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Kinetic Analysis of Fusion of Hemagglutinating Virus of Japan with Erythrocyte Membrane Using Spin-Labeled Phosphatidylcholine

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ABSTRACT: HVJ* (hemagglutinating virus of Japan containing spin-labeled phosphatidylcholine in its envelope around 10 mol %) was adsorbed onto erythrocytes or erythrocyte ghosts at various doses, and the ESR spectrum of the virus–cell system was measured at 37 °C. The peak-height increase for the HVJ*–ghost system was satisfactorily analyzed on the basis of envelope fusion by a first-order kinetic equation with two different rate constants. The rate constant was obtained as $k_1 = 0.84$ min^{-1} and $k_2 = 0.011$ min^{-1}, independent of the virus dose. The fraction of virus fused at the rate constant k_1 decreased with the dose. However, the average number of fast-fusing viruses per cell was nearly independent of the dose, and the value was one to two. The peak-height increase in the HVJ*–erythrocyte system was caused by both envelope fusion and phospholipid exchange catalyzed by the virus-induced hemolyzate. At lower doses, where the virus-induced hemolysis was small and, therefore, the rate of phospholipid exchange was small, the peak-height increase could be analyzed by the same kinetic equation with nearly the same rate constant value for k_1 as that for HVJ*–ghosts. However, the k_2 was larger than that for HVJ*–ghost, owing to the additional transfer by phospholipid exchange.

Fusion of virus envelope with target cell membranes is an essential step in virus infection [see White et al. (1983) and Ohnishi (1985)]. We have been studying envelope fusion using viruses having spin-labeled phosphatidylcholine (PC)*[1] in-

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corporated into their envelope to some high concentrations (ca. 10 mol % of viral phospholipids). Transfer of PC* molecules from the envelope to target membranes was assayed by the ESR peak-height increase due to dilution. The studies have yielded a variety of information, mostly qualitative, on fusions of hemagglutinating virus of Japan (HVJ) or influenza virus with erythrocytes and other cultured cells (Maeda et al., 1975, 1977, 1981a,b; Koyama et al., 1978; Kuroda et al., 1980; Maeda & Ohnishi, 1980; Yoshimura et al., 1982). In the present study we made a detailed kinetic analysis of fusion of HVJ with erythrocyte membranes. Beacuse of rapidity of the fusion reaction, the initial peak-height increase data were collected more carefully and treated more appropriately by fitting the data to a theoretical kinetic equation. It was found that the peak-height increase can be satisfactorily analyzed on the basis of first-order fusion kinetics. An apparent discrepancy from our previous studies (Kuroda et al., 1980) was discussed.

Experimental Procedures

Materials

HVJ, Z strain, was used throughout. Preparation, purification, and radioiodination of the virus were carried out as described previously (Maeda et al., 1975, 1977). The virus concentration was determined as HAU per milliliter. Human erythrocytes were supplied by a blood bank through the courtesy of Dr. Saji and used within 2 weeks after they had been drawn. The cells were washed 3 times with Tricine-buffered saline (140 mM NaCl, 5.4 mM KCl, 20 mM Tricine-HCl, pH 7.6). Ghosts were prepared by hypotonic hemolysis in 5 mM phosphate buffer at pH 8.0, washed twice with the buffer, and incubated in Tricine-buffered saline at 37 °C for 1 h for resealing.

PC* was prepared as described previously (Maeda et al., 1975). Total lipid was extracted from ghosts according to Bligh & Dyer (1959). Lipids were suspended in Tricine-buffered saline and dispersed with a thermomixer or sonicated. The sonicated suspension was centrifuged at 150000*g* for 1 h, and the supernatant was used for the following experiments.

