Interaction of lymphocytes with lipid bilayer membranes: A model for lymphocyte-mediated lysis of target cells

(membrane permeability/ion channels)

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Communicated by Terrell L. Hill, April 21, 1975

ABSTRACT Horizontal lipid bilayer membranes were used as a model system to study lymphocyte-mediated killing of target cells. Dinitrophenylated lipid bilayers can physically support dozens of lymphocytes for periods of over one hour without breakage or increasing the electrical conductance of the membrane. However, in the presence of antibody against Dnp, human lymphocytes rapidly induced increases in membrane conductance of several orders of magnitude without membrane breakage. Such ionic permeability increases occurred only when the membrane voltage was positive on the lymphocyte side, as would be the case with a target cell membrane. The lymphocyte and antibody dependence of this conductance increase parallels that observed for lymphocyte killing of antibody-coated target cells. The results are interpreted as evidence that the primary event in lymphocyte killing of antibody-coated target cells is the creation of ion-conducting channels in the target membrane.

Lymphocytes appear to play a major role in the immunologic rejection of foreign grafts and tumors. Since lymphocytes have also been shown to be able to destroy such foreign cells directly in vitro, interest has focused on the mechanism of this lymphocyte-induced killing (1). Several lines of evidence suggest that lymphocytes destroy their target cells by inducing a breakdown in the permeability barrier to small ions, leading to a subsequent colloid osmotic lysis. Morphological studies of the killing reactions have demonstrated that the cytolytic process is accompanied by a marked swelling of the dying cell (2). The study of target cells internally labeled with molecules of different sizes shows that the smaller molecules are released from the dying cell faster than those of high molecular weight (3–5). High concentrations of macromolecules have been shown to suppress the final release of 51Cr from the target cell (5, 6), but the interpretation of these findings is controversial (7). There has been no evidence to indicate whether the early permeability increase in the target membrane is caused directly by the attacking lymphocyte or is a secondary consequence of some other primary injury.

In order to study the ability of lymphocytes to induce permeability changes in a foreign membrane, we have studied the interaction of human lymphocytes with artificial lipid bilayer membranes. Such “black lipid membranes” have been used extensively to model the permeability properties of naturally occurring membranes and have been especially useful in studying the properties of membrane active drugs (8, 9). The ionic permeability of such membranes can be conveniently studied by electrical measurements. In the present studies we have used an antigenic lipid to form a bilayer membrane, and have examined the ability of nonimmune lymphocytes to induce permeability changes in the presence of antibody bound to the membrane. This system is a model for the antibody-dependent (but complement-independent) killing of target cells mediated by human lymphocytes (reviewed in ref. 1).

Our results demonstrate that lymphocytes can rapidly increase the electrical conductance of the lipid bilayer by several orders of magnitude. Thus, lymphocytes can increase the ionic permeability of a target membrane without any contribution of the target cell cytoplasm or membrane proteins. The attacker cell and antibody requirements for this membrane conductance change closely parallel those for lymphocyte-mediated cytolysis of antibody-coated target cells.

MATERIALS AND METHODS

Materials. Oxidized cholesterol was prepared by the method of Tien et al. (10). Dnp-phosphatidylethanolamine (Dnp-PE) was synthesized from bacterial phosphatidylethanolamine (Pierce Chemical Co.) by the method of Uemura and Kinsky (11). IgG antibodies against Tnp were prepared from hyperimmunized rabbit antisera to Tnp-bovine gamma globulin and purified by affinity chromatography on Dnp-lysine Sepharose (the Tnp and Dnp hapten cross-react). F(ab)2 fragments of these antibodies were prepared by pepsin digestion followed by gel filtration on Sephadex G-150. The hemagglutination titer of the F(ab)2 preparation was equal to that of the undigested antibody. Human lymphocytes were prepared from fresh blood by Ficoll-Hypaque sedimentation (12) and were resuspended in Hank’s balanced salt solution. In some experiments phagocytic monocytes were eliminated (<1%) from these preparations by the nylon column method of Dickler (13). Identical results were obtained with both cell preparations. Preparation of lymphocytes depleted of Fc receptor-bearing cells was achieved by sedimentation onto plastic coated with antigen-antibody complexes (P. Henkart and E. Alexander, manuscript in preparation).

