Differential sensitivity of B lymphocyte populations to IgM receptor ligation is determined by local factors

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Abstract

Ligation of surface IgM on B cells responding to lipopolysaccharide (LPS) suppresses terminal differentiation and high-rate Ig secretion with no effect on proliferation. As shown here, different B cell populations show characteristic mean values of ligand concentration required for 50% inhibition, with Gaussian distributions of sensitivity to IgM receptor ligation that reflect cellular heterogeneity of ‘all-or-none’ inhibitions in single cells. Differential sensitivity of B cell populations to IgM ligation seems to be locally determined by the cellular environment and unrelated to the ‘maturity’ of the responding cells. Thus, while long-lived peritoneal B cells are 3- to 5-fold more resistant than splenic B cells, there is no difference in sensitivity between short- and long-lived B cells in the spleen. Furthermore, while B cells in bone marrow and spleen differ in sensitivity by two orders of magnitude, B cells differentiated in vitro from bone marrow pre-B cells are as resistant as splenic B cells. Moreover, bone marrow cell culture supernatants restore a high level of sensitivity in such cell populations. Differential sensitivity to IgM receptor ligation is reproduced by multivalent nominal antigen, in cell populations that show identical dose–response inhibition curves to direct activation of protein kinase C by phorbol esters. We conclude that the level of sensitivity to IgM ligation is independent of the life span or maturity of the B cell, but differentially regulated in vivo by putative tissue factors.

Introduction

In mammals, a very large Ig diversity (1) imposes mechanisms that select the available repertoires of mature resting B cells and the actual repertoires of Ig-secreting plasmocytes from the genetically determined repertoires emerging in the primary lymphoid organs (2,3). In the adult, all these sets of cells are renewed by the continuous production of B cells in the bone marrow (BM) (4) and the comparison of the cellular pool sizes (5) indicates that only a very small fraction of the emergent repertoire is integrated into the mature compartments (6) suggesting the operation of selection processes. Negative selection of B cells binding ligand with the required avidity was directly confirmed in murine transgenic models (7,8). In normal mice, quantitation of expression of V_H genes and homology families in different B cell compartments (9–11), as well as the analyses of ‘multireactive’ clonal frequencies (12), have both shown that negative selection implicates a large fraction of cells between the emergent and the peripheral repertoires. In turn, studies of serum antibody and plasma cell repertoires have demonstrated the continuous positive selection of a set of reactivities that is characteristic of normal individuals (13,14).

In order to approach the mechanisms of V-region-dependent B cell selection at the transition from the emergent to the available repertoire, we have studied the sensitivity to IgM ligation in BM and splenic B cells. As a model system, we have used anti-µ inhibition of lipopolysaccharide (LPS)-induced differentiation of B cells into Ig-secreting cells (15,16). This experimental system is suitable, since anti-µ antibodies bind to (and potentially inhibit) all IgM⁺ B cells and since LPS can stimulate all B cells under our culture conditions (17,18).

As previously reported (19,20), we also found that immature B cells, present in new-born spleen and adult BM, are much more sensitive to inhibition of differentiation by anti-µ than mature B cells from adult spleen. Furthermore, we have...
extended these findings of differential sensitivity to B cells present in peripheral blood and the peritoneal cavity, and to surface IgM ligation in the absence of Fc receptor engagement. Using the phorbol ester phorbol myristate acetate (PMA), a direct activator of protein kinase C (PKC), which mimics anti-μ inhibition (21,22), we could show that the differences in sensitivity to anti-μ inhibition between splenic and BM B cells concern an early step of intracellular signaling, from surface IgM ligation to PKC activation.

Finally, we have investigated the sensitivity to sIgM ligation of immature B cells generated in vitro from purified BM pre-B cells and found that such cells are as resistant as are peripheral mature B cells, but recover levels of BM sensitivity upon incubation with supernatants of BM cell cultures. These observations question the notion that the sensitivity of newly formed, immature B cells is an inherent cellular property, linked to the stage of differentiation.

**Methods**

**Animals**

The transgenic line B6-Sp6 (23) and control C57BL/6 (B6) littersmates were kept and bred in our animal facilities. The mice were used at 6–20 weeks of age. Treatment with hydroxyurea (HU) was performed as described previously (24): HU (Sigma, St Louis, MO) was dissolved in 0.9% NaCl and administrated i.p. as two doses of 1 mg/g body wt/day, injected 7 h apart for 4 days.

