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ARTICLES

Multiphasic Modulation of Signal Transduction Into T Lymphocytes by Monoiodoacetic Acid as a Sulfhydryl Reagent

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Abstract Actions of monoiodoacetic acid (MIA) as a sulfhydryl reagent on the different stages of the T cell receptor (TCR)–mediated signal transduction were examined. MIA (1 mM) prevented anti-TCR (CD3) monoclonal antibody (mAb)–induced energy-dependent receptor capping but at the same time promoted the anti-CD3 mAb/mitogen-induced tyrosine phosphorylation of the T cell activation-linked cellular proteins of 120, 80, 70, 56, and 40 kDa. Relatively low concentration (0.01 mM) of MIA further promoted anti-CD3 mAb-induced transcription of *c-fos*, production of IL-2, and cell surface expression of IL-2 receptors. The MIA-promoted TCR-mediated IL-2 production actually required signal transduction that could be inhibited by cyclosporin A, genistein, or H-7. In contrast, the same concentration of MIA as promoted the signal transduction for cell activation severely inhibited the anti-CD3 mAb-triggered signal delivery for cell proliferation, selectively at its early stage. We conclude from these results that MIA differentially affects various steps of signaling into T lymphocytes, suggesting that there exist multiple sites of MIA-sensitive or redox-linked control in the signal cascade. © 1995 Wiley-Liss, Inc.

Key words: monoiodoacetic acid, sulfhydryl reagent, modulation of signal transduction, redox-linked

Cross-linkage of cell surface receptors with ligands transduces signals into T lymphocytes for their activation and proliferation [Metzger, 1992; Weiss and Littman, 1994]. Recent evidence has suggested that several redox-linked mechanisms modulate this receptor-mediated intracellular signal transduction [Bauskin et al., 1991; Heffetz and Zick, 1989; Kanner et al., 1992; Devary et al., 1992; Lander et al., 1992, 1993; Staal et al., 1991, 1993; Rahman et al., 1993; Nakamura et al., 1993; Rozsnyay et al., 1993; Nakashima et al., 1994]. However, the sites of the redox-linked controls in the signal cascade are not yet clear. Monoiodoacetic acid (MIA) is a simple sulfhydryl (SH) reagent that enters the cell to inactivate SH enzymes in the cascade of electron transfer for energy production [Nakashima et al., 1982]. The action of MIA has not, however, been studied on various steps of signal delivery. For example, it is still not clear whether the MIA-sensitive energy-dependent mechanism is required for the receptor-mediated intracellular signal transduction [Bourguignon and Bourguinon, 1984; Ratcliffe et al., 1992; Tamura and Nariuchi, 1992]. We here compare the sensitivities to MIA of various stages of signal delivery into T lymphocytes for their activation and proliferation. The results show that inhibition of cross-linkage of receptors by MIA is paradoxically linked to upregulation of the early signal for cell activation, whereas MIA inhibits the signaling for cell proliferation. Multiphasic MIA-sensitive or redox-linked controls have been suggested in the whole signal cascade for activation and proliferation of T lymphocytes.

Abbreviations used: Con A, concanavalin A; mAb, monoclonal antibody; MEM, minimum essential medicine; MIA, monoiodoacetic acid; PKC, protein kinase C; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; PTYR, phosphotyrosine; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SH, sulfhydryl group(s).

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MATERIALS AND METHODS

Cells and Treatment

Single cell suspensions of spleen cells and thymocytes in Eagle's minimum essential me-

dium (MEM) were prepared from C57BL/6 mice as described previously [Nakashima et al., 1982]. A T cell–rich fraction was obtained by passing the spleen cell suspension through a nylonwool column [Nakashima et al., 1982]. Suspensions of cells (1–3 × 10^7/ml) in MEM were used for Western blot and Northern blot assays, and those (1 × 10^7/ml) in RPMI-1640 medium supplemented with 10% FCS (10^7/ml) for cell culture. They were incubated in the presence or absence of anti-CD3 mAb (145-2C11) [Leo et al., 1987], concanavalin A (Con A) (Sigma, St. Louis, MO) or MIA at 37°C before assays. For some experiments cyclosporin A (a gift from Dr. H. Takagi, Nagoya University), genistein (Funakoshi, Tokyo, Japan), and H-7 (Seikagakukogyo, Tokyo, Japan) were added into the culture as inhibitors of calcineurin [Schreiber, 1992], protein tyrosine kinase (PTK) [Akiyama et al., 1987], and protein kinase C (PKC) [Kawamoto and Hidaka, 1984], respectively.

