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Bilayer Lipid Membranes (BLM): Study of Antigen–Antibody Interactions

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Abstract

Previous work of del Castillo and co-workers has shown that bilayer lipid membranes (BLM) can be used as transducers for detection of antigen–antibody reactions. The present experiments extend the previous work by incorporating complement into the BLM system. The results indicate that the antigen–antibody complex or the complement has no ability to affect the BLM system separately, but when carefully combined they will destabilize the BLM even at a much reduced concentration. Further development using the BLM as a tool for investigating immunological reactions is suggested.

Introduction

The bimolecular lipid leaflet, now popularly known as the lipid bilayer, has been increasingly considered as the central component of all biological membranes. This fact is supported by a plethora of recent symposia and publications on biological membranes [1–5]. Concurrently, the study of artificial bilayer membranes as models of biological membranes has been fruitful as evidenced by the publication of monographs and reviews [6–9]. A cogent rationale for investigating artificial bilayer membranes is that the real and much more complex natural counterparts can be better understood at the molecular level, if information is available on simpler, well-defined model systems. This approach is simultaneously useful for testing hypotheses of biological membranes.

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To date, bilayer lipid membranes (BLM) and liposomes (lipid microvesicles) have been widely employed owing to the fact that the structure of these artificial membranes is strikingly similar to the postulated lipid bilayer of biological membranes [1–6]. Among the studies using the BLM system reported recently are electrical properties [10, 11], interfacial tension [12, 13], ion selectivity [14, 15], thickness [16, 17], lipid–protein interaction [18, 19], "excitability" [20, 21], and light-induced effects [22, 23].

In the present paper, we describe a practical and promising application of the BLM system as a model for the study of immunological reactions. The use of a BLM for such a purpose was initiated by del Castillo et al. [24] and was followed by others [25–29]. Among the latter investigators Michaels et al. [25] and Rosenstreich and Blumenthal [26] have clearly shown in their experiments that immune damage to biological membranes can be mimicked in BLM. Related experiments have also been carried out in liposomes [30–38]. It would seem ideal to perform further experiments using BLM to monitor antigen–antibody (Ag–Ab) interactions in view of these findings and current speculations on immune cytolysis where the cell membrane is believed to play a central role [39]. The specificity of Ag–Ab reactions coupled with the sensitivity of electrical measurements developed for the BLM system may provide not only a useful tool for immunology but also a method for testing various proposed mechanisms of membrane-mediated processes including the immune cytolysis already mentioned and cell agglutination. Toward these goals, we have initiated experiments with encouraging preliminary results [40]. The first part of this paper briefly reviews the experimental apparatus and previous immune complex work using the BLM. The pH as well as the ionic strength of the ambient solutions is critical in the enhancement of membrane conductance. The second part presents the results of antigen–antibody and antigen–antibody plus complement interactions with the BLM, and shows that these reactions are specific.

Materials

The lipid used for the BLM was 10 : 1 (vol/vol) of oxidized cholesterol to lecithin. The oxidized cholesterol was prepared according to a published procedure [6]. The L-α-lecithin used was a chloroform solution of 1 g/10 ml and was obtained from the Sigma Chemical Company (St. Louis, Missouri). The antigen–antibody pairs were available in lyophilized form as I-Dent-O-Kits from the Miles Laboratories (Elkhart, Indiana). The complement was obtained in dried form through Hyland Chemicals (Costa Mesa, California). The proteins mentioned previously were reconstituted in water and the

complement was always kept cold and freshly prepared. The bathing solution on both sides of the BLM consisted of sodium chloride (0.145 M), $CaCl_2$ (0.15 mM), and Mg Cl_2 (0.33 mM). Phosphate or Tris HCl buffer concentration was 0.01 M.

