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Functional and physical association of a cell surface phospholipid and interleukin-2 receptor p55(α) subunits

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Abstract

A phosphatidylcholine-like phospholipid expressed in the outer leaflet of the cell membrane shortly after mitogenic activation of T-cells is described, based on the binding of monoclonal antibody 90.60.3. Expression of the 90.60.3 phospholipid antigen in T-cells is activation-dependent. Once expressed, the 90.60.3 phospholipid is in direct physical association with the interleukin-2 (IL-2) binding domain of IL-2 receptor α subunits, but does not affect IL-2 binding. The association is specific, because the 90.60.3 phospholipid is not found in association with other domains of IL-2 receptor α subunits, or near IL-2 receptor β or γ subunits. Culturing cytokine-dependent cell lines in the presence of monoclonal antibody 90.60.3 potentiates IL-2-dependent cell survival and proliferation in a dose-dependent manner. In contrast, IL-4-dependent responses are not potentiated. Taken together, the data suggest that specific plasma membrane phospholipids expressed in the outer leaflet after T-cell activation associate with the IL-2 binding domain of IL-2 receptor α subunits (and perhaps other cytokine receptors), and may play a role in regulating receptor mobility or signal transduction.

Keywords: Cytokine; Receptor; Antibody; Lipid; Membrane

1. Introduction

Hormones and polypeptide growth factors affect proliferation, differentiation, and cell survival by binding to specific receptors. Ligand binding to the extracellular domain of a receptor causes conformational changes, receptor dimerization/oligomerization, and changes in lateral mobility (reviewed in [1]). These events are influenced in part by the lipid composition and the fluidity of the plasma membrane in which the receptors are embedded.

The IL-2 receptor (IL-2R) is composed of distinct ligand binding components and associated signaling components [2–5]. IL-2 is a polypeptide growth factor which regulates proliferation and differentiation of T-cells, B-cells, NK cells, and other cell types. Based on ligand binding, three distinct IL-2R have been described as low ($K_d \sim 10$ nM); intermediate ($K_d \sim 1$ nM); and high affinity ($K_d \sim 10$ pM) [6].

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Three distinct IL-2R cDNAs have been cloned [7] and termed IL-2Rα, IL-2Rβ and IL-2Rγ. The high affinity functional IL-2R consists at least of αβγ subunits associated non-covalently as heterotrimers (reviewed in [8]).

IL-2Rα subunits bind IL-2 with low affinity when expressed in the absence of other subunits [9]. No intrinsic signal transduction has been associated to IL-2Rα subunits. However, IL-2Rα affects the signal transduced by the IL-2Rβγ complex [10]. IL-2Rγ subunits alone do not bind IL-2 with detectable affinity [7]. However IL-2Rγ subunits mediate signal transduction when non-covalently associated with IL-2Rα and IL-2Rβ subunits. The IL-2Rγ subunit can also transduce signals for IL-4 and IL-7 by associating with their selective receptors [11–13]. IL-2Rβ subunits do not bind IL-2 significantly [7,14] and do not transduce signals in the absence of other subunits. However, IL-2Rβγ heterodimers (in the absence of IL-2Rα) bind IL-2 with intermediate affinity ($K_d \sim 1$ nM), and mediate signal transduction [15].

Signal transduction via IL-2R is complex (reviewed in [16–19]); and expression of the three IL-2R subunits does not necessarily correlate with IL-2 responsiveness [20]. Functional evidence exists for IL-2R accessory or adapter molecules, such as PLCγ and PI-3-kinase [21,22]; the *src* family of soluble kinases, and Shc, JAK, and STAT kinases [9,17,18,23,24]. However, these accessory signaling molecules may not be absolutely required [25]. Evidence exists for other putative IL-2R associated components that co-precipitate with or are physically linked with IL-2R subunits on the cell membrane [25–29]. However, these putative IL-2R-associated proteins have not been shown to play a role in signal transduction.

The composition of the plasma membrane has been reported to influence receptor mobility and ligand affinity. We therefore hypothesized that the lipid composition of the activated T-cell may affect the signals of IL-2R or other cytokines. We report a phosphatidylcholine-like phospholipid antigen (recognized by mAb 90.60.3) expressed on the outer leaflet of the plasma membrane in activated T-cells. The 90.60.3 phospholipid is in association with the IL-2-binding domain of IL-2Rα subunits without affecting IL-2 binding. In contrast, the 90.60.3 phospholipid is not physically associated with the IL-2Rβ or IL-2Rγ subunits or with other surface T-cell antigens. The 90.60.3 phospholipid is functionally linked to signal transduction induced by IL-2 because culture with mAb 90.60.3 increases the effect of suboptimal IL-2. IL-3-dependent signals are also enhanced by culture with mAb 90.60.3, but to a much lesser degree; and IL-4-dependent responses are not enhanced at all. This suggests that the 90.60.3 phospholipid is involved preferentially in the IL-2 signal cascade.

Taken together, the data suggest that the phospholipid-like molecule defined by mAb 90.60.3 may represent a membrane component which can associate physically with a specific subdomain of IL-2Rα subunits, and functionally with high affinity IL-2R. Its potential association with other receptors is not excluded.

2. Materials and methods

2.1. Hybridoma production

Female Lewis rats were immunized subcutaneously and intraperitoneally with live CTL-L mouse T-cells grown in logarithmic phase. Splenocytes were fused with SP2/0 myeloma cells [30]. Hybridoma culture supernatant screening was done against HT-2 cells by indirect FACScan analysis with FITC-labeled goat anti-rat-antibody (Sigma, St. Louis, MO). Cells from wells scored positive were subcloned by limiting dilution, expanded in non-selecting media, and tested in proliferation assays described below. A stable subclone which bound a cell surface antigen of CTL-L and HT-2 cells, and affected IL-2 dependent proliferation was identified as rat mAb 90.60.3.

2.2. Cells

Cells used for analyzing expression of 90.60.3 antigen, IL-2Rα, IL-2Rβ, and IL-2Rγ subunits are listed in Table 2A. Cytokine-dependent mouse cells were used for biological assays: IL-2-dependent (and IL-4 responsive) CTL-L and HT-2 cells; IL-4-dependent CT4S cells which were derived from CTL-L [31]; and IL-3-dependent BAF-3 cells. Culture media (RPMI 1640 supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 0.1 mM non-essential amino acids, penicillin, streptomycin, and 5–

10% heat-inactivated FCS) was supplemented with lymphokines as indicated (either 50 U/ml IL-2 (Boehringer Mannheim Biochemicals, Indianapolis, IN); 200 U/ml IL-4 (a gift of Dr. W. Paul, NIH); or 10% WEHI-3B conditioned media containing IL-3).

