Phosphatidylinositol-3 kinase-mediated signals in mice immunized with the 57 kDa major antigenic outer-membrane protein of *Shigella dysenteriae* type 1

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Antigen-specific T-cell signalling via T-cell antigen receptor stimulation was carried out in BALB/c mice immunized with the 57 kDa major antigenic component of *Shigella dysenteriae* 1 outer-membrane proteins. In presence of anti-CD3, the 57 kDa antigen was found to increase the level of IL-2 significantly instead of IL-4. IL-2 production in T cells was consistent with an increase in intracellular free Ca^{2+} $[(\text{Ca}^{2+})_i]$ concentration. The antigen-specific modulation was observed during T-cell signalling, with enhanced release of $[(\text{Ca}^{2+})_i]$. IL-2-receptor stimulation via IL-2 did not significantly induce the release of IL-2 with consistent intracellular Ca^{2+} production. Furthermore, the protein tyrosine kinase was activated during anti-CD3 stimulation, which up-regulated the phosphatidylinositol kinase of p85-mediated serine kinase protein kinase-C of p70.

Phosphoinositide-specific kinases are regulated by the phosphorylation of tyrosine kinase through the activation of the T-cell antigen receptor. The above findings indicate that phosphatidylinositol-3 kinase-mediated signals are up-regulated through $[(\text{Ca}^{2+})_i]$, which is essential for Th1-type responses.

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INTRODUCTION

In shigellosis, cytosolic calcium regulates the level of reactive oxygen species induced by *Shigella dysenteriae* 1 toxin (Kaur *et al.*, 1998). Activation of protein kinase-C (PKC) with decreased endogenous intestinal protection has been reported (Kaur *et al.*, 1997), resulting in damage to the enterocyte membrane, and changes in membrane permeability and fluid secretion. This may be due to the phosphorylation of antagonistic CD8 T cells (Basson *et al.*, 1998), through PKC-mediated signals, rather than agonistic signals (Bommhardt *et al.*, 1997).

The selection of CD4 and CD8 T cells may be derived through distinct signals. Several reports have noted a relationship between signal strength and lineage choice (Kisielow & Von Boehmer, 1995; Germain & Stefanova, 1999; Smith *et al.*, 1997). However, a selecting ligand may not affect the change in development of the CD8 T-cell lineage (Hogquist *et al.*, 1995; Ashton-Rickardt *et al.*, 1994) but rather may alter antibody-receptor expression and signal pathways (Itano *et al.*, 1996; Sharp *et al.*, 1997). It has also been reported that antagonistic antibodies or signals may specify the develop-

ment of CD8 T-cells (Basson *et al.*, 1998), whereas agonistic antibodies to the T-cell antigen receptor (TCR)/CD3 complex may specify the development of CD4 T cells of the Th1 subset (Suzuki *et al.*, 1998).

The TCR up-regulates the PKC-mediated phosphorylation in CD4^+ T cells, which in turn leads to IL-2 production (Matsuyama *et al.*, 1991; Nguyen *et al.*, 1995). Furthermore, receptor-stimulated tyrosine phosphorylation is essential for cytokine production in murine Th1 cells but not Th2 cells (Minoguchi *et al.*, 1999). TCR ligation regulates the intracellular concentration of inositol lipid, a derivative product of phosphatidylinositol 3-kinase (PI3K), $\text{PI}(3,4,5)\text{P}_3$ and $\text{PI}(3,4\text{P})_2$ (Ward *et al.*, 1992). Another experiment revealed that the secondary protein tyrosine kinases, such as Fyn and Zap 70, were activated in Th1 cells just after stimulation with anti-CD3 antibody but not in Th2 cells (Tamura *et al.*, 1995). PI3K-regulated signalling pathways are important for T-cell activation involving up-regulation of phosphoinositide-dependent protein kinases and PKC (Alessi *et al.*, 1998). Although p80 tyrosine phosphorylation was induced during entry of *Shigella flexneri* into epithelial cells (Christoph *et al.*, 1995), the regulatory role of tyrosine phosphorylation in mediating antigen-specific signals towards protection is elusive.

