Characterization of Transformed Cells and Tumors by Proton Nuclear Magnetic Resonance Spectroscopy

Carolyne E. Mountford, George Grossman, Gregory Reid, and Richard M. Fox

School of Biochemistry, University of New South Wales, P. O. Box 1, Kensington, New South Wales 2033 [C. E. M., G. G.], and Ludwig Institute for Cancer Research (Sydney Branch), Blackburn Building, University of Sydney, Sydney, New South Wales 2006 [G. R., R. M. F.], Australia

ABSTRACT

Cultured acute lymphoblastic leukemic cells give a well-resolved proton nuclear magnetic resonance spectrum characteristic of isolated plasma membranes. We demonstrate that the signals, in the spectrum of whole cells, arise predominantly from the plasma membrane and that cells transformed by pokeweed mitogen have membranes which are significantly less rigid than are normal human peripheral blood lymphocytes.

Normal thymus, malignant thymus, and a leukemic T-cell line have been compared by proton nuclear magnetic resonance spin echo experiments, and the normal thymus was found to differ. Cells transformed by the Epstein-Barr virus can also be characterized and shown to differ from the leukemically transformed cells by spin echo experiments.

Since no probe molecule was required to obtain these results, this is the first definitive evidence that the structure and fluidity of the plasma membranes change as a result of transformation of lymphocytes. Proton nuclear magnetic resonance spectroscopy can now be used to compare the effect of different mitogens on T- and B-lymphocytes as well as to monitor the effects of drugs, metals, etc., on the plasma membrane of transformed lymphocytes.

INTRODUCTION

Tumors are composed of neoplastic cells which by definition have lost proliferation control. The transformation of a normal cell to a neoplastic cell can be spontaneous or induced by viruses or carcinogens. Mitogens, which are activators of T- and B-lymphocytes, transform or activate lymphocytes non-neoplastically for one generation only.

Possible changes in the fluidity of the membranes of cells following transformation are currently in dispute. A review article by Nicolau et al. (21) describes some of the electron spin resonance, fluorescence polarization, and NMR experiments undertaken to investigate changes in the fluidity of membranes after transformation. The authors conclude that there is no clear-cut distinction between membrane fluidity of normal and tumor cells and it seems that fluidity is not directly related to tumor transformation.

Membrane fluidity is a function of the rate of motion of the individual component molecules. Since the plasma membrane of lymphocytes is highly specialized for immune responses, such as complement-fixation, antigen-induced histamine release, polar cap formation, formation of antibody-antigen complexes, and cell recognition (28), any changes in the molecular motion of the membrane will affect transport across the membrane and the immunocochemical functions of the cell. Mechanisms responsible for changes in exposed cell surface receptors of transformed cells are not properly understood. It is therefore difficult to determine whether changes, such as an increase in the membrane fluidity, are directly due to the transformation or a coincidental assembly of conditions which have manifested themselves merely because the cell has altered its function with transformation.

While the ultimate aim of transformation studies is to understand tumorigenesis in living cells, it would also be advantageous to have nondestructive methods of monitoring the changes that have occurred at a molecular level in transformed cells. Such techniques would allow subsequent monitoring of any change in response to drugs and modifiers.

NMR spectroscopy, which is nondestructive and is dependent upon molecules having a certain degree of molecular motion for a signal to be observed, is therefore particularly suitable for studying fluid membranes. It also has the advantage that probe molecules do not have to be incorporated into the membranes. The parameters which can be measured are the line width of the resonance and the spin lattice relaxation times of the resonances which are a function not only of the rate of motion of the molecule but also of its environment (9, 29). It has, for example, been realized that cholesterol molecules are able to exert control over the mobility of phospholipids in membranes (7, 24, 26). Thus, the packing of the lipid molecules in the bilayer and the molecular motion contribute to the line width of the resonance and the spin lattice relaxation times. The resonances may also be broadened by phenomena such as field inhomogeneities due to sample inhomogeneity and paramagnetic metal ions which are located in the vicinity of the proton concerned (9).