Lipid Bilayer Membranes. In these experiments we have modified the conventional apparatus for formation of lipid bilayers (8). Our lipid bilayers are horizontal, formed across a 330-nm round hole in the thin bottom of a Teflon cup which is immersed in a small Lucite chamber. The cup and chamber contain Hank’s balanced salt solution (Na+, 159 mM; K+, 5.7 mM; Ca2+, 1.3 mM; Mg2+, 0.8 mM; Cl−, 146 mM; SO42−, 0.8 mM; H2PO4−, 0.4 mM; HPO42−, 0.3 mM; glucose, 5.6 mM), and the membrane is formed by spreading a few microliters of a mixture of oxidized cholesterol (1% w/w) and Dnp-PE (0.2% w/w) in decane across the hole.
with a small paint brush. The horizontal arrangement enables us to observe the bilayer in a phase microscope at X100, and we can readily observe the thinning of the membrane as a wave of slightly higher transparency which closely resembles the wave of secondary black film formation as observed in the conventional apparatus.

Electrical Measurements. The electrical conductance of the membrane separating the two liquid compartments was measured by means of two silver/silver chloride electrodes leading to the circuit shown in Fig. 1. A variable voltage difference was maintained across the membrane by a dc source, and the current flowing in response to this voltage was measured by a current transducer made according to the design of Alvarez et al. (14).

We define the membrane voltage as the potential difference of the upper membrane surface (inside of the cup) with respect to the lower surface (outside of the cup). If we think of the bilayer as being analogous to a target cell membrane, this convention means that the bilayer membrane potential is expressed in the opposite polarity to the convention used to express cellular membrane potentials (inside with respect to outside the cell).

Interaction of Antibody and Lymphocyte with the Membrane. All the experiments reported here were carried out at 23° in Hank’s balanced salt solution with no fetal calf serum. Under these conditions, the antibody-dependent killing of Tnp-erythrocytes by human lymphocytes proceeds nearly as efficiently as at 37° in 10% fetal calf serum.

In most experiments, the membrane was coated with antibody by injection of 5 μl of 1–2 mg/ml of purified antibody directly above the membrane, followed by 20 min of incubation. Alternatively, the membrane could be formed in a solution of 1 μg/ml of antibody. Either method was effective in sensitizing the membrane to lymphocytes. After incubation with antibody, lymphocytes were vigorously injected in a 5-μl volume (of a suspension of 1 X 10^7 cells per cm^2) down onto the membrane with an Oxford sampler micropipet. Cells became visible in the microscope immediately and had finished settling onto the membrane within 10 min. Lymphocytes on the bilayer were counted; those on the thicker "torus" around the edge of the hole were excluded (see Fig. 2).

RESULTS

Stability of Bilayers. Horizontal lipid bilayers can support dozens of lymphocytes for periods of over an hour before membrane breakage occurs. In our experience, lipid bilayers comprised of oxidized cholesterol and Dnp-PE mixture, especially in the presence of anti-Tnp, are more stable than those made of oxidized cholesterol alone. A small membrane formed over a 300-μm hole was used because it appears somewhat more stable than a larger membrane formed over a 1-mm hole, but the latter could support over a hundred cells for periods of up to 30 min. After large (over 100-fold) conductance increases induced by lymphocytes, membranes showed an increased tendency towards an electric breakdown, i.e., breakage induced by an imposed electric field.

Effect of Lymphocytes on Membrane Conductance. The conductance of the oxidized cholesterol-Dnp-PE bilayer membrane is between 10^-7 and 10^-8 mho/cm^2, slightly higher than that of pure oxidized cholesterol membranes. Exposure of such a membrane to anti-Tnp has no effect on this conductance (Fig. 3A and B). When lymphocytes are allowed to settle on antibody-coated membranes, a marked conductance change is observed, beginning 2–20 min after the cells arrive on the membrane. The rate and extent of conductance change are variable from experiment to experiment, and are dependent on the membrane voltage, as discussed below. Fig. 3A and B show typical experiments. In 50% of the experiments, such as that in Fig. 3A, the initial change is a conductance increase of 2–10X, accompanied by a marked increase in the current noise level. After a period of 1–10 min, a more substantial and often rapid conductance increase occurs, between one and three orders of magnitude. Further slow increases in conductance occur after this. Conductance decreases are not seen except in response to negative membrane voltages (see below). Fig. 3B shows a less common pattern of conductance increase, which has been observed in approximately 20% of these experiments. In this case the conductance increase occurs slowly and smoothly. In the other 30% of our experiments, a conductance increase of 100X to 1000X occurred very rapidly, with no prior changes observed. In the presence of active antibody and lymphocytes, we have not failed to obtain conductance increases. The differences in the patterns of conductance do
FIG. 3. Conductance changes caused by lymphocytes acting on antibody-coated lipid bilayers. A and B are typical experiments in which anti-Tnp is added to the medium over the lipid bilayer shortly after formation of the bilayer. Twenty minutes later, 5 X 10^4 lymphocytes are injected into the medium over the membrane, and the number of cells landing on the bilayer (Fig. 2) is indicated (lc here stands for lymphocyte). The number of cells on the membrane increases for several minutes as they sediment onto the membrane. At 30 min, the cells have all settled. The experiment shown in C is a control experiment with no antibody; the cells were added shortly after membrane formation. Thirty lymphocytes remained on the bilayer throughout the experiment. The membrane voltages were held constant at +20 to +60 mv (except A, discussed in the text) until the conductance had increased 100X.

not correlate with other parameters, such as membrane voltage or the number of lymphocytes on the membrane.