The central role of phosphoinositide-specific protein kinase

Abbreviations: $[(\text{Ca}^{2+})_i]$, intracellular calcium; OMP, outer-membrane protein; PI3K, phosphatidylinositol-3 kinase; PKC, protein kinase-C; PTKase, protein tyrosine kinase; TCR, T-cell antigen receptor.

has been identified in mitogenic signal transduction (Gold & De Franco, 1994; Perlmutter *et al.*, 1993) and in mediating the effect of phosphatidylinositol kinase (PTKase) on cell growth and differentiation (Thomas, 1994). The receptor-specific pathways are determined by the substrate specificity of the activated PTKase or by the Src homology 2 (SH2) binding sites of signal-transducing proteins that are phosphorylated by the PTKase. Zap70, a second group of tyrosine kinases, activates the TCR via SH2 domains in the regulatory subunit of PI3K (Delves, 1994).

Antigen alone may not be able to search the specific path through which it can generate antigen-specific responses, though it requires a co-stimulatory molecule (Funaro *et al.*, 1992). Recently, we reported the role of anti-CD3 in mediating Th1-type responses in shigellosis (Sinha & Bagchi, 2004). In continuation, the present study was carried out to identify the signals underlying antigen-specific memory responses to *Shigella* in the presence of anti-CD3 stimulation.

METHODS

Preparation of the 57 kDa OMP from *S. dysenteriae* 1. Outer-membrane proteins (OMPs) were isolated from serovar-specific strains of *S. dysenteriae* 1 by using a standard method (Johnston & Gotschlich, 1974). The strain (PB10) was isolated and identified as described previously (Sinha & Bagchi, 2004) from faeces of patients with bacillary dysentery admitted to paediatric and general wards of the Infectious Diseases Hospital, Kolkata.

The major antigenic fraction (57 kDa) was eluted from gel slices electrophoretically using an electro-eluter (Bio-Rad) as described previously (Sinha *et al.*, 1994). The protein was concentrated using Speed-Vac (Savant SC-210A) and the concentration was measured using 1% BSA as standard, as described by Markwell *et al.* (1978).

To ascertain whether the eluted protein contained trace amounts of bound LPS, the *Limulus* amoebocyte lysate (Sigma) assay was performed using *Escherichia coli* O55:B5 LPS (Sigma) as a control (Yin *et al.*, 1972). The presence of LPS in the eluted component was in picogram concentrations (< 10 pg per 10 µg protein). The negligible amount of LPS did not show any interference in biological responses.

Immunization and specific antibody response. Inbred male 3–4-month-old BALB/c mice were housed in the animal unit of this institute according to the institutional guidelines. They were grouped into immunized and control groups, each containing five mice. Mice were immunized subcutaneously with 25 µg of the 57 kDa antigen emulsified in Freund's incomplete adjuvant at the first, second and third week, followed by a booster dose of 50 µg of the 57 kDa antigen at the fifth week. The serum antibody response for IgG was determined by ELISA (Voller *et al.*, 1978) on days 3, 7, 14 and 28 in immunized mice with respect to day 0 for the control group. The serum antibody responses were measured as the inverse log [ln (x)] of the titre value measured at 492 nm. The serum IgG level in immunized BALB/c mice was significantly elevated and persisted until day 28.

Isolation of T cells. Lymph node cells (from two mice of each group) were isolated by homogenizing the tissues in cold PBS using a siliconized glass homogenizer followed by centrifugation at 400 g for 10 min at 4 °C. The cells were washed twice in supplemented RPMI 1640 medium (Sigma). On average, more than 90% of cells were viable using the trypan blue exclusion method. Finally, 1×10^6 cells ml⁻¹ were suspended in complete RPMI medium supplemented with 10% FBS

(foetal bovine serum) and 100 U gentamicin ml⁻¹. Later, T cells were fractionated from the total cell population using a panning method (Payne *et al.*, 1981). The T cells (5×10^6 cells ml⁻¹) were then washed three times and incubated with 10 µg ml⁻¹ of the 57 kDa antigen for 48 h at 37 °C and 5% CO₂ in complete RPMI 1640 medium. T cells of immunized or non-immunized mice were re-stimulated in duplicate with anti-CD3 antibody (10 µg ml⁻¹; Pharmingen) or rIL-2 (10 µg ml⁻¹; Sigma) in the presence or absence of inhibitor in the same culture conditions for up to 30 min. T cells were pre-incubated with rapamycin (20 ng ml⁻¹), a PI3K inhibitor, for 30 min prior to stimulation via TCR or IL-2-receptor ligands.