We have previously reported that cultured human lymphoblastoid cell lines differ in their 1H NMR spectra. The spectral differences were seen to reflect the stage of differentiation and type of transformation, i.e., EBV or leukemic transformation (19). Thymi from AKR mice, an inbred strain of mouse with an endogenous C-type virus transmitted genetically and resulting in a spontaneously developing T-cell leukemia (15), were also monitored by 1H NMR spectroscopy (19). Carr-Purcell spin echo experiments were seen to reflect the changes taking place in the thymi as the preleukemic period ended and the development of neoplasia occurred (19).

Studies on human peripheral blood lymphocytes and cultured lymphocyte cell lines have now been extended to provide evidence that the well-resolved signals in the spectra of transformed cultured lymphocytes do arise from the plasma membrane. These results show 1H NMR spectroscopy to offer a
valuable noninvasive method of studying intact T- and B-lymphocytes and their isolated plasma membranes.

MATERIALS AND METHODS

Isotopes and Chemicals

Ficoll-Paque was obtained from Pharmacia, Sydney, Australia. Amineothyiostibiouronium bromide was from Calbiochem, Sydney, Australia. iNase I was purchased from Sigma Chemical Co., St Louis, Mo. PBS (8) was from Flow Laboratories, Australia. Gadolinium oxide was obtained from Fluka, Buchs, Switzerland. 15GdCl3 and 52MnCl2 were purchased from the Radiochemical Centre, Amersham, United King-

dom. RPMI-1640 was from Flow Laboratories, Australia. (Glucose from RPMI-1640 is clearly visible in 1H NMR spectrum, but amino acids are not visible at high cell concentrations.)

Cell Preparations

Normal T- and B-Lymphocytes. Fresh human “buffy coat” was supplied by the Red Cross Blood Bank, Sydney, Australia. Lymphocytes and platelets were isolated in an interface layer by centrifugation over Ficoll-Paque (3). After dilution in 0.9% NaCl solution, the lymphocytes were washed free of platelets by repeated centrifugation at 500 x g for 5 min. Fresh sheep RBC were treated with aminomethyliothiouronium bromide for use in the rosetting technique of Madsen and Johnsen (14).

Transformed T- and B-Lymphocytes. Normal peripheral blood lymphocytes were transformed by PWM, a poly dA activator of T- and B-lymphocytes. These cells were prepared as above, and to the final suspension 0.07 M HCI, was added. Both suspensions were incubated for 10 min at 37°. Membrane ghosts were then added to form vesicles after addition of 0.01 volume of 50 mM CaCl2/50 mM MgCl2 and incubation at 37° for 30 min and then counted using a hemocytometer or Coulter Counter. The mem-

branes were washed twice in 0.9% NaCl solution and 3 times in PBS (8) in D2O. The membrane suspension was centrifuged at 1000 x g for 10 min before washings. The membranes were finally suspended in 300 µl PBS/D2O for NMR studies.

Stain for Lipids

Cells were smeared on a slide, dried under a warm air flow, and stained with Sudan IV.

Metal Binding to Isolated Membrane Ghosts

Ghost vesicles prepared from ALL T-cell line CCRF/CEM were washed in 0.9% NaCl solution/20 mM Tris-HCl (pH 7.4) and resus-

pended to 20 ml giving a final concentration of 106 ghosts/ml. To 10 ml of this suspension, 1 ml of 5 mM gadolinium chloride, containing 25 µl of 15GdCl3 (gadolinium, 9.6 µg/ml; 0.12 mCi/ml; in 0.07 M HCl), was added. To the remaining 10 ml of suspension, 1 ml of 5 mM manganese chloride, containing 25 µl of 52MnCl2 (0.1 µCi/ml, carrier free, in 0.1 M HCl), was added. Both suspensions were incubated for 10 min at 37° and then washed twice. The ghosts were then lysed in 5 ml 10 mM Tris-HCl (pH 7.4) for 10 min in ice. The supernatant was drawn off, and the radioactivity of the pellets and supernatants was measured by liquid scintillation.

DNA Analysis

DNA content (per cell) of peripheral blood lymphocytes was mea-

sured using an ICP22 flow cytometer (Ortho Instruments, Westwood, Mass.). The cells were stained with ethidium bromide (27). Approx-

imately 30,000 cells were analyzed for each DNA content histogram. Calculation of percentages of cells in various phases of the cell cycle were made using a curve-fitting method of analysis (16).

