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Immunology

Differential lateral mobility of IgM and IgG receptors in mouse B lymphocyte membranes

(membrane fluidity/electron spin resonance spectroscopy)

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ABSTRACT **Anti-Ig induced redistribution of different Ig subclasses was studied as a function of temperature and correlated with membrane phase transitions as revealed by electron spin resonance spectroscopy. Fluorescein isothiocyanate-coupled anti-IgG2 and anti-IgM antibodies induced patching and capping that proceeded with increasing rates from 2° to 40 ° (measured at 2° intervals). Characteristic temperatures marked the onset of discontinuities in such rate changes. IgG2-bearing lymphocytes displayed discontinuities at 14°, 22°, 28°, and 36°, whereas IgM-bearing lymphocytes displayed discontinuities at 18°, 24°, 32°, and 38°. Electron spin resonance spectroscopy studies using the spin label 2,2-dimethyl-4-butyl-4-pentyl-*N*-oxyloxazolidine, a nitroxide-substituted decane, indicated that these temperatures are a function of hydrocarbon phase separations in the B lymphocyte membrane. With a glucosamine-derivative [2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxyl glucosamide] as a probe restricted to the outer monolayer of the plasma membrane, the temperatures 14° and 28° denoted the onset and end, respectively, of a fluidizing process in the outer monolayers of IgG2-bearing lymphocytes. Temperatures of 18° and 32° denoted these boundaries in IgM-bearing lymphocytes. Inner monolayer transitions are associated with the remaining temperatures. We conclude that membranes of IgM-bearing lymphocytes are less fluid than those of IgG2-bearing lymphocytes.**

The behavior of fluorochrome-labeled anti-Ig reagents at the surface of splenic B lymphocytes has been described in detail by many investigators (1–4). To date, most studies on the mechanical aspects of surface immunoglobulin (sIg) redistribution in response to antibody have examined sIg movement primarily as a function of time at a few selected temperatures. We have used an alternate approach—namely, the investigation of membrane surface behavior as a function of temperature over a constant increment of time. This tactic has been successfully exploited by a number of investigators for correlating changes in membrane function with changes in membrane physical state (5–7). Changes in the physical state of membranes with changing temperature can be monitored by measuring the partitioning of electron spin resonance (ESR) spin labels (or fluorescent labels) between the aqueous and the hydrocarbon (i.e., membrane) milieu of a membrane suspension.

Arrhenius plots of partitioning ratios of probe in hydrocarbon to probe in water versus 1/K show discontinuities at characteristic temperatures at which changes in state (i.e., melting or freezing) occur. These discontinuities arise because the probes used are more soluble in fluid membrane hydrocarbon than in frozen hydrocarbon. Whereas a pure lipid system undergoes a change in state (frozen → fluid) over a discrete and narrow temperature range, observations on binary lipid systems show that "melting" occurs over a wider range of temperatures. The boundaries of this process, termed lateral phase separation, are defined as "characteristic temperatures" t_l and t_h that mark the onset and completion of lipid melting: frozen $\xrightarrow{t_l}$ frozen plus fluid domains $\xrightarrow{t_h}$ fluid. Recent evidence suggests that the asymmetric distribution of lipids and proteins across the bilayer membrane of animal cells may result in a physically asymmetric bilayer (8). In accordance with this model, it has been shown that the inner and outer monolayers of the membrane bilayer display two nonidentical sets of characteristic temperatures (9–11).

To determine the extent to which sIg modulation (patching and capping) is dependent on the physical state of the B lymphocyte membrane, studies were initiated to compare and correlate anti-Ig induced redistribution of sIg with changes in the physical state of the plasma membrane. This approach has provided new insight into the types of molecular associations and interactions that exist between the sIg receptors on B lymphocytes and the membrane hydrocarbon core through which they pass.

