Leukemic-Like Membrane Properties Acquired by B Lymphocytes When Depleted of 185,000-Dalton Macromolecular Insoluble Cold Globulin

By Mary A. Simmonds, Gloria Sobczak, and Stephen P. Hauptman

Human peripheral blood lymphocytes can be phenotypically identified by the presence of one or both of two proteins, 225,000-dalton macromolecular insoluble cold globulin (225-MICG) and 185,000-dalton MICG (185-MICG). T cells synthesize and insert into their plasma membrane 225-MICG, null cells 185-MICG, and B cells both 225 and 185-MICG. In contrast, the monoclonal B cells of chronic lymphocytic leukemia are characterized by the presence of 225-MICG and the absence of 185-MICG. We have recently found it possible to chemically deplete 185-MICG from viable normal B cells by treating them with diisopropylfluorophosphate (DFP), thus making normal B cells phenotypically resemble leukemic cells. In the present report we determined whether certain peculiar properties of these leukemic cells would be associated with the normal B cells chemically depleted of 185-MICG. In normal B cells, SIg diffuses in the lipid bilayer to form clusters and caps under appropriate conditions, while in chronic lymphocytic leukemia (CLL) cells this does not occur. Normal B cells depleted of 185-MICG fail to undergo capping of SIg or surface MICG under appropriate conditions. Both DFP-treated B cells and CLL cells tend to rupture when smeared on a glass slide. Both CLL cells and DFP-treated B cells fail to secrete 225-MICG after it has been synthesized intracellularly. The relationship of these findings to the mechanisms of secretion and capping are discussed.

MATERIALS AND METHODS
Isolation of Human Lymphocytes and Subpopulations

Peripheral blood mononuclear cells (PBL) from normal donors and CLL patients were separated from 30 ml of heparinized whole blood or from byproducts of platelethpheresis (kindly donated by the Thomas Jefferson University Hospital Donor Center) by Ficoll-Hypaque centrifugation (FHC). T cells in these preparations were enumerated by rosette formation with neuraminidase-treated sheep red blood cells (SRBC) and separated from B cells by rosette sedimentation on FH in RPMI-1640, as previously described. The T lymphocytes contained less than 1% SIg^+ cells, and 97% of these cells formed rosettes with SRBC, i.e., were E^+. B lymphocytes contained 93% SIg^- cells and less than 1% E^- cells. Surface Ig^- lymphocytes, i.e., B cells, were identified by direct immunofluorescence (IF) as previously shown. If cells were to be left overnight, they were routinely placed in 10% fetal calf serum (FCS) at 4°C; otherwise, there was a considerable loss of 185-MICG. All experiments with CLL cells were done after PBL were depleted of T cells and rather than referring to these cells as CLL B cells, we will use CLL cells. The population of patients with leukemia has been previously detailed.

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Isolation of MICG by Cold Precipitation

Isolated cells were lysed in isotonic buffer containing 0.5% Nonidet P-40 (NP-40) and 0.12 M iodoacetamide for 20 min at 4°C, as previously described. In some experiments, DL-isopropyl-fluorophosphate (DFP) (Calbiochem-Behring Corp., La Jolla, Calif.) at a final concentration of 5 μM was added immediately after NP-40, while controls received 5 μM of propanol. The cell lysates were centrifuged to 105,000 g for 30 min at 4°C, the pellet discarded, and the supernatant incubated at 4°C for 16 hr. The cold precipitate was washed and solubilized in an SDS-urea buffer. Further details of 225 and 185-MICG cold precipitation, preparation of antisera, and SDS polyacrylamide gel electrophoresis (SDS-PAGE) (5%) have been previously reported. Solubilized precipitates were reduced with 0.5 M 2-mercaptoethanol for 1 hr at 37°C, alkylated with 0.65 M iodoacetamide, and then dialyzed. Protein was quantitated as previously described and equal amounts of protein (20–50 μg) applied to gels that were to be directly compared.

Cell Culture Conditions for Protein Synthesis and Secretion

Single cell suspensions (10–50 × 10^6) were incubated in 3 ml of Eagle's minimum essential medium (MEM) minus leucine (Grand Island Biological Co., Grand Island, N.Y.), without FCS, with 50 μCi of 3H-leucine (specific activity 111.2 Ci/mole from New England Nuclear, Boston, Mass.) as described. The cells were incubated for 4 hr at 37°C in a mixture of 95% air-5% CO2 in a humidified atmosphere. The radiolabeled cell suspension was then immersed in ice water, made 0.06 M with iodoacetamide, and centrifuged at 400 g for 10 min. The supernatants were collected and termed "secretions." The cells were washed extensively and resuspended in 1.5 ml of isotonic buffer, pH 7.2.