Spin-labeling of virus was carried out as described previously (Maeda et al., 1975). Briefly, virus (10000–20000 HAU) was suspended in 1 mL of sonicated dispersion of PC* (1 mM), incubated for 5 h at 37 °C, and washed with Tricine-buffered saline containing bovine serum albumin (10 mg/mL) and then with Tricine-buffered saline. The spin-labeled HVJ (HVJ*) contained PC* by about 10 mol % of the viral phospholipid and had essentially the same hemagglutination titer and only somewhat lowered hemolytic activity. Spin-labeling of erythrocytes with a small concentration of PC* was carried out by incubation of a mixture of equal volumes of the cell pellet and sonicated dispersion of PC*–cholesterol (1 mM each) for a shorter period, 0.5–1 h at 37 °C, followed by washings. Spin-labeled ghosts were prepared by hypotonic lysis of spin-labeled erythrocytes.

Methods

Assay of Transfer of PC from Virus Envelope to Erythrocyte Membranes.* This was done essentially as described previously (Maeda et al., 1975, 1977; Kuroda et al., 1980) with some improvements for quantitative kinetic analysis. HVJ* was centrifuged at a low speed (3000 rpm for 15 min) before use to separate it from aggregates. A total of 0.9 mL of HVJ* at various concentrations was mixed with 0.3 mL of erythrocytes [10% (v/v)] or ghosts [20% (v/v)] at 0 °C and kept for 15 min for adsorption. The mixture was added with 9 mL of Tricine-buffered saline and centrifuged for 5 min at 2000 rpm at 4 °C to remove unadsorbed virus. The pellet was sucked into a quartz capillary tube in a cold room at 4 °C, not at room temperature (about 23 °C) as in the previous assays, to avoid occurrence of fusion during the handling. Immediately after transfer of the sample tube into the insert in the cavity, the inside temperature being preset at 37 °C, the ESR spectrum or its central peak height was measured repeatedly (every 10 s or longer) on a JEOLCO Model FE-2 spectrometer. It took some time for the sample temperature to reach 37 °C from 4 °C after the transfer. An estimate for the time delay was made by measuring the ESR peak height of a sample (HVJ*). The peak height increased to a steady value in about 60 s. Integration of ESR spectra was done on an EC-100 computer connected on-line to the spectrometer.

ESR Peak-Height Increase on Dilution of PC in Membranes.* HVJ* contained around 10 mol % of PC* in its envelope. If the virus fused with erythrocyte membrane and the envelope lipid mixed with erythrocyte membrane lipid completely, the concentration of PC* will greatly decrease because of a much larger amount of unlabeled erythrocyte lipid as compared with viral lipid. This causes a large increase in the ESR peak height. The increase factor can be estimated by comparing the peak height for PC* incorporated into erythrocyte membranes at a very small concentration with that of HVJ*. For quantitative comparisons, we took normalized peak height; that is, the central peak height divided by the double integrated area of the whole spectrum and equivalent to the central peak height per a fixed number of spin-labels. The normalized peak height for erythrocytes (H_R) or erythrocyte ghosts (H_G) containing a small concentration of PC* was 0.8 or 1.0, respectively. That for HVJ* (H_V) was 0.15 for most preparations. Therefore, the peak-height increase accompanied by envelope fusion should be at a factor of $f = (H_R \text{ or } H_G)/H_V = 5.3$ or 6.7.

The basic principle on which the spin label assay relies is therefore the concentration dependence of the ESR spectrum. As the concentration increases, the spin–spin interaction increases, resulting in broadening of the spectrum and, as the consequence, a decrease in the peak height. A model experiment using liposomes made of erythrocyte total lipid and various concentrations of PC* showed that the normalized peak height decaresed to 0.61 at m (mol % of PC*) = 2, to 0.32 at $m = 5$, and to 0.15 at $m = 10$, as compared with 1.0 at $m = 0.2$. This result indicates that the peak-height increase factor on dilution of PC* in the liposome membrane from 10% to 0.2% is 1.0/0.15 = 6.7. Note that the increase factor does not much depend on the host membrane.