NMR Spectroscopy

1H NMR spectra were recorded at 37° on the following instruments operating in the pulsed Fourier transform mode: a Bruker WM-400 spectrometer equipped with an Aspect 2000 computer; a Bruker HX-270 spectrometer equipped with a Nicolet 1180 computer; or a Jeol FX100 spectrometer equipped with a Texas Instruments TI880B com-

puter. All peaks are referred to trimethylsilylpropane-sulfonic acid (TSP) as an internal standard. Spectra were recorded with the residual HOD peak suppressed by either selective gated irradiation (12) or selective irradiation (23). Data were accumulated with the samples spinning which caused the cells to line the side of the NMR tube.

CPA Pulse Sequence. This pulse sequence (90°-r-180°-r-echo) (4) separates resonances according to their T2 relaxation times, multiplet structure, and coupling constants. The resonances of large rigid mole-

cules with short T2 disappear from the spectrum, whereas resonances from small mobile molecules remain.

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Anhydrous manganese sulphate was dissolved in D$_2$O, and the pH was adjusted to 6.5 with NaOD. Gadolinium oxide was dissolved in 10 M HCl and evaporated to dryness. The residues were dissolved in D$_2$O, and the pH was adjusted to 6.5 with NaOD.

RESULTS

$^1$H NMR spectra have been recorded for normal peripheral blood T- and B-lymphocytes; cultured ALL T-, ALL B-, ALL pre-B, ALL null, and EBV-transformed lymphocytes; and thymi from normal and AKR mice. Real and spin echo spectra (with varying delays between pulses) have been collected for several cell lines in each of the above categories, and some interesting comparisons have been made.

The $^1$H NMR spectrum of a normal human peripheral blood B-lymphocyte is predominantly that of a lipid bilayer (Chart 1C). The spectrum contains resonances from the lipid —N(CH$_3$)$_3^+$, —(CH$_2$)$_n$—, and —CH$_3$ groups at 3.2, 1.2, and 0.9 ppm, respectively (5). Carbohydrate resonances may be observed at 4.0 to 4.5 ppm, and acetyl resonances from the carbohydrate may be seen at 2.0 to 2.2 ppm. The carbohydrate regions of these spectra may be attributed to cellular components since the cells were washed and resuspended in PBS. This lipid spectrum is common to all B-lymphocytes and transformed T-lymphocytes. However, differences in the line widths of the lipid resonances and the carbohydrate and amino acid components of the cells (Table 1) are apparent when the spectra of normal and transformed lymphocytes are compared.

Comparison of the $^1$H NMR Spectra of Normal and Mitogenically and Leukemically Transformed Lymphocytes

The $^1$H NMR spectrum of the ALL B-cell line BALM, shown in Chart 1A, is typical of that observed for leukemically transformed B- and pre-B-lymphocytes. Normal human peripheral blood B-lymphocytes, suspended in PBS, give a $^1$H NMR spectrum similar to that observed for leukemically transformed cells (Chart 1C), although the —(CH$_2$)$_n$—, —N(CH$_3$)$_3^+$, and —CH=CH— resonances at 1.2, 3.2, and 5.4 ppm, respectively, are considerably broader (Table 1). This difference is indicative of a decrease in the mobility of the lipid fatty acid chain. The broad —CH=CH— resonance at 5.4 ppm could also be due to the presence of smaller amounts of unsaturated lipid.

After the normal B-lymphocyte is mitogenically transformed by exposure to PWM for 5 days, the line widths of the lipid resonances in the $^1$H NMR spectrum (Chart 1B) can be seen to be comparable with the leukemic lymphocyte spectrum (Chart 1X) except for the —N(CH$_3$)$_3^+$ resonance which is not as clearly resolved. The lymphocyte stimulation was monitored by flow cytometry where the cells could be seen to enter S phase indicating that DNA replication is occurring (Chart 2).

By contrast, normal human peripheral blood T-lymphocytes have a poorly resolved $^1$H NMR spectrum which is characteristic of neither lipid nor protein (Chart 3C). However, after the normal T-lymphocyte is mitogenically transformed by PWM for 5 days, the $^1$H NMR spectrum has the characteristic lipid spectrum (Chart 3B) and is directly comparable with the spectrum of the ALL T-cell line CCRF/CEM (Chart 3A). Line widths observed for the lipid resonances in each spectrum are shown in Table 1.