Detergent Lysis of Radiolabeled Cells and Immune Precipitation of Cell Lysates and Secretions

Desoxycholate (Fisher Scientific Co., Fairlawn, N.J.) was added to the radiolabeled cell suspension to yield a final concentration of 0.5% (v/v), and the mixture was made 0.12 M with respect to iodoacetamide and 1000 U/ml of Trasylol added. After incubation for 20 min at 4°C, the resultant cell lysate was centrifuged at 105,000 g for 30 min at 4°C and the pellet discarded. Radiolabeled lysates and secretions in a volume of 1.6 ml or 3.0 ml, respectively, were immune precipitated by the direct technique using anti-225-MICG antiserum (250 μl), anti-185-MICG antiserum (250 μl), or antiserum with reactivity toward both 225- and 185-MICG, i.e., anti-225 + 185-MICG antiserum (250 μl) in antibody excess. Incubation of antiserum with cell lysates proceeded at 4°C for 16 hr. Under these conditions, MICG proteins remained soluble, enabling MICG to be immune precipitated from whole cell lysates. The precipitates were washed and dissolved in SDS-urea buffer.

Immunofluorescence

Indirect immunofluorescence (IF) was performed on viable PBL and subpopulations to detect surface MICG (SMICG) as previously detailed. Chronic lymphocytic leukemic cells were recently shown to synthesize only 225-MICG, while normal B cells synthesize both 225- and 185-MICG. When normal B lymphocytes were treated with 5 μM DFP, they were found to be depleted of 185-MICG, thus phenotypically resembling CLL cells. This is illustrated in Fig. 1, where DFP-treated B cells were lysed and cold precipitated. On SDS-PAGE only the 225,000-dalton band was stained (Fig. 1). Similarly, cold-precipitated lysates from CLL cells demonstrated...
the absence of 185-MICG (Fig. 1), in contrast to B cells reacted with 5 μM propanol, which demonstrated both 225- and 185-MICG (Fig. 1). We have also studied the B cells from 15 other laboratory controls for the presence of these proteins following DFP or propanol treatment. In each case, propanol treatment of normal B cells followed by cold precipitation of the lysates yielded two bands on SDS-PAGE, i.e., a 225,000-dalton band and a 185,000-dalton protein band. However, DFP treatment of B cells yielded only the 225,000-dalton protein from each of the B-cell controls in spite of the application of 100 μg of protein on these latter gels. When B cells were incubated with radiolabeled amino acid and treated with 5 μM DFP they were also shown to lack 185-MICG after cold precipitation, while 225-MICG was synthesized in normal amounts. Control B cells radiolabeled and incubated with 5 μM propanol were shown to synthesize both 225- and 185-MICG. These results were reproduced in 5 other B-cell controls. It should be noted that 5 μM DFP added immediately after lysis of B cells also induces the loss of 185-MICG in the cold precipitate, as we have shown in four separate experiments.

To determine whether DFP results in the loss of protein(s) other than 185-MICG, the NP-40 lysate of DFP (5 μM) treated B cells was precipitated with trichloracetic acid at a final concentration of 10% and compared to that of propanol (5 μM) treated B cells. The only difference noted was the absence of 185-MICG in the DFP-treated cells, out of at least 50 bands identified on stained SDS gels. Control T cells were also treated with 5 μM DFP or propanol, lysed and cold precipitated. These studies demonstrated an intact 225-MICG on SDS-PAGE, regardless of whether DFP was added before or after cell lysis.

Verification of the absence of 185-MICG in B cells after incubation with 5 μM DFP was sought utilizing immunofluorescent microscopy. When isolated B cells were incubated for 1 hr with 5 μM DFP, immunofluorescence utilizing antibody to 185-MICG revealed 1%-3% 185-MICG+ cells, while 98% of the propanol-treated B cells were 185-MICG+ (Table 1). These findings contrast with the results obtained with antisera to 225-MICG, which stained the cell surface of 90% of propanol-treated B cells and 90% of DFP-treated B cells with equal intensity. Three representative experiments are shown in Table 1. Similar results were obtained from B cells derived from 4 other donors. As a control, T lymphocytes were treated with 5 μM propanol or DFP and 225-MICG detected by immunofluorescence using anti-225-MICG antibody (not shown). A mean of 98% of the T cells were stained in a ringed pattern in the presence of either DFP or propanol in five laboratory personnel.