ELISA for IL-2 and IL-4. The assay was done as described in the World Health Organization manual (WHO, 1999). In brief, 96-well microplates (Nunc) coated with 0.5 µg capture antibody per well in PBS (pH 7.2) were incubated overnight at 4 °C in a refrigerator. The next day, the plates were washed twice with washing buffer (PBS-T with 1% BSA) at room temperature. The non-specific sites were then blocked with 5% BSA (IgG-free) in PBS for 30 min at 37 °C. The plates were washed three times with washing buffer and incubated for 2 h at 37 °C with different serial dilutions (twofold) of standard and differently stimulated cultures (described above), in duplicate, in PBS. The wells were again washed three times with washing buffer and incubated with biotinylated antibody (1 µg ml⁻¹) in PBS. Unbound antibody was removed by washing three times with washing buffer. One hundred microlitres of a 1:2000 dilution of streptavidin-horseradish peroxidase conjugate was added to each well. After washing three times, the colour was developed after adding 50 µl per well of 0.1% substrate [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] in 0.1 M sodium citrate buffer (pH 4.5) and 0.1% H₂O₂ for 15 min at 37 °C in the dark. Finally, the reaction was stopped by adding 20 µl 10% SDS. The absorbance was recorded at 492 nm in an ELISA reader (Bio-Rad). Calculations were performed to estimate the concentrations of IL-2 and IL-4 in the culture supernatant.

Intracellular calcium measurements. The intracellular calcium [(Ca²⁺)_i] level was measured using Fura-2 fluorescence as described by Grynkiewicz *et al.* (1985). Briefly, stimulated cells [10^6 (ml culture)⁻¹] were washed twice with BSS (pH 7.2) and incubated in a Ca²⁺ buffer [136 mM NaCl, 4.8 mM KCl, 5 mM glucose, 1 mM CaCl₂, 20 mM HEPES (pH 7.4)] supplemented with 10 µM Fura-2 in dimethyl-sulfoxide (DMSO) for 45 min at 37 °C in the dark. The cells were then washed twice with BSS and resuspended in Ca²⁺ buffer containing BSA (1 mg ml⁻¹). Finally, the cells were in suspension (without BSA) and were exposed to different T-cell antigen stimulants at different time points in a cuvette at 37 °C using a spectrofluorometer (Hitachi-3010). Phorbol myristate acetate (PMA; Sigma) was used as a positive control.

Data were recorded as the relative ratio of fluorescence at excitation wavelengths of 340 and 495 nm (slit, 5 nm), with emission measured at 484 nm (slit 5 nm). After each measurement, maximal and minimal fluorescence were assessed by addition of 20 mM 0.1% Triton-X followed by 5 mM MnCl₂.

PTKase assay. The PTKase activity in the sample after re-stimulation was measured using a commercial quantitative assay kit for PTKase (Sigma). The poly-Glu-Tyr (PGT) substrate (Sigma) was used to probe the phosphotyrosine-specific mAb-conjugated horseradish peroxidase (HRP). The PTKase was extrapolated from the epidermal growth factor receptor (EGFR) activity graph (absorbance at 492 nm vs. units EGFR activity).

Immunoprecipitation. The cell suspension (10^6 cells ml⁻¹) was centrifuged at low speed and incubated for 30 min in 200 µl cold RIPA lysis buffer [1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Tris pH 7.2, 1 mM ethylene glycol tetra-acetic acid (EGTA)] containing protease inhibitors [1 ml lysis buffer contain-

ing 10 μ l 200 mM phenylmethanesulfonyl fluoride (PMSF) in acetone, 10 μ l 0.1 M sodium orthovanadate in deionized water, 1 μ l 10 mg leupeptin ml^{-1} in deionized water, 1 μ l 10 mg aprotinin ml^{-1} in deionized water and 1 μ l 1 mg pepstatin ml^{-1} in methanol; stock solutions were stored at -20°C]. The lysates were centrifuged and supernatant was collected. The protein concentrations were adjusted to 500 $\mu\text{g ml}^{-1}$ in RIPA lysis buffer. Lysates were pre-incubated with 50 μ l of Protein A-Sepharose 4CL beads (Sigma) in PBS for 20 min. The supernatant was centrifuged and incubated for 3 h with 100 μ l of different stimulated culture supernatants followed by a 1 h incubation with 30 μ l of protein-A at 4°C in refrigerator. After centrifugation, the immunoprecipitate was washed twice in lysis buffer and once with cold PBS. One hundred microlitres of glycerol sodium dodecyl sulfate (GSD) solution (10 ml glycerol, 30 ml 10 % SDS, 5 ml 0.5 M Tris pH 6.8, 10 mg bromophenol blue, 55 ml H_2O) was added and heated at 100°C for 5 min. Twenty microlitres of the samples were separated by electrophoresis on a 7.5 % SDS-polyacrylamide gel and immunoblotted as described next.