Apparatus

The electrical measurements were made using a Keithley 610BR electrometer and a Keithley picoammeter (Model 417) connected as shown in Fig. 1. The resistor (R_i) can be selected as 0 Ω or 10^5–10^{10} Ω by factors of 10. The applied voltage (E_i) was variable from −100 to 100 mV. The capacitor (C) was sometimes used during thinning to reduce breakage and is 0.01 μF. Switches S_1 to S_4 are voltage-activated microrelays of very high resistance in the open position and are available through Potter and Brumfield. If the current measurement is desired, S_1 and S_3 are activated while S_2 and S_4 are left open; if the voltage measurement is desired, the opposite selection is made. In this way it is possible to manipulate the BLM measuring apparatus with no disturbance to the BLM and without inducing spurious voltage or instabilities. The overload circuit was designed to protect the picoammeter if the BLM ruptures when the current is being measured. The circuit is shown

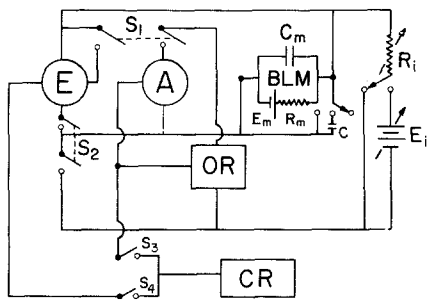

Figure 1. Measuring circuit for electrical measurements in bilayer lipid membranes (BLM). R_i is a resistor ranging from 0 or 10^5–10^{10} Ω. E_i is an external battery variable from −100 to 100 mV. C is a 0.01 mfd capacitor, E a Keithley electrometer (610C), A a Keithley picoammeter (Model 417). S_1, S_2, S_3, S_4 are voltage-activated switches, used to select the voltage or current measurement mode and to switch the chart recorder from one instrument to the other. C_m, R_m, and E_m refer to the capacity, resistance, and voltage of the BLM and are drawn as an equivalent circuit for a BLM. CR is the chart recorder, OR the overload protection circuit that prevents current surges to the picoammeter, which is described in Fig. 2.

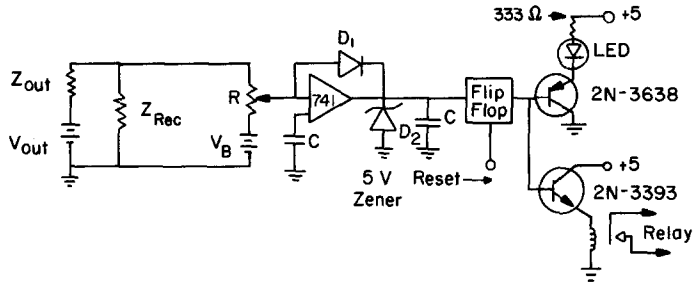

Figure 2. Current overload protector. Z and V refer to the chart recorder (*CR* in Fig. 1) output terminal of the picoammeter. Z_{rec} is a chart recorder and R and V_B are used to bias the recorder output to cross zero when the picoammeter suffers a current overload. The 741 operation amplifier is used as a comparator and the output voltage is limited to range from 0 to 5 V by diodes D_1 and D_2. When the picoammeter is overloaded, the 741 comparator output switches from 0 to +5 V, which will cause the flip-flop output to drop from 6 V to zero. The light-emitting diode (LED) trouble indicator will turn on due to current flow in the 2N3638 and the relay will open due to loss of current in the 2N3393. The relay is connected to the input of the picoammeter as shown in Fig. 1. The reset is used to restore the flip-flop after the current overload is eliminated.

in Fig. 2. The input resistance (R) to the operational amplifier (741) is chosen to be 5 MΩ, and does not interfere with the meter output impedance (Z) or the chart recorder impedance (Z_{rec}) at this value. The bias voltage (V_B) is adjusted so that the potential into the operational amplifier will cross zero at the desired limiting voltage. The diode (D_1) limits the operational amplifier output to positive voltage while the zener diode (D_2) ensures that the output will not exceed the 5 V input maximum for the flip-flop. The capacitors, designated by C, are 0.1 μF and are inserted to cut down spurious noise. The flip-flop is connected to be at $+V_{max}$ when the current is subthreshold, and to drop to zero when the current exceeds the maximum value. When the voltage is zero the transistor switch 2N3393 NPN turns off while the transistor switch 2N3638 PNP turns on. This provides for the removal of the picoammeter from the circuit by deactivating a relay and simultaneously turning on a light-emitting diode (LED) as a trouble indicator.

