Poor Mixed Lymphocyte Reaction Stimulatory Capacity of Leukemic B Cells of Chronic Lymphocytic Leukemia Patients Despite the Presence of Ia Antigens

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ABSTRACT The human Ia-like antigens, selectively expressed on B lymphocytes, are now recognized to be closely associated with, or identical to, the gene products of the major histocompatibility complex responsible for stimulation in the mixed lymphocyte reaction. The leukemic B lymphocytes of patients with chronic lymphocytic leukemia express these antigens very well. In the present study they were readily detected by several techniques utilizing both allo- and heteroantiser. However, the leukemic B cells from most patients were found to be extremely poor stimulating cells in the mixed lymphocyte reaction. This was particularly apparent when comparisons were made on a B-cell basis with isolated normal B lymphocytes.

Leukemic cell death, abnormal kinetics of leukemic cell-mediated stimulation, and serum or cellular suppressor factors do not appear to explain these findings. Studies comparing cells from a leukemic patient with those of her HLA identical sibling and results of mixed lymphocyte reactions between normal and leukemic subjects discordant for D-region-associated Ia antigens ruled out genetic explanations for the differences observed. Experiments with normal peripheral blood mononuclear cells depleted of T cells and monocytes exclude the quantitative deficiency of monocytes which is found in the peripheral blood of most leukemic patients as an explanation.

The present results with chronic lymphocytic leukemia cells indicate that the mere expression of the Ia-like antigens by cell populations does not render them effective stimulators. The accumulated evidence obtained indicate that abnormalities, particularly of membrane function and metabolism, known to occur in chronic lymphocytic leukemia lymphocytes may be involved in the poor stimulatory capacity of the leukemic B cells.

INTRODUCTION

Human B lymphocytes selectively express alloantigens with a restricted tissue distribution. The alloantigens' extensive polymorphism, linkage to the major histocompatibility complex (MHC) and two-chain glycoprotein structure have indicated that they represent the human equivalent of the murine Ia antigens. They are detected by alloantibodies (1) found in the sera of multiparous women and transplant recipients and by heteroseras (2-4) prepared by injecting rabbits with B-lymphocyte membrane fractions. These antigens have usually been termed "Ia-like"; for simplicity, they will be referred to here as Ia antigens. Evidence has been obtained that Ia alloantibodies will block a one-way mixed lymphocyte reaction (MLR) when directed against determinants expressed on the stimulator cell populations (5) and that treatment of the stimulator population with heteroantisera is also effective (6). In addition, a close relationship exists between MLR typing using homozygous typing cells and serological typing using alloantibodies directed against Ia (7). Thus, it has been concluded that Ia antigens are gene products of the MHC region (HLA-D) responsible for MLR stimulation, or are intimately associated with these gene products. For this reason certain Ia antigens

1 Abbreviations used in this paper: CLL, chronic lymphocytic leukemia; Dr, HLA-D region associated; E, sheep erythrocyte; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; PBM, peripheral blood mononuclear cells.
detected are designated as D-region-associated (Dr) antigens. The expression of Ia antigens by B lymphocytes and results of experiments with isolated cell populations have led to the conclusion that B lymphocytes are capable of stimulating in the MLR (8–11). Most cases of chronic lymphocytic leukemia (CLL) represent a neoplastic proliferation of B lymphocytes (12) which, like their normal counterparts, express membrane immunoglobulin (Ig) and Ia antigens (2, 3, 13). Therefore, it was of interest to examine the stimulatory capacity of these cells which can be obtained readily in large amounts and in a highly purified state.