Immunoblotting. After electrophoresis the gel was transferred onto nitrocellulose paper for 4 h at 40 mA at 4°C in the refrigerator. Then blotted nitrocellulose paper was washed twice in TBS-T (Tris-buffered saline Tween-20) and residual binding sites on the membrane were blocked by incubating with 5 % BSA for 1 h at room temperature. After incubation, it was rinsed twice with washing buffer (TBS-T with 1 % BSA) and the filter was probed by incubating at room temperature for 1 h with anti-PKC or anti-phosphotyrosine or anti-PI3K antibody (Sigma) diluted in TBS. Again the nitrocellulose paper was washed twice with washing buffer. The blotted nitrocellulose paper was then incubated for 1 h with horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibodies (1:15 000 dilution). Finally, immunoreactive bands were visualized with diaminobenzidine (DAB, 0.5 mg ml^{-1}) substrate containing 0.01 % H_2O_2 .

Statistical analysis. Two-way analysis of variance and Student's *t*-test were performed to compare the effect of stimulants (in duplicate) for each variable in immunized and control groups of mice. The data for immunoassays were processed using a software package (Epistat) to generate a curve using linear regression analysis and expressed as mean $\pm$ SE for three consecutive experiments.

RESULTS

Cytokine responses to anti-CD3 stimulation

Increased IL-2 (1734 ng ml^{-1}) and IL-4 (1433 ng ml^{-1}) responses were found in anti-CD3-activated T cells 7 days after the mice were immunized. This was noted to be significantly higher ($P < 0.05$) when compared with other stimulated T cells and unstimulated T cells of immunized mice and controls. On day 28 of immunization, the IL-2 level was increased and the IL-4 level was decreasing in anti-CD3-activated T cells of immunized mice. Insignificant responses were found in other stimulated culture supernatants (Fig. 1). In control groups, the same responses were seen in anti-CD3-stimulated cells due to the presence of the antigen-specific stimulant, although the responses were found to be significantly different when compared with other stimulated groups.

Anti-CD3-induced T-cell signalling

Intracellular free Ca^{2+} release during T-cell activation. The maximal $[(\text{Ca}^{2+})_i]$ level was observed at 1 min of anti-CD3

Fig. 1. Kinetics and pattern of IL-2 (◆) and IL-4 (−) release in the culture supernatant of differently stimulated (a, PHA; b, rIL-2; c, anti-CD3) and unstimulated (d) T cells co-cultured with antigen-presenting cells of immunized BALB/c mice and non-immunized mice as control. Bars indicate SEM.

stimulation (Fig. 2). It was significant at the 95 % confidence limit when compared with other stimulated and unstimulated T cells of non-immunized mice. The level of $[(\text{Ca}^{2+})_i]$ was maintained until 2.5 min of such stimulation, whereas insignificant levels of $[(\text{Ca}^{2+})_i]$ were observed in other stimulated and unstimulated T cells of immunized BALB/c mice.

PTKase activation. The activation of PTKase in T cells of immunized BALB/c mice was consistently high, with the

Fig. 2. Intracellular Ca^{2+} levels at various time points of differently stimulated (PMA, ■; rIL-2, ▲; anti-CD3, ×) and unstimulated (◆) T-cell cultures of non-immunized (a) and immunized (b) BALB/c mice.

elevation of the $[Ca^{2+}]_i$ concentration after both anti-CD3 and rIL-2 stimulation. The PTKase response initiated by anti-CD3 in immunized mice was comparable to that of rIL-2 (Fig. 3). This elevation in PTKase level fluctuated and was insignificant when compared with the stimulated and unstimulated T cells of non-immunized mice.

PKC-mediated PI3K regulation in anti-CD3-activated T cells. Immunoblot analysis using anti-PI3K and anti-PKC was undertaken to examine the changes in the pattern of PI3K-dependent kinase phosphorylation of PKC upon activation of the T cells via the TCR complex. It was observed that a unique pattern of kinase protein was secreted during T-cell activation in BALB/c mice immunized with the 57 kDa antigenic OMP of *S. dysenteriae* 1 (Fig. 4). Immunoprecipi-

Fig. 3. Changes in PTKase release were noted after 10 min in differently stimulated and unstimulated T cells of BALB/c mice immunized with 57 kDa antigenic OMP of *S. dysenteriae* 1 (□) and non-immunized mice (■). Bars indicate SEM.

tates of anti-CD3- or rIL-2-stimulated T-cell lysates of immunized mice were seen to have three major bands, namely 70, 85 and 110 kDa proteins (Fig. 4a, lanes 3 & 5). However, immunoprecipitates of the T-cell extract of stimulated non-immunized mice were seen to have only one major band, at 85 kDa (Fig. 4, lanes 8 & 9). Protein bands at 70 kDa and 110 kDa were consistently observed in immunoprecipitates of anti-CD3- or rIL-2- stimulated T-cell extracts of immunized mice.