When the switches (S_2 and S_4) are in the voltage mode (on position), the BLM is represented by an equivalent circuit consisting of a capacitor (C_m) in parallel with a series resistor (R_m) and the BLM voltage (E_m) as shown in Fig. 1. Thus, the electrometer voltage (E) is related to the other parameters in the circuit diagram by

$$E=\frac{E_m}{1+R_m/R_i}-\frac{E_i}{1+R_i/R_m} \tag{1}$$

If R_i is much larger than R_m, which is done in practice by setting it at infinity, one will measure E_m. If R_m is much larger than R_i, one can obtain the applied voltage E_i. Knowing these extreme voltages, one can measure the voltage at intermittent values for R_i and obtain several values for the BLM resistance through the equation

$$R_m = R_i\left(\frac{E_m - E}{E + E_i}\right) \tag{2}$$

The capacity of the BLM can be obtained by measuring the RC decay curve. For a given charging curve, one obtains

$$E(t) = E_{\max}\left[1 - exp\left(-\frac{t}{R_p C_m}\right)\right] \tag{3}$$

where $E(t)$ is the charging curve voltage at time t, $E_{\max}$ is the final charging curve voltage, and $R_p = (R_i R_m)/(R_i + R_m)$. In this paper, the voltage change appeared across the BLM and C_m was calculated by the equation

$$C_m = \frac{-t}{\ln(1 - E(t)/E_{\max})R_p} \tag{4}$$

The time constant was large, and usually two readings were made 10 sec apart and the capacity value was averaged.

Results and Discussion

As mentioned in the Introduction, pioneering in using the BLM system as a tool for detection of antigen–antibody reactions was the work reported by del Castillo et al. [24]. These authors observed that immunological reactions involving protein molecules dissolved in one side of the bathing solution of the BLM caused large and reversible reductions in the membrane electrical impedance. In their immunological experiments, del Castillo and co-workers used BLM formed from brain lipids. The transient transverse electrical impedance of the BLM was measured. Human and bovine serum albumins, ovalbumin, and human serum albumin diazotized with sulfanilic acid were used as antigens and corresponding immune sera as antibodies. No attempt to heat-deactivate complement was made. Separate addition of albumin or immune serum did not affect the BLM, but addition of a protein after the corresponding antigen or antibody was in contact with the membrane caused a sudden reduction in the electrical impedance of the BLM. Later work by Wobschall and McKeon [27] showed that complement was

needed in addition to immune complexes to cause a steady state resistance decrease in the BLM. A recent interesting paper by Wolf et al. [29] is pertinent to these studies. The present experiment also finds that the BLM resistance can be exquisitely sensitive to specific antigen–antibody complexes, in the presence of complement. The BLM resistance has also been shown to decrease reversibly in the presence of antigen. The membrane in this condition is stable and can last for hours. The addition of specific antibody will rapidly cause the resistance and capacity of the BLM to return to their original values. The antibody stabilization effect follows a titration curve. When excessive antibody is added, the BLM once again becomes unstable.

Ab and Ag Studies

The antigen or antibody was first separately interacted with the BLM to ascertain the proper pH, temperature, and electric field needed for stable, reproducible BLM studies. Bovine serum (BS), human serum (HS), rabbit anti-bovine serum (RABS), and rabbit anti-human serum (RAHS) were reconstituted in their lyophilized form and kept at 0°C for use within 1 hr. The Teflon cup used to support the BLM was cleaned with chloroform and soap between experiments and the membranes were checked for good stability and high resistance before an experiment was initiated. A Teflon-coated magnetic stirrer was used. Between 0.01 and 0.1 mg/ml of protein was needed to destabilize the BLM and the resistance was inversely proportional to the concentration. The BLM capacitance increased linearly with protein concentration. Only slight negative voltages were generated at the low protein concentrations. The effect of the proteins on the resistance, voltage, and capacity of the BLM depends on pH. Studies using phosphate buffer (0.01 M) at pH 6.5, 7.4, and 7.8 indicated that the BLM was most stable at pH 7.4. Addition of 0.1 mg/ml or protein caused small negative voltages at the lower pH. The specific antigen–antibody interaction is now presented.