METHODS

Cell preparation and isolation of lymphocyte subpopulations. Heparinized peripheral blood samples were obtained from normal volunteers and patients with CLL. Mononuclear cells were isolated by standard Ficoll-Hypaque methods (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.). Cell yields were always >60% and consisted of >95% mononuclear cells; viability, as assessed by trypan blue exclusion, was always >95%. B-Cell purification was achieved by eliminating T cells forming sheep erythrocyte (E) rosettes with neuraminidase-treated E (14). The nonpelleting (interface cells) contained <2.5% of cells capable of forming E rosettes. This was followed by monocyte deple- tion using one of three techniques: 10^2 peripheral blood mononuclear (PB M) cells were incubated at 37°C with 40–80 mg of carbonyl iron (superfine, GAF Corp., New York). After 1-h incubation, the iron-laden monocytes were retained by a magnet and the lymphocytes were harvested. Monocytes were also removed by allowing them to adhere to plastic flasks for 1–2 h in RPMI 1640–10% fetal calf serum at 37°C. The third method of monocyte depletion employed human erythrocytes sensitized by human anti-Rh allosera. The sensitized cells form rosettes with monocytes and third population cells (15) which can be removed by gradient centrifugation. B cells bearing surface IgM were isolated from T-cell- and monocyte-depleted PB M according to their capacity to form rosettes with purified anti-IgM antibodies covalently linked to bovine erythrocytes by the CrClk method. Briefly, the cells were rosetted with the anti-IgM bovine erythrocyte reagent for 1 h at 4°C followed by two Ficoll-Hypaque gradient centrifugations. The pellet obtained was shock-lysed with an (NH4)Cl-tris buffer and washed in phosphate-buffered saline before culturing.

Cell Characterization. Rhodamine-conjugated F(ab')2 fragments specific for IgM, IgG, IgD, and IgA were prepared and used for surface staining of lymphocytes as previously described (16). IA alloantigens were detected with sera obtained from multiparous women in an indirect fluorescence assay using F(ab')2 fragments of rabbit anti-human Ig anti serum, as described (1). Rabbit antisera against purified human Ia antigens were made as described previously (2) and used to prepare rhodamine-conjugated F(ab')2 fragments for lymphocyte surface staining. It has been shown that both the allo- and heteroantisera detect the same populations of cells and have similar properties, including precipitation of a bimolecular complex whose components have mol wt of 28,000 and 37,000 daltons (2).

T cells were enumerated by rosette formation with neuraminidase-treated E. Fc receptors were detected by immune complex binding (2) and rosette formation between Fc-bearing lymphocytes and ox erythrocytes sensitized by rabbit anti-ox erythrocytes, according to Haegert et al. (17). Monocytes were enumerated by their appearance in Wright-stained preparations, latex ingestion (2), and by detection of endogenous (18) peroxidase activity.

Mixed lymphocyte cultures. Unidirectional MLR were performed using a slight modification of the procedure described by Hartzman et al. (19). Lymphocytes were resuspended in RPMI 1640 supplemented with penicillin, streptomycin, and 20% heat-inactivated AB Rh+ serum obtained from healthy nontransfused males. Stimulatory lymphocytes were irradiated with 3,000 rads. The indicated number of stimulator cells and 1.5 x 10^6 responding cells were mixed in flat-bottomed wells (Microtest II, Falcon Plastics, Oxnard, Calif). Each culture was established in triplicate. After a 120-h incubation at 37°C in 5% CO₂ humidified atmosphere, 2 μCi of [3H]thymidine was added to each culture. After an additional 16 h, the cultures were harvested on an automated sample harvester and processed for scintillation counting. To be considered an acceptable experiment, the coefficient of variation of the triplicates had to be <20%.

Experimental design. In each experiment the MLR responses of PB M from two normal subjects to a leukemic stimulator were measured. In addition, the response of each normal subject to the other normal serving as a stimulator was determined. This experimental design allowed the direct comparison of the abilities of normal and leukemic PB M to stimulate the same responder. Thus, the autologous counts (responder’s MLR response to his own irradiated cells) are the same for the simultaneously tested stimulators. Hence, in most cases stimulation indices were not tabulated.

In some experiments two leukemic subjects were tested simultaneously, allowing comparison of their capacities to stimulate the same responders.

Stimulator cells were tested at two-fold dilutions varying from a concentration of 10^4–3.12 x 10^5 cells/ml.

RESULTS

Characterization of CLL cases. Patients were classified as having CLL on the basis of having a lymphocytosis of >10,000 lymphocytes/mm^3 consisting predominantly of mature small lymphocytes and a clinical picture consistent with this diagnosis. In most instances the lymphocyte counts were >30,000. The patients were untreated or on ≤10 mg/d of prednisone at the time their lymphocytes were studied.