PKC-mediated phosphorylation of the 85 kDa protein (p85) was initiated in both of the stimulated T-cell extracts of 57 kDa OMP-immunized mice. Moreover, it was found to be reproducible and comparable after anti-CD3 stimulation up to the maximal time of 25 min (Fig. 5a). On the other hand, it was observed that a significant change in phosphorylation of PI3K gave a 70 kDa protein (p70) that reacted with the anti-PI3K-specific antibody, while generation of PI3K was inhibited by rIL-2 stimulation (Fig. 5b). The phosphorylation of PI3K-regulated PKC was sustained up to 30 min of anti-CD3 stimulation (Fig. 5). However, the inhibition of PI3K prevented rIL-2-induced phosphorylation of PKC during T-cell activation. This suggests that PI3K-mediated signals are generated and up-regulated by PKC via TCR activation.

TCR-induced PKC and PTKase regulation by PI3K. An analysis was undertaken to find out whether PI3K may have a role in T-cell signalling by TCR activation in these immunized mice. Further, whether the anti-CD3 ligand induced T-cell phosphorylation in the presence or absence of a PI3K inhibitor, i.e. rapamycin, was also investigated. The phosphorylation of PKC or PTKase was prevented in the presence of rapamycin (Fig. 6). Immunoprecipitated p70 or p85 of anti-CD3-stimulated T-cell lysate from immunized mice did not react with either the anti-PKC or anti-phosphotyrosine antibody. rIL-2 in the presence of inhibitor did not show any significant changes in the phosphorylation of PKC, but rather blocked the tyrosine kinase phosphorylation (Fig. 6, lane 3). Rapamycin prevented the ligand-induced phosphorylation of tyrosine kinases.

DISCUSSION

It has been reported that PTKase activation helps in host cell invasion following infection by *Shigella* (Minoguchi *et al.*, 1999) and PKC-mediated signals up-regulate the phosphorylation of antagonistic $CD8^+$ T cells, which results in membrane permeability and fluid secretion (Kaur *et al.*, 1998). Recently, we reported that anti-CD3 could significantly stimulate the macrophage migration toward the activated response for $CD4^+$ T-cell with enhanced IL-2 secretion. The proliferation of $CD4^+$ T cells was found to be dominating over the $CD8^+$ T cells after anti-CD3 stimulation in BALB/c mice immunized with the 57 kDa antigen (Sinha & Bagchi, 2004). On the other hand, the expression of CD22 was also recorded as higher in mice after 57 kDa antigenic immunization, indicating a T-cell dependent humoral response (Bagchi & Sinha, 2004). In continuation to this report, an attempt was

Fig. 4. Immunoprecipitated protein profile, at 7.5% resolving gel, of differently stimulated T cells of BALB/c mice immunized with 57 kDa antigenic OMP of *S. dysenteriae* 1 (a) and non-immunized mice (b). Lanes: 1, molecular mass marker (Bio-Rad); 2 & 7, immunoprecipitate of unstimulated T-cell lysates; 3, 5 & 8, 9, immunoprecipitate after rIL-2 or anti-CD3 stimulation, respectively; 4, 6 & 10, pre-immunoprecipitated lysates after rIL-2 or anti-CD3 stimulation, respectively.

Fig. 5. Anti-CD3 activation of PI3K-regulated protein kinase PKC in BALB/c mice immunized with 57 kDa antigenic OMP of *S. dysenteriae* 1. Immunoprecipitates of T-cell lysates were resolved at 10% SDS-PAGE and analysed by Western blot at different time points of anti-CD3 antibody or rIL-2 stimulation using specific anti-PKC (a) and anti-PI3K (b) antibodies. Unstimulated and stimulated T cells of non-immunized mice are not shown as they seemed to be weakly reactive. US, unstimulated.

