Membrane Fusion Activity of Influenza Virus. Effects of Gangliosides and Negatively Charged Phospholipids in Target Liposomes†

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ABSTRACT: Fusion of influenza virus with liposomes composed of negatively charged phospholipids differs from fusion with biological membranes or zwitterionic liposomes with ganglioside receptors [Stegmann, T., Hoekstra, D., Scherphof, G., & Wilschut, J. (1986) *J. Biol. Chem. 261*, 10966-10969]. In this study, we investigated how the kinetics and extent of fusion of influenza virus, monitored with a fluorescence resonance energy-transfer assay, are influenced by the surface charge and the presence of receptors on liposomal membranes. The results were analyzed in terms of a mass action kinetic model, providing separate rate constants for the initial virus-liposome adhesion, or aggregation, and for the actual fusion reaction. Incorporation of increasing amounts of cardiolipin (CL) or phosphatidylserine (PS) into otherwise zwitterionic phosphatidylcholine (PC)/phosphatidylethanolamine (PE) vesicles results in a gradual shift of the pH threshold of fusion to neutral, relative to the pH threshold obtained with PC/PE vesicles containing the ganglioside G_{D1a}, while also the rate of fusion increases. This indicates the emergence of a fusion mechanism not involving the well-documented conformational change in the viral hemagglutinin (HA). However, only with pure CL liposomes this nonphysiological fusion reaction dominates the overall fusion process; with pure PS or with zwitterionic vesicles containing CL or PS, the contribution of the nonphysiological fusion reaction is small. Accordingly, preincubation of the virus alone at low pH results in a rapid inactivation of the viral fusion capacity toward all liposome compositions studied, except pure CL liposomes. The results of the kinetic analyses show that with pure CL liposomes the rates of both virus-liposome adhesion and fusion are considerably higher than with all other liposome compositions studied. With pure PS vesicles or zwitterionic vesicles containing CL or PS, the rate of virus-liposome adhesion and the number of virus particles involved in the fusion process are higher than the corresponding parameters for PC/PE/G_{D1a} liposomes, but the rate of the fusion reaction itself is the same. Incorporation of cholesterol in PC/PE/G_{D1a} liposomes causes a relative enhancement of the rate of the fusion reaction itself, while relative to PC/PE liposomes the G_{D1a}, serving as a receptor for the virus, only causes the rate of adhesion to increase. These results indicate that a specific interaction of the virus with a receptor on the target membrane does not influence the characteristics of the fusion reaction itself.

Influenza virus is internalized by its host cell through a process of endocytosis (Matlin et al., 1981; Yoshimura & Ohnishi, 1984; Stegmann et al., 1987a; Wilschut & Stegmann, 1988), after binding to specific sialic acid containing receptors on the cellular plasma membrane (Carroll et al., 1981; Bergelson et al., 1982). Induced by the low pH inside the endosome (Tycko & Maxfield, 1982), the viral membrane then fuses with the endosomal membrane. Both the initial binding of the virus to the plasma membrane (Rogers et al., 1983) as well as the subsequent low-pH-induced fusion activity in the endosome (White et al., 1982a) are mediated by the viral membrane glycoprotein hemagglutinin (HA).[1] At or near the pH of fusion, this protein undergoes a conformational change, which is crucial to expression of its fusion activity (Skehel et al., 1982; Doms et al., 1986; Daniels et al., 1985).

We have previously studied the kinetics of fusion of influenza virus with erythrocyte ghosts or liposomes consisting of zwitterionic lipids and a ganglioside receptor (Stegmann et al., 1986). Using these target membranes for fusion, we found a very steep dependence of the initial rate of fusion on pH, resembling the pH dependence of the conformational change in the HA and that of hemolysis induced by the virus. A low-pH preincubation of the virus alone led to a rapid loss of fusion activity toward these target membranes. In contrast, a deviating pH dependence, more gradual and with a threshold shifted to neutral pH, was observed for fusion of the virus with liposomes consisting of negatively charged lipids, particularly cardiolipin (CL). Furthermore, the observed fusion activity toward these negatively charged liposomes was virtually insensitive to a low-pH preincubation of the virus alone, and fusion was much faster. It was concluded that fusion with liposomes carrying a net negative surface charge does not reflect the biological fusion activity of the virus. Recent observations by Chejanovsky et al. (1986) support this view. Using reconstituted viral envelopes that contained the binding but not the fusion protein of Sendai virus, they observed fusion with pure phosphatidylserine (PS) vesicles at low pH. Similar

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[1] Abbreviations: CL, cardiolipin; Chol, cholesterol; G_{D1a}, disialoganglioside; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; *N*-Rh-PE, *N*-(Lissamine rhodamine B sulfonyl)phosphatidylethanolamine; PC, phosphatidylcholine (dioleoyl); PE, phosphatidylethanolamine (dioleoyl); PS, phosphatidylserine; RET, resonance energy transfer.