The surface markers of PB M obtained from representative leukemic patients are shown in Table I. All patients studied had <30% lymphocytes forming E rosettes and in all cases at least 70% of the lymphocytes expressed Fc receptors. Hence, these patients were considered to have B-cell leukemias despite the fact that only 11 of 21 expressed readily detectable surface membrane Ig. In most instances the percentage of monocytes in their PB M was extremely low.

Consistent with prior studies of CLL (2, 3, 13), 70% of the cells in these E-rosette-negative leukemias were

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TABLE I
MLR Stimulatory Capacity and Surface Markers of PBM from Representative CLL Cases

<table>
<thead>
<tr>
<th></th>
<th>Cell count x 10^6</th>
<th>E rosette</th>
<th>Ia</th>
<th>Surface membrane Ig</th>
<th>Fe</th>
<th>Mono</th>
<th>Maximal stimulation by normal</th>
<th>Stimulation by CLL at concentration where normal gives maximal stimulation/Maximal stimulation by normal*</th>
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<tr>
<td>A</td>
<td>900</td>
<td>&lt;5</td>
<td>&gt;95</td>
<td>&gt;95</td>
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<td>&lt;1</td>
<td>0.06</td>
<td></td>
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<tr>
<td>B</td>
<td>150</td>
<td>1</td>
<td>96</td>
<td>85</td>
<td>92</td>
<td>&lt;1</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>130</td>
<td>8</td>
<td>90</td>
<td>&lt;5</td>
<td>90</td>
<td>7</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>120</td>
<td>5</td>
<td>96</td>
<td>92</td>
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<tr>
<td>E</td>
<td>119</td>
<td>15</td>
<td>87</td>
<td>&lt;5</td>
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<td>5</td>
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<td></td>
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<tr>
<td>F</td>
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<td>G</td>
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<td>86</td>
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<td>2</td>
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<td>22</td>
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<td>2</td>
<td>85</td>
<td>12</td>
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</table>

* Maximal normal stimulation 60,000–125,000 cpm.

Ia-positive, further evidence of their B-cell origin. In contrast, only 5–20% (mean 10%) of peripheral blood lymphocytes isolated from normal subjects express Ia. Because monocytes are for the most part also Ia-positive, 10–35% (mean 23%) of PBM express Ia.

Comparison of MLR stimulatory capacities of PBM isolated from normal and leukemic subjects. Dose-response curves generated by PBM obtained from 21 cases of CLL were examined. Cells from many of the leukemic patients were tested on multiple occasions, allowing 94 comparisons of the MLR stimulatory capacities of leukemic and normal PBM.

In 82 of the 94 comparisons (representing results with 19 leukemic subjects) the leukemic PBM gave rise to a pattern of MLR stimulation significantly different from normal PBM. As shown in Fig. 1, when used as stimulators, the normal cells gave increasing stimulation up to a stimulator concentration of 1.25–2.5 x 10^6/ml. Further increase of stimulator concentration led to a decrease in stimulation. In contrast, the leukemic cells stimulated less effectively than the normal PBM at stimulator concentrations at which the normal PBM generated maximal stimulation. Stimulation increased with increasing leukemic stimulator cell concentration up to 1–2 x 10^7 cells/ml (Fig. 1).

There was considerable heterogeneity in the stimulatory capacity of the cells obtained from these 19 leukemic subjects (Table I). Thus, at the stimulator cell concentration at which normal PBM gave maximal stimulation PBM from these leukemic subjects gener-

![Figure 1](image-url)
ated between 5 and 64% of the stimulation generated by the simultaneously tested normal subject. In 45 of the 82 comparisons, the leukemic cells generated <25% of the MLR response generated by the simultaneously tested PBM obtained from normal subjects and in only 15 of the comparisons did the stimulation generated by leukemic cells exceed 50% of that generated by the normal cells. The variation was also seen at stimulator cell concentration of 10^7 cells/ml. However, at this concentration, because of the contrasts in the dose-response curves, in over half of the comparisons the leukemic cells generated more stimulation than did the normal PBM, whereas in only 29 comparisons did they generate <50%. There was no clear relation between stimulatory capacity of the leukemias and cell count, percent of cells forming E rosettes, percent of monocytes or presence or absence of readily detectable surface membrane Ig on the leukemic B cells (Table 1).