MATERIALS AND METHODS

Ig receptor modulation

Cell Suspensions. Spleen cells from CBA/J mice, 2–4 months old, were suspended at 10^7 cells per ml in RPMI-1640 (Gibco) buffered with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Calbiochem) and layered over lymphocyte separation medium (specific gravity, 1.077) (Bionetics). After centrifugation at 2000 × *g* at room temperature for 15 min, the interface layer of cells yielded a population that was greater than 95% viable, greater than 95% lymphoid (primarily small to medium lymphocytes), and virtually devoid of erythrocytes. The lymphocytes were washed and resuspended in RPMI-1640 at 5×10^7 cells per ml and 0.1 ml samples were placed into 1-ml centrifuge tubes (Fisher Scientific).

Fluorescent Antibody Staining of Lymphocyte sIg. The fluorescein isothiocyanate (FITC)-coupled IgG fractions of goat antisera against mouse IgM and mouse IgG2 were purchased from Meloy Labs. FITC-anti-Ig (5–10 μg) was added to the 0.1-ml cell samples, each pre-equilibrated to a specific water-bath temperature. At concentrations of anti-IgM or anti-IgG2 from 5 to 500 μg/ml, the same characteristic temperatures were found (see below), indicating no direct dependence of the observed receptor behavior on antiserum dilution. Incubation for either 5 or 15 min followed. At the end of the incubation period, 0.9 ml of cold 4% paraformaldehyde was added to each tube, and the cells were left at ice-bath temperature for at least 30

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Abbreviations: sIg, surface immunoglobulin; ESR, electron spin resonance; FITC, fluorescein isothiocyanate; 5N10, 2,2-dimethyl-4-butyl-4-pentyl-*N*-oxyloxazolidine; 12NS-GA, 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxyl derivative of glucosamine.

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min. Each sample was then washed three times with 1.0 ml of 0.01 M phosphate-buffered saline at pH 7 in a Fisher model 59 centrifuge (1000 × *g*, 1 min) and resuspended in the same buffer for microscopic examination.

Fluorescence Microscopy. Lymphocytes binding fluorescent anti-Ig antibodies were observed with a Leitz Ortholux fluorescence microscope equipped with a Ploem-type epi-illumination system, two BG-38 filters (red suppression), a 475-nm cutoff filter (UV removal), and two KP490 interference filters. Ig-positive lymphocytes were scored as "capped" if fluorescence was restricted to *less* than half the cell surface, "patched" if an irregular pattern of binding existed on *more* than half the cell surface, or "diffuse" if surface staining appeared smooth with no irregularities. Between 100 and 200 Ig-positive lymphocytes were counted in each sample. Frequencies of capped, patched, and diffuse lymphocytes were expressed as a percentage of the total number of stained lymphocytes and plotted versus temperature.

Characterization of Anti-IgM and Anti-IgG2 Sera. The specificities of the two antisera were analyzed by determining their ability to cap-off or "strip" sIg in unique subpopulations of the spleen. Receptor stripping involved incubating unfractionated spleen cells (5×10^6 cells per ml) at 37° in RPMI-1640 (Gibco) buffered with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid. The FITC-coupled IgG fractions of goat antisera against mouse IgM or mouse IgG2 (Meloy Labs) were added to the spleen cultures at a final concentration of 50 μg/ml. At the end of 8 hr, the cells were harvested and washed several times in phosphate-buffered saline. They were then incubated with the original FITC-coupled anti-IgM and anti-IgG2 at 4° for 30 min, fixed with 4% paraformaldehyde, and then scored for IgM- or IgG2-positive cells.

ESR studies

Cell Suspensions. Preparation of splenocytes was identical to that in the sIg studies except that the cells were treated with an AKR anti-Thy 1.2 ascites fluid (Bionetics) plus guinea pig complement (BBL) with cytotoxic activity against CBA thymocytes and T lymphocytes. The two-step incubation scheme was that described by Owen and Raff (12). The remaining cells were passed through lymphocyte separation medium and suspended at a final density of 4×10^8 cells per ml. They were found to be 85–95% Ig positive and depleted of erythrocytes, granulocytes, and dead cells.

Spin Labels. Two different spin-labeled compounds were used in this work. The spin label 2,2-dimethyl-4-butyl-4-pentyl-*N*-oxyloxazolidine (5N10), a nitroxide-substituted decane (a gift from A. D. Keith, Pennsylvania State University), was used to detect inner and outer membrane monolayer phase transitions (9). Another spin-labeled compound was a derivative of 12-nitroxide stearic acid (Syva),2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxyl glucosamide (12NS-GA) (11). This fatty acid derivative, because of its very polar sugar moiety, has been shown to be capable of detecting phase transitions associated only with the outer monolayer of sealed plasma membrane bilayers (11).