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results have been obtained by Klappe et al. (1986), using intact Sendai virus. In all likelihood, this low-pH fusion process is different from the biological fusion activity of Sendai virus.

In this study, we have investigated which specific steps in the fusion reaction between influenza virus and liposomes are affected by the incorporation of negatively charged phospholipids or gangliosides in an otherwise zwitterionic liposomal bilayer. The kinetics and extent of fusion of the virus with the liposomes were determined, using a fluorescence resonance energy-transfer (RET) assay (Struck et al., 1981; Stegmann et al., 1985), and the results were analyzed in terms of a mass action kinetic model (Nir et al., 1986a,b). This model views the overall fusion reaction as a second-order aggregation process followed by a first-order fusion reaction, yielding separate rate constants for both steps (Nir et al., 1983; Bentz et al., 1983).

Materials and Methods

Chemicals. *N*-(Lissamine rhodamine B sulfonyl)phosphatidylethanolamine (*N*-Rh-PE), *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (*N*-NBD-PE), cardiolipin (CL), phosphatidylserine (PS), dioleoylphosphatidylethanolamine (PE), and dioleoylphosphatidylcholine (PC) were purchased from Avanti Polar Lipids, Inc. (Pelham, AL). Disialoganglioside (G_{D1a}) was from Supelco Inc. (Bellefonte, PA) and cholesterol (Chol) from Sigma (St. Louis, MO).

Virus and Liposomes. The X-47 strain of influenza virus was propagated in the allantoic cavity of embryonated eggs, as described before (Stegmann et al., 1985). After isolation by differential centrifugation, the virus was purified by sucrose gradient centrifugation (Nir et al., 1986a) and stored at −80 °C. Viral phospholipid phosphate was determined, after extraction of membrane lipids (Folch et al., 1957), by phosphate analysis (Böttcher et al., 1961). Protein was determined according to Peterson (1977). Liposomes (large unilamellar vesicles) were prepared by reverse-phase evaporation (Szoka & Papahadjopoulos, 1978) with some minor modifications (Wilschut et al., 1980) and sized by extrusion through polycarbonate filters with a pore size of 0.2 μm (Olson et al., 1979). After extrusion, any remaining larger or multilamellar liposomes were removed by centrifugation for 15 min at 12000*g*. The lipid concentration in the supernatant was determined by phosphate analysis (Böttcher et al., 1961).

Binding Experiments. For binding experiments, virus was added to liposomes, containing the RET probes, in "fusion" buffer (see below) at a 1:1 ratio (5 nmol of membrane phospholipid phosphorus or equivalent) and incubated for 15 min at 0 °C. The mixture was centrifuged at 0 °C for 15 min at 12000*g*. In the absence of liposomes, about 80–85% of the virus was pelleted by this procedure. After addition of Triton X-100 to pellet and supernatant and proper dilution, *N*-NBD-PE fluorescence was measured as described below. Binding was calculated as the percentage of total fluorescence recovered in the pellet. Preincubations of the virus were performed in a small volume (60 μL) of fusion buffer at the indicated pH and temperature.

Chemical Modification of Gangliosides. Conversion of the sialic acid of G_{D1a}, incorporated in liposomes, to its eight- or seven-carbon analogue was carried out according to Suttajit and Winzler (1971). Briefly, sodium periodate in a 2-fold molar excess over the sialic acid was incubated with the liposomes for 2 h at 0 °C in the dark. An excess of glycerol was added to terminate the reaction. After removal of the glycerol by dialysis, Na_2CO_3 and NaCl were added to final concentrations of 0.1 and 0.15 M, respectively. Subsequently, a freshly prepared solution of $NaBH_4$ was added to a borohydride concentration of 0.13 M. After 2 h at 0 °C, the reaction was terminated by the slow addition of an excess of cold glacial acetic acid. The preparation was dialyzed against 145 mM NaCl/2.5 mM HEPES, pH 7.4. Sialic acid was determined according to Warren (1959).

Fusion Experiments. For the RET fusion assay (Struck et al., 1981), 0.6 mol % each of *N*-NBD-PE and *N*-Rh-PE were incorporated in the bilayer of the liposomes. Measurements were carried out, under continuous stirring, at 37 °C in 1.4 mL (final volume) of 135 mM NaCl, 15 mM sodium citrate, 10 mM MES, and 5 mM HEPES (fusion buffer), set to various pH values. The increase in fluorescence, due to dilution of the fluorophores into the viral membrane upon fusion, was recorded at excitation and emission wavelengths of 465 and 530 nm, respectively, using an SLM-8000 fluorometer (SLM/Aminco, Urbana, IL). For calibration of the fluorescence scale, the initial residual fluorescence of the liposomes was set to zero and the fluorescence at infinite probe dilution to 100%. The latter value was determined by addition of Triton X-100 (0.5% v/v) to the liposomes and subsequent correction of the fluorescence intensity for sample dilution and the effect of Triton on the quantum yield of the *N*-NBD-PE (Struck et al., 1981). It is important to note that, when the initial concentrations of *N*-NBD-PE and *N*-Rh-PE are 0.6 mol % each, the fluorescence of *N*-NBD-PE increases linearly with probe dilution (Struck et al., 1981; Driessen et al., 1985).