Immunoblot Assay of Phosphotyrosine (PTYR)-Containing Proteins

SDS-PAGE sodium dodecyl sulfate (SDS)-Polyacrylamide gel electrophoresis (PAGE) and immunoblots were performed as described [Hamaguchi et al., 1988; Nakashima et al., 1991]. Briefly, suspensions of cells were lysed by adding an equal volume of ×2 sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% 2-ME, 10% glycerol) and heated in boiled water for 3 min. The cell lysates, containing 50 μg of proteins per lane, were applied on SDS–12% polyacrylamide gels. After electrophoresis, proteins were transferred electrophoretically to polyvinylidene difluoride membrane (Millipore, Bedford, MA) and stained with affinity-purified anti-PTYR rabbit antibody [Hamaguchi et al., 1988] followed by ^{125}I-labeled protein A (ICN, Irvine, CA). Autoradiography was performed on X-ray film for 15–48 h. All the protein bands developed were confirmed to be PTYR-specific from selective inhibition of development by adding free PTYR before reaction with anti-PTYR [Nakashima et al., 1991].

RNA Isolation and Northern Blot Analysis

Northern blot analysis was done according to the method described [Iwamoto et al., 1990]. Briefly, the cells (3 × 10^7 cells/sample) were lysed with solution D (4 M guanidin, 0.5 mM N-lauroylsareosine, 2.5 mM $Na_3C_6H_5O_7 \cdot 2H_2O$) and total RNA was extracted. The RNA preparation (10 μg/lane) was electorphoresed on 1% agarose gels containing 1.1 M formaldehyde. The RNA was transferred to Hybond-N nylon membranes (Amersham, Tokyo, Japan), and mRNA was detected by ^{32}P-labeled probe.

Cell Growth and Cytokine Assay

[^{3}H]thymidine (37 kBq/well) was added for the last 4 h of culture of spleen cells (10^6 cells/well) in 96-well plates. After harvesting the cells on filter paper the radioactivity (cpm) was determined by a liquid scintillation counter.

Cytokine (IL-2) activity in culture supernatant was assayed by use of IL-2–dependent CTLL-2 cells as described [Zhang et al., 1993]. CTLL-2 cells (5 × 10^3 cells/well) were distributed in 96-well microplates with culture supernatant in 50% of the medium. After 24 h incubation at 37°C, the CTLL-2 culture was added with 37 kBq/well [^{3}H]thymidine for overnight incubation, and cells were harvested for measurement of radioactivity. Preliminary study showed that addition of 0.005 mM or less MIA into CTLL-2 cells never promoted but slightly (up to 20%) inhibited the growth of CTLL-2 cells. This eliminated the possibility that any growth promotion by supernatant from the culture added with 0.01 mM MIA was mediated by the carried-over MIA.

Immunofluorescence and Laser Flow Cytometry

For observation of capping/aggregation of CD3, cells were first incubated with anti-CD3 mAb at 4°C for 20 min, followed by another 30 min incubation at 37°C with FITC-labeled anti-Ig goat antibody (Tago, Burlingham, CA). The cells were fixed with 1% paraformaldehyde and mounted on slide glass for observation under a fluorescence microscope.

For measurement of the level of expression of IL-2R, cells were incubated first with anti-IL-2R alpha mAb (7D4) (donated by Dr. Y. Yoshikai, Nagoya University) and then stained with FITC-conjugated anti-Ig antibody. As control, another set of cells were stained with FITC-conjugated anti-Ig antibody only. In order to discriminate T cells from B cells in the cytogram, cells were further stained with PE-labeled anti-Thy-1 (Becton Dickinson, Mountain View, CA). The cells were stained by each antibody incubating for 40 min at 4°C and analysed on an EPICS Profile flow cytometer (Coulter, Hieleah, FL). Mean FITC fluorescence of Thy-1$^+$ T cells from 5,000 spleen cells was measured for both experimental

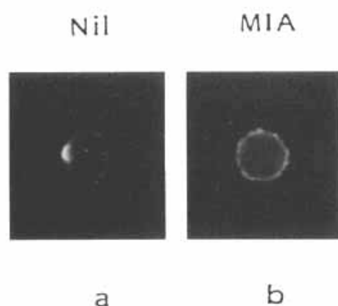


Fig. 1. Inhibition of antibody-induced capping of CD3 on T lymphocytes by MIA. Nylonwool column–passed splenic T lymphocytes were first incubated in the absence (**a**) or presence (**b**) of 1 mM MIA for 5 min and were then reacted with anti-CD3 mAb and FITC-labeled anti-Ig antibody as described in Materials and Methods. More than 100 cells were scored for each sample under a fluorescence microscope, which all showed capping (a) or its inhibition (b); the picture of a single cell for each sample is presented as representive.

(anti-IL-2R + anti-Ig) and control (anti-Ig only) groups. The difference between mean values of the two groups was calculated and presented as an arbitrary index for IL-2R expression.