Normal T-cells were isolated from thymus, spleen, or lymph nodes of mice (CBA/Ca (H-2^k), C57BL/6 (H-2^b) and BALB/c (H-2^d) (NIH, Betheseda, MD). Cells were analyzed directly or after mitogenic stimulation with ConA; with anti-TCR Abs (anti-TCRβ mAb clone H57-597 [32], anti-CD3ε mAb clone 500A2 [33], and cross-linking with syngeneic T-depleted irradiated splenocytes); or with phorbol ester (PMA, 50 ng/ml) and Ca^{2+} ionophore (ionomycin, 1 μg/ml)).

2.3. Proliferation and survival assays

Five thousand cells/well were added to serial dilutions of recombinant cytokine in 96-well plates (Costar). The MTT assay modified as described [34] was used to quantitate proliferation and survival of cytokine-dependent cells (the MTT assay is a measure of both events). One unit/ml of IL-2 was arbitrarily defined as the concentration required to support the half-maximal growth of HT-2 cells over a 24–36 h incubation period.

2.4. Antibodies, fluorescent labels, and flow cytometry

Cultured or normal cells (5×10^5 cells/tube, >95% viable) were suspended in FACS buffer (PBS, 1% BSA, 0.1% Na azide) and immunostained. After further washing, cells were analyzed on a FACScan (Becton Dickinson, Mountain View, CA). Immunostaining for surface 90.60.3 antigen used either diluted 90.60.3 ascitic fluid, followed by 0.5 μg/tube of FITC-coupled goat $(Fab')_2$ anti-rat Ig (Pierce, Rockford, IL); or mAb 90.60.3-biotin followed by with 0.5 μg/tube of Red613-coupled avidin or FITC-coupled avidin (Gibco BRL). Biotinylation was performed with long arm biotin (Pierce) as per manufacturer's instructions. For two color staining (CD4 or CD8/90.60.3 or p55α), primary labeling with mAb 90.60.3 (90.60.3-biotin plus Avidin-FITC) or anti-IL-2Rα mAb 7D4 (7D4-FITC) was followed with 50 μg/tube rat Ig (Sigma, St. Louis, MO) for blocking; together with anti-CD4 PE (clone YTS, Gibco BRL, Gaithersburg, MD) or anti-CD8 Red613 (clone 53-6.7, Gibco BRL). mAb 7D4-FITC was a gift of Dr. T.R. Malek (University of Miami). Other biotinylated antibodies used (e.g. to assess the purity of T-cells) included anti-αβ TCR (clone H57-597, PharMingen); anti-CD3-ε (clone 145-2C11, PharMingen); the anti-IL-2Rα mAbs 7D4 (PharMingen), AMT13, and 5A2 [35]; the anti-class I MHC mAb M1.42; anti-IL-2Rβ mAb TMβ1 and anti-IL-2Rγ mAb TUGm2 [20].

2.5. Blocking/competition assays

HT-2 or CTL-L cell suspensions were first bound with saturating concentrations of anti-IL-2Rα mAbs 7D4, 3C7, AMT-13, or 5A2; anti-IL-2Rβ mAb TMβ1, anti-IL-2Rγ mAb TUGm2; anti-class I MHC mAb M1.42; with 50 μg/ml rat Ig; with IL-2 (1000 U/ml); or with mAb 90.60.3. After washing, the binding of the same ligands (directly labeled or biotinylated) was tested by FACScan analysis as described above. Competition of IL-2 was assessed using ^{125}I[IL-2] in Scatchard plot analysis.

2.6. ELISA

Test compounds for 90.60.3 binding assays were prepared at a concentration of 0.25 mg/ml and stored at −20°C. Soluble in methanol: β-oleoyl-γ-palmitoyl-phosphatidylcholine (POPC), phosphatidylethanolamine (PEA), diheptanoyl-L-α-phosphatidylcholine (DHPC), didecanoyl-L-α-phosphatidylcholine (DDPC), and dilauroyl-L-α-phosphatidylcholine (DLPC) (Sigma, St. Louis, MO), sphingomyelin (SM) and sphingosine (Matreya, Pleasant Gap, PA); dioctanoyl-L-α-phosphatidylcholine (DOPC), dimyristoyl-L-α-phosphatidylcholine (DMPC), dipalmitoyl-L-α-phosphatidylcholine (DPPC), and distearoyl-L-α-phosphatidylcholine (DSPC) (Avanti Polar Lipids, Alabaster, AL). Soluble in water: choline chloride (Sigma, St. Louis, MO) and dibutyroyl-L-α-phosphatidylcholine (DBPC) (Avanti Polar Lipids, Alabaster, AL). Soluble in dimethylsulfoxide (DMSO): C2-ceramide (Sigma, St. Louis, MO; a gift from Dr. B. Collier (McGill University)). Solvent selection and solubility was strictly dependent on the length of the lipid tail.

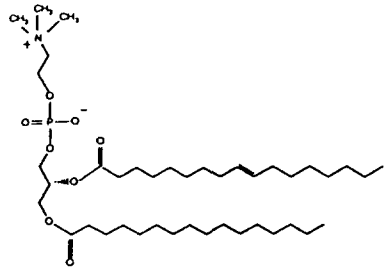
Table 1
Identification of the minimal antigenic epitope of mAb 90.60.3

(A)	Test antigen	C-Lipid chain(s)	% Binding ± S.E.M.
1	POPC	16, 18[a]	100.0 ± 0.0
2	Phosphatidylethanolamine	16, 18[a]	17.6 ± 2.2
3	Choline Chloride	no lipid	−5.7 ± 1.9
4	Sphingomyelin	18[a]	7.6 ± 0.8
5	Sphingosine	16[a]	−5.9 ± 3.3
6	C2-Ceramide	16[a]	0.4 ± 0.0
7	DBPC	4, 4	2.4 ± 1.2
8	DHPC	6, 6	−0.4 ± 0.8
9	DOPC	8, 8	1.4 ± 0.7
10	DDPC	10, 10	2.8 ± 0.7
11	DLPC	12, 12	16.5 ± 1.6
12	DMPC	14, 14	13.3 ± 1.1
13	DPPC	16, 16	8.1 ± 0.8
14	DSPC	18, 18	14.2 ± 1.5