Kinetic Analysis of ESR Peak-Height Increase Based on Envelope Fusion. When HVJ* fused with erythrocyte membrane, the PC* molecules would rapidly diffuse into the membrane and become greatly diluted. The lateral diffusion is quite rapid, with the diffusion constant on the order of 10^{-9} cm^2 s^{-1} (Kapitza & Sackmann, 1980). If the lateral diffusion was much faster then the envelope fusion, which is found to be the case, the spectrum during the fusion reaction should be sum of that of HVJ* remaining unfused and that of PC* transferred into erythrocyte membrane by fusion. The spec-

[1] Abbreviations: PC, phosphatidylcholine; PC*, PC with 12-doxylstearate attached at the 2-position; HVJ, hemagglutinating virus of Japan, synonym of Sendai virus; HVJ*, HVJ containing PC* in the envelope; HAU, hemagglutinating unit; Tricine-HCl, *N*-[tris(hydroxymethyl)methyl]glycine hydrochloride; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy.

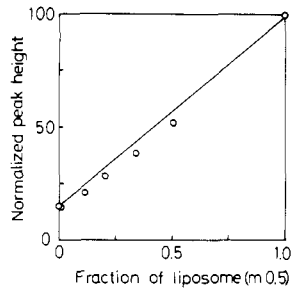

FIGURE 1: Central peak height of the ESR spectrum synthesized by addition at various ratios of spectra of erythrocyte total lipid liposomes containing 0.5% of PC* and 13.2% of PC*. The normalized peak height is given in a relative scale as a function of the fraction of liposomes containing 0.5% of PC*.

trum is therefore a composite. The central peak height of the composite spectrum can be assumed to be sum of those of the two components. This is a reasonable assumption when the fusion reaction progressed to considerable extents. For smaller extents of fusion, however, this may be a poor assumption since the peak position for HVJ* does not coincide to that for PC* in erythrocyte membrane because of the broadening in the former. To test its validity, we added ESR spectra of liposomes containing a small (0.5%) and a high (13.2%) concentration of PC* at various ratios and plotted the central peak height of the synthesized spectrum against fraction of the component ($m = 0.5$) in Figure 1. The data showed approximate proportionality between them.

The envelope fusion can be assumed to follow first-order kinetics (Ohnishi & Maeda, 1982):

$$dn/dt = k_f (n_0 - n) \quad (1)$$

$$n/n_0 = 1 - \exp(-k_f t) \quad (2)$$

where n is the number of fused virus particles, n_0 is the number of initially adsorbed virus particles, and k_f is the rate constant or probability of fusion. The ESR central peak height h can be given as a sum of those for PC* in virus and in erythrocyte, within the limit of proportionality shown in Figure 1:

$$h = (n_0 - n)H_V + nH_G \quad (3)$$

By dividing both sides by the initial peak height h_0 ($=n_0H_V$)

$$h/h_0 = (1 - n/n_0) + (n/n_0)(H_G/H_V) \quad (4)$$

and by using eq 2

$$h/h_0 = f - (f - 1) \exp(-k_f t) \quad (5)$$

where f ($=H_G/H_V$) is the peak-height increase factor on fusion of a virus with erythrocyte membrane. The fraction of fused virus F ($=n/n_0$) can be obtained from h/h_0 by

$$F = (h/h_0 - 1)/(f - 1) \quad (6)$$

If there were two populations of virus fusing at different rates, then

$$h/h_0 = f - (f - 1)[\alpha \exp(-k_1 t) + (1 - \alpha) \exp(-k_2 t)] \quad (7)$$

where k_1 and k_2 are the fusion rate constants and α is the fraction of virus with k_1.

In practice, there was a time delay (ca. 60 s) in the rise of sample temperature from 4 to 37 °C, during which the ESR peak height should be smaller than that at 37 °C, on one hand, and the envelope fusion would have occurred to some extents, on the other hand. For calibration of these effects, we may introduce an effective time zero, t_e, into the exponent of the kinetic equation. For example, eq 7 becomes

$$h/h_0 = f - (f - 1)(\alpha \exp[-k_1(t - t_e)] + (1 - \alpha) \exp[-k_2(t - t_e)]) \quad (8)$$

In the following analysis, we did not use data before 60 s. The value for t_e was either made adjustable or fixed at 60 s.