RESULTS

Effect of MIA on Capping of CD3

Figure 1 confirmed the known action of MIA to inhibit the antibody-induced capping of T cell receptors (TCR/CD3) through inactivating SH enzymes in the electron transport system. The capping was inhibited severely by addition of 1 mM MIA prior to anti-CD3 mAb (Fig. 1b) and partially or marginally by 0.01–0.1 mM MIA (not shown). MIA did not, however, prevent formation of patchy aggregates of receptors (Fig. 1b) that should occur directly through energy-independent cross-linkage of receptors.

Effect of MIA on Signaling for Protein Tyrosine Phosphorylation

A study was conducted to see whether the previous treatment of T cells with MIA before anti-CD3 mAb or Con A would affect the subsequent tyrosine phosphorylation of cellular proteins. The results are shown in Figure 2. As previously reported [Nakashima et al., 1991, 1993], cross-linkage of CD3 on thymocytes or splenic T lymphocytes by anti-CD3 mAb or stimulation with Con A induced low grade or marginal level of increase in tyrosine phosphorylation of 120, 80, 70, 56, and 40 kDa proteins, particularly of the 40 kDa protein, at a short time (2 min) after the cross-linkage (Fig. 2A–C, lane 3). Treatment of thymocytes and spleen cells with 1 mM MIA alone barely induced protein phosphorylation that was more than the background level (Fig. 2A–C, lane 2). To our surprise, previous treatment of thymocytes (Fig. 2A, lane 7; 2B, lane 4) and spleen cells (2C, lane 4) with 1 mM MIA before agonist (anti-CD3 mAb or Con A) never inhibited but rather accelerated the phosphorylation of the same set of proteins (120, 80, 70, and 40 kDa) as those phosphorylated by the stimulation with agonist alone, particularly of the 40 kDa protein. The anti-CD3 mAb-induced phosphorylation of the 40 kDa protein was less extensively promoted by 0.001–0.1 mM MIA (Fig. 2A, lanes 4–6). Addition of MIA immediately before anti-CD3 mAb was also effective for promoting the anti-CD3–induced protein tyrosine phosphorylation (*not* shown). In a time course study (Fig. 2D), longer (5–20 min) exposure of the lymphocytes to 1 mM MIA alone induced marginal increase of tyrosine phosphorylation of 120, 80, 70, 56, and 40 kDa proteins. The anti-CD3 mAb-induced tyrosine phoshorylation of 120, 80, 70, 56, and 40 kDa proteins was also peaked at 5 or 20 min, but the peak phosphorylation levels of these proteins were low. Interestingly, the most extensive acceleration by MIA occurred at a later time (20 min) with the aCD3 mAb-induced protein tyrosine phoshorylation (Fig. 2D, lane 13). The accelerated phosphorylation thereafter subsided toward 60 min (not shown). These results argued against the view that the anti-CD3 mAb-induced signaling for protein tyrosine phosphorylation indispensably requires the energy-dependent capping of the receptors. On the contrary, they suggest that the signal pathway is subjected to a unique MIA-sensitive regulatory mechanism.

Effect of MIA on Signaling for Immediate Early Gene Transcription

Further study was conducted to see whether MIA would also affect the signal for lymphocyte activation. Incubation of splenic lymphocytes for more than 5 h in the medium containing >0.1 mM MIA was lethal to 50% of the cells, due to the general toxicity of MIA. We therefore examined the effect of a lower concentration of MIA on the anti-CD3 mAb-induced signal for gene activation. As shown in Figure 3, 0.01 mM MIA accelerated the anti-CD3 mAb-induced transcription of c-*fos* as an immediate early gene. MIA alone was, however, ineffective for inducing transcription *of c-fos. The anti*-CD3 mAb-