Theoretical Analysis. Details of the theoretical analysis of fusion between influenza virus and liposomes as monitored with the RET assay have been presented elsewhere (Nir et al., 1986a). Briefly, the overall fusion process is considered to consist of a second-order adhesion or aggregation reaction of virus particles (V) with liposomes (L) followed by a first-order fusion reaction:

$$V + L \underset{d}{\overset{c}{\rightleftharpoons}} VL \xrightarrow{f} F \qquad (1)$$

in which F denotes the fusion product and *c*, *d*, and *f* the aggregation, dissociation, and fusion rate constants, respectively. Previously, we have demonstrated that a virus particle can fuse with several CL liposomes but that a fusion product cannot fuse with a virus particle (Nir et al., 1986a). This was also found in the present study using liposomes of various compositions, as determined from the final extents of fluorescence increase at various liposome to virus ratios between 16:1 and 1:4 (results not shown). Assuming that all liposomes, even those bound to virus particles which do not participate in fusion, are available for binding to and fusion with an active virus particle, and that a fraction (α) of the virus is unable to fuse, the final extent of fused virus particles was calculated according to eq 4 in Nir et al. (1986b).

The rate constants for aggregation and fusion were determined from the kinetics of fluorescence increase at several particle concentrations, as described in Nir et al. (1986a,b). An extension was introduced to account for virus inactivation as described in Nir et al. (1987).

Results

pH Dependence of Virus Fusion with PS-Containing Liposomes. The characteristics of fusion of influenza virus with pure CL liposomes deviate from those observed using biological or zwitterionic liposomal target membranes (Stegmann et al., 1986). The most important difference is the pH dependence of the fusion reaction. To determine whether this deviating behavior is a general property of negatively charged phospholipid vesicles, we investigated the effects of increasing

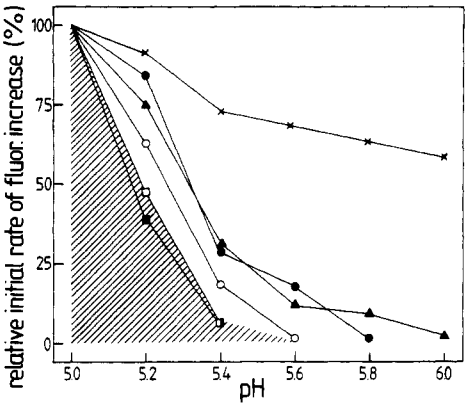

FIGURE 1: Fusion of influenza virus with liposomes consisting of pure CL (×), pure PS (▲), or PC/PE (molar ratio 2:1) containing increasing mole fractions of PS: 10% (■), 20% (□), 40% (○), and 80% (●). Fusion was measured as a function of pH. The initial rate of fusion is plotted relative to the optimal rate of pH 5.0 for each of the liposome compositions. The hatched area marks the pH dependence of the virus with human erythrocyte ghosts (Stegmann et al., 1986). Fusion was measured at 37 °C at a 1:1 ratio of virus to lipsomes, 5 μM membrane phospholipid phosphorus each.

concentrations of phosphatidylserine (PS) in PC/PE (molar ratio 2:1) liposomes on their fusion with the virus. At all pH values studied, the initial rate of fusion increased with increasing negative surface charge on the liposomes. This point will be addressed below. Figure 1 presents initial rates of fusion for the various liposome compositions as a function of pH, relative to the respective rates at pH 5.0. For comparison, the pH dependence of fusion with erythrocyte ghosts and pure CL liposomes is also presented in Figure 1. With increasing mole fractions of PS in the liposomes, starting at 40 mol %, the threshold pH for fusion shifted to neutral pH. However, the shift in pH dependence relative to that of fusion with biological membranes was much less pronounced for PS-containing liposomes than for pure CL liposomes (Figure 1). Even with pure PS vesicles, no fusion was observed above pH 6.0. The pH dependence of fusion with PC/PE vesicles containing 20 or 40 mol % CL was very similar to that of pure PS (results not shown).

Role of Gangliosides. It has been argued that gangliosides, such as G_{D1a}, rather than acting as specific receptors for the virus, merely introduce a negative surface charge on target liposomes, comparable to the effect of acidic phospholipids (Haywood & Boyer, 1986). We determined the pH dependence of virus fusion with PC/PE vesicles containing 5 mol % G_{D1a} and found it to be identical with the pH dependence of virus fusion with erythrocyte ghosts (results not shown), which argues against a nonspecific electrostatic effect of the ganglioside.