**Cells and cultures**

Cell suspensions from spleen, BM and peritoneal cavity were prepared as described earlier (3,6), and cultured at 10^6 cells/well in 0.2 ml flat-bottomed microtiter plates, containing different concentrations of antibodies or antigen together with 6×10^9 irradiated (2000 rad) S17 stromal cells (25,26) in OPTI-MEM medium (Bioproducts, Rockland, ME), supplemented with L-glutamine, penicillin, streptomycin (all from Flow, Irvine, UK), 2-mercaptoethanol (5×10^−4 M) (Merck, Darmstadt, Germany), gentamycin (Unilabo, Paris, France), 10 mM HEPES buffer, pH 7.3 (Sigma) and 10% FCS (Boehringer, Mannheim, Germany). After 1–2 h, 25 µg/ml LPS from Salmonella typhimurium (Difco, St Louis, MO) was added and the cells harvested on day 2 for [3H]thymidine uptake, on day 4 or 5 for cellular assays of Ig secretion (see below) and on day 7 for ELISA determination of secreted Ig accumulated in culture supernatants (see below).

**Culture of pre-B cells and generation of immature B cells in vitro.**

BM cells from adult C57BL/6 mice were cultured in the presence of recombinant mouse IL-7 (supernatant from J558-IL-7) and irradiated (2000 rad) S17 cells for 10 days. Remaining slg^+ cells were removed by panning (using goat anti-mouse IgM). Thereafter the cells were allowed to differentiate for 3 days in the presence of S17 cells but in the absence of IL-7, resulting in a heterogeneous population of pre-B cells and slgM^+ B cells, the majority of which (85%) were immature B cells as judged by high expression of slgM and negative for slgD.

In experiments involving pre-incubation of the newly differentiated cells (Fig. 7), at day 2 of culture without IL-7, the medium was supplemented with a supernatant (two-thirds final concentration) from bulk BM cultures (9×10^6 cells/ml in normal medium for 3 days) and further incubated for 2 more days.

**Antibodies and antigen.**

The mAb used in this study have been described in (27). MB86 (anti-µ^b_), RS3.1 (anti-µ^b_), b-7 6 (anti-µ), 187.1 (anti-κ L chain) and RA3-682 (anti-CD45R, anti-B220) were used either unlabeled or conjugated with biotin or FITC as indicated. Biotin-labeled goat anti-mouse IgM and monoclonal phycoerythrin (PE)-labeled anti-mouse IgD were purchased from Southern Biotechnology Associates (Birmingham, AL). Polyclonal anti-mouse Ig antibodies were purified from a sheep antiserum (kindly donated by Dr A. Kelus at the Basel Institute for Immunology, Basel, Switzerland) by affinity chromatography on Sepharose-bound normal mouse IgG. For elimination of slg^+ cells by panning, a polyclonal goat anti-IgM from Sigma was used. TNP-BSA was prepared as described by Rittenberg and Campbell (28), using BSA and 3,4,6-trinitrobenzene sulfonic acid from Sigma.

**Flow cytometry**

Flow cytometric analyses were performed as described before (3,29). Briefly, single-cell suspensions of BM, spleen or peritoneal cells were stained with FITC-labeled mAb (all anti-Ig antibodies and the plates saturated with PBS containing 1% BSA). Dead cells were excluded from analysis by using propidium iodide gating as described (29). Analyses were performed on a FACScan (Becton Dickinson, Sunnyvale, CA) interfaced to a Hewlett-Packard computer (HP900) using the FACScan research software programs.