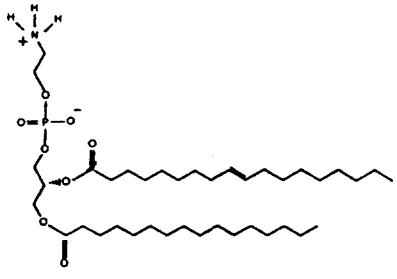
(B)	Test antigen	% Binding ± S.E.M.	
		+PEA lipids	+SM lipids
1	Choline	32.9 ± 1.6	not tested
2	Sphingomyelin	92.7 ± 7.4	not tested
3	DBPC	26.8 ± 3.9	9.4 ± 3.1
4	DHPC	19.7 ± 0.5	1.2 ± 2.0
5	DOPC	27.5 ± 0.5	0.4 ± 3.1
6	DDPC	38.0 ± 1.8	1.8 ± 2.3
7	DLPC	57.5 ± 3.3	27.7 ± 6.4
8	DMPC	60.6 ± 1.5	49.4 ± 9.6
9	DPPC	26.5 ± 0.4	46.9 ± 7.8
10	DSPC	8.6 ± 1.1	30.1 ± 0.9

ELISAs were done with mAb 90.60.3 or control rat Ig. For detailed lipid structures see Fig. 1. (A) Single test lipid. The indicated lipids were immobilized onto 96-well plates. The length of each carbon lipid chain(s) is shown. OD values from ELISAs are expressed as percent binding standardized to the binding of POPC (100%). Data from 4–20 independent experiments; $n = 4$–8 wells/experiment; ± S.E.M. (B) Assembly of the epitope by combination of antigens. The indicated Ags were combined either with phosphatidylethanolamine (+PEA lipid), or with sphingomyelin (+SM lipid). OD values from ELISAs were standardized to the binding of POPC in the same plates (100%). Data from 3–6 independent experiments; $n = 8$ wells/experiment; ± S.E.M.
[a]C = C double bond in the lipid chain.

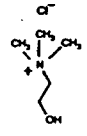
The above compounds were further diluted in water (usually 1:25). Each compound (~0.66 nmol; 50 μl/well final volume) was immobilized by drying onto polystyrene 96-well plates (VWR-Canlab, Ville Mont-Royal, Que.). Plates were blocked for 2 h at room temperature (blocking buffer (BB): PBS pH 7.4, 1% BSA). mAb 90.60.3 or control rat Ig (primary Abs) were diluted in BB to 2–4 μg/ml. mAb 90.60.3 (test) and rat Ig (background control) were added to each well (~100 ng/well) containing test compounds or no compounds (background control). After 1 h at room temperature, plates were washed three times with BB, followed by 1 h incubation with peroxidase-conjugated affinity-purified goat anti-rat Ig (H+L) secondary antibody (~25 ng/well) (Sigma, St. Louis, MO). After washing three times with BB and three times with PBS, the peroxidase substrate ABTS (2,2′-azinobis(3-ethylbenzthiazoline-sulfonic acid)) (Sigma, St. Louis, MO) was added (freshly prepared (0.4 mg/ml) in 0.062 M citric acid, 0.076 M Na_2HPO_4, 5 μl/ml 30% hydrogen peroxide). The enzymatic reaction was stopped by adding an equal volume of 2% oxalic acid after 5–15 min. Absorbance was measured at 414 nm in a microplate reader (Bio-Rad Laboratories, Mississauga, Ont.). Optical density (OD) values minus background control



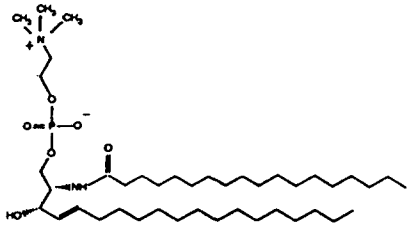
L - α - phosphatidylcholine, β - oleoyl - γ -palmitoyl (C18:1, [cis] - 9 / C16:0)



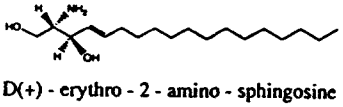
L - α - phosphatidylethanolamine, β - oleoyl - γ - palmitoyl (C18:1, [cis] - 9 / C16:0)



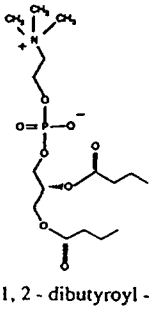
choline chloride



sphingomyelin, stearoyl



D(+) - erythro - 2 - amino - sphingosine



1, 2 - dibutyroyl - sn - glycero - 3 - phosphorylcholine

Fig. 1. Structures of the phospholipids bound by mAb 90.60.3.

were calculated. The OD of primary rat Ig or mAb 90.60.3 without test antigen in the well were near zero. All values obtained with control rat Ig with test antigen in the well were near zero.

3. Results

3.1. Biochemical characterization of the 90.60.3 antigen reveals a phospholipid

Biochemical tests failed to consistently detect a polypeptide recognized by mAb 90.60.3 (data not shown). Thus, we hypothesized that the 90.60.3 antigen might be a cell surface phospholipid moiety (Fig. 1). Several phospholipids were tested in ELISA for specific binding to mAb 90.60.3 (Table 1A). Combinations of two phospholipids were also tested by mixing them prior to ELISA (Table 1B).

β-Oleoyl-γ-palmitoyl-phosphatidylcholine (POPC) was the strongest binding phospholipid. POPC binding by mAb 90.60.3 consistently produced values OD > 0.500; while POPC binding by control rat Ig produced values OD < 0.040. Thus, the absorbance of POPC was standardized to 100% to allow direct comparisons of all compounds (Table 1A, row 1).

β-Oleoyl-γ-palmitoyl-phosphatidylethanolamine (PEA) is related to POPC, except that ethanolamine ($^{+}NH_3$) substitutes the choline ($^{+}N(CH_3)_3$) head of POPC (see Fig. 1). PEA had low binding to mAb 90.60.3 (Table 1A, row 2), suggesting that the choline head of POPC is an important moiety. Choline had no specific binding to mAb 90.60.3 (Table 1A, row 3), suggesting that the rest of POPC is also important.

Sphingomyelin (SM) is a compound related to POPC, except that an hydroxyl group, a peptide bond, and a C=C double bond substitute two ester bonds and a ketone in POPC (see Fig. 1). SM had low but significant specific binding to mAb 90.60.3 (Table 1A, row 4). This suggests either that the esters or the ketone in POPC may be important, or that the substitutants in SM interfere with mAb 90.60.3 binding. Sphingosine and C2-ceramide had no specific binding to mAb 90.60.3 (Table 1A, rows 5 and 6), as expected because they have no choline head and a single lipid chain.