In order to further investigate the specificity of the interaction of the G_{D1a} with the virus, we chemically shortened the sialic acid of the ganglioside, converting it to its eight- or seven-carbon analogue, leaving the charge of the molecule unchanged, as described under Materials and Methods. After oxidation, the sialic acid content was 25% and, after subsequent reduction, 85% of the original. Oxidation drastically affected the fusion activity, and the activity was not restored upon reduction (Figure 2). Fusion of the virus with the chemically modified liposomes was faster than fusion with PC/PE liposomes without a ganglioside receptor. This can be entirely attributed to the 25% residual unaffected sialic acid.

Loss of Fusion Activity of Influenza Virus after a Preincubation of the Virus Alone at Low pH. In addition to its deviating pH dependence profile, fusion of the virus with pure

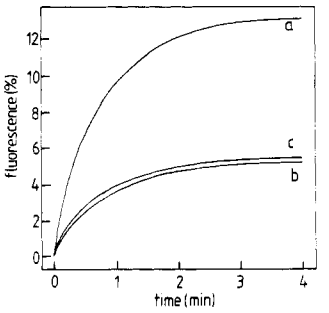

FIGURE 2: Fusion of influenza virus with PC/PE (molar ratio 2:1) liposomes containing 5 mol % G_{D1a} (curve a) or with G_{D1a}-containing liposomes that had previously been subjected to oxidation (curve b) or oxidation and reduction (curve c). Fusion was measured as described in the legend to Figure 1.

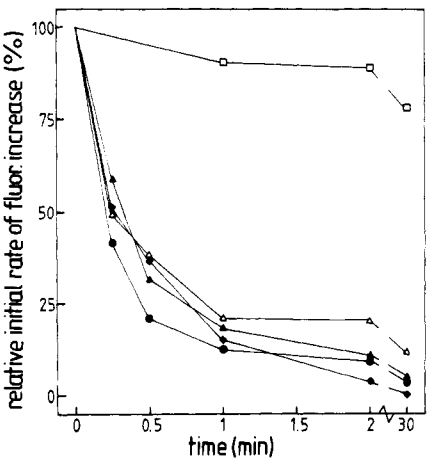

FIGURE 3: Effect of preincubation of influenza virus at pH 5.0 and 37 °C on its subsequent fusion at pH 5.0 and 37 °C with liposomes of various compositions. Liposomes consisted of pure CL (□), pure PS (●), or PC/PE (molar ratio 2:1) containing 40 mol % CL (△), 40 mol % PS (▲), or 5 mol % G_{D1a} (♦). Fusion was measured as described in the legend to Figure 1. The initial rate of fluorescence increase obtained without preincubation of the virus was set to 100%.

CL liposomes also differs from fusion with erythrocyte ghosts in that it is hardly affected by a low-pH preincubation at 37 °C of the virus alone; such a preincubation results in a rapid loss of fusion capacity toward ghosts (Stegmann et al., 1986). Figure 3 shows that pure PS vesicles or zwitterionic vesicles containing either CL, PS, or GD_{1a} are similar to erythrocyte ghosts in this respect. With all these liposome compositions, we observed a rapid and dramatic reduction of fusion due to a preincubation of the virus alone at pH 5.0. Aggregation of the virus particles among themselves was not found to be involved in inactivation, as studied by using light-scattering or absorbance techniques. Furthermore, the rate of viral inactivation was independent of the virus concentration during preincubation at low pH (results not shown), indicating that the inactivation process is of first order with respect to the virus particle concentration.

Extent of Fusion. As mentioned above, increasing concentrations of PS or CL in otherwise zwitterionic target liposomes produce an enhancement of the overall rate of fusion with influenza virus. In order to delineate whether this stimulatory effect arises at the level of the kinetics of virus–liposome adhesion or at the level of the fusion reaction itself, we carried out extensive kinetic analyses of the fusion of the virus with liposomes of various compositions. As a starting point in the kinetic analysis, we determined the extent to which virus fused with the liposomes at optimal conditions for fusion, i.e., pH 5.0 and 37 °C (Stegmann et al., 1986). The liposomes

Table I: Extent of Fusion of Influenza Virus with Zwitterionic Liposomes Containing CL, PS, G_{D1a}, or G_{D1a} and Cholesterol

liposome composition[a]	final level of N-NBD-PE fluorescence (%) experimental[b] virus, control	virus, preincubated[d] at pH 5.0 for 1 min	virus, preincubated[d] at pH 5.0 for 30 min	calculated[c]	active virus (%)
pure CL	44 (46)	45	46	39	100
PC/PE/CL (40%)	36 (45)	36	36	36	80
PC/PE/CL (20%)	37 (36)	30	35	35	70
pure PS	44 (46)	41	37	39	100
PC/PE/PS (80%)	43 (44)	42	36	38	90
PC/PE/PS (40%)	34 (31)	32	29	36	80
PC/PE/G_{D1a} (5%)	21 (24)	17	11	17–27	20–40[e]
PC/PE/G_{D1a} (5%)/ Chol (40%)	19 (nd)	19	12	17–29	20–45[e]
PC/PE	19 (20)	14	15	17–27	20–40[e]