Detection of Temperature-Dependent Fluidity Changes in Lymphocyte Membranes. Twenty-five microliters of cell suspension was loaded into a capillary tube (sealed at one end) with 0.5 μl of 5N10 (10 mM) or 12NS-GA (10 mM) and 1.0 μl of $K_3Fe(CN)_6$ (10 mM). The capillary was then placed in a Varian E-104 Century Series ESR spectrometer and equilibrated to a predetermined temperature. The ESR spectrum was taken over a 100-gauss (1×10^{-4} tesla) range in a 4-min scan. Spectra were taken every 0.5° with increasing temperature from 5° to 42°. Cavity temperature was monitored with a copper-Constantan thermocouple attached to a Doric digital temperature indicator. The amount of spin label in polar (h_P) or hydrocarbon (h_H) environments was determined by measuring the amplitudes of the split high-field line (10). The logarithm of the ratio h_H/h_P was graphed in an Arrhenius plot versus the reciprocal of the absolute temperature [$1/T$(K)]. ESR data plotted in this way were analyzed by computer for deviations from linearity ($P < 0.05$) (11). Discontinuities, such as changes in slope, are indicative of temperatures at which changes in membrane physical state occur (13).

RESULTS

Temperature-Dependent Fluidity Changes in B Lymphocyte Membranes Detected With ESR. Biochemical studies performed by a number of workers have demonstrated lipid and protein compositional differences between the inner and outer monolayers of certain plasma membrane systems (8, 14). These differences were shown by Wisnieski *et al.* (9–11) to create nonidentical sets of phase boundary temperatures (t_l and t_h) for each monolayer. Thus, in a homogeneous population of cells, a total of *four* phase-transition temperatures should be observed: one pair associated with the onset and completion of the phase transition process in each monolayer. In order to assign transition temperatures to the outer or inner membrane monolayer, two spin-labeled compounds were used: 5N10, which demonstrates a characteristic association with both monolayers, and 12NS-GA, which preferentially associates with the outer membrane monolayer only. No obvious perturbation due to the probes was indicated by the observation that final concentrations of each probe of 0.1 mM to 2 mM gave similar results. With 5N10, ESR analysis of splenic lymphocyte populations that were greater than 90% sIg positive showed seven to eight characteristic temperatures (Fig. 1). Because the number of discontinuities observed was twice the expected four, the data suggested that the sIg-positive lymphocytes consisted of at least two distinct populations. Four discontinuities were observed with 12NS-GA, again twice the number normally seen with sealed membrane systems.

The obviousness of a particular transition temperature, determined visually as a break in the Arrhenius plot, may vary between experiments. For this reason, computerized slope and intercept analysis was used to establish confidence limits for deviations from linearity. Only transition temperatures that were consistently detected by the computer as significant at the $P < 0.05$ level are reported here.

Temperature Dependence of Receptor Mobility. The splenic B lymphocyte population was heterogeneous, containing lymphocytes at various stages of differentiation. The eight discontinuities revealed by ESR could be explained if the membrane properties of cells changed during differentiation, resulting in at least two distinct populations. Experiments with sIgM- and sIgG-bearing lymphocytes seemed a reasonable starting point for examining this possibility. A characterization of the antisera used in the experiments described below is summarized by data shown in Table 1. The monospecificity of each antiserum was demonstrated by its ability to strip receptors from the surface of its own sIg-positive population but *not* from the surface of the other sIg-bearing population of the second isotype. In addition, there was no indication that the sIg detected was a species bound by an Fc receptor. Following stripping of receptors and subsequent culturing in the absence of anti-Ig, the original frequencies of sIgM- and sIgG2-bearing lymphocytes were observed. An anti-IgG1 reagent, prepared in the same way as these antisera, bound to virtually no splenocytes. This argues against the fluorescent IgG fractions of the antisera binding directly to cells via Fc receptors.