To test the possible role of the length of the lipid

chain, we tested a variety of phospholipids (DBPC, DHPC, DOPC, DDPC, DLPC, DMPC, DPPC, DSPC). These lipids contain chains ranging in length from C4 to C18 (Table 1A, rows 7–14); and mimic POPC in every respect except that the lipid chains lack a C=C double bond (see Fig. 1). DBPC, DHPC, DOPC, and DDPC (C4–C10 lipid chain lengths) have no specific binding to mAb 90.60.3 (Table 1A, rows 7–10), while DLPC, DMPC, DPPC, and DSPC (C12–C18 lipid chain lengths) have low, but significant, specific binding to mAb 90.60.3 (Table 1A, rows 11–14). This suggests that the required lipid chain length is at least 12 carbons. The presence of the C=C double bond is also important for binding mAb 90.60.3

Taken together, these data indicate that the minimal antigen is comprised of three moieties: (1) choline; (2) a diester linkage; and (3) two lipid chains of 12–18 carbons in length containing at least one C=C double bond. The C=C double bond may contribute to changes in geometry, flexibility, or a propensity to rotate or flip the lipid chain.

3.2. *The 90.60.3 phospholipid antigen can be assembled in vitro*

Next, we hypothesized that the three moieties that comprise the 90.60.3 antigen could be reconstituted in vitro by mixing the appropriate phospholipids. It was expected that each of the moieties could be oriented in a manner suitable for mAB 90.60.3 binding; or that induced fit by mAb 90.60.3 could force assembly.

Different compounds were mixed and tested by ELISA for binding to mAb 90.60.3 (Table 1B). A combination of choline+PEA resulted in ~33% of mAb 90.60.3 binding compared to POPC (Table 1B, row 1). This would be expected if addition of choline compensated for the chemical difference between PEA and POPC. Assembly is evident because PEA alone had low binding and choline alone had no specific binding to mAb 90.60.3 (Table 1A, rows 2 and 3). A mixture of SM+PEA restores ~93% of mAb 90.60.3 binding (Table 1B, row 2). In contrast, mAb 90.60.3 binding to each SM or PEA was very low (8 and 18%, respectively, Table 1A, rows 2 and 4).

The series of phospholipids DBPC, DHPC, DOPC, DDPC, DLPC, DMPC, DPPC, and DSPC (C4–C18 in length) were also tested in combination with PEA or SM (Table 1B, rows 3–10). The most efficient combination is that of DMPC+PEA or DMPC+SM (Table 1B, row 8). DLPC+PEA also binds efficiently (Table 1B, row 7), as does DPPC+SM (Table 1B, row 9).

Therefore, it seems that mAb 90.60.3 recognizes a phospholipid-like molecule similar to POPC; or a combination of phospholipid mixtures expressed in the plasma membrane.

3.3. *Characterization of the biological activity of mAb 90.60.3*

The screening used to isolate hybridoma 90.60.3 used conditioned media containing mAb 90.60.3. The conditioned media synergized with suboptimal concentrations of IL-2 in increasing IL-2-dependent HT-2 and CTL-L proliferation as assessed by ^{3}H-thymidine incorporation assays and MTT colorimetric assays (data not shown). These assays were repeated using purified mAb 90.60.3 to confirm that they were due to the mAb (Fig. 2).

Serial dilutions of mAb 90.60.3 with constant 0.1 U/ml IL-2 were tested in MTT assays (Fig. 2A). An estimated 1 nM (~1 μg/ml) mAb 90.60.3 afforded optimal synergy with 0.1 U/ml IL-2. Control cultures with no antibody added (data not shown), or with equimolar concentrations of rat Ig (Fig. 2A) did not synergize with IL-2, and showed biological responses typical of simple IL-2 dilutions.

A constant concentration of 1 nM (1 μg/ml) purified mAb 90.60.3 was tested versus serial dilutions of IL-2 (Fig. 2B). Synergy was observed at suboptimal concentrations of IL-2 (ranging from 0.005–0.15 U/ml), and resulted in increased HT-2 cell survival and proliferation to ~50% of the maximum achieved with optimal IL-2 concentrations. The maximal response achieved at optimal IL-2 concentrations was not affected by mAb 90.60.3. Control assays with purified rat Ig added to cultures (Fig. 2B) did not synergize with IL-2; and yield profiles typical of simple IL-2 dilutions.

Similar tests were performed with the IL-3-dependent BAF-3 cell line. Some synergy between mAb 90.60.3 and low concentrations of IL-3 (ranging from 0.005 to 0.15 U/ml) afforded increased cell sur-

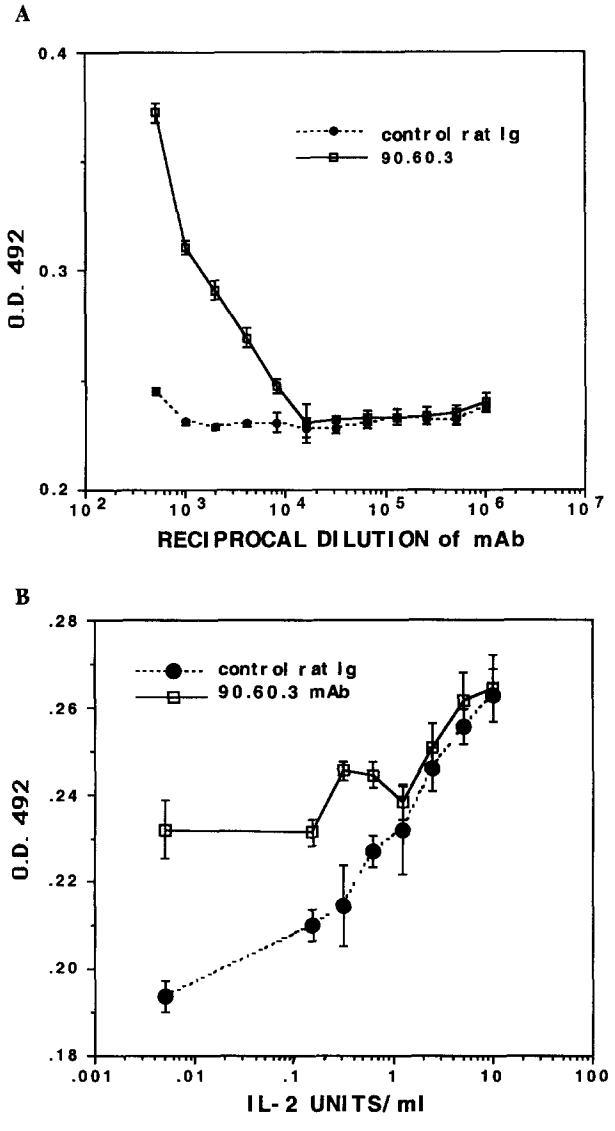


Fig. 2. Biological synergy of mAb 90.60.3 and IL-2. Cells were cultured with the indicated ligands. After 24–36 h proliferation was assessed by the MTT method. Similar results were obtained with ^{3}H-thymidine incorporation (not shown). (A) Response of HT-2 cells to dilutions of 90.60.3 mAb or rat Ig and constant 0.1 U/ml IL-2. (B) Response of HT-2 cells to increasing doses of IL-2 and constant 1 μg/ml 90.60.3 mAb or rat Ig.