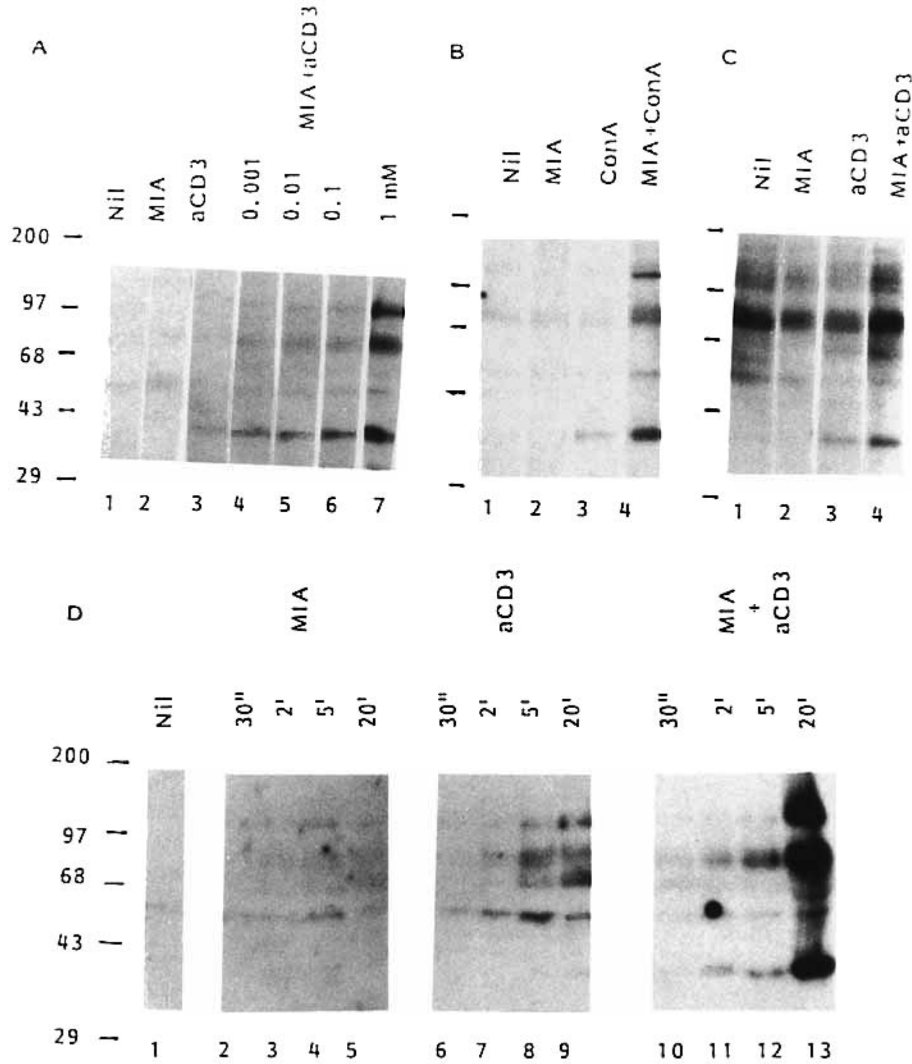


Fig. 2. MIA accelerates the anti-CD3 mAb/Con A–induced signal delivery for protein tyrosine phosphorylation. **A–C:** Thymocytes (A,B) and spleen cells (C) were incubated for 2 min in the presence or absence of 0.001–1 mM (A) or 1 mM (B,C) MIA and then stimulated with anti-CD3 mAb (ascites 1:1,000) or Con A (10 μg/ml) for 2 min. These were lysed for immunoblot analysis against anti-PTYR. Lane 1: Nil control. Lane 2: MIA alone. Lane 3: ACD3 mAb alone. Lanes 4–7: MIA followed by anti-CD3. **D:** Time course of MIA-mediated acceleration of anti-CD3 mAb-induced protein tyrosine phosphorylation. Thymocytes were incubated for 30 s to 20 min in the presence of 1 mM MIA alone (lanes 2–5), anti-CD3 mAb alone (lanes 6–9), or both MIA and anti-CD3 mAb (lanes 10–13). These cells were lysed for immunoblot analysis against anti-PTYR. Lane 1: Nil control. Positions of molecular markers (kDa) are indicated on the left. Shown are representatives of six experiments with consistent results.

induced c-*fos* transcription that was accelerated by MIA was peaked at 15 min (Fig. 3) and thereafter decreased toward 45 min, when the enhancing effect of MIA became marginal (not shown).

Modulation by MIA of Early Signal for Cytokine Production and Cytokine Receptor Expression

Low concentrations (0.001–0.01 mM) of MIA were also found to promote the anti-CD3 mAb-induced elevation of cytokine (IL-2) activity in the culture supernatant (Fig. 4). The promotion was first detected at as early as 6 h after the start of culture (up to five times increase). The IL-2 activity in the culture with 0.01 mM MIA peaked at 12 h and then decreased slowly toward 5 days later. This marked increase in IL-2 activity in the culture stimulated by both anti-CD3 mAb and MIA was inhibited by addition of cyclosporin A, genistein, or H-7 (Fig. 5), suggesting the involvement of intensified signal for IL-2 production.