**Spot-ELISA**

Numbers of IgM-secreting cells were enumerated using a modification (3) of the Sedgwick and Holt technique (30). Briefly, flat-bottomed ELISA plates were coated with either sheep anti-mouse Ig antibodies or monoclonal anti-allotypic antibodies and the plates saturated with PBS containing 1% gelatin. Lymphoid cells harvested on day 4 or 5 of culture were suspended in RPMI 1640 complete medium containing 2% FCS and distributed to the microwells at appropriate concentrations. The plates were incubated for 5–6 h at 37°C in a humidified 5% CO₂ atmosphere and the cells thereafter removed by flicking the plate followed by lysis with 0.05% Tween 20 in water. After extensive washing with PBS containing 0.05% Tween 20, biotinylated goat anti-mouse IgM antibody was added to each well. After incubation and subsequent washing, streptavidin labeled with alkaline phosphatase (Southern Biotechnology Associates) was added and the plates further incubated for an additional hour. After washing, the revealing substrate (2.3 mM of 5-bromo-4-chloro-3-indolyl phosphate; Sigma) diluted in 2-amino-2-methyl-1-propanol buffer (Merck) was added. After incubation for 2 h at 37°C, the traces of Ig-secreting cells were revealed as blue spots and counted. For every test, duplicate dilution series were used and the frequency of secreting cells was...
determined. In some experiments, after development and counting, the blue spots were dissolved by adding 100 µl DMSO and the optical density at 650 nm (OD650) was recorded using an ELISA reader as described (31).

ELISA
Culture supernatants were assayed for IgM content with the ELISA technique. ELISA plates that were coated overnight with b-7-6 antibodies (anti-µ), and then saturated with PBS-1% gelatin, received dilution’s of culture supernatants to be tested. After incubation (2 h, room temperature) and washing, peroxidase-labeled goat anti-mouse IgM antibodies were added. After incubation (1 h, room temperature) and washing, bound antibodies were revealed with the substrate o-phenylenediamine and H2O2. The reaction was stopped after 20 min by addition of 10% SDS and the absorbency read at 450 nm in a Titertek multiscan spectrometer (Flow). The concentration of IgM in culture supernatants were calculated by using standard curves obtained with purified monoclonal IgM in the same assay.

Proliferation
[3H]Thymidine (Amersham, Buckinghamshire, UK; sp. act. 5 Ci/mmol) uptake was performed at the indicated times by adding 1 µCi per culture, 16 h before harvest. Pulsed cultures were harvested on glass-fiber filter papers using a Skatron 96-well microtiterplate harvester (Flow). After drying, the filters were immersed in scintillation fluid and counted in a Rack-Beta Plate liquid scintillation counter (Pharmacia-LKB, Bromma, Sweden). The data represent the mean of four cultures expressed as c.p.m. per culture. The SEM never exceeded 10%.

Results
Characterization of the experimental model system
Since a variety of effects have been described for the action of anti-Ig reagents on B cell proliferation and differentiation (15,16,20,32,33-35), it was important to first characterize our experimental model system for IgM-dependent in vitro inhibition of terminal differentiation.

B cells from different organs of C57BL/6 mice (IgMβ allotype) were incubated for 2 h with increasing doses of monoclonal anti-µβ or anti-µα antibodies and then stimulated by LPS in the continuous presence of the IgM ligand. After 2, 4 and 7 days respectively the cultures were tested for proliferation ([3H]thymidine uptake), for differentiation to IgM-secreting cells (Spot-ELISA), as well as for total amounts of IgM secreted in the culture supernatants (ELISA). Three main findings should be stressed. First, anti-µβ (but not the anti-µα) antibodies inhibit LPS-induced differentiation of splenic B cells into high rate IgM-secreting cells. As shown in Fig. 1(A), the number of IgM-secreting cells, as well as the concentrations of secreted IgM accumulated in the culture supernatants during 7 days, were markedly reduced at anti-µβ antibody concentrations of 100 ng/ml and almost completely abolished at 1 µg/ml. The inability of anti-µα to inhibit B cells from C57Bl/6 was specific, since this mAb preparation readily suppresses µα-expressing B cells in similar experiments (27). Second, the anti-µ antibody treatment does not affect proliferation induced by LPS in the same cultures where B cell differentiation to IgM secretion is completely suppressed (Fig. 1B). Third, anti-µ inhibition is an ‘all-or-none’ phenomenon at the single cell level. Thus, by using a modification of Spot-ELISA (31), which measures total IgM secreted by a given number of spot-forming cells, we could derive a slope representing the quantity of Ig produced per IgM-secreting B cell and thus ascertain whether or not anti-µ antibody treatment affects the rate of IgM secretion per cell. As can be seen in Fig. 1(C), we obtained overlapping straight lines with data from both control and anti-µ treated cultures which were almost completely inhibited for the numbers of Ig-secreting cells. This strongly suggests that remaining secreting B cells in inhibited cultures produce IgM at the same rate per cell as B cells stimulated by LPS in the absence of anti-µ mAb. We conclude that, in the presence of anti-µ mAb, LPS-stimulated B cells seem to have only two alternatives: normal rate of secretion or no detectable secretion of IgM at all. In turn, this observation confirms that the respective cellular controls operate at early stages in the activation process (15).