Conductance increases were observed with as few as five cells and commonly with less than 20 cells. Since the lymphocyte preparations contain 10–30% cells bearing Fc receptors, and since these cells are responsible for killing of antibody-coated target cells or induction of this membrane conductance increase (see below), we have inferred that a large percentage of Fc receptor-bearing cells are probably capable of causing this conductance increase.

Fig. 4 shows the actual current recording of a lymphocyte-induced initial slow conductance increase during an experiment with no detectable noise. Small step increases in current corresponding to conductance increases of about 10^{-11} mho are apparent in this record. Similar discrete conductance changes have been seen in bilayer membranes in the presence of antibiotics for which there is good evidence that pores form in the membranes (15, 16). These have been interpreted as the opening of individual channels.

Voltage Dependence of the Conductance Increase. The membrane conductance increases described are highly dependent on the polarity of the membrane voltage. These increases occur only when the potential of the lymphocyte side is positive; when a negative membrane voltage is imposed at the start of an experiment, the conductance does not increase in spite of the presence of antibody and many lymphocytes. If, after such a period of negative voltage, the polarity is reversed, a conductance change is observed within 15 min, indicating that no permanent barrier to conductance changes has been established. Negative voltage pulses reversed part of the conductance increase induced by a positive pulse. Thus, in Fig. 3A an initial increase in conductance occurred while the membrane was held at +60 mv (38 min). At 41 min the voltage was reversed to -60 mv, and the conductance returned to its previous value. When the membrane voltage was returned to +60 mv at 45 min, the conductance again increased. Negative pulses also decrease the current noise level observed during the initial phase of the lymphocyte-induced electrical changes. A further example of this voltage dependence is shown in Fig. 5, which shows the membrane current (not conductance) recording after a conductance increase of 1000X had occurred. When the membrane voltage polarity was reversed at 6.5 min, the negative current decreased on 4 μA/cm^2, indicating a conductance decrease. The current appeared to stabilize at this level however, indicating that only part of the previous
Table 1. Antibody and lymphocyte dependence of membrane conductance increase

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Lymphocytes</th>
<th>% ¹¹⁴Cr release by Tnp-RBC (no. of Exps.*)</th>
<th>Membrane conductance increase (no. of Exps.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti-Tnp</td>
<td>Human PBL</td>
<td>100</td>
<td>250x -20,000x (12)</td>
</tr>
<tr>
<td>None</td>
<td>Human PBL</td>
<td>0-2 (4)</td>
<td>0-10x (6)</td>
</tr>
<tr>
<td>Anti-Tnp-F(ab)₂</td>
<td>Human PBL</td>
<td>4-5 (2)</td>
<td>0 (1)</td>
</tr>
<tr>
<td>Anti-Tnp</td>
<td>None</td>
<td>-1-8 (4)</td>
<td>0-5x (2†)</td>
</tr>
<tr>
<td>Anti Tnp</td>
<td>Fc receptor negative</td>
<td>3-15 (4)</td>
<td>0 (1)</td>
</tr>
</tbody>
</table>

RBC, red blood cells; PBL, peripheral blood lymphocytes.
* Relative to complete system (line 1).
† In each experiment the membrane is incubated with antibody alone for 20 min; conductance increases greater than 5X were never seen. In two experiments the membranes were incubated with antibody alone for 1 hr.

conductance increase could be reversed by reversing the voltage. At 83 min the voltage was returned to a positive value, inducing a rapid conductance increase indicated by the rising current. A second negative voltage pulse at 9.4 min again resulted in a partial reversal of the previous conductance increase. This example is typical of the behavior of the conductance after high values had been established by antibody and lymphocytes.

Initial conductance increases were routinely observed at membrane potentials between +20 and +60 mv, and in all the experiments the imposed potential was held constant at a value in this range until the conductance had increased by two orders of magnitude. After the initial conductance increases, further increases could generally be induced by potentials higher than about +10 mv; the higher the potential, the more rapid was the observed conductance increase.

