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Membrane Fluidity and Lipid Hapten Structure of Liposomes Affect Calcium Signals in Antigen-Specific B Cells

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ABSTRACT: Antigen-specific B-cell clones directed against a 2,4,6-trinitrophenyl (TNP) hapten have been established [Hamano et al. (1990) *J. Immunol. 144*, 811–815]. We measured here the cytosolic free calcium ion concentration ($[Ca^{2+}]_i$) in these B-cell clones after antigen stimulation. Trinitrophenylated liposomes with different length spacers between TNP and phosphatidylethanolamine (TNP-C_n-PE) increased cytosolic free calcium concentration in TNP-specific B cells (clone TP67.21). The magnitude of calcium signals depended on the length of the spacer. TNP-C_6-PE in dipalmitoylphosphatidylcholine (DPPC) liposomes triggered larger calcium signals in B cells than TNP-C_n-PE with n = 0, 4, 8, or 12. The magnitude of the calcium signals was strongly dependent on the fluidity of the liposome membranes. TNP-C_6-PE in the solid DPPC liposomes triggered the calcium signals in B cells 50–100 times as efficiently as TNP-C_6-PE in the fluid dimyristoylphosphatidylcholine liposomes. The difference between the solid liposomes and the fluid liposomes was more pronounced in triggering calcium signals in B cells than in antibody binding to these liposomes.

Membrane forms of immunoglobulin (mIg) serve as antigen receptors on B lymphocytes. Following antigen binding, the membrane immunoglobulin transduces transmembrane signals into the B cells. These signals play important roles in the inactivation of immature B cells, a process that contributes to tolerance to self, and in the activation of mature B cells to produce antibodies (Defranco et al., 1989; Cambier & Ransom, 1987; DeFranco, 1987). However, antigen-specific B cells are difficult to isolate and utilize because of their small numbers. Thus, many studies on the B cell signal transduction have been done by examining the effects of anti-immunoglobulin antibodies (Cambier & Ransom, 1987; DeFranco et al., 1989).

Recently, Hamano et al. established antigen-specific B-cell clones directed against 2,4,6-trinitrophenyl (TNP)[1] hapten (Hamano et al., 1987, 1990). These B-cell hybridomas have served as good models for the study of B-cell activation and differentiation. These antigen-specific B-cell clones are also attractive models of membrane-bound antigen recognition by B cells. Liposomes sensitized with lipid haptens have been extensively studied by many authors (Six et al., 1973; Brulet & McConnell, 1977; Dancey et al., 1979; Balakrishnan et al., 1982; Ho & Huang, 1985; Kimura et al., 1990). However, most studies have focused on the interaction between the hapten-sensitized liposomes and antibody molecules. Unfortunately, there has been no systematic study of the interaction between liposomes bearing lipid haptens and their membrane-bound receptor molecules (membrane immunoglobulins) in B lymphocytes.

In this paper it will be shown that trinitrophenylated liposomes increase the cytosolic free calcium concentration in TNP-specific B cells. The magnitude of the calcium signals was dramatically dependent on both the membrane fluidity and the spacer lengths of the lipid haptens.

Materials and Methods

Materials. L-α-Dimyristoylphosphatidylcholine (DMPC) and L-α-dipalmitoylphosphatidylcholine (DPPC) were purchased from Avanti (Birmingham, AL). 2,4,6-trinitrophenylated (TNP) phosphatidylethanolamine with different length spacers were prepared by a previous method (Okada et al., 1982).

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[1] Abbreviations: DMPC, L-α-dimyristoylphosphatidylcholine; DPPC, L-α-dipalmitoylphosphatidylcholine; TNP, 2,4,6-trinitrophenyl; PE, L-α-dipalmitoylphosphatidylethanolamine; TNP-C_0-PE, TNP-PE; TNP-C_4-PE, TNP-aminobutyryl-PE; TNP-C_6-PE, TNP-aminocaproyl-PE; TNP-C_8-PE, TNP-aminocapryl-PE; TNP-C_{12}-PE, TNP-aminolauryl-PE; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PBS, phosphate-buffered saline; FCS, fetal calf serum; BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin.