[a] In the mixed liposomes, the PC to PE molar ratio was 2:1; the percentages indicated in parentheses are mole percent. [b] Stable levels of fluorescence after incubation of influenza virus with liposomes at a 1:1 ratio (5 μM membrane phospholipid phosphorus each) at pH 5.0 and 37 °C; the numbers in parentheses represent data obtained at 25 μM membrane phospholipid phosphorus each. The stable levels were reached after 10–30 min (upper six liposome compositions) or after 3–5 h (others). [c] Calculated according to eq 4 in Nir et al. (1986b), using the percentage of active virus given in the last column of the table. [d] Virus alone was preincubated at pH 5.0 and 37 °C. [e] The lower percentage gave the best fit for the final extent of fusion.

consisted of PC and PE in a 2:1 molar ratio. To this composition was added CL to molar concentrations of 20% and 40%, PS to 40% and 80%, G_{D1a} to 5%, or Chol and G_{D1a} to 40% and 5%, respectively. For comparison, liposomes consisting of pure PS or CL only were also investigated.

The final extents of fluorescence increase at a 1:1 ratio of virus to liposomes are shown in Table I. For all liposomes investigated, the stable extents of fluorescence increase were essentially independent of the initial virus and liposome concentrations (Table I), indicating that fusion had indeed reached its final extent. Incubations at various virus to liposome ratios (results not shown) corroborated earlier results (Nir et al., 1986a) indicating that one virus particle can fuse with several liposomes but that the fusion products cannot fuse with additional virus particles. Fusion of the virus with liposomes containing zwitterionic phospholipids, particularly the ones containing only PC/PE or PC/PE and G_{D1a}, left a fraction of the available virus particles unfused. The fraction of virus particles actively involved in fusion was also determined from the extent of fluorescence increase at ratios of virus to liposomes varying from 4:1 to 1:16 (see Materials and Methods). The estimates are reasonably reliable as can be seen by comparing the experimental final levels of fluorescence increase with the corresponding values calculated according to eq 4 in Nir et al. (1986b) (Table I).

Analysis of the Kinetics of Fusion: Effect of Low-pH-Induced Viral Inactivation. Knowing the fraction of the virus particles active in fusion and the number of virus particles per fusion product (one), in principle we could analyze the kinetics of the fusion reaction in terms of the mass action kinetic model developed previously (Nir et al., 1986a). However, during the course of the fusion process any unfused virus particles can be expected to rapidly lose their fusion activity (see Figure 3). This problem was not encountered in the analysis of viral fusion with pure CL liposomes (Nir et al., 1986a), as this fusion reaction is hardly affected by a low-pH preincubation of the virus alone (Figure 3).

The data in Figure 3 are inconclusive as to whether, in the overall fusion reaction, the aggregation or the fusion step or both are affected by a low-pH preincubation of the virus alone. Table II presents evidence indicating that the aggregation or binding step is not affected. Most of the activity of the virus to bind the liposomes was retained after a preincubation of the virus alone at pH 5.0, 37 °C, for 5 min, with the exception of the binding activity toward PC/PE liposomes. However, the latter liposomes bind to the virus via a different mechanism than vesicles containing acidic phospholipids or G_{D1a} (Stegmann et al., 1987).

Table II: Binding of Liposomes to Influenza Virus at pH 5.0

liposome composition[a]	binding (%)[b] virus, control	virus, preincubated at pH 5.0
pure CL	100	100
PC/PE/CL (40%)	100	100
PC/PE/CL (20%)	92	85
pure PS	100	100
PC/PE/PS (80%)	100	100
PC/PE/PS (40%)	100	99
PC/PE/G_{D1a} (5%)	98	91
PC/PE/G_{D1a} (5%)/Chol (40%)	94	93
PC/PE	74	9

[a] In the mixed liposomes, the PC to PE molar ratio was 2:1; the percentages indicated in parentheses are mole percent. [b] Binding was determined at 0 °C, as described under Materials and Methods, at a 1:1 ratio of virus to liposomes (5 μM membrane phospholipid phosphorus each). [c] Virus alone was preincubated at pH 5.0 and 37 °C during 5 min.