The preceding paragraph makes it clear that the antigen and antibody can interact individually with the BLM to produce voltage, resistance, and capacity changes. The resistance of the BLM will be used as the primary indicator of activity because it often changes several orders of magnitude as compared to relatively smaller changes in the voltage and capacity.

Complement fixation triggered by the antigen–antibody complex has been observed with the BLM system [25]. These experiments are repeated, and the slight effect on steady state voltage and capacity are presented as well. To demonstrate complement fixation, small aliquots of rabbit anti-bovine serum and bovine serum at concentrations of 1 mg/ml were heated

separately to 56° C for 15 min to remove all complement activity. The rabbit anti-bovine serum was added in a 3 : 2 ratio to the bovine serum and the resulting mixture was allowed to stand in a warm water bath. This process helps to form antigen–antibody complexes. The complexed antigen–antibody solution was diluted a thousand-fold and added to the inner cup of the BLM solution. Stirring was ensured to be adequate for complete distribution using

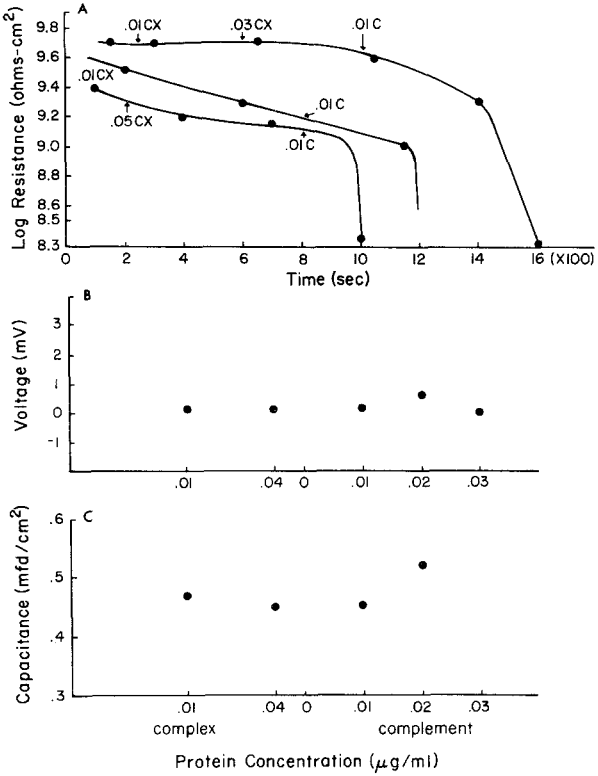

Figure 3. The destabilizing effect of rabbit complement when added to a BLM system containing BS-RABS antigen–antibody complexes. A 10 : 1 oxidized cholesterol–lecithin BLM formed at 22°C in a magnesium–calcium–sodium chloride solution as described in the text and phosphate buffered to pH 7.4 is used. (A) The BLM resistance on a linear scale with the horizontal axis indicating real time. Arrows indicate addition of the antigen–antibody complex (CX) or rabbit complement (C) with units of microgram per milliliter. (B) The BLM voltage and (C) the BLM capacity. These data were obtained in three runs and averaged. The horizontal axis indicates first the complex concentration (in μg/ml) and then the complement concentration added after the complex (in μg/ml).

a magnetic stirrer. The BLM system was left undisturbed and electrically shorted out for at least 5 min while the antigen–antibody complexes were being absorbed onto the BLM. Freshly prepared complement kept at 0°C was added to the inner cup solution of magnesium–calcium saline which was buffered to pH 7.4. The BLM resistance would gradually decrease over a period of 5 min under these conditions. The reaction resulted in very little voltage generation and almost no capacity change, as shown in Figs. 3B and 3C. Four individual experiments, all giving small voltage and capacity changes, are averaged to give the results presented here. Figure 3A shows the resistance of the BLM as a function of time with the addition of complex or complement marked C. The speed at which the complement will affect the