These characteristics, as described for splenic B cells, also apply for B cells from the peritoneal cavity as well as for BM B cells (Fig. 1B and C).

The sensitivity to anti-µ inhibition varies among different B cell populations
B cells from different organs were stimulated by LPS in the presence of increasing concentrations of monoclonal anti-µ allotype antibodies and then tested by Spot-ELISA for numbers of IgM-secreting cells after 4 days of culture. For each population, the concentration of anti-µ mAb resulting in a 50% inhibition of the control LPS response was evaluated (dotted line in Fig. 2). As can be seen in Fig. 2, BM B cells from adult mice were 25 to 100 times more sensitive to anti-µ-mediated suppression of the LPS-induced terminal maturation than were splenic B cells or peripheral blood B cells from the same animals. Furthermore, splenic B cells from newborn animals were as sensitive as BM B cells from adult mice. As for adult spleen cells, the suppression is specific and does not concern proliferation. In addition, anti-B220 mAb at 10 µg/ml had no effect on the LPS stimulation of either B cell population. Similar results were obtained by employing ELISA tests, determining the total amount of IgM secreted into culture supernatants (data not shown).

One possible explanation for this differential sensitivity to IgM ligation could be that the density and/or the total number of surface IgM molecules are different in these various B cell populations. However, after direct staining with FITC-labeled anti-µ antibodies FACS analyses revealed broad profiles where the differences in fluorescence intensities between the various B cell populations tested show considerable overlap and thus can not alone account for the differential sensitivity to IgM ligation (3 and data not shown).

Different sensitivity to nominal antigen of splenic and BM B cells from the B6-SP6 transgenic mouse line
In the B6-SP6 line (23), which is a C57BL/6 mouse (IgMβ allotype) transgenic for a complete IgMα molecule derived from a TNP-specific BALB/c hybridoma (36), >90% of the
Fig. 1. Characterization of the experimental assay. (A) Specificity of anti-µ antibody suppression of LPS-driven B cell differentiation into IgM-secreting cells, as revealed by both Spot-ELISA and ELISA. C57BL/6 spleen cells were cultured with LPS in the presence of different concentrations of anti-µ^b (○) or anti-µ^a (□) mAb. At day 4, the numbers of IgM-secreting cells were determined by Spot-ELISA (top part) and at day 7, culture supernatants were scored for secreted IgM by ELISA (bottom part). Control LPS-stimulated and non-stimulated cultures contained respectively 82,000 and 1000 IgM-secreting cells, whereas cultures with no LPS but in the presence of 10 µg/ml of anti-µ^b resulted in 1500 IgM-secreting cells. In ELISA, control LPS cultures contained 65 µg/ml IgM, whereas non-stimulated culture supernatants contained 2 µg/ml IgM and cultures receiving only anti-µ^b (10 µg/ml) contained 1 µg/ml IgM. (B) Unimpaired proliferation of B cells in cultures where anti-µ antibodies suppress B cell differentiation into IgM-secreting cells. Cultures identical to those of (A), containing B cells from spleen (top part), BM (middle part) and peritoneal cavity (lower part) were scored for [3H]thymidine uptake on day 2 after stimulation with LPS in the presence of anti-µ^b (○) or anti-µ^a (□) mAb. Control cultures gave the following [3H]thymidine uptake (c.p.m. per culture). Spleen: LPS only, 82,400; no LPS, 5100; anti-µ^b alone, 7200. BM: LPS only, 45,700; no LPS, 2200; anti-µ^b alone, 4200. Peritoneal cavity: LPS only, 19,000; no LPS, 5200; anti-µ^b alone, 4200. (C) Cells escaping inhibition by anti-µ antibodies secrete as much IgM as cells in LPS control cultures. Five days after LPS stimulation in the absence (○) or presence (□) of anti-µ concentrations that yielded 95% inhibition of the control LPS response, cells were diluted in spot-ELISA to reveal a countable number of spots, which after enumeration were dissolved in DMSO for determination of the optical density at 650 nm (OD_{650}).