vival and proliferation to ~20% of that achieved with optimal IL-3 concentrations (Fig. 3). Synergy was evident at suboptimal concentrations of IL-3, but the maximal response afforded by optimal IL-3 concentrations was not increased. Addition of control rat Ig had no effect in these assays.

In contrast, mAb 90.60.3 did not potentiate the survival and proliferation of IL-4-dependent CT4S cells in response to IL-4. The dose response of CT4S cells to IL-4 was identical whether rat Ig, mAb 90.60.3 or no rat Ig was added to the cultures (data not shown). Similarly, the survival and proliferation of CTL-L cells (IL-2 dependent, but are IL-4 responsive) were not potentiated by mAb 90.60.3 at any dose of IL-4 (data not shown).

3.4. IL-2R subunits and the 90.60.3 phospholipid are independently expressed

Binding of mAb 90.60.3 to the outer leaflet of the plasma membrane was surveyed in a variety of mouse and human cell lines by flow cytometry. Moreover, given the synergy of mAb 90.60.3 with IL-2 we explored whether mAb 90.60.3 recognized any of the three IL-2R subunits (Table 2A).

Flow cytometric analysis of intact cells revealed high levels of mAb 90.60.3 binding to the surface of the R1.1 thymoma cell line. R1.1 cells do not express IL-2Rα protein or mRNA, do not react with several anti-IL-2Rα mAbs (e.g. mAb 7D4 (Table 2A), 3C7, AMT-13, or 5A2 (data not shown)), and do not bind ^{125}I[IL-2] (data not shown). Thus, R1.1 cells do not express IL-2Rα subunits, but do express the 90.60.3 phospholipid.

EL4 cells do not express surface 90.60.3 phospholipid or IL-2Rα subunits, but do express IL-2Rβγ. Expression of IL-2Rα subunits in EL4 cells (upon stable transfection of mouse IL-2Rα cDNA) [28] permits binding of anti-IL-2Rα mAbs (mAbs 7D4, 3C7, AMT-13, and 5A2 (data not shown)). In contrast,

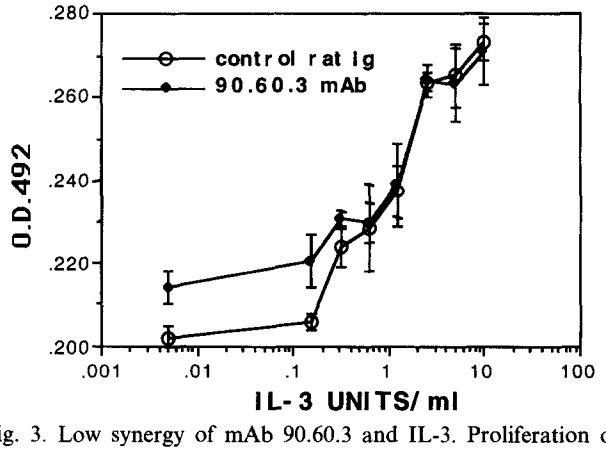


Fig. 3. Low synergy of mAb 90.60.3 and IL-3. Proliferation of BAF cells to increasing doses of IL-3 (WEHI cell conditioned supernatant) in the presence of mAb 90.60.3 or rat Ig control.

Table 2
Phenotypic analysis of IL-2R subunits and 90.60.3 Ag

(A) Cell lines	90.60.3 mAb$^+$	anti-IL-2Rα	anti-IL-2Rβ	anti-IL-2Rγ
HT-2 (IL-2 dependent mouse T-cell)	high	high	low	low
CTL-L (IL-2 dependent mouse T-cell)	high	high	low	low
CT4.S (IL-4 dependent mouse T-cell)	intermediate	low	not tested	low
BAF-3 (IL-3 dependent mouse pre-B-cell)	intermediate	low	negative	low
R1.1 (mouse thymoma)	high	negative	low	not tested
EL4 (mouse thymoma)	negative	negative	low	low
EL4J-3.4 (IL-2Rα transfected EL4)	negative	high	low	low
TS1 (IL-9 dependent mouse T-cell)	negative	negative	negative	high

(B) Mitogen	90.60.3$^+$		7D4$^+$ anti-IL-2Rα	
	CD4$^+$	CD8$^+$	CD4$^+$	CD8$^+$
ConA				
0 h	7	6.2	4	3.3
9 h	3	4	28	21
18 h	16	25	22	27
27 h	35	29	46	42
36 h	34	36	47	44
αTCR mAb				
0 h	8.3	5.2	4.8	1.69
24 h	37.6	33.3	76	90
36 h	60.4	38.1	69.3	75.8
PMA+ionomycin				
0 h	6.24	3.44	6.97	2.6
24 h	9.2	16	68.5	73
36 h	33.9	53.3	97.6	96.5

(A) Cell lines. Cell lines were tested by indirect immunofluorescence analysis in a FACScan with rat mAbs 90.60.3, 7D4 (anti-IL-2Rα), TMβ1 (anti-IL-2Rβ), or TUGm2 (anti-IL-2Rγ). All cloned cell lines exhibit uniform staining. Relative fluorescence intensity is indicated as high, intermediate, low, or negative. See Fig. 4 for experimental details. (B) Cultured splenic T-cells. Freshly isolated T-cells (purity >90%) were analyzed by FACScan before or after mitogenic stimulation. Two color staining was performed with mAbs 90.60.3 or 7D4; and directly labeled anti-CD4 or anti-CD8. Data shown is from the CBA/Ca strain, and was reproducible with cells isolated from Balb/c and C57BL6 strains (data not shown). Data shown are percent of cells stained.

mAb 90.60.3 does not bind the IL-2Rα transfected EL4 cells. Thus, mAb 90.60.3 does not bind IL-2Rα subunits, and expression of IL-2Rα does not inevitably result in co-expression of the 90.60.3 phospholipid (Table 2A).