Subsequently, fusion measurements were performed over a range of virus and liposome concentrations, with or without a low-pH preincubation of the virus, using pure PS or PC/PE/G_{D1a} target liposomes. Consistent with the results in Table II, the data could be fit by employing the same aggregation rate constant, irrespective of whether the virus had been preincubated or not. On the other hand, a decay of the fusion rate constant with time according to

$$f(t) = f(0)/(1 + \gamma t)^2 \quad (2)$$

had to be assumed. In this equation, $f(t)$ is the fusion rate constant at time t, $f(0)$ the initial fusion rate constant, and γ the decay rate constant. The simulation yielded reduced f values for preincubated virus, in accordance with eq 2. Similar decay rate constants were found for both liposome types as the fusion target (γ = 0.014–0.02 s^{-1}).

Aggregation and fusion rate constants were then determined for all other liposome compositions (Table III), using the estimated percentage of active particles (Table I), and approximately the same decay rate constant for all compositions, except CL liposomes. As mentioned above, fusion activity toward pure CL liposomes is hardly affected by a low-pH preincubation of the virus alone (Figure 3). Good fit of the simulated fluorescence increase with the experimental data was thus obtained for all types of liposomes at different concentrations (Figure 4). For zwitterionic liposomes with or without G_{D1a}, we employed a value of 40–45% actively fusing virus particles initially to obtain an optimal fit, but the final extents of fluorescence increase were best explained by a value of 20%. The difference presumably represents the complete loss of fusion activity of a fraction of the virus during the course of the process, due to the effect of low pH on unfused virus particles.

It must be emphasized that with the liposomes containing negatively charged phospholipids the decay of the fusion rate constant applies only to the initial stages of the inactivation process. Some residual fusion activity toward negatively charged liposomes is retained, even after 30 min of preincu-

Table III: Analysis of Kinetics and Extent of Fusion of Influenza Virus with Zwitterionic Liposomes Containing CL, PS, G_{D1a}, or G_{D1a} and Cholesterol at pH 5.0[a]

liposome composition[b]	active virus (%)	aggregation rate constant, c ($M^{-1}s^{-1}$)	initial fusion rate constant, f (s^{-1})	fusion rate decay constant,[d] γ (s^{-1})	initial rate of fusion (% fluor/min)
pure CL	100	7.5×10^8	0.9	0	80
PC/PE/CL (40%)	80	5.0×10^8	0.25	0.013	47
PC/PE/CL (20%)	70	4.0×10^8	0.20	0.02	15
pure PS	100	4.8×10^8	0.15	0.012	25
PC/PE/PS (80%)	90	4.5×10^8	0.14	0.016	24
PC/PE/PS (40%)	80	4.4×10^8	0.10	0.016	16
PC/PE/G_{D1a} (5%)	20–40[c]	3.5×10^8	0.20	0.014	18
PC/PE/G_{D1a} (5%)/Chol (40%)	20–45[c]	2.5×10^8	0.50	0.015	19
PC/PE	20–40[c]	1.2×10^8	0.25	0.015	3.5

[a] Fusion was measured at pH 5.0 and 37 °C and analyzed as described in the text. The dissociation rate constant d had the same value throughout (0.01–0.02 s^{-1}). The estimated uncertainties are 30%, 20%, and 20% for f, c, and γ, respectively. [b] In mixed liposomes, the molar ratio of PC to PE was 2:1. The percentages indicated in parentheses are mole percent. [c] The higher value was used in the simulation of the kinetics. [d] See eq 2.

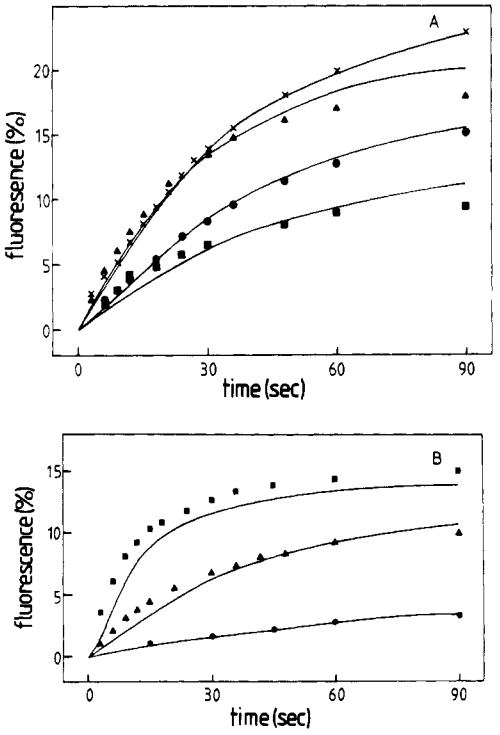

FIGURE 4: Kinetic simulation of fusion between influenza virus and liposomes of various compositions. Fusion was measured at pH 5.0 and 37 °C. The data points represent experimentally determined values and the drawn lines the theoretical simulation calculated by using the parameters presented in Table III. (A) Liposomes consisted of pure PS (×) or PC/PE (molar ratio 2:1) containing 40 mol % CL (▲), 40 mol % PS (●), or 5 mol % G_{D1a} and 40 mol % Chol (■). Fusion was measured at a 1:1 ratio of virus and liposomes, 5 μM each of membrane phospholipid phosphorus. (B) Liposomes consisted of PC/PE (molar ratio 2:1) containing 5 mol % G_{D1a}. Fusion was measured at a 1:1 ratio of virus to liposomes at concentrations of 0.5 μM (●), 5 μM (▲), or 25 μM (■) membrane phospholipid phosphorus each.

bation (Figure 3). Thus, the final extents of fusion with these liposomes were not extensively affected by preincubation of the virus alone (Table I).