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splenic and BM B cells express only the transgenic IgM^a molecule because of allelic exclusion (3). In such a mouse, therefore, anti-µ antibodies can be replaced by a non-antibody ligand for surface IgM, represented by the nominal antigen TNP. If used in a multivalent form (TNP-BSA) to allow for cross-linking of surface IgM, this could give indications on the general physiological significance of IgM ligation. The results from such an experiment are illustrated in Fig. 3, where anti-µ mAb has been substituted by the antigen TNP-BSA. As can be seen, the LPS-induced terminal maturation of splenic B cells from B6-SP6 is ~50 times more resistant to inhibition by nominal antigen than BM B cells from the same mouse. These observations strongly suggest that the differential sensitivity to anti-µ of BM and splenic B cells applies to all ligands binding (and cross-linking) surface IgM. Furthermore, this experimental strategy excludes that the differences recorded are due to different levels of Fc receptor expression in the various B cell populations. Finally, since transgenic B cells lack surface IgD (3,37), the findings demonstrate that both induction and suppression of B cell differentiation may occur in the absence of IgD expression, and are actually regulated independently of such receptors.

BM and splenic B cells show the same sensitivity to suppression by PMA

Since IgM ligation may mediate activation of PKC (38,39) the differential sensitivity described above could be due to variations in the relative levels of B cell PKC expression or activity. Since direct activation of PKC by phorbol esters (PMA) can mimick the inhibitory effects of anti-µ mAb on LPS-induced B cell differentiation (21,22), we tested the sensitivity of BM and splenic B cells to different concentrations of PMA. As can be seen in Fig. 4, contrary to sIgM-ligation-dependent inhibition, splenic and BM B cells stimulated by LPS display similar sensitivity to inhibition by PMA. Furthermore, in bulk cultures of LPS-stimulated spleen cells, anti-µ mAb at 5 µg/ml or PMA at 5 ng/ml resulted in 10–20% enhanced numbers of activated B cell blasts as compared to cultures receiving LPS alone. However, <1% of these blasts secreted IgM to form a spot (data not shown). If IgM ligation indeed results in PKC activation, these findings suggest that comparable IgM ligation in BM and splenic B cells results in different levels of PKC activation. Thus, B cell populations may differ, with respect to signals generated by surface IgM ligation, and this difference is then located at an early point after ligand binding
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Fig. 3. Polyvalent nominal antigen is as efficient in suppressing LPS-induced B cell differentiation as anti-IgM antibodies, and reveals the same difference in sensitivity between BM and adult splenic B cells. Spleen (●) and BM (○) cells from the IgM transgenic mouse B6-Sp6 were cultured with LPS in the absence or presence of different concentrations of TNP-BSA and assayed for IgM-secreting cells by ESA at day 5 of culture.

Fig. 4. Direct activation of PKC results in the same inhibition of LPS-induced differentiation to Ig secretion in BM and splenic B cells. Spleen (●) and BM (○) cells from C57BL/6 adult mice were cultured with LPS in the absence or presence of different concentrations of PMA for 5 days, at which time the number of IgM-secreting cells was determined by Spot-ELISA. The 50% inhibition is indicated by the dotted line.

Level of sensitivity to anti-µ inhibition is independent of B cell life span

In order to assess the physiological characteristics of B cell populations that are associated with their level of sensitivity to sIgM ligation, we tested whether cellular life span would correlate with the threshold of anti-µ-induced suppression. Peritoneal B cells have been characterized as long-lived cells, whereas B splenocytes are mostly composed of the short-lived populations (40). However, injection of the anti-mitotic drug HU results in a drastic depletion of all dividing cells and leads to elimination of splenic short-lived B lymphocytes and therefore in enrichment for long-lived populations (41). As can be seen in Fig. 5, splenic B cells isolated from HU-treated or untreated animals display the same sensitivity as peritoneal cells from the untreated animals. These results indicate therefore that life span does not correlate with sensitivity to sIgM ligation.