Likewise, expression of IL-2Rβ and IL-2Rγ subunits do not result in expression of the 90.60.3 phospholipid (Table 2A). mAb 90.60.3 binds to BAF-3 cells which are negative for IL-2Rβ receptors. COS fibroblasts transfected with mouse IL-2Rα or IL-2Rβ cDNAs showed no reactivity with mAb 90.60.3, while they did bind the appropriate anti-IL-2Rα and anti-IL-2Rβ Abs (data not shown). In addition, mAb 90.60.3 did not bind the IL-9-dependent T-cell line TS1 (Table 2A) which expresses high levels of IL-2Rγ subunits [10].

These data strongly indicate that the antigen recognized by mAb 90.60.3 is not the IL-2Rα, IL-2Rβ, or IL-2Rγ subunits. Furthermore, mAb 90.60.3 did not bind some lymphoid cell lines including 2B4 T-cell hybridoma, SP2/0 B-cell myeloma, T-ALL human leukemia, Jurkat human T-cells, THP1 promonocytic cells, and CD34$^+$ cells TF1 and K61a cells; or a variety of non-lymphoid cells of fibroblastoid, epithelial, and neuronal origin (data not shown).

3.5. *Expression of the 90.60.3 phospholipid in the outer leaflet of normal T-cells*

We next analyzed 90.60.3 expression in freshly isolated T-cells from BALB/c, C57BL/6 and CBA/Ca mice (Table 2B). Surface expression was detected

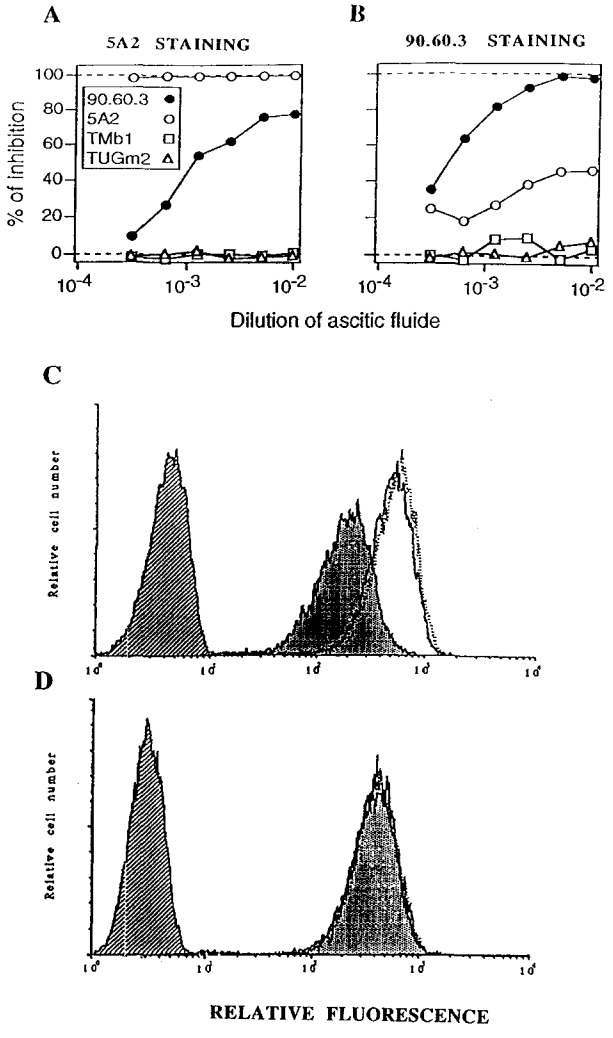


Fig. 4. Proximity of 90.60.3 phospholipid and the IL-2 binding domain of IL-2Rα. Various mAbs were tested for cross-inhibition of cell surface binding. Saturating concentrations of each mAb were pre-determined (data not shown) and used. (A) Inhibition of anti-IL-2Rα mAb 5A2 binding by mAb 90.60.3. Anti-IL-2Rβ and IL-2Rγ mAbs had no effect in mAb 5A2 binding. (B) Inhibition of mAb 90.60.3 by anti-IL-2Rα mAb 5A2. Anti-IL-2Rβ and IL-2Rγ mAbs had no effect in mAb 90.60.3 binding. (C) mAb 90.60.3 inhibited the binding of anti-IL-2Rα mAb AMT-13 by more than 50% (solid line, filled), while anti-IL-2Rα mAbs 7D4 or 2E4 (dashed and solid lines, empty) had no effect in mAb AMT-13 binding. (D) Saturating amounts of mAb M1.42.6 to class I MHC, 50 μg rat IgG, or 20% fetal calf serum used as controls, none of which affected subsequent binding of AMT-13 mAb by >5%. For a complete summary see Table 3.

on a small percentage (5–10%) of T-cells freshly isolated from thymus, from spleen, or from lymph nodes. Surface expression of the 90.60.3 phospholipid rose to 30–60% of all T-cells after 36 h of in vitro mitogenic stimulation with either Concanavalin A, anti-CD3 (mAb 500A2), or PMA and ionomycin. Both the $CD4^+$ and $CD8^+$ subsets were induced to express 90.60.3 phospholipid upon activation. Furthermore, the 90.60.3 phospholipid was also detected on a subpopulation of $CD4^-$ $CD8^-$ cells from spleen and lymph nodes 24–36 h after activation with PMA and ionomycin (data not shown). We have not tried to identify these cells, which are likely to include B-cells.

3.6. *Association of 90.60.3 phospholipids and IL-2Rα subunits*

A potential association of IL-2R subunits with the 90.60.3 phospholipid was studied to evaluate whether the functional synergy of mAb 90.60.3 and IL-2 was germane to IL-2R signaling.

Thus, we studied whether mAb 90.60.3, mAb 5A2 (anti-IL-2Rα), mAb TMβ1 (anti-IL-2Rβ), and TUGm2 (anti-IL-2Rγ) would reciprocally affect each other's binding to the cell surface (Fig. 4A,B). This study was done by FACScan analysis, measuring heterologous blocking of one antibody upon the binding of a different antibody which was directly fluorescinated or biotinylated. Control 'self competition' gauged homologous blocking.

The binding of labeled mAb 5A2 was progressively reduced by increasing concentrations of mAb 90.60.3 (Fig. 4A). In control experiments, the binding of labeled mAb 5A2 was efficiently cold-competed by unlabeled 5A2; but mAbs TMβ1 and TUGm2 had no effect. In the reciprocal assay, binding of labeled mAb 90.60.3 was reduced by increasing concentrations of mAb 5A2 (Fig. 4B). In control assays, the binding of labeled mAb 90.60.3 was efficiently cold-competed by unlabeled mAb 90.60.3; but mAbs TMβ1 and TUGm2 had no effect.