The value h_0 can be obtained by extrapolation of the data points to time zero, as done in our previous studies (Kuroda et al., 1980). This method can give a correct value provided that the fusion occurred slowly. However, since the fusion was found to occur rapidly especially in the initial phase, we employed different methods for the estimation in the present analysis. First, h_0 was obtained as the product of the double-integrated area of the spectrum (DA) for HVJ*–cell at any time of the fusion reaction with the normalized peak height for HVJ* being

$$h_0 = H_V(\text{DA for HVJ*–cell}) \quad (9)$$

The double-integrated area is proportional to the number of spin-labels in the specimen and should therefore be constant during fusion even though the marked change in the spectral shape. This constancy was actually observed within 3.5% error during the 45-min assay at 37 °C. The constancy also indicates the small range of error in the double integration. This method should give a correct h_0 value for the series of experiments and, therefore, a correct h/h_0 value irrespective of fusion. This is true even when the fusion reaction finished before the first ESR measurement. In this case, we can obtain the degree of fusion from the h/h_0 value although not the rate constant.

As a second method, h_0 was obtained as an adjustable parameter in the data fitting to the theoretical kinetic equation. The least-squares data fitting was done by using the SORD M243 computer.

Quenching Experiment. A quenching experiment was carried out to obtain data on the fast fusion reaction. A pellet of HVJ*–ghosts in a centrifuge tube was incubated at 37 °C for varying periods and quenched by putting the tube into ice. The pellet was then sucked into a quartz capillary tube at 4 °C, and the ESR spectrum was measured at 15 °C where practically no fusion reactions occurred. The peak-height increase factor f at this temperature was smaller than that at 37 °C, being about 80%.

RESULTS

Envelope Fusion with Erythrocyte Ghosts. Figure 2 shows the ESR peak-height increase for the HVJ*–ghost system at various doses of HVJ*. The experimental data can be fitted very well by eq 8 on the basis of two populations of virus, but not by eq 5 with a single fusion rate constant. The full-line curves in Figure 2 were drawn by eq 8 with the best fit parameter values for α, k_1, k_2, t_e. The h_0 value was obtained by the double-integration method (eq 9) and the f value by the ratio of normalized peak heights H_G/H_V, 6.6 in this case. The parameter values are summarized in Table I.

Similarly, good fitting of the data to eq 8 can be made by taking h_0, α, k_1, and k_2 as the adjustable parameters and using a fixed value for t_e (60 s). The best fit parameter values are also tabulated in Table I. The h_0 values were close to those obtained by the double-integration method. The k_1 value agreed quite well, and the α and k_2 values agreed satisfactorily with those obtained from other set of parameters, respectively.

The obtained rate constants k_1 and k_2 were constant in that range of virus dose (Figure 3A): the average values were $k_1 = 0.84$ min^{-1} and $k_2 = 0.011$ min^{-1}. On the other hand, the

Table I: Kinetic Parameters for Envelope Fusion of HVJ with Erythrocyte Membranes at 37 °C[a]

HVJ* dose (HAU/mL)	k_1 (min^{-1})		k_2 (min^{-1})		α		h_0	
	a	b	a	b	a	b	a	b
101	0.87	0.85	0.021	0.019	0.53	0.56	463	450
203	0.90	0.90	0.0099	0.011	0.30	0.31	582	560
406	0.71	0.71	0.0093	0.011	0.17	0.20	420	392
812	0.72	0.73	0.0088	0.011	0.12	0.16	434	371
1623	0.83	0.80	0.0083	0.0086	0.081	0.085	761	748

[a] HVJ* at the indicated final concentration was mixed with erythrocyte ghosts (5%, final), kept at 0 °C for 15 min, and centrifuged. The ESR central peak-height increase at 37 °C was analyzed by eq 8 with two different sets of adjustable parameters: (a) α, k_1, k_2, and t_e and (b) h_0, α, k_1, and k_2. In (a), h_0 was estimated by the double-integration method (eq 9), and in (b), t_e = 60 s was fixed. f was estimated by the ratio of normalized peak heights H_G/H_V, 6.6 for this series of experiments. The parameter value for t_e in (a) was 61.1 (101 HAU/mL), 61.5 (203 HAU/mL), 65.8 (406 HAU/mL), 75.8 (812 HAU/mL), and 63.3 (1623 HAU/mL) s.