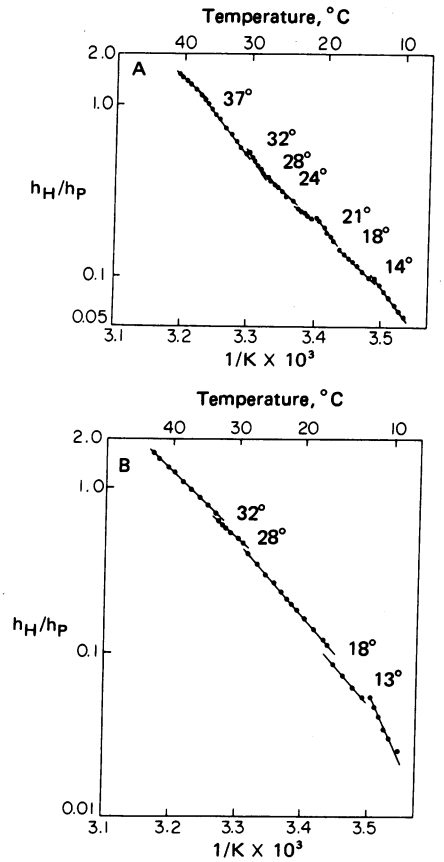

FIG. 1. Characteristic temperatures for the partitioning of 5N10 (*A*) or 12NS-GA (*B*) between the fluid hydrocarbon phase of B lymphocyte membranes and the surrounding aqueous environment. The ratio h_H/h_P represents the partitioning of spin label between hydrocarbon and polar domains of the cell suspension. Because nitroxide-spin-label partitioning favors fluid over frozen hydrocarbon, membrane melting behavior can be monitored. All transition temperatures were obtained by computer analysis of the Arrhenius plots ($P < 0.05$).

The results outlined below showed that FITC-labeled goat antibody against mouse IgM induced sIg redistribution patterns in IgM-bearing cells at different temperatures than did FITC-labeled antibody directed against mouse IgG2. The latter induced patch formation on the surface of 5–10% of the IgG2-bearing lymphocytes after incubation for 15 min at 2°, leaving 90% with diffuse binding (Fig. 2). When the temperature was increased by 2° increments to 10°, there was a concomitant increase, to about 80%, in the numbers of patched lymphocytes found after 15 min-exposures. Between 14° and 18°, the percentage of patched lymphocytes decreased sharply as the temperature was increased. The decrease in percentage of patched lymphocytes in this latter temperature range corresponded with the appearance of increasing numbers of capped lymphocytes. A plateau in the percentage of patched or capped lymphocytes existed between 18° and 22°. Then, an increase in the rate of surface activity began at 22° and plateaued between 24° and 26°, until the temperature reached 28°, when increased receptor movement was again observed. This increased rate of surface activity again leveled off until 36° was reached which marked the onset of a final increase in the rate of induced receptor redistribution, resulting in an IgG2-bearing population that was greater than 95% capped.

The percentage of patched or capped lymphocytes was scored at 15 min so that the disappearance of diffuse lymphocytes into patched lymphocytes could be observed. Shorter times of incubation with anti-Ig obscured this activity. However, because 15-min incubations at temperatures above 30° resulted in the completion of relevant receptor redistribution, 5-min incubations had to be used to clarify the activity occurring at the higher temperatures. Comparison of redistribution activity after 5- and 15-min incubations (Figs. 2 *A* and *B* and 3 *A* and *B*) showed that both sIgM- and sIgG-bearing lymphocytes displayed a discontinuity in the 28°–32° range, independent of the time of incubation. The proportion of lymphocytes patched or capped was different after 5- versus 15-min

Table 1. Specificity of stripping and regrowth of sIg on splenic lymphocytes

Initial stripping treatment	% spleen lymphocytes stained with labeled antisera after strip or regrowth*			
	FITC-anti-IgM, %		FITC-anti-IgG2, %	
	Stripped	Regrown	Stripped	Regrown
None	—	38	—	7
Anti-IgM	4	36	7	6
Anti-IgG2	37	40	<1	8

* Receptor stripping involved incubating spleen lymphocytes (5×10^6 cells per ml) in RPMI-1640 at 37° with IgG fractions (50 μg/ml) of the indicated antisera. At the end of 8 hr of such an incubation, the lymphocytes were harvested, washed, stained for sIg at 4°, fixed, and then scored for percent stained. Receptor regrowth involved harvesting the stripped cells, washing them three to five times in RPMI-1640, and then culturing them for 10–14 hr without anti-Ig serum present. They were then harvested and treated like the stripped population to determine the frequencies of each sIg-positive subpopulation.