These data suggest an association of the 90.60.3 phospholipid with IL-2Rα subunits; and suggests the absence of an association of the 90.60.3 phospholipid with IL-2Rβ and IL-2Rγ subunits.

3.7. *The 90.60.3 phospholipid is linked to the IL-2 binding domain of IL-2Rα*

Given the putative link of the 90.60.3 phospholipid

and IL-2Rα, we studied this association in more detail. For that purpose, several antibodies that recognize IL-2Rα subunits are available. Previous epitope mapping of anti-IL-2Rα antibodies have defined three clusters [35,36]. In cluster I, mAbs AMT-13, 5A2, and 3C7 bind to epitopes overlapping with the IL-2 binding site on IL-2Rα. Their binding is reciprocally inhibited by binding of IL-2, or by any of these mAbs. In cluster II, epitopes recognized by mAbs 7D4 and 2E4 are distinct from the binding site of IL-2 on IL-2Rα. In cluster III, mAb PC61 binding reduces IL-2 binding to IL-2Rα by inducing conformational changes rather than by steric hindrance. These published data were reproduced as internal controls in assays summarized in Table 3.

FACScan assays tested whether mAb 90.60.3 or mAbs directed to each of the IL-2Rα clusters reciprocally inhibit each other (Fig. 4C,D). Binding of mAb 90.60.3 led to a greater than 50% reduction of binding of mAb AMT-13 to HT-2 cells (Fig. 4C). The specificity of inhibition is shown by the inability of mAb 90.60.3 to affect binding of mAbs 7D4 or 2E4 (Fig. 4C), nor did 2E4 or 7D4 mAbs inhibit binding of mAb 90.60.3 (data not shown, see Table 3). Further controls included saturating amounts of mAb M1.42.6 against MHC class I, 50 μg/ml rat Ig, or 20% fetal calf serum, none of which affected subsequent binding of any antibody by $>5\%$ (Fig. 4D).

A summary of results are presented in Table 3, and demonstrate that binding of mAb 90.60.3 leads to a significant inhibition of subsequent binding of all anti-IL-2Rα mAbs which interact with the IL-2 binding domain of this subunit (cluster 1). In the reciprocal experiment, binding of mAbs against cluster 1 significantly inhibited binding of mAb 90.60.3. In control assays, none of the mAbs against different clusters of IL-2Rα or other mAbs reciprocally affected mAb 90.60.3 binding.

The effect of prior binding of IL-2 on the binding of mAb 90.60.3 or antibodies to IL-2R was also tested by indirect immunofluorescence. IL-2 did not reduce binding of mAb 90.60.3 (Table 3). Similarly, previous binding of 90.60.3 mAb had no effect on binding of ^{125}I[IL-2] to high or low affinity receptors on HT-2 cells as measured in Scatchard plot analysis (data not shown). In contrast, in control experiments IL-2 blocked binding of all antibodies of cluster I (AMT-13, 5A2, 3C7), but not of those of cluster II (7D4, 2E4) (Table 3) as previously reported. We interpret these results to mean that the 90.60.3 phospholipid is in contact with IL-2Rα in a specific and invariant orientation towards the face of IL-2Rα which binds IL-2, and away from the domain(s) relevant for binding other anti-IL-2R mAbs.

4. Discussion

We have characterized biochemically and functionally an activation marker related to POPC phospholipids, present on the outer leaflet of the cell membrane. In cell cultures, binding of the phospholipid with 90.60.3 mAb results in enhanced signal transduction via IL-2R.

Table 3
Specific inhibition of anti-IL-2Rα cluster 1 mAb binding by mAb 90.60.3

Cold competitor	Labeled ligand						
	FITC-7D4	Biotin-3C7	Biotin-AMT13	Biotin-5A2	Biotin-M1.42	^{125}I[IL-2]	Biotin-90.60
7D4 (anti-IL-2Rα)	++++	–	–	NT	–	–	–
3C7 (anti-IL-2Rα)	–	++++	+++	NT	–	+++	+++
AMT13 (anti-IL-2Rα)	–	+++	++++	+++	–	++	+++
5A2 (anti-IL-2Rα)	NT	NT	+++	++++	–	NT	+++
M1.42 (anti-MHC)	–	–	–	–	++++	–	–
IL-2	–	+++	+++	NT	–	++++	–
90.60.3	–	+++	+++	++	–	–	++++

Summary of the results presented in Fig. 3. Blocking/inhibition studies were performed as described in Section 2. All ligands were biotinylated, with the exception of 7D4 (which was directly fluorescinated), and radiolabeled ^{125}I[IL-2]. After washing, the biotinylated antibodies were detected with avidin-FITC. ^{125}I[IL-2] cpm were counted. Data represents inhibition of maximal binding in arbitrary units: ++++, $>50\%$ inhibition; +++, 35–50% inhibition; ++, 15–35% inhibition; –, $<5\%$ inhibition; NT, not tested.

4.1. The 90.60.3 antigen

The minimal 90.60.3 antigen is a POPC-like phospholipid comprised of: (1) choline; (2) a diester linkage; and (3) two lipid chains of 12–18 carbons in length containing at least one C=C double bond. Choline and the diester linkage could be exposed on the outer leaflet of the plasma membrane as components of several molecules. However, it is unclear how lipid chains with C=C double bonds could be exposed to the hydrophilic environment and made available for binding by mAb 90.60.3. It is also intriguing that while all cells have POPC on the plasma membrane, mostly T-lymphoid cells are bound by mAb 90.60.3.

One possibility that may account for mAb 90.60.3 binding is that some tails containing a C=C double bond bend at angles of ~130° within the lipid bilayer. Bending alters the chemistry and the state of the lipid–non-lipid monolayer interface [37–39]. If the 90.60.3 antigen is POPC, perhaps activated T-cells have the ability to flip lipids or to expose the lipid C=C double bonds more efficiently than other cells. However, it is also possible that the 90.60.3 antigen is distinct from POPC and this issue awaits resolution. It is noteworthy that antibodies against lipids are rare, but there are mAbs that discriminate between POPC and PEA [40,41].