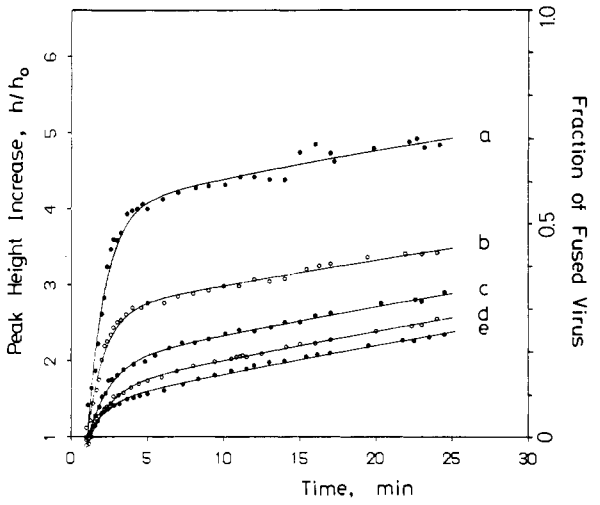

FIGURE 2: ESR peak-height increase for the HVJ*–ghost system at various doses of HVJ*; 101 (a), 203 (b), 406 (c), 812 (d), and 1623 HAU/mL (e). HVJ* at the indicated final concentration was mixed with erythrocyte ghost at 5% (v/v) final, and the ESR spectrum of the pellet was measured at 37 °C. The central peak height h divided by the initial peak height h_0 is plotted against time. The full-line curve was drawn by eq 8 with the best fit parameter values for α, k_1, k_2, and t_e. On the right ordinate, the fraction of fused virus calculated from h/h_0 is given.

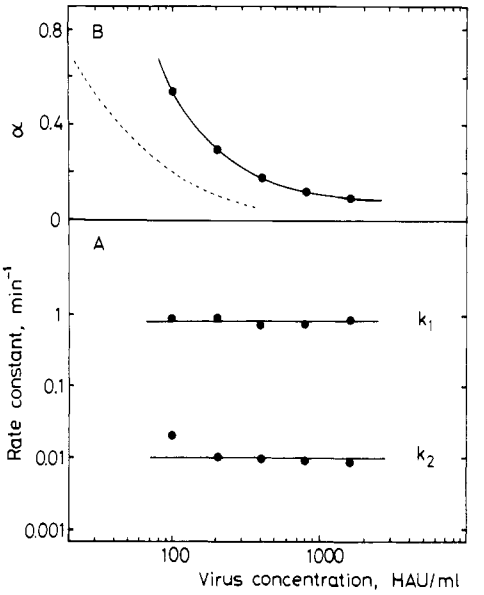

FIGURE 3: Fusion rate constants k_1 and k_2 (A) and the fraction of virus fusing at the rate constant k_1, α (B), are plotted against virus dose. HVJ* at the indicated final concentration was mixed with erythrocyte ghost at 5% v/v final (or erythrocyte at 2.5% v/v), and the ESR peak height increase at 37 °C was analyzed by eq 8. The dotted curve in (B) shows α for the HVJ*–erythrocyte system.

α value was greatly dependent on the virus dose, decreasing with it (Figure 3B). A characteristic feature in Figure 2, a much larger increase in the initial peak height for smaller doses, is due to a larger α value for the smaller dose systems. The slow peak-height increase in the later stage (after ca. 5 min), common to all systems, is characterized by the slower rate constant k_2.

The fraction of fused virus was calculated by eq 6 and given on the right ordinate in Figure 2. The fusion occurred to 50% completion in 1–2 min for the smallest dose system, followed by a gradual slow increase. Fusion for the highest dose proceeded only 25% completion even after 25 min owing to the relative preponderance of the slow component.

The average number of fused virus per cell was calculated as a product of the fraction of fused virus and the average number of adsorbed virus per cell. For the latter data, the fraction of adsorbed virus was measured, with radiolabeled virus, after incubation of various concentrations of virus with a fixed number of erythrocytes at 4 °C. Further, the average number of virus fused at the rate constant k_1 per cell was obtained by using the α value. The result given in Table II shows that, in contrast to the great dependence of α with dose, the number of virus fused at k_1 was rather independent of the dose with a value of one to two per cell.