The two leukemic patients whose cells generated a dose-response curve similar to those generated by the simultaneously tested normals were patients Ei (Fig. 2) and Se, whose leukemic B cells have been shown to be capable of differentiation both in vivo and in vitro (20). Similar results were obtained when patient Ei was retested on multiple occasions, despite an increase in the leukocyte count from 17,000 to 45,000 and a decrease in monocytes from 13 to 3%.

Several factors were excluded as the explanation for the different results obtained with leukemic and normal PBM. Stimulator cell viability, as assessed by trypan blue exclusion, of irradiated cells cultured in the absence of responder cells was >80% after 2 d. After a 6-d culture, viability ranged from 40 to 60% but was no different for leukemic and normal cells. As leukemic cells gave minimal MLR response, they could be used as stimulators without irradiation. However, omission of irradiation did not change their stimulatory capacity. Substitution of plasma from leukemic or normal subjects for AB serum did not alter the results of the experiments.

Results obtained with cell counts and blast counts were consistent with those found with [3H]thymidine uptake in the three MLR in which they were compared. Although most normal subjects used in these experiments were younger than the leukemic patients, results obtained using four normal subjects over the age of 60 as donors of the stimulator cells were essentially the same as when younger subjects were used. These included one experiment in which the stimulatory capacity of a leukemia subject’s cells was compared to that of her sister who was of similar age.

The kinetics of the MLR was studied for four leukemic subjects. Harvesting of cultures at 3 and 8 d significantly decreased the amount of stimulation generated by the simultaneously studied normal subject but failed to increase the stimulation generated by the leukemic cells.

In several of the MLR studied, the normal responder lacked one or more Dr antigens expressed in the leukemic stimulator. Despite these alloantigenic differences, implying D-locus disparities, the leukemic stimulators generated the typical “leukemic pattern” of low MLR stimulation.

Fig. 3 depicts the contrasting stimulatory capacities of PBM from a leukemic subject and her HLA identical sister; the leukemic sibling gave a typical leukemic pattern of MLR stimulation, whereas her normal sibling generated a normal pattern of stimulation. The same contrasting reactivity was given by the leukemic sibling’s and normal sibling’s cells when tested against several normal responders. These findings were of particular significance because by several lines of evidence, these siblings were MHC identical. These criteria included, in addition to HLA identity, identical typing by a panel of Dr antisera, and lack of

![Figure 2](image-url)  

**Figure 2.** MLR stimulatory capacity of PBM from leukemic subject Ei (○) and a normal subject (●). Autologous counts were ≤800.
MLR response of the normal sibling's lymphocytes at cell concentration (10^7/ml) at which this leukemic subject generated a response in unrelated normal subjects.

Comparison of MLR stimulatory capacities of purified normal B lymphocytes and leukemic B lymphocytes. In initial experiments with preparations of normal B cells for comparison with CLL B cells, the contrast between the normal and leukemic cells was even more striking than the contrast between the whole PBM and leukemic cells. However, these preparations of B cells obtained by removal of E-rosetting T cells were contaminated by other cells including monocytes. In view of the possible role of these cells in the MLR stressed in other reports (21–23) and because they are so decreased in the peripheral blood of most leukemic patients, the stimulatory capacity of normal B lymphocytes depleted of monocytes by plastic adherence, carbonyl iron or a combination of the two were examined. In all cases monocyte depletion to levels comparable to or lower than those found in leukemic B cells either had little effect on the stimulatory capacity or actually enhanced it, especially at higher cell concentrations. Evidence was obtained that the falloff in the stimulation curve obtained with normal PBM was at least in part a result of monocyte-mediated inhibition.

However, even B cells purified in this way were contaminated by other cells, possibly including the human equivalent of the murine dendritic cell known to be positive for Ia antigens which has been proposed as the major stimulator cell in the murine MLR (24). It was, therefore, important to obtain normal B cells in a very high state of purity for these experiments. Recently, it has been demonstrated that erythrocytes coupled to purified anti-IgM antibodies form rosettes with B lymphocytes with membrane IgM and can be used to isolate extremely pure IgM-bearing B cells. These preparations contain no detectable monocytes.