To further explore the specificity of DFP treatment...
Table 1. Absence of 185-MICG After DFP Treatment of B Cells*

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Antiserum</th>
<th>Percent Immunofluorescent-Positive Cells</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Propanol</td>
</tr>
<tr>
<td>1</td>
<td>Anti-185-MICG</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>Anti-225-MICG</td>
<td>92</td>
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<tr>
<td></td>
<td>Anti-Ig</td>
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<td>Anti-185-MICG</td>
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<td>Anti-Ig</td>
<td>93</td>
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<tr>
<td>3</td>
<td>Anti-185-MICG</td>
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<td>Anti-Ig</td>
<td>92</td>
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*Isolated B cells (50 x 10^6/3 ml) were treated with either propanol or DFP at a final concentration of 5 μM for 1 hr at 37°C, followed by the examination of these viable cells with the antisera noted above and immunofluorescent microscopy.

†From 300 to 400 cells were counted on each slide and the results expressed as the ratio of the number of immunofluorescent-positive cells to the number of phase contrast cells (x 100).

‡Each number refers to a separate control of donor B cells.

on B cells we determined the presence or absence of SIg utilizing immunofluorescent antiserum to Ig (Table 1). Surface Ig was detected on propanol-treated B cells, DFP (5 μM) reacted CLL cells (not shown), and DFP-incubated B cells. There was a ringed staining pattern in 91% of the B cells, with and without DFP, in all three experiments.

Surface Ig on CLL cells cannot be capped under appropriate conditions, i.e., with bivalent antibody to Ig and incubation at 37°C. In the next experiment we compared the ability to induce capping of SIg and surface 225-MICG in control B cells, DFP-treated B cells, and CLL cells (Fig. 2). To detect capping on viable cells we utilized anti-Ig antibody at 37°C.

Surface Ig on normal B cells treated with propanol migrated to one pole (capped), while DFP-incubated (5 μM) B cells and CLL cells did not undergo capping of SIg (Fig. 2 A–C). In these experiments we studied isolated B cells from 12 normal donors. We found that the number of capped cells in the propanol-treated group ranged from 85%–90% of the fluorescent-positive B cells, whereas the number of capped cells from the B-cell group treated with DFP ranged from 0%–6%. When surface 225-MICG was examined for capping with anti-225-MICG antibody, results similar to SIg were obtained. Capping of surface 225-MICG was demonstrated in control B cells. However, 225-MICG could not be induced to undergo capping in either DFP-reacted B cells or in CLL cells.

In recent studies we have shown that CLL cells, in contrast to normal B cells, lacked the ability to secrete 225-MICG into the culture medium. Synthesis experiments were performed to determine if a similar

Fig. 2. Absence of SIg capping following DFP treatment of B cells. B cells or CLL cells were treated with propanol or DFP for 1 hr at 37°C. After washing, the cells were reacted with GAHIgM (FITC) for 30 min at 37°C, and a drop of the cell suspension fixed on a slide. The cells were examined with an epifluorescent microscope with selective filters for FITC and phase microscopy. (A) B cells treated with propanol; (B) B cells treated with DFP; (C) CLL cells treated with propanol.
situation accompanied DFP-reacted B cells. Radiolabeled cells were incubated with either DFP or propanol and the lysates and secretions precipitated with anti-225 + 185-MICG antiserum and subjected to SDS-PAGE (Fig. 3). Two radioactive peaks, 225,000 and 185,000 daltons, were immune precipitated from the lysate and secretion of propanol-treated B cells (Fig. 3 A and C). Chronic lymphocytic leukemia B cells demonstrated only a 225,000-dalton peak in the cell lysate, and there was no 225-MICG in the secretion (not shown). When normal B cells were labeled and the viable cells reacted with 5 μM DFP and then lysed, only a 225,000-dalton peak was present in the lysate (Fig. 3B). Furthermore, 225-MICG was absent in the secretion of these labeled B cells treated with DFP (Fig. 3D). We have performed identical experiments in 6 other controls, and in each case, there was an absence of 185-MICG intracellularly and an absence of secretion of 225-MICG. As a control, the addition of DFP to secretions collected from normal B cells did not alter the ability to detect the 225- and 185-MICG proteins.