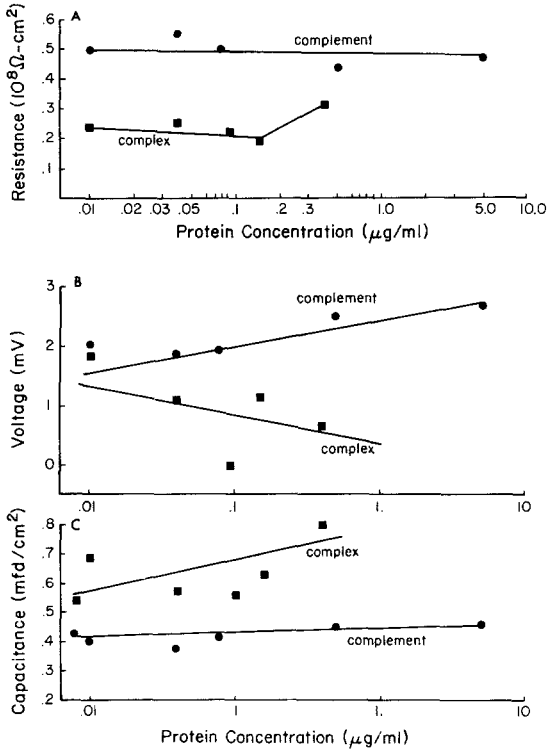

Figure 4. The lack of effect of complement or antigen–antibody complex when added separately. The experimental conditions are identical to those in Fig. 3: (A) BLM resistance, (B) BLM voltage, and (C) BLM capacity. These null reactions resulted from addition of protein which extends up to 5 μg/ml, or about 100 times the concentrations used to give successful reactions in Fig. 3.

membrane increases with the concentration of antigen–antibody complex for the low concentration shown.

Identical solutions of the complement and antigen–antibody complex were tested separately for membrane activity; they showed no ability to affect membrane resistance even after the protein concentration was increased a 100-fold above that necessary for complex–complement reaction. Figures 4A–4C show the resistance on a linear scale, voltage, and capacity changes of a BLM when fresh complement and antigen–antibody complex were added separately. The concentrations were started small at around 10 ng/ml, and were increased to 5 mg/ml with no appreciable change in the parameters measured. Three nearly identical experiments using complement addition were made and the results were averaged. These results indicate that the antigen–antibody complex or the complement has no ability to affect the BLM system separately, but when carefully combined they will destabilize the BLM even at a much reduced concentration.

Antigen–Antibody Interactions on the BLM

The BLM has been successfully used as a substrate to observe interactions of antibody with antigens. The antigen (HS or BS) and antibody (RAHS or RABS) protein solutions were heated to 56°C for 15 min to destroy complement activity. The pH was maintained at pH 7.4 by a phosphate buffer in a magnesium–calcium–sodium chloride solution, as described earlier. The addition of antigen resulted in destabilization of the BLM at concentrations of 0.4–0.8 μg/ml as described earlier. Instead of further decreasing the BLM resistance as in the previous experiments by the addition of more antigen, small quantities of antibody were added. Figures 5A and 5B show the dramatic resistance and capacitance increase, respectively, upon addition of antibody to the inner BLM cup along with the destabilizing antigen. It is not necessary to add equivalent amounts of antibody to a given amount of antigen in order to observe the restabilization effect. Small concentrations of the antibody can be titrated against the antigen and after their concentrations become equal the BLM starts to destabilize again, as it does when antibody is added alone. This unexpected result was not observed at higher protein concentrations of around 50 μg/ml simply because the reaction between each protein and the membrane was always sufficient to cause a marked membrane reaction. It was also possible that at high protein concentrations, the entire reaction was occurring in the bathing solution, leaving no material for the membrane. When the protein concentration is reduced, the membrane is envisioned as an amplifier that can collect small quantities