In addition to promotion of IL-2 production, 0.01 mM of MIA also accelerated cell surface

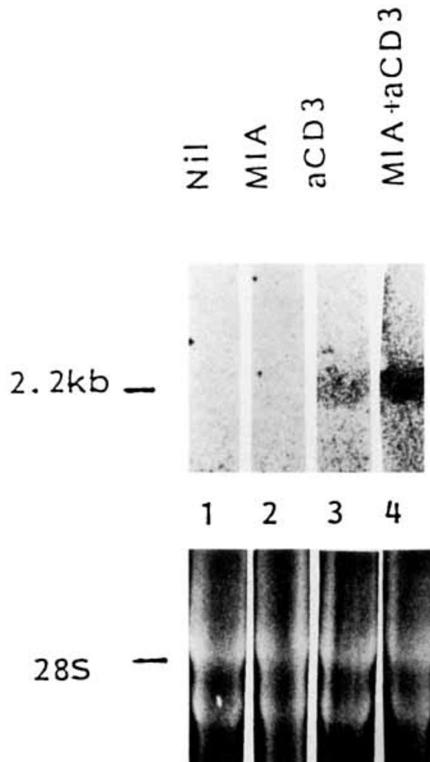


Fig. 3. MIA accelerates the anti-CD3 mAb-induced transcription of c-*fos*. Spleen cells (2×10^7/ml) were incubated in the presence of 0.01 mM MIA (lane 2), anti-CD3 mAb (lane 3), or both MIA and anti-CD3 mAb (lane 4) for 15 min and then lysed to extract RNA for Northern blotting. Lane 1: Nil control. Ethidium bromide staining of total RNA (bottom) shows the equal loading. The positions of the message of c-*fos* (2.2 kb) and 28S ribosomal RNA are indicated on the left.

expression of cytokine receptors (IL-2R alpha) (Fig. 6), most markedly on day 1 of culture.

Inhibition by MIA of Early Signal for Cell Proliferation

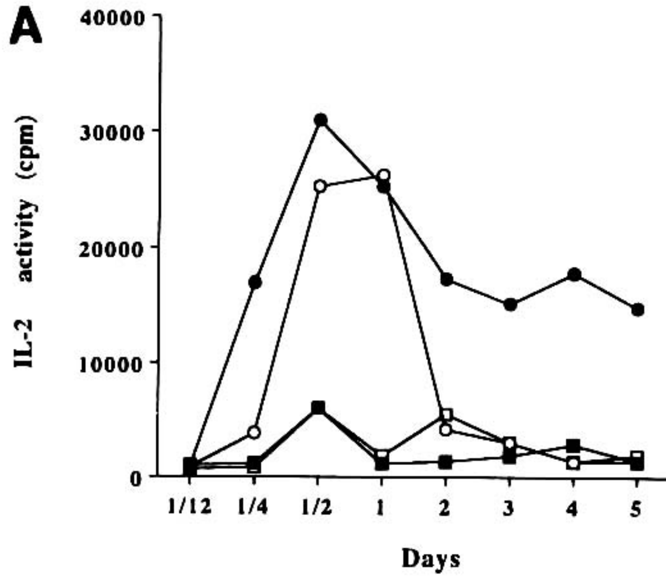
The same concentrations (0.001–0.01 mM) of MIA as promoted cytokine production and cytokine receptor expression, however, inhibited the proliferation response of spleen cells to anti-CD3 mAb (Fig. 7). Interestingly, the inhibition occurred when 0.01 mM MIA was added at 5 h or earlier but not at 24 h or later after the stimulation with anti-CD3 mAb (Fig. 8). This result suggested that the inhibition was restricted to the anti-CD3 mAb-induced early signal for DNA synthesis.

DISCUSSION

One of the several noted observations in this study was the extensive promotion of antireceptor antibody-induced protein tyrosine phosphorylation by MIA, which inhibited the energy-dependent membrane activity for capping of receptors. The pattern of phosphorylated protein bands developed by the apparent synergy between anti-CD3 mAb and MIA was similar to that induced by other procedures to augment the anti-CD3 mAb-mediated signal such as co-cross-linking of CD3 and Thy-1 [Nakashima et al., 1991, 1993] and pretreatment of cells with phosphatidylinositol-specific phospholipase C before anti-CD3 mAb [Rahman et al., 1992]. Particularly, accelerated phosphorylation of the 40 kDa protein, a unique T cell activation–linked protein that was previously discriminated from the 43/41 kDa MAP kinases by direct comparison of their mobilities in SDS-PAGE [Nakashima et al., 1993], was noted in all cases. These results characterize the action of MIA as an upregulator of the TCR-mediated signal. They in turn prove that the TCR-mediated signal for protein tyrosine phosphorylation does not require the energy-dependent membrane activity, although it may require the energy-independent patchy aggregation of receptors (Fig. 1b).