The spectra of the cultured lymphocytes are well resolved above 270 MHz, and differences are observed between the individual cell lines and the cell classes. However, when spin echo experiments are used to select certain resonances from the real spectrum according to the relaxation rate of the nuclei in question, some interesting observations can be made.

Spin Echo Experiments Discriminate between Transformed and Normal Lymphocytes

Variations in the Relaxation Rates of Cellular Components. Spin echo experiments are able to differentiate between leu-
Chart 2. Changes in the DNA distribution of peripheral blood B-cells following culture with PWM. Channel number corresponds to relative fluorescence intensity (DNA content); ordinate, number of cells. The peak between Channels 10 and 20 represents chick RBC, used as an internal standard. A, unstimulated cells; B, after 5 days of culture with PWM. C.V., coefficient of variation.

Chart 4 shows the CPA spectra ($\tau = 60$ msec) of the EBV-transformed cell line GK and the ALL T-cell line 8402. Resonances at 2.0 to 2.6 ppm have been tentatively assigned as acetyl resonances from surface carbohydrate since they are broadened with Gd$^{3+}$ along with the external phospholipid $-\text{N}($CH$_3$)$_3^+$ resonance (Chart 7A). Resonance A marked in Chart 4 is similar in both cell lines, while Resonance B is well resolved only in the EBV-transformed cell line. Resonance C is found in both types of transformed cell lines. Each of these spectra is common for the cells in each of its classes. The additional broad resonance apparent in the GK spectrum at $\approx 1$ ppm could be partly due to the residual free amino acids in the culture medium, since EBV-transformed cells have been shown to utilize the supply of culture medium more slowly than do ALL T-cells.  

Normal Thymus Compared with a T-Cell Tumor and Cultured Leukemic T-Cells. Spectra obtained from normal rat thymus, 23-week-old AKR thymus, and ALL T-cell line CCRF/CEM can be seen to be composed of the same resonances with differing degrees of spectral resolution. However, CPA ($\tau = 60$ msec) experiments clearly distinguish between the normal thymus and malignant thymus (Chart 5). The 23-week-old AKR mouse thymus which is known to have undergone leukemic transformation (19) has a spin echo spectrum comparable with that of the ALL T-cell line 8402.

The line width of the resonances in the spectrum of the cultured T-cell line is considerably less than that observed for the tumor. This phenomenon can be expected since the cells...
in the tumor are closely packed and are held together by connective tissue. The size of the complex, cell, or organ can influence the line width of the resonance in question. While the membrane lipids in nonaggregated cells have an intrinsic molecular motion the same as those firmly embedded in connective tissue, the overall rate of motion of the aggregate is slower. This difference in motion is manifested in the line width of the resonance in the NMR spectrum.

The intensity of the \(-\text{N}(\text{CH}_3)_3^+\) resonance in the CPA spectrum of the AKR mouse thymus is also considerably less than the normal thymus or the cultured T-cells, indicating a reduction in the mobility of the phospholipid head group or different levels of free choline.

The normal thymus has no resonance which could be attributable to the \(-\text{CH}_2\) or \(-\text{CH}_3\) resonances from the fatty acid chain but, unlike the AKR mouse thymus, it does have a well-resolved choline resonance at 3.2 ppm.

Resonances A, B, and C (between 2 and 2.6 ppm), shown in Chart 4, which clearly discriminate between neoplastic and normal T-cells, are broadened by Gd\(^{3+}\) (see later) and are therefore likely to be from surface carbohydrate or protein.

Identification of the Lipid Bilayer in the NMR Spectrum

As previously discussed, the \(^1\text{H}\) NMR experiment is dependent upon the molecules, the signals of which are observed, having a certain degree of mobility. For the line width of lipid resonances to decrease after the lymphocyte has been stimulated or transformed, one or more of the lipid moieties in the cell has become more mobile or there has been lipid synthesized during transformation. While these experiments demonstrate quite clearly that the \(^1\text{H}\) NMR spectrum in Charts 1A, 1B, 2A, and 2B are characteristic of transformed or stimulated cells, they do not indicate where in the cell the lipids are located that give rise to these signals.