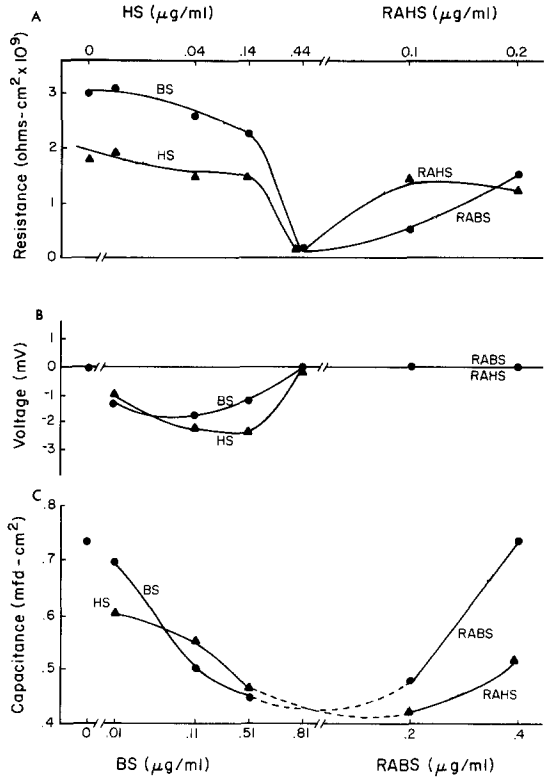

Figure 5. The stabilizing effect of antibody on BLM which have been destabilized by antigen. The experimental conditions are identical to those described in Fig. 3: (A) BLM resistance, (B) BLM voltage, and (C) BLM capacity. The two horizontal scales refer to the BS and RABS concentration (in μg/ml) (Fig. 5C) or to the HS and RAHS (in μg/ml) (Fig. 5A) and were matched together for easy comparison of results. Note that Ab addition results in the recovery of the BLM resistance and to some extent the capacity with antibody addition. The antibody by itself will destabilize and break the BLM.

of protein and hold it in a two-dimensional matrix until its molecular counterpart could be joined to it. The joining could relieve stresses in the membrane surface resulting in an altered resistance. The restabilization of the BLM happens quickly, within a few seconds at most, and so it probably occurs right at the membrane surface as opposed to involving antigen which is present in the solution. One could otherwise think that the antibody complexes with solution antigen which could restore the BLM resistance by

deabsorption of antigen from the BLM into a lower antigen concentration medium. If the antibody interacts at the surface, it could activate complement which then interacts with the BLM by either forming a stable channel or rupturing the membrane.

Conclusions

The central idea behind this study of antigen–antibody reactions is to demonstrate that many of the classical experiments which are performed routinely in immunology can also be employed using the artificial BLM system. The basic precipitin reaction depends on the bonding of the bivalent antibodies with the multivalent antigen and thereby acting as a cement to build up a large lattice of proteins. When the complex is large enough it will precipitate out, giving a visual test for the antigen–antibody complex. Another standard reaction is the complement fixation reaction, where complement serves as a membrane lysing agent when activated in the presence of calcium, magnesium, and sodium ions.

The experiments described herein have reproduced these reactions using a BLM as a two-dimensional matrix upon which the delicate molecular immune reactions can take place. The electrical method of measuring the resistance, voltage, and capacity of the BLM while the reaction occurs is demonstrated to be an appropriate and extremely sensitive method to observe immunological reactions. The reaction of the antigen or antibody protein with the BLM was destabilizing at concentrations of 0.4–0.8 μg/ml. The effect of complement on the electrical properties of a BLM–immune complex system is studied [25, 27], and the capacity and voltage data are also presented. The interaction of complement-inactivated immune sera with the BLM was studied and a specific prolonged recovery and stabilization reaction was observed when the antibody was added to a BLM with an antigen (protein) induced low resistance. The capacity of the BLM also exhibits a reversible decrease. The BLM again becomes unstable when excessive antibody is added.

Further extensions of this work now in progress include development of the antigen–antibody into a laboratory tool which can be used to identify and quantify the antigen. The application of electric fields to the BLM in an attempt to manipulate solution proteins and enhance the immune reactions observed thus far is also being studied. By proper selection of BLM lipid and proteins and by carefully controlling experimental conditions, one can obtain precise and unambiguous physical measurements of correlated changes in BLM properties, which may be

useful for the understanding of a variety of cellular activities such as the immunological reactions [39–41].

Acknowledgment

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