The demonstrated synergy between antireceptor antibody and MIA for inducing protein tyrosine phosphorylation might result from inhibition of the capping-mediated decay of receptors. This does not seem, however, the case, because inhibition of capping alone by sodium azide or cytochalacin B barely upregulated the phosphorylation (data not shown). Recent studies have shown that receptor PTK such as Ltk at ER and nonreceptor PTK such as $p56^{lck}$ and $p60^{c\text{-}src}$ could be activated by a number of SH reagents and oxidative stress such as iodoacetamide [Bauskin et al., 1991], diamide [Nakamura et al., 1993], $HgCl_2$ [Nakashima et al., 1994], and ultraviolet [Devary et al., 1992] through yet unclarified redox-linked mechanisms. These reagents and oxidative stress have also been shown to inactivate protein tyrosine phosphatases (PTP) that carry the SH residues essential for their enzyme activity [George and Parker, 1990; Staal et al., 1993]. MIA probably displays similar actions to ultimately accelerate the protein tyrosine phosphorylation, although the exact molecular mechanism remains to be clarified. Our present results therefore demonstrate that both anti-CD3 mAb-induced protein tyrosine phosphorylation and its upregulation by MIA occur independently of energy-dependent membrane activity.

The action of MIA differed from that of $HgCl_2$ as another SH reagent in two points. First,



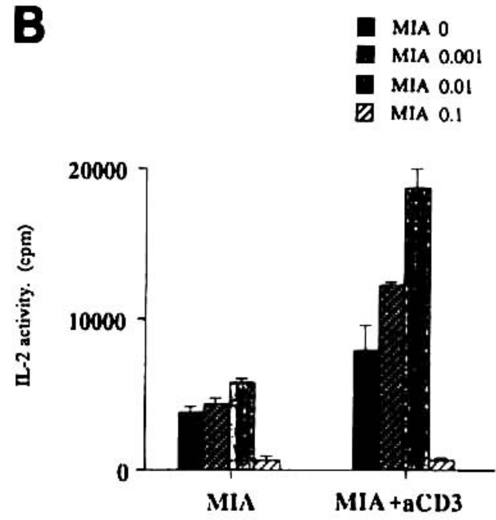


Fig. 4. MIA accelerates the anti-CD3 mAb-induced increase in the cytokine activity in culture. **A:** Spleen cells were incubated in vitro in the absence (open squares) or presence (solid squares) of 0.01 mM MIA, anti-CD3 mAb (open circles), or both MIA and anti-CD3 mAb (solid circles). The supernatants of cultures were obtained at indicated times to measure the IL-2 activity. Each point shows the mean of triplicate assays and SD was $<20\%$ in every determination. Differences of values between the last two groups on day 1/4 (6 h), 1/2 (12 h), and 2–5 are statistically significant by Student's *t*-test ($P < 0.05$). Shown is a representative of four experiments. **B:** Concentration-effect relation. Spleen cells were incubated for 5 days in the presence or absence of the indicated concentrations of MIA, with or without anti-CD3 mAb. Each column represents the mean ± SD of triplicate cultures.

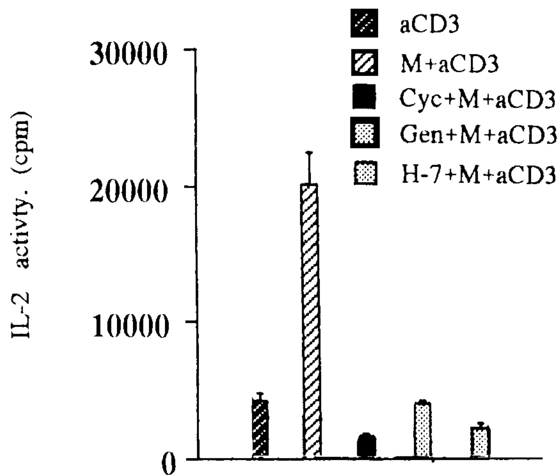


Fig. 5. The increase in the cytokine activity in the MIA plus anti-CD3 mAb-stimulated culture is signal delivery–dependent. Spleen cells were incubated in vitro for 5 days in the presence of anti-CD3 mAb alone (the column on the left end) or both anti-CD3 mAb and 0.01 mM MIA (other columns). Some of these cultures (three columns on the right) were added with 1 μg/ml cyclosporin A, 1 μg/ml genistein, or 30 mM H-7 at the start of cell cultivation. The supernatant of the culture was obtained to measure IL-2 activity. Each column shows the mean ± SD of triplicate cultures.

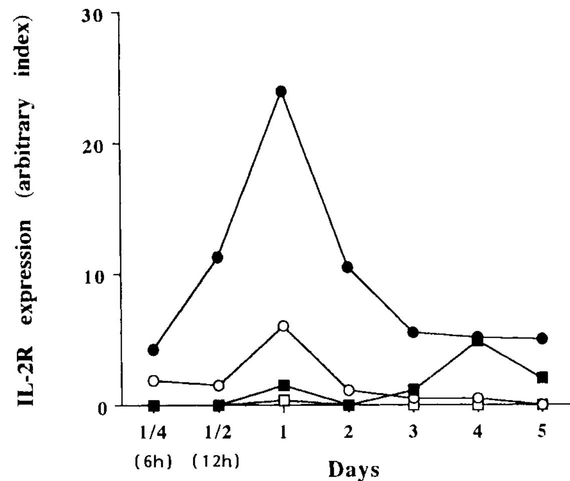


Fig. 6. MIA promotes anti-CD3 mAb-induced cytokine receptor expression. Spleen cells were incubated in vitro in the absence (open squares) or presence (solid squares) of 0.01 mM MIA, anti-CD3 mAb (open circles), or both MIA and anti-CD3 (solid circles). The level of cell surface expression of IL-2R on cultured T lymphocytes was measured at indicated times. Each point shows the value of the arbitrary index calculated from the mean fluorescence levels of $>1{,}000$ cells. Shown is a representative of three experiments.