Fig. 6. Rapamycin blocks the TCR of PKC and phosphotyrosine kinases. T cells of immunized BALB/c mice were stimulated with anti-CD3 or rIL-2 for 10 min following a 30 min pre-incubation in the presence (1 & 3) or absence (2 & 4) of rapamycin, a PI3K inhibitor. Immunoprecipitates of T-cell lysates were resolved at 10% SDS-PAGE followed by Western blot analysis using specific anti-PKC (a) and anti-phosphotyrosine kinase (b) antibodies. Unstimulated T-cell immunoprecipitates did not show any reaction against the antibodies used.

made to determine the antigen-specific signals by monitoring anti-CD3-induced Th1- or Th2-type responses in BALB/c mice immunized with the 57 kDa major antigenic OMP of *S. dysenteriae* 1. Anti-CD3 significantly proliferated the memory T-cell population, enabling them to release cytokines through antigen-specific signals (Kruisbeek, 1991) towards *Shigella*, which was evident by producing the change in kinetics of IL-2 rather than IL-4 (Fig. 1). Cholera or shigella toxins have been observed to block phosphatidylinositol 4,5 biphosphate (PIP₂) breakdown pathway or protein tyrosine kinase activation that exerted no effect on Th2 stimulated with anti-CD3 (Tamura *et al.*, 1993). Elevated $[(Ca^{2+})_i]$ concen-

trations and generation of inositol phosphates were detected following such stimulation of T cells producing IL-2, whereas there was a lack of IL-4-producing T cells.

Phosphoinositide-dependent protein kinase plays a central role in mitogenic signal transduction, leading to regulation of the serine phosphorylation of the PKC and the tyrosine phosphorylation of the PTKase. Although PTKase has an important role in host cell invasion and fluid accumulation by up-regulating the PKC, nevertheless it is also involved in phosphatidylinositol 4,5 triphosphate breakdown to inositol triphosphate (IP₃) during selection of Th1 responses. In shigellosis, PKC-mediated signals are generated as a result of selecting only CD8⁺ cells rather than CD4⁺ cells (Basson *et al.*, 1998). Hence, there is a need for an immunomodulator, which can modulate a biased signal. We observed that the level of $[(Ca^{2+})_i]$ and PTKase after such stimulation was significantly higher than that in non-immunized mice (Figs 2 and 3). When the T cells were stimulated with anti-CD3 antibody, immunoprecipitates of kinase-activated proteins of 70, 85 and 110 kDa molecular mass were revealed using protein A-Sepharose 4CL beads (Fig. 4). p70 of PI3K is induced by p85 of activated PKC, as revealed by immunoblot using anti-PI3K or anti-PKC antibodies (Fig. 5). TCRs might regulate the activity of diacylglycerol-regulated kinases of PKC with intracellular Ca^{2+} release.

PI3K inhibitors revealed that the PI3K-mediated signalling pathway is important for T-cell activation (Lafont *et al.*, 2000) via TCR ligation. We noted that two specific p70 and p85 signal-transducing proteins were phosphorylated by

PKC or PTKase, and that TCR- or IL-2-induced phosphorylation of tyrosine kinases was prevented in the presence of PI3K inhibitor (Fig. 6). Inhibition of PI3K prevents the TCR-induced phosphorylation of the PTKase. It is revealed that the p70 structural domain binds with high affinity to phosphotyrosine residue, poly-Glu-Tyr (PGT) substrate.

The above data also support the hypothesis that these second messengers are detected only in Th1 clones via the TCR complex (Gajewski *et al.*, 1990). We have also noted PI3K up-regulated anti-CD3-induced phosphorylation of PKC during memory T-cell activation. Earlier, we reported the anti-CD3-induced TCR activation by maintaining the homeostasis of CD4⁺ T cells with increased IL-2 levels in immunized mice following *Shigella* infection (Sinha & Bagchi, 2004). Further, we assumed that IP3-mediated signals up-regulated the phosphorylation of memory CD4⁺ T-cell selection with increased IL-2 levels in 57 kDa OMP-sensitized mice. The two regulatory transducing proteins were activated p70 and p85 in anti-CD3-induced immunized mice, indicating that PI3K binds to activated PTKase via the SH2 domain of the p70 regulatory subunit and is up-regulated by phosphorylation of p85 serine kinase PKC. However, our data suggested that PI3K signals are up-regulated via the TCR complex, where the level of PTKase involved in the phosphorylation of Th1-type cells of Th0 subsets is regulated through [(Ca²⁺)i].

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