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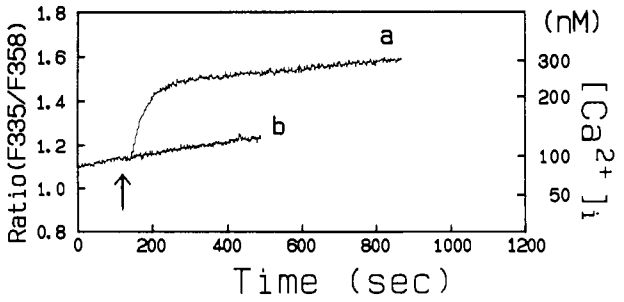

FIGURE 1: Time courses of changes of the $[Ca^{2+}]_i$ in fura-2 loaded B cells (TP67.21) in response to DPPC liposomes with 1% TNP-C_6-PE (8 nM) at 37 °C. The abscissa shows the time after antigen stimulation. Liposomes were added at the point indicated by an arrow. (a) Stimulated with 99% DPPC liposomes with 1% TNP-C_6-PE. (b) Stimulated with 100% DPPC liposomes without lipid haptens (a control experiment).

TNP-Specific B-Cell Hybridomas. TNP-specific B-cell hybridomas (TP67.21) have been established and as described previously (Hamano et al., 1987). TP67.21 stands for a cell clone. The cells were grown in a RPMI 1640 medium with 5% FCS.

Labeling of Fura-2AM. Fura-2AM (Dojin, Kumamoto) was incorporated into the B-cell hybridomas (TP67.21) in culture medium. B cells (10^6 cells/mL) were incubated for 30 min at 37 °C with 5 μM fura-2AM. After incubation, cells were centrifuged for 1 min at 1000 rpm in a Hitachi centrifuge model CR5B2. The cells were washed with 10 mM HEPES buffer (10 mM HEPES, 140 mM NaCl, and 5 mM KCl, pH 7.2, with 0.1% BSA) and resuspended in 10 mM HEPES buffer containing 1 mM $CaCl_2$ for fluorescence measurements.

Preparation of Small Unilamellar Liposomes. Small unilamellar liposomes were prepared by the injection of an ethanolic solution of a mixture of phospholipid (DPPC or DMPC) and a TNP-lipid hapten into PBS (10 mM sodium phosphate and 150 mM NaCl, pH 7.2) at 70 °C (Batzri & Korn, 1973; Kremer et al., 1977; Kimura et al., 1990). The entrapment efficiency of small unilamellar vesicles was checked by the trapped umbeliferone phosphate method (Six et al., 1974).

Fluorescence Measurements. Fluorescence spectra were measured with a Shimadzu spectrophotometer RF-5000 with a temperature control bath (Shimadzu TB-85). The time-dependent fluorescence intensity changes of the fura-2 loaded cells were measured with the excitation wavelength at 335 or 358 nm and the emission wavelength at 500 nm at 37 °C. Here, the fluorescence ratio (F_{335}/F_{358}) was collected every 2 s. Estimation of $[Ca^{2+}]_i$ from fura-2 fluorescence data was based on a previously described method (Grynkiewicz et al., 1985; Utsunomiya et al., 1990; Teshima et al., 1990).

Results

Calcium Signals in Antigen-Specific B Cells by Hapten-Sensitized Liposomes. As shown in Figure 1a, addition of DPPC liposomes with TNP-C_6-PE to TNP-specific B cells (TP67.21) resulted in a clear-cut elevation in the concentration of cytosolic free calcium ions $[Ca^{2+}]_i$. In this experiment, we used liposomes made of 99 mol % DPPC and 1 mol % TNP-C_6-PE. A similar elevation in $[Ca^{2+}]_i$ of B cells (TP67.21) was triggered by TNP-conjugated KLH (Hamakawa et al., unpublished results). In control experiments, we added liposomes made of 100% DPPC (without TNP-lipid) to the B cells (TP67.21); no significant $[Ca^{2+}]_i$ change was observed (Figure 1b). In another control experiment, we measured the response of fura-2-loaded nonspecific B cells (WEHI231 and BAL17) to DPPC liposomes containing 1% TNP-C_6-PE; no significant $[Ca^{2+}]_i$ was observed (data not shown).