$HgCl_2$ (1 mM) induced ligand-independent aggregation of cell surface receptors, rather than inhibition of capping of receptors. Second, $HgCl_2$ did not specifically promote the anti-CD3 mAb-induced tyrosine phosphorylation of the 40 kDa protein [Nakashima et al., 1994] (data not

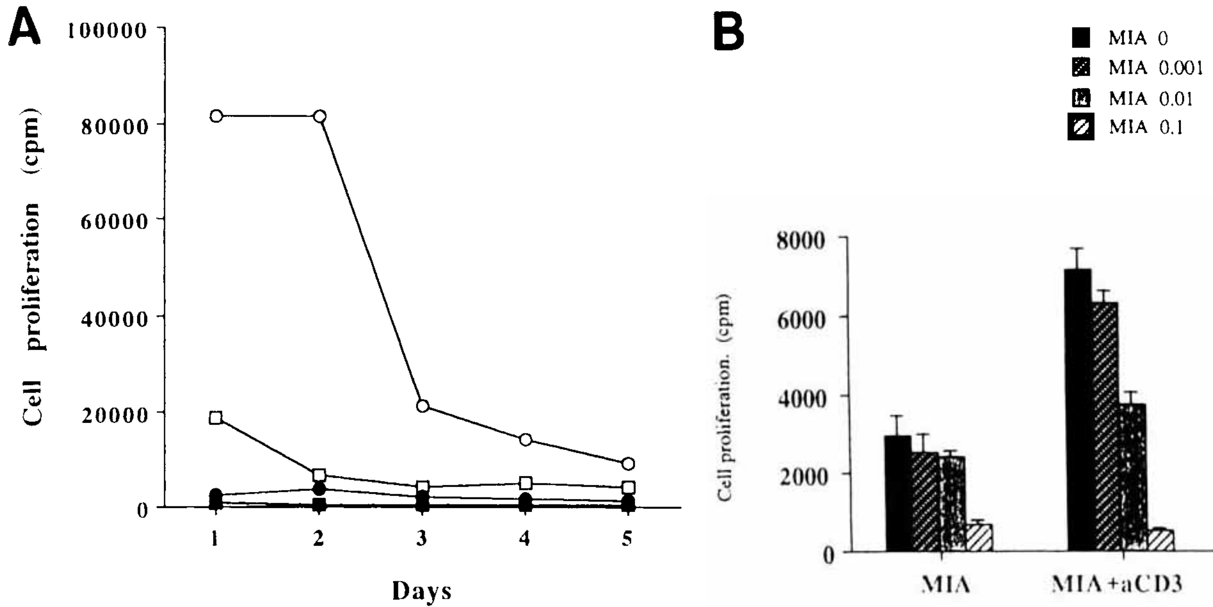


Fig. 7. MIA inhibits the proliferation response of spleen cells to anti-CD3 mAb. **A:** Spleen cells were incubated in vitro in the absence (open squares) or presence (solid squares) of 0.01 mM MIA, anti-CD3 mAb (open circles), or both MIA and anti-CD3 (solid circles). [^{3}H]thymidine uptake was measured at the indicated times. Each point shows the mean of triplicate cultures, and SD was <20% in every determination. The difference of values between the last two groups on days 1–5 was statistically significant by Student's *t*-test ($P < 0.05$). Shown is a representative of four experiments. **B:** Concentration-effect relation. Spleen cells were incubated for 5 days in the presence or absence of the indicated concentrations of MIA with or without anti-CD3 mAb. Each column shows the mean ± SD of triplicate cultures.