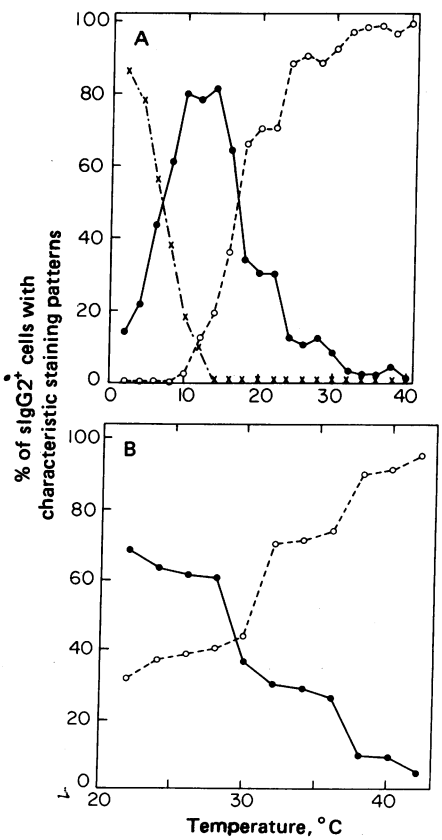

FIG. 2. Extent of splenocyte receptor movement induced at various temperatures (2°–40°) during 15-min (*A*) or 5-min (*B*) exposures to FITC-coupled goat anti-mouse-IgG2 sera (50 μg/ml per 10^7 cells). Incubations were terminated by addition of 10 volumes of 4% paraformaldehyde. Between 100 and 200 IgG2-positive lymphocytes were scored as diffuse (x), patched (●), or capped (○).

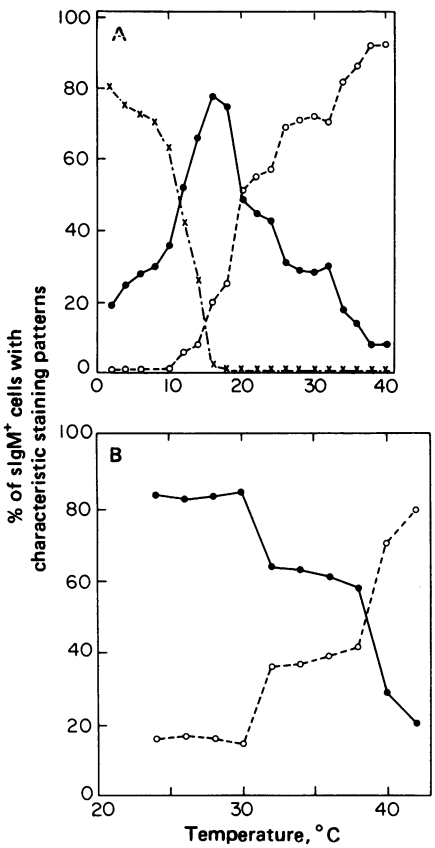

FIG. 3. Extent of splenocyte receptor movement induced at various temperatures (2°–40°) during 15-min (*A*) or 5-min (*B*) exposures to FITC-coupled goat anti-mouse-IgM (50 μg/ml per 10^7 cells). Incubations were terminated by addition of 10 volumes of 4% paraformaldehyde. Between 100 and 200 IgM-positive lymphocytes were scored as diffuse, patched, or capped. Symbols are as in Fig. 2.

incubations, owing to a time-dependent statistical process; nevertheless, the characteristic temperatures at which discontinuities were observed were identical, indicating a time-independent physical process.