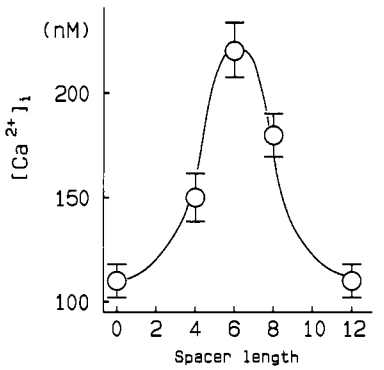

FIGURE 2: Levels of the $[Ca^{2+}]_i$ in B cells at 5 min after the stimulation with TNP-liposomes with different length spacers. The levels of the $[Ca^{2+}]_i$ are plotted against the length of the spacers. Each $[Ca^{2+}]_i$ level was an averaged value of four individual measurements. Standard deviations are given by vertical bars.

When 1 mM EGTA was in the external medium (without $CaCl_2$), intracellular $[Ca^{2+}]_i$ levels decreased. This indicates that the elevation in $[Ca^{2+}]_i$ of B cells (TP67.21) was mediated by the release of Ca^{2+} from intracellular stores and also by the entry of extracellular Ca^{2+}.

Calcium Signals in B Cells Induced by the Hapten-Sensitized Liposomes with Different Length Spacers. We next measured the concentration of the cytosolic free calcium ions $[Ca^{2+}]_i$ in B cells after the addition of the hapten-sensitized liposomes with different length spacers between TNP and phosphatidylethanolamine (TNP-C_n-PE). The efficiency of the $[Ca^{2+}]_i$ in B cells (TP67.21) was strongly dependent on the spacer length. TNP-C_6-PE in DPPC liposome membranes increased intracellular $[Ca^{2+}]_i$ levels in B cells more than TNP-C_n-PE with n = 0, 4, 8, or 12 in DPPC liposomes. The $[Ca^{2+}]_i$ levels of B cells at 5 min after stimulation with TNP-liposomes are shown in Figure 2. The results show that derivatives containing a shorter spacer (TNP-C_0-PE or TNP-C_4-PE) or a longer spacer (TNP-C_8-PE or TNP-C_{12}-PE) were less effective in triggering calcium signals in B cells than a derivative containing an intermediate length spacer (TNP-C_6-PE). The results suggest that membrane-bound B-cell antigen receptors preferentially respond to TNP lipid haptens with an intermediate length spacer.

We and others (Kimura et al., 1990; Dancey et al., 1979) have shown that anti-TNP (or anti-DNP) antibody in solution bound preferentially to the hapten-sensitized liposomes with the intermediate length spacer. Thus, the present results indicate that the membrane forms of immunoglobulin (mIg) as well as the secreted forms (sIg) interact most efficiently with hapten-sensitized liposomes with the intermediate length spacer. In addition, the results showed that the liposomes with the intermediate length spacer triggered the activation of the antigen-specific B cells with the highest efficiency.

Effects of Liposome Membrane Fluidity on Calcium Signals in B Cells. Levels of calcium signals in antigen-specific B cells (TP67.21) triggered by solid DPPC liposomes containing TNP-C_6-PE were affected by the concentration of TNP-lipid. In Figure 3 the levels of $[Ca^{2+}]_i$ induced in B cells at 5 min after antigen stimulation are plotted against the concentration of TNP-C_6-PE in the liposomes. A level of 0.5–2% TNP-C_6-PE in DPPC liposomes induced calcium signals in B cells similar to those of 1% TNP-C_6-PE in DPPC liposomes. However, 0.1% TNP-C_6-PE in DPPC liposomes was less effective in increasing $[Ca^{2+}]_i$ levels in B cells than 1% TNP-C_6-PE, and 0.02% TNP-C_6-PE in the DPPC liposomes was not effective on the $[Ca^{2+}]_i$ in B cells. In the case of fluid DMPC liposomes, the magnitude of calcium signals

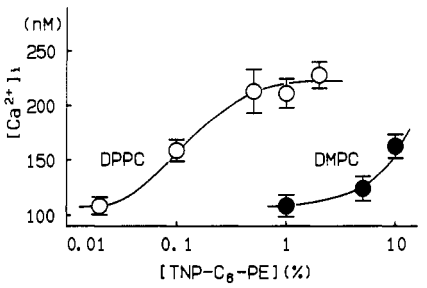

FIGURE 3: Levels of the $[Ca^{2+}]_i$ in B cells at 5 min after antigen stimulation plotted against the concentration of TNP-C_6-PE in liposomes. Open circles are for DPPC liposomes and closed circles for DMPC liposomes. Each $[Ca^{2+}]_i$ level was an averaged value of four individual measurements. Standard deviations are given by vertical bars.