Effect of the Liposome Composition on the Kinetics of Fusion. Table III summarizes the results of the analysis of fusion kinetics. The overall initial rate of fluorescence increase as observed in the fluorometer upon fusion is considerably faster for liposomes with a net negative surface charge than for PC/PE liposomes. Table III demonstrates that this is mainly due to an increase in the number of virus particles that are actively engaged in fusion and an increased rate constant of aggregation. With all liposomes containing negatively charged phospholipids, a high percentage of the virus particles (70–100%) is actively engaged in fusion, whereas with the other lipid compositions, as discussed above, this fraction is only 40–45%. For all liposomes containing negatively charged phospholiids, the aggregation rate constant is approximately 4-fold higher than the aggregation rate constant for PC/PE liposomes; for pure CL liposomes, the value is even 6-fold higher. By contrast, except for the case of pure CL, the rate constant of the fusion reaction itself is not enhanced by the presence of negatively charged phospholipids in the target vesicles.

Incorporation in zwitterionic liposomes of G_{D1a} as a specific receptor for the virus does not increase the number of virus particles active in fusion, nor the fusion rate constant. The aggregation rate constant, however, increases 3-fold in the presence of only 5 mol % of the ganglioside in otherwise zwitterionic liposomes, suggesting an efficient interaction. Incorporation of cholesterol results in a 2-fold enhancement of the fusion rate constant. Within the limits of uncertainty, we found a similar, very, low value for the dissociation rate constant for all liposome compositions (0.01–0.025 s^{-1}).

Discussion

Effect of Negatively Charged Phospholipids in Target Liposomes on the Fusion Activity of Influenza Virus. Several recent studies have indicated that the low-pH-induced fusion of certain enveloped viruses or reconstituted virosomes with liposomes containing negatively charged phospholipids does not properly reflect the biological fusion characteristics of the viruses (Stegmann et al., 1986; Amselem et al., 1986; Klappe et al., 1986; Nir et al., 1986b; Chejanovsky et al., 1986; Eidelman et al., 1984; Metsikkö et al., 1986). Our present study indicates that fusion of influenza virus with pure PS liposomes and with PS- or CL-containing zwitterionic liposomes, in contrast to fusion of the virus with pure CL liposomes (Stegmann et al., 1985, 1986; Nir et al., 1986a), has characteristics that are similar to fusion of the virus with biological target membranes. First, the pH threshold of fusion is shifted only slightly toward neutral pH, even in the case of pure PS (Figure 1). Second, fusion is highly sensitive to a low-pH preincubation of the virus alone. We have presented evidence indicating that this inactivation, occurring in the absence of target membranes, is a result of the low-pH-induced conformational change in the viral HA (Stegmann et al., 1987b). This conformational change results in exposure of the hydrophobic N-terminus of the HA_2 subunit of the molecule (Skehel et al., 1982; Sato et al., 1983; Doms et al., 1985). In the presence of a fusion target for the virus, this hydrophobic segment of HA_2 is thought to trigger the membrane fusion reaction, presumably through penetration of the segment into the target membrane (White et al., 1983; Doms et al., 1985,

1986; Harter et al., 1988). In the absence of target membranes, the exposure presumably leads to clustering of the HA molecules or penetration of the hydrophobic segment into the viral membrane, resulting in an irreversible loss of fusion activity (Junankar & Cherry, 1986; Stegmann et al., 1987b). Therefore, our present observation of fusion of the virus with liposomes being strongly affected by a low-pH preincubation of the virus alone (Figure 3) indicates that this fusion reaction occurs predominantly via a mechanism involving the exposure of the hydrophobic segment of the HA.

The results of the kinetic analysis, summarized in Table III, are completely in line with this conclusion. The rate constant of the fusion reaction itself is not affected by the inclusion of negatively charged phospholipids in otherwise zwitterionic target liposomes (Table III). The stimulatory effect on the overall fusion reaction (Table III) is entirely due to an increase in the number of virus particles participating in the fusion process and an increase in the rate of virus–liposome aggregation. The increase in the number of virus particles that fuse with negatively charged liposomes will be discussed below, as it relates directly to the question as to why only a fraction of the virus has the capacity to fuse with zwitterionic liposomes with or without gangliosides (Table III). The increased rate of aggregation is most probably due to electrostatic interactions between the virus and the liposomes.