Fig. 4 shows the results of a typical experiment with such a B-cell preparation (of >97% purity) contrasted with CLL cells, >90% of which expressed surface membrane IgM. The normal B cells give a very good stimulation that contrasts strikingly with the CLL cells.

A number of comparisons were also carried out with B lymphoblastoid cell line cells also consisting predominantly of Ia-positive cells bearing IgM. Here again the poor reactivity of the CLL cells was evident.

Relation of Ia antigen expression to MLR stimulatory capacity. B lymphocytes from the leukemic patients appeared to stain as brightly as normal B lymphocytes with fluorochrome-conjugated anti-Ia heterosera and, using indirect fluorescence, with allo-antibodies. The expression of these antigens by lymphocytes from patient Ei, which gave normal stimulation in the MLR, and by those from J, which were poor stimulators, was investigated in more detail. These leukemic lymphocytes were stained in parallel with normal B lymphocytes, using serial dilutions of

![Figure 3](image-url) A leukemic subject, E (○) and her MHC identical normal sibling (●) generate dissimilar MLR in an unrelated normal responder. Autologous counts were ≤900.

![Figure 4](image-url) Comparison between stimulatory capacity of highly purified normal B cells (●) and CLL B cells (▲) in a typical MLR experiment. The whole normal PBM effect is also shown (○). Autologous counts were ≤1,200.
rhodamine-conjugated F(ab')₂ fragments of anti-Ia heterosera. Percentage of positive cells and intensity of fluorescence at each dilution were scored by an independent observer. The staining properties of the leukemic cells from the two patients were indistinguishable despite their dissimilar MLR stimulatory capacities; both reacted at least as strongly as the normal B cells.

The capacity of the above two leukemic B cells to absorb anti-Ia heterosera was also compared to that of purified normal B lymphocytes. Graded numbers of cells from each of the three cell preparations were added to 100 µl of diluted fluorochrome-conjugated F(ab')₂ fragments of anti-Ia heterosera in U-bottomed microplates. After 2 h at 4°C the plates were centrifuged and the supernates’ ability to stain cells from a B-lymphoblastoid cell line and normal B cells was measured. On the basis of the number of cells required to absorb out fluorescence activity, cells from E1 were 80–100% as efficient as normal B lymphocytes; those from J were 116–140% as efficient as normal B lymphocytes.

**Stimulatory capacity of mixtures of PBM isolated from normal and leukemic subjects.** To investigate the possibility that PBM isolated from leukemic patients contained a population capable of inhibiting a normal MLR or lacked an accessory cell required for stimulation, the stimulatory capacities of mixtures of normal and leukemic cells were examined. In seven such experiments, using cells from different leukemic subjects, the observed values were well within ±33% of those calculated with only occasional exceptions. Thus, there was no evidence of inhibition or reconstitution. The results obtained with a mixture of cells from the leukemic and normal HLA identical siblings are shown in Table II and clearly demonstrate the absence of a suppressor effect of the leukemic population.

In related experiments it was found that depletion of residual T cells from leukemic PBM in two cases did not enhance the stimulatory capacity of the remaining B lymphocytes. Hence, specific MLR suppressor T cells could not be demonstrated in the leukemic population.

**DISCUSSION**

When used as stimulators in the MLR in the system described above, PBM prepared from normal subjects give increasing stimulation with increasing stimulator concentration until maximal stimulation is reached at 1.25–2.5 x 10⁶ cells/ml (stimulator responder ratio = 1 or 2). As stimulator cell concentration is further increased, stimulation falls off. In contrast, PBM isolated from most subjects with CLL give relatively low stimulation at the concentrations at which normal PBM generate maximal stimulation, and the stimulation increases with increasing stimulator concentrations up to 1–2 x 10⁷ cells/ml. This deficient stimulatory capacity was most striking when CLL cells were compared on a cell to cell basis with normal B cells isolated by several techniques. The normal B cells gave a strong stimulation while the CLL B cells at similar concentrations had little or no effect.