In summary, characteristic temperatures that marked the onset of an increased rate of IgG2 receptor movement were observed at 14°, 22°, 28°, and 36°. However, when the same experiment was performed with anti-IgM (Fig. 3), the corresponding temperatures for IgM receptor movement were 18°, 24°, 32°, and 38°. The observed temperatures were accurate to within 1° as determined by 13 individual experiments. These results indicate that the membranes of IgM-bearing lymphocytes permit receptor movement comparable to IgG2 lymphocytes only at higher temperatures.

Correlation of sIg Receptor Mobility with Temperature-Dependent Membrane Phase Transitions. Because the characteristic temperatures observed in the receptor movement studies correlated exquisitely with ESR-derived characteristic temperatures (Fig. 4), we conclude that temperature-dependent receptor mobility, at least in part, reflects changes in the hydrocarbon structure of the B lymphocyte membrane. Comparison of data generated from patching–capping studies with the data derived by ESR studies with 5N10 versus 12NS-GA suggest that, for sIgM-bearing lymphocytes, 24° and 38° mark the beginning (t_l inner) and the end (t_h inner) of an inner monolayer melting process, whereas 18° (t_l outer) and 32° (t_h outer) mark the boundaries of a similar process in the outer monolayer (Table 2). For IgG2-bearing lymphocytes, the characteristic temperature assignments for the inner monolayer

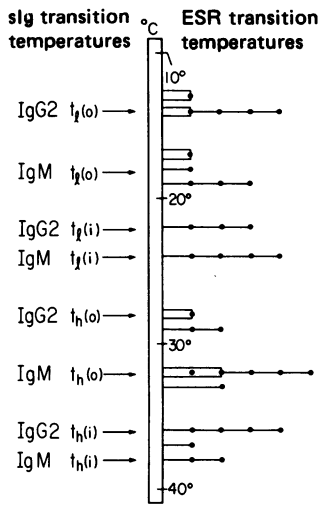

FIG. 4. Correlation between sIg movement experiments and ESR experiments. Arrows to the left of the temperature scale indicate temperatures in which discontinuities in the rates of sIg mobility were observed. Membrane phase transition temperatures ($P < 0.05$) revealed by computer analysis of ESR spectral data are indicated to the right of the temperature scale. ●—● represents each experiment in which 5N10 revealed transitions occurring in both monolayers of the plasma membrane. ●▬● represents each outer monolayer transition revealed by 12NS-GA.

are 22° and 36°, whereas those for the outer monolayer are 14° and 28°. Examination of the temperatures associated with the two sIg-positive lymphocyte populations revealed that IgM receptors reside in membranes that are less fluid than those containing IgG receptors. Furthermore, because the physical changes at characteristic temperatures of *both* monolayers were detected in the receptor modulation studies, the influence of both monolayers of the membrane seems to be exerted on receptor behavior. The capping process, of course, with its complex relationship to energy dependence and interactions with cytoskeletal structures, could also reflect the influence of changes in bilayer fluidity at a number of levels in addition to the direct effect on the diffusion rates of membrane receptors.

DISCUSSION

IgG-Bearing and IgM-Bearing Lymphocytes Have Different Membrane Transition Temperatures. We have shown that the membranes of IgG-bearing lymphocytes allow receptor redistribution to occur at lower temperatures than do the membranes of IgM-bearing lymphocytes in response to multivalent anti-Ig. The temperature-dependent behavior of receptors on sIgM- and sIgG-positive lymphocytes has been shown to be strikingly different by virtue of characteristic breaks and shoulders in plots of receptor behavior versus temperature (Table 2). It should be noted that the membranes of sIgG-bearing lymphocytes demonstrate characteristic temperatures similar to the rather universal temperatures observed in various other systems (9). sIgM-Positive lymphocytes are unusual in this regard, demonstrating higher characteristic temperatures than other cell membrane systems studied. It should also be pointed out that, in the purified B lymphocyte suspension (anti-θ treated, density fractionated), the ratio of cells present was 75 $sIgM^+/20$ $sIgG2^+/5$ non-sIg^+. Thus, the IgG-bearers and the contaminating non-sIg-positive lymphocytes may demonstrate membrane transitions at the same temperatures. However, the predominating ESR signal should emanate from the IgG2-bearing lymphocytes, with the contribution made by the other lymphocyte type at most reinforcing the signal.

