t or V_0 . The value of kc for Sarcoma-180 cells were 3×10^{-10} and $3 \times 10^{-11} \text{ cm}^3/\text{min} \cdot \text{pfu}$, respectively with time and virus-concentration as the variable functions. The kc value was roughly estimated to be $10^{-13} \sim 10^{-14} \text{ cm}^3/\text{min} \cdot \text{virus-particle}$ by assuming that 1 pfu equals 1000 particles, as described above.

Discussion

The histograms in Fig. 1 can be interpreted as the distributions of cells which bound varying amounts of virus on the

Fig. 3. Decrease of the negative ratio with time at a constant virus concentration.

Sarcoma-180 cells adsorbed in 2×10^8 pfu/ml at 4°C and 0.5 ml of the cell suspension were extracted at each point in time. After reacting with FITC-labeled IgG, the samples were measured by the cytoflowmeter.

Fig. 4. Change of the negative ratio with virus concentration at a constant duration of adsorption.

Sarcoma-180 cells adsorbed viruses at various concentrations at 4°C for thirty min. After reacting with FITC-labeled IgG, the samples were measured by the cytoflowmeter.

plasma membrane. Shifting of the histogram to the right on an abscissa of virus concentration means an increased amount of virus has been bound to individual cells. The cells at the mode of histogram are considered to be the typical cell group with virus-binding activity. When there

is no further shifting (panel 9 in Fig. 1), binding sites in this cell line are probably saturated. At saturation the diversions of the histograms seemed to depend on the relationship between virus and cells. Data from other cell lines have different diversions (not shown).

Plotting the binding equilibrium for $1/r$ vs input-virus concentration yields the dissociation constant (K_d). Two K_d s can be obtained from the slopes in Fig. 2 meaning there are two classes of receptors on the cells, high affinity and low affinity receptors. This is compatible with the data from Shlegel et al. (1982), in which K_d s of Vero cells to Vesicular Stomatitis virus were analyzed by a Scatchard plot with a computer program. The present analysis is simpler, easier to calculate, and does not involve a radioisotope. An initial rate constant, K_R , presented by Lonberg-Holm et al. (1976), describes the affinity of a cell to a virus (Morishima et al. 1983). The K_R has the dimensions of $\text{cm}^3/\text{min} \cdot \text{cell}$ which describes the average rate for all receptors on a cell, but not the rate of an individual receptor. On the other hand, kc has different dimensions of $\text{cm}^3/\text{min} \cdot \text{virus-particle}$ and describes the rate for a single virus. As a single virus-particle is thought to bind to a single receptor, the dimensions of kc can be converted to $\text{cm}^3/\text{min} \cdot \text{receptor-site}$. As schematically shown in Fig. 5, K_R represents the binding velocity of a virus to any sites on the cell, while " kc " represents the velocity to the single virus receptor. The ratio of K_R and " kc " (site/cell) represents the number of equivalent receptor sites for a cell. The relationship of the two constants is considered to be expressed as follows.

$$K_R = n \cdot kc \quad (n, kc; \text{the number and rate constant of receptors with a high rate constant})$$

The functions derived by Adam and Delbruck (1968) and Berg and Percell (1977)

Fig. 5. Schematic representation of the difference between K_R and kc . K_R represents the highest rate constant at which a virus particle can bind to any receptors on the cell membrane.

" kc " represents a rate constant for binding of which a single virus particle binds to the virus receptor with the high rate constant.

also expressed the relationship between the number of receptors per cell and the binding rate constant, but they have different dimensions due to the method of derivation of the original function.

The cytoflowmeter can theoretically detect a cell which binds a single virus particle in our system. When a cell binds a single virus particle the positive ratio increases. Therefore " kc " can be interpreted to represent the highest rate constant among all the receptor classes on a cell.

When considering the specificity or susceptibility of the organ *in vivo*, the efficiency of the infection under low multiplicity of infection (m.o.i.) must be taken into account, because a single infectious virus can be adequate to infect a cell. Thus a high rate constant for the receptors on a cell should be taken into account when susceptibility of a cell is discussed. The measurement of " kc " is useful to evaluate the susceptibility of a cell. In conclusion, cytoflowmetrical analysis of receptors contributes to the analysis of receptor classes and to the estimation of the binding rate constant for susceptibility of a cell by virus. This method will be applied to other types of receptors from cell-or receptor-side.

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