Lipids are found in the plasma membrane, nuclear membrane, Golgi bodies, endoplasmic reticulum, and mitochondrial membrane of cells. Some cells are also known to have lipid-containing vesicles or liposomes. The possibility that the lipid signals arise from liposomes was discounted after experiments were undertaken to stain any such liposomes with Sudan IV and none were observed. Mitochondrial membranes were also discounted, since electron microscopy quite clearly demonstrated that leukemically transformed cells in culture possessed very few mitochondria. Thus, the most likely location of the lipids, which had been mobilized by the transformation of the cell, was either the nuclear or plasma membranes.

Isolation of Plasma Membrane. The 400-MHz \(^1\text{H}\) NMR spectra of plasma membrane ghosts, prepared from the EBV-transformed cell line JP, are compared with the spectra of the intact cells in Chart 6. The signal/noise ratio in the spectrum of the isolated membranes is lower than that observed for the intact cells due to a smaller volume of the sample. Nevertheless, the spectra are directly comparable.

Use of Paramagnetic Probes to Identify the Resonances in the NMR Spectrum. Gadolinium and manganese ions are paramagnetic and broaden the resonances in the NMR spectrum of the nearby nuclei (2). Gadolinium chloride, when titrated into \(5 \times 10^7\) ALL T-cells CCRF/CEM suspended in PBS (Chart 7A), selectively broadens certain resonances in the spectrum. After 0.25 mM GdCl\(_3\) had been added, no further broadening occurred; however, at this concentration of GdCl\(_3\), all fatty acid resonances are broadened by approximately 50%. This is a
Fig. 7. $^1H$ NMR spectra (100 MHz) of ALL T-cell line CCRF/CEM ($5 \times 10^8$ cells) in PBS/D$_2$O: A, with increasing amount of GdCl$_3$; B, with increasing amounts of MnCl$_2$. Spectra were recorded at 37° in 17 min on a Jeol FX-100 NMR spectrometer. The residual HOD peak was suppressed by selective gated irradiation (12).

Table 2
Location of isotopes after incubation with ALL T-cells

<table>
<thead>
<tr>
<th>Location of isotopes (%)</th>
<th>$^{153}$Gd</th>
<th>$^{54}$Mn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma membrane</td>
<td>92</td>
<td>34</td>
</tr>
<tr>
<td>Inside the cell</td>
<td>8</td>
<td>66</td>
</tr>
</tbody>
</table>

typical example of the experiment as described by Bergelson and Barsukov (2), in which only the external phospholipid molecules in the bilayer are broadened because the Gd$^{3+}$ ions bind to the external face of the lipid bilayer and are unable to pass through.

Manganese ions, on the other hand (Chart 7B), broaden the resonances in the spectrum nonselectively because the metal ions pass through the lipid bilayer. In contrast to the effect of the Gd$^{3+}$ ions, the Mn$^{2+}$ ions do not cause the $-\text{N(CH}_3\text{)}_3+$ resonance to broaden and decrease in intensity.

In order to determine if the lipid bilayer affected by these paramagnetic probes was the plasma membrane, intact membrane ghosts were prepared from ALL B-cell line BALM and exposed to either $^{152}$Gd$^{3+}$ or $^{54}$Mn$^{2+}$. Table 2 shows the location of these metals after incubation with the membrane ghost. These data establish that the $^{54}$Mn$^{2+}$ was able to pass through the plasma membrane whereas the $^{152}$Gd$^{3+}$ bound to the outside of the membrane ghost which is consistent with the results obtained by NMR spectroscopy.

DISCUSSION

The $^1H$ NMR spectrum observed for human lymphocytes is composed of well-resolved resonances from a lipid bilayer (5). Few nontransformed or stimulated cells provide such a well-resolved spectrum; however, normal human peripheral blood B-lymphocytes have been shown to be an exception (Chart 1C) and provide a spectrum as well resolved as those obtained for chicken embryo fibroblasts (20).