Table 2. Inner and outer monolayer temperature assignments related to discontinuities in sIg behavior

Boundary*	Temperature, °C	
	sIgM	sIgG2
t_h (inner)	38†	36†
t_h (outer)	32‡	28‡
t_l (inner)	24†	22†
t_l (outer)	18‡	14‡

* t_h (inner) and t_l (inner) refer to the upper and lower temperature boundaries, respectively, of a membrane phase transition process in the inner monolayers of IgM- or IgG2-bearing lymphocytes. t_h (outer) and t_l (outer) refer to the upper and lower temperature boundaries, respectively, of a membrane phase transition process in the outer monolayers of IgM- or IgG2-bearing lymphocytes.

† Temperatures also observed with ESR data generated with B lymphocyte-enriched spleen cells with the probe 5N10 (detects inner and outer monolayer transitons).

‡ Temperatures also observed with ESR data generated with B lymphocyte-enriched spleen cells with the probe 5N10 or 12NS-GA (detects outer monolayer transitions only).

Inner and Outer Monolayers Show Independent Phase Transitions. Our results suggest the following model. When IgG-bearing lymphocytes are maintained at a temperature below 14°, both inner and outer monolayer constituents are in an ordered, rigid state. When warmed above 14°, the components of the outer monolayer begin to melt, establishing an equilibrium between melted and unmelted components of that monolayer. Meanwhile, the inner monolayer, being different in its composition, remains relatively rigid. As the temperature is further increased beyond 22°, the inner monolayer components begin an analogous transition process. At temperatures above 28°, the outer monolayer has reached a randomized (fluid) state, with the inner monolayer reaching this state only at temperatures above 36°. A comparable transition process could be described for IgM-bearing lymphocytes. These transition temperatures are accurate to within ±1° as determined from multiple experiments.

As the temperature increases, the overall resistance to lateral movement of membrane-associated proteins would be expected to decrease as the hydrocarbon components become more fluid. The effect of passing through a temperature associated with a membrane phase transition would be a relatively large increase in the rate of protein movement corresponding to the increase in membrane fluidity as a result of the melting process. When the extent of protein movement is plotted versus increasing temperature, membrane transitions would be seen as discontinuities in which the expected rate of movement is exceeded by the observed rate. So, for example, in an actual curve (Fig. 2) generated by plotting percentage of sIgG2 lymphocytes patched versus temperature, the plateau at 10°–14° represents a range of temperature at which little or no fluidity change is discernible by measuring the 15-min endpoint of receptor redistribution. The number of points included in this temperature range is dictated by a physical process. When the incubation time is changed, the *level* of the plateau changes, but the temperature *range* in which it is found remains the same (above and unpublished data). In this case, as the 14° transition boundary t_l(outer) is passed, the extent of receptor movement increases until another plateau is reached. This new plateau marks the end of the enhanced receptor mobility created by the onset of the lateral phase separation. A similar process could be described for the other discintinuities in the curve.

Membrane Changes during Development. It is remarkable that the ESR studies should have revealed eight characteristic transition temperatures, suggesting that the spleen cell population consists of two homogeneous B lymphocyte types (sIgM positive and sIgG2 positive) rather than the heterogeneous range of differentiated B lymphocyte types known to be present in the spleen (15, 16). The sIgM-bearing population predominates (~35–45%) and is comprised largely of lymphocytes bearing both sIgM and sIgD. The ESR temperature assignments associated with IgM-bearing lymphocytes, therefore, are presumably for the sIgM–sIgD receptor bearers, whereas separate detection of the relatively minor sIgM-bearing populations (sIgM–sIgG$^+$, sIgM$^+$ only) is beyond the resolution of these experiments. Even the minority population (about 5%) with sIgG on its surface is quite heterogeneous, with many members also bearing sIgM or sIgD. Despite these limitations inherent in only examining the predominant B lymphocyte populations, our results point to a clear difference in the fluidity of the membrane of any sIgG-bearing lymphocyte compared to one lacking sIgG. The accession of IgG to the cell surface, which represents a distinct maturation state in B lymphocytes, correlates with altered membrane physical state. These results provide a basis for future studies concerning lymphocyte activation and development.

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