Antibody and Lymphocyte Dependence of the Membrane Conductance Increases. Lymphocytes resting on the membrane in the absence of antibody do not induce significant conductance changes, as shown in Fig. 3C. Antibody dependence is also a characteristic of cytotoxicity of target cells by nonimmune lymphocytes as shown in Table 1. Similarly, F(ab)₂ anti-Tnp antibodies, prepared from the purified anti-Tnp preparation used in all our experiments, did not sensitize the Dnp-membrane to conductance increases, although their antigen binding ability appeared intact.

In the absence of lymphocytes, the antibody-coated membranes have a stable low conductance, and the only conductance increases observed were those immediately preceding membrane breakage. Lymphocyte preparations contained 10-20% monocytes in most experiments. Identical results were obtained with a monocyte-depleted, purified lymphocyte preparation. This is in accord with previous findings (P. Henkart and I. Yust, in preparation) on the antibody-dependent killing of Tnp-human erythrocytes, which showed that purified lymphocytes are as effective killer cells as the monocyte-containing cell populations. Likewise, the lymphocyte cell population depleted of Fc receptor bearing cells is inactive both in killing antibody-coated target cells and in increasing the conductance of antibody-coated membranes.

These experiments, summarized in Table 1, show that the lymphocyte and antibody dependence of the membrane conductance increase is identical to that of target cell lysis. These results support the hypothesis that the membrane permeability increase we observe is the initial event in the destruction of target cells by lymphocytes.

Variation of the Lipid Composition of the Membranes. Lipid bilayers composed of oxidized cholesterol alone were not stable when lymphocytes were added; breakage generally occurred within 10 min. In our apparatus, membranes composed of Dnp-PE alone, or PE alone, did not thin to form bilayers. No conductance changes were observed when lymphocytes plus anti-Tnp were added to these membranes. Thus, it would appear that thick membranes containing Dnp-PE and coated by antibody are not affected by the lymphocytes under conditions where thin membranes show conductance increases. Alternatively, it may be that cholesterol is required for the lymphocyte-mediated conductance increase.

DISCUSSION

The data presented clearly demonstrate that lymphocytes possess the ability to increase the permeability of lipid bilayer membranes to one or more of the ions of balanced salt solution under the same conditions as required for the killing of target cells. Extrapolating from this model to the lysis of target cells, our observations have two important implications concerning the mechanism of cell killing. First, lymphocytes can act on membranes directly to cause permeability increases without the participation of other target cell components. Thus, it seems plausible that the observed early increases in target cell permeability (3-5) represent primary damage to the membrane and are not the secondary consequence of cell injury elsewhere. Second, target cell membrane protein is not necessary for the lymphocyte-induced permeability increase. Thus, the mechanism of permeability increase does not necessarily involve the loss of control of preexisting permeability pathways.

Three alternative mechanisms that could explain the observed lymphocyte-induced electrical conductance increases are shown in Fig. 6. The fusion mechanism shown at the bottom would result in a conductance increase mediated by the existing ion-conducting pathways in the lymphocyte membrane. This mechanism would predict discrete conductance changes of the order of 10⁻⁶ to 10⁻¹¹ mho associated with each fusion event since naturally occurring plasma membranes have conductances ranging from 10⁻² to 10⁻⁸ mho/cm². The variety of patterns of conductance increase observed is, however, difficult to explain by the fusion mechanism.

The channel and carrier mechanisms are parallel in many respects and are based on the interpretation of studies of the interaction of antibiotics and lipid bilayers (15). A carrier is a membrane component which combines with the substance being transported to form a complex with a lipophilic exterior or capable of crossing the membrane. A channel is a specialized region that provides a low resistance diffusion path for ions or nonelectrolytes to cross the membrane. Our data suggest that the lymphocyte-induced conductance increase is mediated by a channel-forming mechanism on the basis of
the target membrane; (2) this substance is very rapidly inactivated; or (3) this substance possesses a specificity component. The present results suggest another mechanism that could account for part or all of the observed specificity. Some channel-forming compounds act in a highly cooperative fashion, i.e., the induced conductance is dependent on the concentration of the channel former raised to a high power, apparently because a functional channel is composed of a number of molecules aggregated together within the membrane (9, 17). Thus, the observed specificity could be due to the fact that the lymphocyte releases a soluble channel-forming substance whose activity is highly concentration-dependent, and it is only in the immediate vicinity of the attacking lymphocyte that the active material reaches effective concentrations.

Conductance increases somewhat similar to those we have reported have been observed in lipid bilayers treated with antibody and complement by Barfort et al. (18) and recently confirmed by Wobschall and McKeon (19). It would appear that both forms of immunologic damage to cells, humoral and cellular, may be initiated by a membrane permeability increase due to the formation of pores in the membrane.

We thank Ms. Judith Magnotta and Ms. Cynthia Neels for their excellent technical assistance, and Dr. William Terry for helpful discussions.