The two leukemic subjects whose PBM showed near normal behavior when used as stimulators in the MLR are of special interest because the leukemic B cells are perhaps more normal than those in other cases of CLL in that they have been shown to be capable of differentiating into plasma cells synthesizing monoclonal macroglobulins in vivo and in vitro (20).

Previous studies of the MLR stimulatory capacities of CLL lymphocytes have reported both normal (25–27) and decreased stimulatory capacity (28, 29). However, it is apparent in all of the studies that when the data are recalculated on a per B-cell basis, the MLR generated by CLL subjects are abnormal. On comparing the dose-response curves obtained with isolated normal B lymphocytes and those obtained with the PBM isolated from most cases of CLL, it is clear that even a low proportion of residual normal B lymphocytes in the leukemic PBM could give rise to most of the observed stimulation obtained at higher concentration of cells.

Poor viability of leukemic cells, increased sensitivity to irradiation, dissociation of thymidine uptake from blast transformation, increased age of leukemic subjects, kinetic difference between stimulation by leukemic and normal cells and requirements for special plasma factors were ruled out as explanations for the
Poor stimulatory capacity of the leukemic PBM. No evidence for cells capable of suppressing the MLR was found in the leukemic PBM.

PBM isolated from patients with CLL contain a decreased proportion of monocytes, cells which are required, in small numbers, to support the MLR (21, 22). However, normal B cells depleted of monocytes by a variety of techniques so that they contained a percentage of monocytes similar to or lower than that found in the leukemic PBM were excellent stimulators. Monocyte depletion, however, did lead to an attenuation of the decrease in stimulation at high stimulator concentrations seen with normal but not leukemic PBM.

In a recent study, Steinman and Witmer (24) reported that for mouse spleen the primary stimulatory cells in the MLR is an Ia-positive dendritic cell, and they brought into question a significant role for B cells. Previous work (8–11) appearing to demonstrate the strong stimulatory capacity of the B cell in the human MLR did not utilize B cells purified sufficiently to eliminate minor populations of active contaminating cells, such as the dendritic cells. However, the new technique of isolating B cells using anti-IgM-coated erythrocytes allowed isolation of B cells free of contaminating Ia-positive non-B cells. Conclusive evidence for the strong stimulatory capacity of normal B cells was obtained, ruling out the B-cell nature of CLL lymphocytes as the explanation for their poor stimulatory capacity.

Because of the identity of, or close relationship between, Ia antigens and the MHC determinants (LD) responsible for stimulation in the MLR, the expression of these antigens by the leukemic cells was examined. These cells were found to express Ia antigens as strongly as normal B lymphocytes as assessed by fluorescent staining and absorption studies. This is consistent with previous results using anti-Ia hetero sera and the fluorescence-activated cell sorter (13). The alloantigenic portions of the Ia molecules were also found to be expressed normally on CLL B cells in other studies (2, 30), and the bimolecular complexes precipitated from normal and leukemic B cells shown to be identical (30).

Recently, several diseases have been found to be associated with Ia and/or LD antigens (31), and this group may include CLL (32). Results with the MHC identical siblings and the failure of the leukemic cells to stimulate any of the large number of genetically heterogenous normal responders, some of whom lacked Dr antigens expressed by cells of the leukemic subjects they were tested against, argue against a genetic explanation for the decreased stimulatory capacity of leukemic cells.

To serve as a stimulator in the MLR, it is likely that a cell must meet certain metabolic and membrane functional requirements in addition to possessing the D locus antigens (33, 34). Many abnormalities in these parameters have been documented in CLL (35). Of particular interest is the recent finding that CLL B cells are permeable to merocyanine a compound which is excluded by normal lymphocytes (36). The cells from one of the two subjects mentioned above which were capable of giving a more normal MLR and differentiating in vitro were also unique in not being permeable to merocyanine; the other similar patient was not available for testing. Further investigation will be required to determine whether such membrane or other metabolic defects explain the results of this study. At present, however, it can be concluded that leukemic B cells, despite their strong expression of Ia antigens, differ from their normal counterparts by being poor stimulators in the MLR.

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