The rate constant value 0.84 min^{-1} for the fast component indicates the half-time of fusion to be 0.83 min. Our analysis was based on the peak-height increase data after 1 min of

Table II: Estimated Number of Virus Fused at the Rate Constant of 0.84 min^{-1} per Cell[a]

HVJ dose (HAU/mL)	mixing ratio of virus to cell	no. of adsorbed virus per cell	α	no. of virus fused at 0.84 min^{-1} per cell
101	4.8	~2	0.55	1.1
203	9.6	4.2	0.31	1.3
406	19.2	7.6	0.19	1.4
812	38.4	14	0.14	1.9
1623	76.8	25	0.083	2.1

[a] Calculated by α (taken from Table I) multiplied by average number of adsorbed virus per cell. The latter was obtained by using the fraction of the adsorbed virus and by taking 1 HAU = 2.4 × 10^7 virions (Okada et al., 1961) and 10^{10} cells contained in 1 mL of packed erythrocytes.

insertion of sample tube into the cavity, because of incomplete temperature equilibration within that time interval. To confirm the kinetic data for the fast component, a quenching experiment was carried out as described under Methods. The peak-height increase data for the HVJ*–ghost system at a dose of 26 HAU/mL were collected up to 7 min at 37 °C by the quenching experiment and analyzed by eq 8 by using experimentally obtained values for h_0 and f (6.13) and by putting t_e = 0 in this case. The least-squares fitting gave a value of 0.83 min^{-1} for k_1, which is in excellent agreement with that obtained in the above analysis (0.84 min^{-1}). The α value was

Table III: Kinetic Analysis of ESR Peak-Height Increase for the HVJ*–Erythrocyte System in the Lower Dose Range by Envelope Fusion[a]

HVJ* dose (HAU/mL)	k_1 (min^{-1})		k_2 (min^{-1})		α		h_0	
	a	b	a	b	a	b	a	b
12.5	nd	0.79	nd	0.047	nd	0.57	nd	995
25	0.87	0.83	0.037	0.045	0.35	0.36	331	309
51	0.98	0.96	0.034	0.047	0.21	0.24	422	368
101	0.88	0.99	0.040	0.050	0.081	0.083	509	457

[a] HVJ* at the indicated final concentration was mixed with erythrocytes (2.5% v/v), kept at 0 °C for 15 min, and centrifuged. The ESR peak-height increase at 37 °C was analyzed by eq 8 with two different sets of adjustable parameters: (a) α, k_1, k_2, and t_e and (b) h_0, α, k_1, and k_2. In (a), h_0 was obtained by the double-integration method. The parameter value for t_e (s) was 66.6 (25 HAU/mL), 71.8 (51 HAU/mL), and 78.7 (101 HAU/mL). In (b), t_e was fixed at 60 s. f was 6.6 for this series of experiments. nd was not determined.