In comparison with the other negatively charged target liposomes used in this study, CL liposomes display a deviating behavior. There is a major shift in the pH dependence of virus fusion with CL liposomes (Figure 1), the fusion reaction is virtually insensitive to preexposure of the virus alone to low pH (Figure 3), and the fusion rate constant for CL liposomes is significantly larger than for all the other liposome compositions (Table III). It is unclear what causes the aberrant behavior of pure CL liposomes. However, it is interesting that it is strongly moderated by inclusion of zwitterionic phospholipids in the vesicles: Not only do the characteristics of the fusion reaction resemble more the biological fusion characteristics of the virus, there is also a decrease in the initial rate of the overall fusion reaction (Table III). This latter observation indicates that with pure CL liposomes a nonphysiological fusion mechanism, independent of the conformational change in the viral HA, is superimposed on the physiological fusion mechanism, whereas with the other liposome compositions the contribution of this nonphysiological mechanism is sufficiently reduced to reveal the biologically relevant fusion process with its specific characteristics (Figures 1 and 2). Conversely, after a preincubation of the virus at pH 5.0, which abolishes its biological fusion activity, the residual activity is probably due to the unaffected nonphysiological fusion mechanism. With CL liposomes, this residual activity is high (Figure 3). With the other negatively charged liposomes, the residual activity is very low (Figure 3), but yet it does result eventually in a high final extent of fusion (Table III).

Effect of Gangliosides and Cholesterol. The very specific interaction of influenza virus with G_{D1a}-containing PC/PE liposomes (Figure 2) results in an enhanced rate of aggregation relative to the rate of aggregation between the virus and liposomes consisting of PC/PE only (Table III). However, the fusion reaction per se is not affected. Incorporation of cholesterol in G_{D1a}-containing liposomes stimulates the rate of fusion 2-fold, but the aggregation rate constant and the number of virus particles involved in the fusion process remain unchanged. Evidently, unlike fusion of Semliki Forest virus (Kielian & Helenius, 1984), fusion of influenza does not display an absolute requirement for the presence of cholesterol in the target membrane. The same conclusion has been reached by White et al. (1982b).

Besides the nature of the interaction, the most important difference between zwitterionic liposomes, with or without ganglioside receptors, on the one hand, and liposomes containing negatively charged phospholipids, on the other hand, is the number of virus particles that are able to fuse with the liposomes. We do not know why not all of the virus particles have the capacity to fuse with biological membranes or zwitterionic liposomes. However, heterogeneity in the virus population must be involved, since the fraction of the virus that is able to fuse is essentially independent of the initial virus to liposome ratio (Table I).

It is remarkable that, although the initial interaction of the virus with negatively charged liposomes is electrostatic in nature and thus differs from the specific binding to GD_{1a}-containing zwitterionic liposomes or biological target membranes, the subsequent fusion reaction exhibits largely the same characteristics. Recently, Daniels et al. (1987) have described several receptor binding mutants of influenza virus, exhibiting concomitant altered fusion characteristics. Our present results suggest that these altered fusion characteristics may have been independent effects of the mutations in the receptor binding site of the HA on the fusion reaction, as the specific interaction of the HA with its receptor does not seem to influence the characteristics of the HA-induced fusion reaction.

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High-Yield Purification of Platelet-Derived Endothelial Cell Growth Factor: Structural Characterization and Establishment of a Specific Antiserum

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ABSTRACT: Platelet-derived endothelial cell growth factor (PD-ECGF) is a 45-kDa protein that stimulates the growth of endothelial cells [Miyazono, K., et al. (1987) *J. Biol. Chem. 262*, 4098-4103]. Here, we describe a method to purify large quantities of PD-ECGF from human platelet lysate at a high yield (14% overall recovery). The purification method involves five steps, using high-performance liquid chromatography grade hydroxylapatite and hydrophobic chromatographies as the two final steps. The purified material contained two major components of apparent molecular weight values of 46 000 and 44 000. These components coeluted in a high-resolving reversed-phase chromatography and were found to give similar peptide maps after treatments with staphylococcal V8 protease, suggesting that the 44-kDa form is related to the 46-kDa molecule. Partial tryptic digestion of native PD-ECGF revealed that the molecule contains a trypsin-resistant domain of 37-39 kDa. A rabbit antiserum was produced against the purified material and was found to specifically recognize PD-ECGF in immunoblotting. When added to the cell culture medium, an immunoglobulin fraction of the antiserum neutralized the activity of purified PD-ECGF. Furthermore, it completely neutralized the endothelial cell mitogenic activity of platelet lysate, indicating that PD-ECGF is the only mitogen in platelet lysate for this cell type.

Factors with the ability to stimulate the formation of blood vessels have attracted interest as causative agents in angiogenesis of normal and malignant tissues [for a review, see Folkman and Klagsbrun (1987)]. The most well-characterized

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