in B cells was greatly decreased. 1% TNP-C_6-PE in DMPC liposomes did not affect $[Ca^{2+}]_i$ in B cells, as shown in Figure 3. 5% TNP-C_6-PE in DMPC liposomes increased slightly the $[Ca^{2+}]_i$ in B cells; and 10% TNP-C_6-PE in DMPC liposomes increased the $[Ca^{2+}]_i$ response of B cells; however, they were less effective than 0.5% TNP-C_6-PE in DPPC liposomes. Thus, Figure 3 indicates that TNP-C_6-PE in the solid DPPC liposomes triggered the calcium signals in B cells 50–100 times more efficiently than TNP-C_6-PE in fluid DMPC liposomes.

Discussion

Lipid hapten-dependent triggering of calcium signals in B cells was strongly dependent on the length of linker chain (spacer) connecting the hapten to the head group of the lipid. Derivatives containing short or long spacers were less effective in activating B cells than that of an intermediate length spacer. This may be due to differences in the binding affinities between the membrane forms of immunoglobulin (mIg) and the haptenized liposomes with different length spacers. It has previously been shown that the secreted forms of immunoglobulin (sIg) bound preferentially to liposomes with intermediate length spacers (Brulet & McConnell, 1977; Dancey et al., 1979; Balakrishnan et al., 1982; Kimura et al., 1990). The reason for this was explained by our recent fluorescence energy transfer measurements (Kimura et al., 1990).

In the present experiments, we showed that the calcium signals in B cells were highly dependent on the fluidity of liposome membranes. TNP-C_6-PE in the solid DPPC liposomes triggered the calcium signals in B cells 50–100 times more efficiently as TNP-C_6-PE in fluid DMPC liposomes. This must be partly due to the affinity differences between TNP-spacers and the bilayer surfaces in both liposomes (Kimura et al., 1990). The binding affinity of TNP-spacer groups (without phospholipids) was slightly higher to the fluid liposomes than to the solid liposomes [Balakrishnan et al., 1982; Kimura et al., 1990; Kimura and Nakanishi (unpublished results)]. Thus, the lipid hapten in the fluid liposomes is not able to be easily transferred from the bilayer surfaces (unavailable for antibody binding) to the aqueous solution (available for antibody binding) as we described in a previous paper (Kimura et al., 1990). Actually, we observed that anti-TNP IgGs bound to the solid TNP-liposomes a few times higher than to the fluid TNP-liposomes [Balakrishnan et al., 1982; Kimura et al., 1990; Kimura and Nakanishi (unpublished results)]. In the present experiments, however, the solid liposomes induced the calcium signals in B cells with much higher efficiency than the fluid liposomes did.

It is thought that the transmembrane signal transduction in B cells occurs in the following pathways (Cambier & Ransom, 1987). Cross-linking of the membrane immunoglobulin by antigen prompts the hydrolysis of polyphosphoinositides by phospholipase C, yielding inositol trisphosphate and diacylglycerol. Then, inositol trisphosphate stimulates the release of Ca^{2+} from the endoplasmic reticulum and Ca^{2+} influx into cytoplasm (Berridge & Irvine, 1984). Cross-linking of membrane immunoglobulin is able to be mimicked by specific divalent reagents, such as anti-immunoglobulin antibodies. Thus, the triggering of the calcium signals is induced by cross-linking membrane immunoglobulin (Pozzan et al., 1982; Ransom et al., 1986). Compared with the fluid liposomes, the solid liposomes were more effective in triggering the calcium signals of B cells than in antibody binding to them.

Registry No. Ca^{2+}, 7440-70-2; TNP-C_0-PE, 87706-96-5; TNP-C_4-PE, 136706-28-0; TNP-C_6-PE, 136706-29-1; TNP-C_8-PE, 136706-30-4; TNP-C_{12}-PE, 136706-31-5.

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