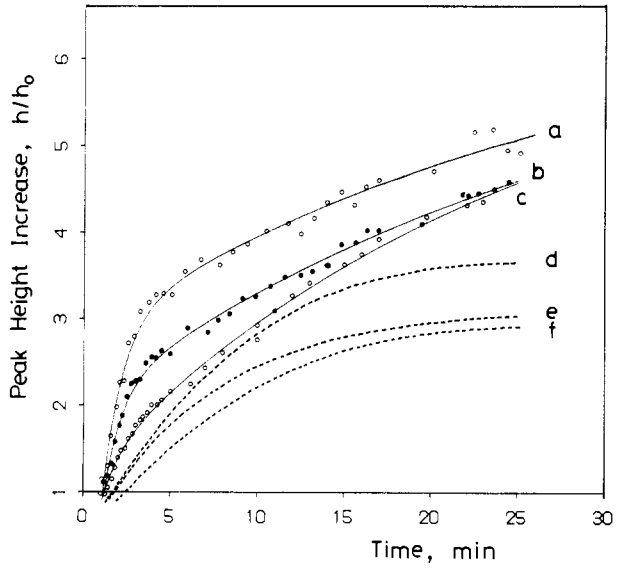

FIGURE 4: ESR peak-height increase for the HVJ*–erythrocyte system at various doses of HVJ*: 25 (a), 51 (b), 101 (c), 406 (d), 812 (e), and 1623 HAU/mL (f). HVJ* at the indicated final concentration was mixed with erythrocyte at 2.5% v/v final, and the ESR spectrum of the pellet was measured at 37 °C. The central peak height h divided by the initial peak height h_0 is plotted against time. The full-line curve in (a)–(c) was drawn by eq 8 with the best fit parameter values for α, k_1, k_2, and t_e.

0.41. k_2 was obtained as 0.048 min^{-1}, which , however, may not be so accurate because of lack of enough data for longer time incubations.

Envelope Fusion with Erythrocytes. The ESR peak-height increase for the HVJ*–erythrocyte system at various doses of HVJ* is shown in Figure 4. Data in the lower dose range (≤100 HAU/mL) can be analyzed satisfactorily by eq 8. The curves a–c were drawn by using the best fit parameter values for α, k_1, k_2, and t_e. Similarly, good fitting can also be made by using another set of parameter values for h_0, α, k_1, and k_2. The two sets of parameter values agreed satisfactorily with each other (see Table III).

The rate constants k_1 and k_2 were constant in that range of virus doses. The average value for k_1, 0.88 min^{-1}, was essentially the same as that for the HVJ*–ghost system. The average value for k_2, 0.039 min^{-1}, was however larger than that for the HVJ*–ghost system. This discrepancy can be attributed to transfer of PC* by the hemolyzate-catalyzed phospholipid exchange, in addition to the envelope fusion. It was found that human erythrocyte contained PC transfer protein in the cytoplasm that catalyzed exchange of PC between membranes (Kuroda & Ohnishi, 1983). Therefore, when HVJ caused hemolysis as a result of envelope fusion, the hemolyzate catalyzed PC exchange between the viral and erythrocyte membranes. The effect of the phospholipid exchange on the k_1 value was small since the rate of the exchange was smaller than k_1 and larger than k_2 in that dose range (K. Kuroda et al., unpublished results). On the other hand, the transfer in the HVJ*–ghost system was caused predominantly by envelope fusion since ghosts did not contain cytoplasm any more.

The α value decreased with dose, as in the case of the HVJ*–ghost system. However, there is a shift in the dose dependence between them; the decrease occurred in a lower dose range for the HVJ*–erythrocyte system (see dotted curve in Figure 3B).

Data for HVJ*–erythrocytes at higher doses (≥200 HAU/mL) (Figure 4d–f) should be analyzed on the basis of both envelope fusion and phospholipid exchange since the virus-induced hemolysis was extensive and the rate of hemolyzate-catalyzed exchange became large. When the data were analyzed by eq 8, the faster rate constant value ranged from 0.09 to 0.14 min^{-1} and had a tendency to increase with the dose. The slower rate constant became negative for a few cases. These results suggest inapplicability of eq 8 for these systems. Instead, analysis based on the phospholipid exchange gave a reasonable fit (K. Kuroda et al., unpublished results).

Discussion

ESR peak-height increase data for HVJ*–ghost in a wide range of virus doses can be satisfactorily analyzed by first-order kinetics based on envelope fusion. A fraction of the adsorbed virus fused with erythrocyte membrane rapidly at a rate constant of 0.84 min^{-1} at 37 °C. The other fraction had much smaller rate constant of 0.011 min^{-1}.

The fraction of the fast-fusing virus greatly decreased with the dose (Figure 3B). However, the average number of fast-fusing virus was nearly independent of it with a value of one to two per cell. A possible interpretation for this is that when a few virus particles fused with erythrocyte membrane, the fusion caused some change in the membrane that inhibited further fusion of the rest of virus particles. Another explanation is to relate this to heterogeneity on the cell surface sites for the virus binding and fusion. Virus bound to a limited number of sites may fuse rapidly. Both sialoglycoprotein (glycophorin) and sialoglycolipids can bind HVJ, but their effectiveness for fusion may be different.