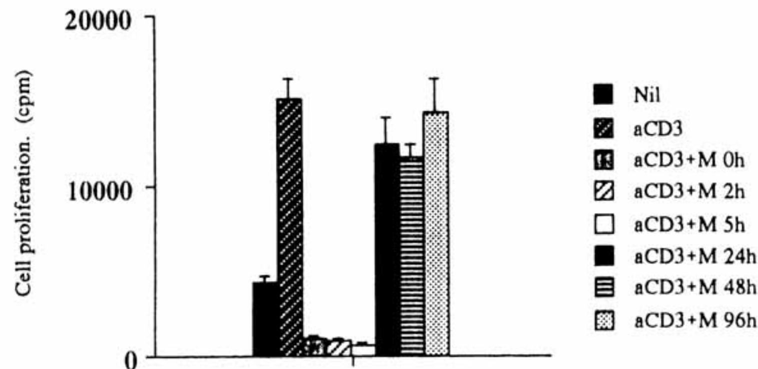


Fig. 8. MIA selectively inhibits the early signal delivery for cell proliferation. Spleen cells were incubated in vitro for 5 days in the absence (the column on the left end) or presence of anti-CD3 mAb (other columns), and 0.01 mM MIA was added at the indicated times (0, 2, 5, 24, 48, 96 h) after the start of cell cultivation. [^{3}H]thymidine uptake was measured at the last day of culture. Each column shows the mean of triplicate cultures ± SD. Shown is a representative of two experiments.

shown). The differential actions of MIA and $HgCl_2$ on lymphocyte signal transduction may be linked to the distinct molecular nature of the two SH reagents. MIA, a monovalent SH reagent, can enter the cell to inactivate SH enzymes in the cascade of electron transfer. On the other hand, the primary target of $HgCl_2$ could be cell surface proteins to be directly cross-linked. This raises the possibility of stage-specific control of the signal transduction by the redox mechanism.

Although the concentration of MIA that was most active for accelerating protein tyrosine phosphorylation (1 mM) was lethal to the lymphocytes because of its general toxicity, relatively low concentrations (0.001–0.01 mM) of MIA, which more or less intensified the TCR-mediated signal for tyrosine phosphorylation of

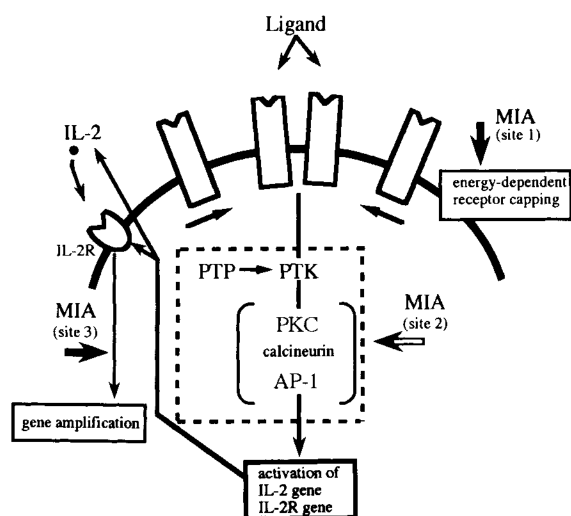


Fig. 9. The sites of action of MIA in the TCR-mediated signal transduction for activation and proliferation of T lymphocytes (a summary). In the cascade of TCR-mediated signal transduction, three MIA-sensitive sites have been demonstrated for regulation, two of them (site 1: energy-dependent membrane activity for receptor capping; site 3: signaling for DNA synthesis in the cell cycle) for downregulation and one (site 2: signaling for gene activation including genistein-sensitive PTK, H-7–sensitive PKC, and cyclosporin-sensitive calcineurin) for upregulation. In addition, potential inactivation of PTP by MIA may be linked to upregulation of protein tyrosine phosphorylation. Large thick arrow, downregulation; open arrow, upregulation.

the 40 kDa protein, have been shown to modulate the later signal for lymphocyte activation. These concentrations of MIA potentiated the signals for antireceptor mAb-induced *c-fos* transcription and for cytokine production and cytokine receptor expression. The MIA-accelerated anti-CD3 mAb-induced signal for IL-2 production was shown to be inhibited by genistein, H-7, and cyclosporin A. This suggested that MIA upregulated the signal in connection with the actions of PTK, PKC, and calcineurin.

The same concentration of MIA as upregulated cell activation was found to inhibit the antireceptor antibody-induced cell proliferation. This may suggest that the step of the signal delivery to promote the cell cycle for DNA synthesis involves some crucial element that must be inactivated by MIA. The action of MIA to inhibit a cell cycle–linked signal element for gene amplification contrasts its action to upregulate the signal mediated by PTK, PKC, and calcineurin for gene activation. This action of MIA in part mimics the action of rapamycin that preferentially inhibits the signal element needed for cell cycle [Brown et al., 1994]. The present result thus suggests that some element in the signal cascade for cell cycle is highly sensitive to sulfhydryl modification.

As summarized in Figure 9, our results suggest that there are multiple sites of action of MIA in the signal delivery for lymphocyte activation and proliferation of lymphocytes, which are affected by MIA in different modes. This in turn suggests that the TCR-mediated signal cascade of T lymphocytes for activation and proliferation is subject to multiphasic regulation by MIA-susceptible redox-linked mechanisms, although MIA may also affect other structures.

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