4.2. Expression of the 90.60.3 phospholipid

The 90.60.3 phospholipid is a marker expressed on the cell surface after T-cell activation. It is induced on mouse T-cells as early as 18 h post-activation in vitro and maximally by 36 h. The increase in 90.60.3 phospholipid expression is distinct, but parallel to that of IL-2Rα subunits and transferrin receptors (data not shown). Interestingly, the kinetics of 90.60.3 phospholipid expression also parallels IL-2 responsiveness by activated T-cells.

The 90.60.3 marker is not the IL-2Rαβγ polypeptides. Since IL-2 does not affect the binding of mAb 90.60.3 and mAb 90.60.3 does not affect the affinity of IL-2, it is unlikely that the 90.60.3 antigen represents a new IL-2 binding subunit. Furthermore, cells expressing 90.60.3 phospholipid do not bind IL-2 in the absence of IL-2R subunits, and not all IL-2 responsive cells express 90.60.3 phospholipids.

4.3. Physical association of 90.60.3 and IL-2Rα subunits

Cross-inhibition of binding between antibodies to distinct, but membrane-associated structures is a common phenomenon, and the degree of this inhibition has been used to study the spatial relationships between different subunits of multiple subunit receptors [42–44]. Based on cross-inhibition studies, we have observed that the 90.60.3 phospholipid can associate with a specific domain of IL-2Rα which is involved in IL-2 binding.

We have assumed that the ability of 90.60.3 mAb to block some anti-IL-2Rα mAbs is based on steric hindrance, a possibility supported by the fact that IL-2 and mAb 90.60.3 do not block each other's binding. However, we have been unable to consistently co-immunoprecipitate 90.60.3 phospholipids and IL-2R subunits (data not shown). This may be due to technical difficulties or because IL-2R subunit–phospholipid association is not stable to detergent lysis.

4.4. Functional association of 90.60.3 and IL-2R

Likely the phospholipid recognized by mAb 90.60.3 is not involved in IL-2 binding but in IL-2 function. The 90.60.3 antigen seems to associate with cell surface IL-2Rα subunits and synergizes functionally with low levels of IL-2 to increase IL-2-dependent survival and proliferation.

Cell culture with mAb 90.60.3 does not allow for long-term responses (>3 days) in the complete absence of IL-2. Thus, the mAb does not act as a cytokine or growth factor. Rather, the mAb potentiates the biological activity of IL-2. While mAb 90.60.3 did not affect the affinity of IL-2R for IL-2, we have not yet compared IL-2 affinities in 90.60.3 positive versus 90.60.3 negative cells. It would be interesting to do so because there is a precedent wherein the affinity of epidermal growth factor (EGF) was affected by lipid composition. The introduction of cholesterol or phosphatidylinositol lipids (but not phosphatidylserine or phosphatidic acid) into DOPC vesicles resulted in a 6–18-fold increase in the affinity of the EGF receptor for EGF [54].

It is also possible that the short-term responses by HT-2 and CTL-L cells in the absence of IL-2 simply

reflects a carry over of IL-2 which remains bound to the cells even after washing. Thus, an alternative possibility is that the apoptotic mechanism induced on HT-2 and CTL-L cells after IL-2 withdrawal may be delayed by mAb 90.60.3, but IL-2 is absolutely required for long-term growth. Indeed, lipid peroxidation is a marker of apoptosis, and perhaps mAb 90.60.3 binds to or sequesters peroxidated phospholipids.

4.5. 90.60.3 Phospholipid cell surface density

Taken together, our observations suggest that mAb 90.60.3 synergizes with IL-2 to affords trophic signals. Synergy of IL-2 and mAb 90.60.3 in culture required high surface density of 90.60.3 phospholipids, which is directly related to logarithmic cell growth. Nutrient depleted or cell cycle arrested cells express >500-fold reduced 90.60.3 density (data not shown). Consistent, mAb 90.60.3+IL-2 synergy did not occur when surface expression of 90.60.3 phospholipids were low after partial starvation of the cells (data not shown). Perhaps optimal growth conditions are required for high expression of the 90.60.3 phospholipid and consequent function.

The ability of mAb 90.60.3 to potentiate IL-2 but not IL-4 remains to be studied further. It is intriguing that IL-4 responses are not potentiated, even in cells such as HT-2 or CTL-L which possess the machinery required to respond. Experiments using the IL-4 dependent (and IL-2 responsive) CT4-S cell line, derived from CTL-L cells [31], demonstrated that the pathways for signaling in response to mAb 90.60.3 binding are intact in the presence of IL-2, but fail to function in response to IL-4. The only phenotypic differences observed on the CT4-S line when compared to the parental CTL-L line were: (1) reduced levels of IL-2Rα; (2) reduced levels of the 90.60.3 phospholipid; and (3) they are more adherent to plastic.

These three differences may account for lack of synergy in CT4-S cells, because perhaps IL-2Rα subunits or IL-2 itself are required for high density of surface expression of the 90.60.3 phospholipid (in analogy to IL-2 upregulation of IL-2Rα subunits [45]). However, our data argue against this possibility because EL4 cells expressing high affinity IL-2R after transfection with IL-2Rα cDNA do not express the 90.60.3 phospholipid, even after culture with IL-2.

Biological assays also indicated that co-culture with mAb 90.60.3 causes a significant but poor synergy with IL-3, and failed to synergize with IL-12 (data not shown). This was surprising, as both IL-3 and IL-12 are known to potentiate IL-2 responses in non-T-cells. We have not tested whether mAb 90.60.3 synergizes with IL-7 as would be expected for a IL-2Rγ subunit mediated signal.

4.6. Potential role of lipids in IL-2 signals

The functional association of lipids and IL-2 or IL-2 receptors has been suggested [46–49]. Similarly, there are other mAbs not directed to IL-2R that affect IL-2 responses [50,51]. However these molecules affect responses negatively as opposed to mAb 90.60.3 which affects IL-2 responses positively.

The lipids sphingomyelin and ceramide, and lipid peroxidation play a role in apoptotic cell death [52,53]. Thus, an mAb against a lipid may protect cells from apoptosis, as does mAb 90.60.3 in culture. While the *biological* effect of mAb 90.60.3 may be attributed to selective inhibition of lipid peroxidation or to inhibition of sphingomyelin metabolism; the *binding* characteristics of mAb 90.60.3 and a selective blockade of anti-IL-2Rα antibodies suggest otherwise.

The 90.60.3 phospholipid seems to play a role in specific IL-2 signaling, but its mechanism of action remains unknown. We are interested in identifying the signal transduction mechanism and in exploring whether protein phosphorylation is involved. Taken together, the data suggest that the 90.60.3 phospholipid is a novel structure distinct from IL-2 receptors, but capable of associating with this (and perhaps other) lymphokine receptors.

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