The slow component, k_2 = 0.011 min^{-1}, can be assigned to slow fusion for the other population of virus. Alternatively, the component may be assigned to slow exchange of PC* between virus envelope and erythrocyte membrane (uncatalyzed by hemolyzate). Similar slow transfer of PC* has been observed between fusion-inactive virus (trypsinized HVJ, for example) and erythrocyte membranes, which was assigned to be due to phospholipid exchange between the two membranes (Maeda et al., 1981a). In the present system, the slow exchange may be between either bound or released HVJ*, or both, and erythrocyte membranes. Bound viruses are released by the action of viral neuraminidase, and the release can be extensive. For example, ca. 60% of virus was released during 10 min at 37 °C under the experimental conditions of Volsky

& Loyter (1978). Since the released virus cannot fuse with cell membranes, this would greatly reduce the value for fraction of fused virus. Kinetically, for smaller degrees of transfer, the peak-height increase by phospholipid exchange is not so different from that by envelope fusion.

The ESR peak-height increase for HVJ*–erythrocyte was caused by envelope fusion as well as phospholipid exchange catalyzed by the virus-induced hemolyzate. The latter contribution increased with the progress of hemolysis since the exchange rate was proportional to the concentration of PC transfer protein. For low doses of HVJ* (≤100 HAU/mL) where the virus-induced hemolysis was small, the data could be analyzed by eq 8 on the basis of envelope fusion, and the rate constant value for K_1 was nearly the same as that for HVJ*–ghost. However, the slower component was affected by the additional transfer due to phospholipid exchange since the exchange rate was larger than k_2 even in that low dose range.

A characteristic difference in the peak-height increase between HVJ*–erythrocyte and HVJ*–ghost systems at comparable doses was a smaller initial increase and a larger later increase in the former (compare Figures 2a and 4b, for example). These differences can now be well explained: the larger later increase by the additional transfer by phospholipid exchange and the smaller initial increase by the smaller α value at comparable doses (see Figure 3B). In our previous papers, we explained the larger later increase by enhancement of envelope fusion and/or phospholipid exchange caused by the virus-induced modification of erythrocyte membranes (Kuroda et al., 1981).

The improvements made in the present study to measure the earlier part of fusion revealed the fast component of fusion. Its presence and the rate constant value for the component were confirmed by a quenching experiment. Previous studies could not detect this component fully because of sampling at 23 °C, during which the fast reaction occurred to considerable extents. This also lead to erroneous estimation of h_0 by extrapolation, a larger value than the correct one. The analysis gave a smaller rate constant, 0.08 min^{-1}, for fusion (Ohnishi & Maeda, 1982). Another spin-label assay using Tempo-labeled choline also gave a smaller value of 0.085 min^{-1} (Maeda et al., 1981a; Ohnishi & Maeda, 1982), probably by the same reasons. Lyles & Landsberger (1979) obtained 0.1 min^{-1} by a spin-label assay utilizing change in the overall splitting value for PC*.

In our previous paper (Kuroda et al., 1980), we showed that the ESR peak-height increase for the HVJ*–erythrocyte system became larger as the dose of HVJ* increased, while in the present study we obtained the opposite tendency (Figure 4). This decrepancy can also be explained by the incomplete detection of early data points and by the dose dependency of α. The previous estimate of h_0 by extrapolation gave larger values at smaller doses, because of more fraction of fast-fusing virus, while it gave correct value at larger doses because of preponderance of the slow component. As a consequence, the peak-height increase h/h_0 obtained by using the h_0 value would give smaller values for the smaller dose systems. If we used the peak-height data only after 2 min in the present study, estimated h_0 by extrapolation, and replotted h/h_0 with the h_0 value, we obtained a qualitatively similar dose dependency of the peak-height increase to that in our previous paper.

In conclusion, it is shown that a fraction of HVJ adsorbed on erythrocyte membrane fused rapidly at a rate constant of 0.84 min^{-1} at 37 °C, independent of the virus dose and irrespective of erythrocytes or ghosts as the target.

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