Immature B cells generated in vitro from pre-B cells are as resistant to sIg cross-linking as are mature peripheral B cells

Differential sensitivity of B cell populations is currently linked with the respective maturation stage (42–45). Therefore, in order to determine the sensitivity of a pure immature B cell population, pre-B cells from BM were expanded in vitro in the presence of the BM stromal cell line S17 and recombinant murine IL-7. When IL-7 is removed from the culture, the proliferating pre-B cells differentiate into immature IgM−IgD−B cells (Fig. 5A).

Surprisingly, such immature B cells generated in vitro were considerably more resistant to anti-IgM-mediated suppression than were B cells isolated from BM ex vivo. Actually, as can
be seen in Fig. 6(B), B cells generated in vitro were as resistant to anti-IgM treatment as were splenic B cells. Thus the correlation between immaturity of the B cell population and the sensitivity to anti-Ig-mediated suppression of LPS-driven Ig secretion breaks down.

Soluble factor(s) produced by total BM cells enhance sensitivity to sIgM ligation of in vitro generated B cells

The observation that immature B cells, generated in vitro from expanded pre-B cells on cloned stromal cells and IL-7, are considerably more resistant than BM B cells isolated ex vivo could suggest that these defined culture conditions lack putative factors that determine high levels of B cell sensitivity to slgM-mediated signals in the BM environment. In order to address directly this hypothesis, in vitro differentiated B cells were incubated with bulk BM culture supernatants for 2 days and their sensitivity to slgM ligation tested as before. Strikingly, B cells that have been exposed to factors produced by total BM cells acquire a sensitive phenotype that translates as a 1 log difference in the concentration of anti-µ necessary to achieve a 50% inhibition (Fig. 7). This result demonstrates that some BM soluble factor(s) can modulate B cell sensitivity to slgM ligation.

Discussion

This paper demonstrates major differences between BM and peripheral B cell populations in their sensitivity to surface IgM ligation (and cross-linking), as measured by the subsequent effects on the LPS-induced differentiation into high-rate Ig-secreting cells. When B cells isolated ex vivo were compared, B cells from adult BM and newborn spleen were found to be 25- to 100-fold more sensitive to slg cross-linking than mature peripheral B cells (isolated from spleen, peritoneal cavity or peripheral blood). These results confirm and extend previous studies (45), but are at variance with others (46) concluding on the inability of anti-Ig treatments to inhibit peritoneal B cells. Thus, although peritoneal B cells display the higher level of resistance among all the B cell populations tested here, they are nevertheless completely suppressed by 1 µg/ml of anti-µ mAb.

Anti-Ig reagents have been the preferred ligands in this type of experiment (15,16), but we show here that similar
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The threshold for Ig-mediated signaling in BM B cells and another that would make peripheral B cells less sensitive for signaling. The observation that in vitro generated immature B cells are considerably more resistant than ex vivo isolated BM B cells favors the former alternative. Furthermore, the activity of a sensitivity-inducing soluble factor(s) in BM cell cultures demonstrated here suggests its operation in vivo.

An argument for the hypothesis above is contributed by the dose–response curves for inhibition which, being of a markedly broad appearance, indicate cellular heterogeneity in the response to a homogeneous ligand. There is indeed no difference in broadness between anti-µ-treated normal cells and the clonal populations of B6-Sp6 transgenic B cells treated with TNP-BSA. While some degree of cellular heterogeneity within each population may also concern sIgM levels (3), these differences are not sufficient to explain the differential sensitivity of BM and spleen. Furthermore, the present results show that some 30% of the BM B cells display sensitivity levels that are comparable to those of the spleen cell population.

In short, we postulate a developmentally regulated and tissue-specific factor(s), such as a cytokine or a soluble component in a specific receptor/ligand interaction, that sets the level of B cell sensitivity to sIgM-mediated signaling. This mechanism would provide the immune system with a capability of functionally regulating specific B cells all the way from their production in BM to their terminal differentiation into Ig-secreting cells.

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Abbreviations

BM bone marrow
LPS lipopolysaccharide
PE phycoerythrin
PKC protein kinase C
PMA phorbol-12-myristate-13-acetate

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