A frequently seen, but poorly understood, phenomenon in CLL is the presence of “smudge or basket cells” on the peripheral blood smear (Fig. 4A). When normal T and B cells are similarly smeared, no smudging is observed (Fig. 4B), nor is smudging observed when normal T cells are treated with 5 μM DFP prior to smearing. In contrast, 45%–57% of the B cells from 12 CLL patients (not shown) and 35%–49% of the DFP-treated B cells from 7 normal controls smudged when smeared on glass slides (Fig. 4C).

**DISCUSSION**

The findings in this study indicate that DFP physicochemically and antigenically depletes 185,000-dalton MICG from B cells, endowing them with many properties usually attributed to CLL cells. The physical depletion of 185-MICG was demonstrated on SDS-PAGE after lysates from DFP-treated B cells were cold precipitated. Studies utilizing immune precipitation of radiolabeled lysates showed that after 185-MICG was synthesized, DFP was able to alter it, making it unreactive with the appropriate antiserum. These findings were confirmed by immunofluorescence, which showed the absence of 185-MICG on the B-cell surface after incubation with DFP.

Since normal B cells depleted of 185-MICG phenotypically resemble CLL cells, we determined whether they also developed some unusual characteristics normally identified with these leukemic cells. Surface Ig on normal B cells diffuses in the lipid bilayer to form clusters and caps under appropriate conditions, i.e., temperature of >20°C and bivalent antibody. A property of CLL cells is the absence of redistribution (capping) of SIg under appropriate conditions. In this regard, B cells depleted of 185-MICG by DFP also failed to undergo capping of SIg after exposure to antibody at 37°C. Surface 225-MICG on DFP-treated B cells was also incapable of being redistributed.
Similarly, 225-MICG on CLL cells could not be capped.

An additional property shared by the DFP B cell and the CLL cell is the “smudge” phenomenon, i.e., when smeared on a glass slide, certain lymphocytes rupture, producing “basket-like” cells. We have no explanation for why only 35% of DFP-reacted B cells and 45% of CLL cells smudged when smeared. It may be related to the force applied while smearing the cells or to the thickness of the drop of cells before smearing.

The final likeness identified between DFP-treated B cells and CLL cells is the absence of MICG secretion. Radiolabeled B lymphocytes treated with propanol, as a control, demonstrated labeled 225- and 185-MICG in the cell lysate and secretion. In contrast, radiolabeled B cells treated with DFP showed only 225-MICG, while the secretion from these cells lacked 225-MICG. These results are analogous to the findings in CLL cells, i.e., the labeled lysate contains only 225-MICG, and there is no detectable 225-MICG in the secretion from these cells.14

The mechanism of action of DFP in relationship to the disappearance of 185-MICG is speculative at present. Diisopropyl fluorophosphate is a serine protease (esterase) inhibitor, which irreversibly inactivates certain enzymes, i.e., serine proteases, by covalently binding to the serine residue in the active site.34 At least two possibilities could explain the loss of 185-MICG. First, DFP could cause the inactivation of a serine esterase that acts as an inhibitor in and/or on normal B cells. This enzyme would normally react with and inactivate a protease in B cells that is specific for 185-MICG. When the inhibitor is inactivated by DFP, the protease would be free to cleave 185-MICG. This proposal contrasts with the more usual, direct consequence of DFP, i.e., inhibition of proteolysis due to serine proteases.35 In keeping with this latter mechanism, DFP would inhibit a protease in normal B cells that cleaves 225-MICG to 185-MICG, thus 185-MICG would be a breakdown product of 225-MICG. Studies are currently in progress to determine the mechanism of action of DFP on 185-MICG.

The present studies suggest a possible biologic function for 185-MICG. Like CLL cells, DFP-treated B cells lack 185-MICG, rupture when smeared on a slide, lack the ability to form caps, and lack the capacity to secrete 225-MICG. These phenomena have in common their dependence on an intact plasma membrane and cytoskeletal system. In lymphocytes, as well as in other motile cells, a microfilament and microtubular system underlies the plasma membrane.36,39 It has been suggested that cell movement,
secretion, and capping may be controlled by a contractile mechanism similar, but not identical, to that which induces muscle contraction in striated muscle. It is possible, although admittedly speculative, that 185-MICG serves as a bridge connecting cytoskeleton components to integral membrane proteins without which the cell is unable to undergo capping or secretion. Studies are in progress to determine the validity of this speculation.

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