The lipid bilayer in the NMR spectrum was identified as being the plasma membrane by 2 independent series of experiments. Firstly, the lipid bilayer observed in NMR spectrum was shown to be affected by the paramagnetic metal ions Mn$^{2+}$ and Gd$^{3+}$ (Chart 7). The Gd$^{3+}$ selectively broadened the external phospholipids of the bilayer, whereas the Mn$^{2+}$ broadened both internal and external components of the bilayer. The data thus provide evidence that the Mn$^{2+}$ passes through the lipid bilayer in question whereas the Gd$^{3+}$ binds to the outside and is not able to pass through. Radioisotope labeling with $^{153}$Gd$^{3+}$ and $^{54}$Mn$^{2+}$ confirmed that Gd$^{3+}$ bound to the plasma membrane and did not pass through to the center of the membrane ghost (Table 2).

Further direct evidence that it is the plasma membrane of the lymphocytes observed in the NMR spectrum was obtained by comparing the spectra of isolated membrane ghosts and the intact cells. The spectra were directly comparable (Chart 6).

Since it was conceivable that the fluid plasma membrane observed for ALL cells could be a function of cells being cultured, the possibility was examined by monitoring normal human lymphocytes isolated from human serum before and
after transformation with PWM. This possibility was discounted when the $^1$H NMR spectrum of normal human peripheral blood lymphocytes became well resolved after stimulation with PWM, indicative of increased lateral mobility in the membrane as the cell size increased.

After stimulation with PWM, the $\text{--}[\text{N(CH}_3\text{)}_3]^+$ resonances retained the same relative intensity as those observed in the spectrum of the normal lymphocyte. This is in contrast to the ALL B- and T-cell spectra where the $\text{--}[\text{N(CH}_3\text{)}_3]^+$ resonances have a significantly smaller line width (Table 1). Analysis of the choline content of these 3 samples showed no increase in the total choline present in the ALL B-cell BALM or the mitogen-stimulated lymphocyte.\(^6\)

Thus, we have demonstrated that signals in the $^1$H NMR spectrum of lymphocytes arise predominantly from the plasma membrane and that an increase in the fluidity of the plasma membrane of normal lymphocytes occurs after stimulation. This strongly suggests that the unusually fluid plasma membrane of leukemic lymphocytes is a manifestation of the cells being transformed.

Any increase in the membrane fluidity will be accompanied by a change in the behavior of the membrane-bound proteins; e.g., lymphocyte transformation induced by plant mitogens results in a marked increase in the cell size and an increase in the number of lectin receptor sites (25). This increase in the lateral mobility of lectin-binding sites on the transformed cell surface is also similar to the increase in lateral fluidity of certain antigens observed in transformed cells (22). Thus, the increase in membrane fluidity reported here may be due to changes in the organization of the membrane lipids brought about by the increase in the cell size (1, 6) or in the composition of the membrane lipids (18) or a reduction in the membrane constraints (10, 22).

The increase in membrane fluidity observed on transformation or stimulation of lymphocytes is accompanied by an increase in fatty acid chain lengths.\(^6\) This finding is inconsistent with the data reported for model membranes (13). The question therefore still remains as to whether the increase in the membrane fluidity is the cause or the result of the altered behavior of the cell surface components.

Spin echo experiments have been shown to be useful in distinguishing normal and leukemic lymphocytes. To illustrate this, we selected 2 samples where pulse sequence experiments were used to select certain resonances from the rest of the normal spectrum according to their relaxation rate. The CPA experiments demonstrated a difference between normal and transformed T-lymphocytes (Chart 6) and between virally and leukemically transformed cells (Chart 5). Resonances B and C in the CPA spectra of these cells, which are clearly different, are likely to be acetyl resonances from surface carbohydrate since they were broadened on exposure of the cells to 0.1 mM Gd\(^{3+}\). Thus, the $^1$H NMR experiment also has the potential to demonstrate some cell surface changes which accompany the changes in membrane fluidity.

NMR is able to monitor changes in the fluidity of the plasma membranes in leukemically and virally transformed lymphocytes. It is therefore now possible to study the effect on the membrane of chemotherapeutic agents, metals, and other biological and immunological phenomena by this method.

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\(^6\) G. Grossman and C. E. Mountford, unpublished data.

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