Inhibition of Ly-6A Antigen Expression Prevents T Cell Activation

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Summary

Antisense oligonucleotides complementary to the 5' end of the mRNA encoding the Ly-6A protein were used to block the expression of that protein. Using this approach we could inhibit the expression of Ly-6A by 60–80% in antigen-primed lymph node (LN) T cells as well as in the D10 T cell clone. Inhibition of Ly-6 expression resulted in the inability to restimulate in vitro, antigen-primed T cells. It also blocked the activation of normal spleen cells by Con A, monoclonal antibody (mAb) to CD3, and mAb to Ly-6. In contrast, stimulation of normal spleen cells with the pharmacological agents PMA + ionomycin were unaffected by the inhibition of Ly-6 expression. Similar results were obtained with the D10 T cell clone; stimulation with Con A + interleukin 1 (IL-1), antigen-presenting cells (APC), or the clonotypic antibody + IL-1 was greatly reduced in the presence of antisense oligonucleotides to Ly-6. Stimulation with PMA + ionomycin was again unaffected. We also studied the effect of antisense oligonucleotides on stimulation of preactivated D10 cells. Preactivation of D10 cells with Con A + IL-1 renders them receptive to secondary stimulation by other lymphokines. In this case, antisense oligonucleotides to Ly-6 had no effect on secondary activation with IL-2, IL-4 + IL-1, or PMA + ionomycin. We conclude from these studies that Ly-6 expression is required for T cell receptor (TCR)-mediated T cell activation.

The murine Ly-6 alloantigens are encoded by members of a multigene family located on chromosome 15 (1–3). The Ly-6 alloantigens are small phosphatidylinositol (PI)[1]-anchored, membrane glycoproteins with a molecular weight of 15,000–18,000 (4–6). Members of the Ly-6 family are expressed on the surface of a large number of different cell types, including T cells, B cells, thymocytes, macrophages, neutrophils, and BM cells (7, 8). Recent studies have shown that the expression of the Ly-6A-encoded protein is associated with CD4+ T cell activation and the acquisition of immunocompetence during T cell maturation in the thymus (9–13). It has been postulated that Ly-6 antigens, specifically Ly-6A, are involved in the transduction of signals originating in the CD3-TCR in CD4+ cells. We addressed this question by specifically inhibiting Ly-6A antigen expression using antisense oligonucleotides, and here we show that inhibition of Ly-6A antigen expression resulted in a dramatic inhibition of antigen- and mitogen-driven T cell activation without affecting lymphokine or pharmacologically induced T cell response. Thus,

it appears as though the Ly-6A antigen plays an important role in physiological T cell activation through the CD3/TCR complex.

Materials and Methods

Oligonucleotides. Antisense (5'-AGTGTGAGAAGTGTCCAT-3') and two control oligonucleotides (5'-GGTCAACGGTGAGGC-CAT-3', 5'-TCACACTCTTCACAGGTA-3') were synthesized using a DNA synthesizer (model 380A; Applied Biosystems, Foster City, CA). Oligonucleotides were purified on OPC columns (Applied Biosystems) according to the standard procedure recommended by the company, or by polyacrylamide gel electrophoresis followed by elution and passage over a Sephadex G-50 column (Pharmacia Fine Chemicals, Piscataway, NJ). After purification, oligonucleotides were resuspended in PBS before use.

Mice. BALB/c and C3H/HeJ female mice were purchased from the Jackson Laboratories, Bar Harbor, Maine.

T Cell Proliferation Assay. Mice were primed with 100 μg of fowl gamma globulin (FGG; Cappel Laboratories, Malvern, PA) in CFA (Difco Laboratories, Detroit, MI) in the hind footpad. 8 d later, draining popliteal lymph nodes were removed and 3 × 10⁵ lymph node cells were cultured in 100 μl of RPMI-1640 media

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[1] *Abbreviations used in this paper:* FGG, fowl gamma globulin; PI, phosphatidylinositol; PPD, purified protein derivative of tuberculin.

(Gibco Laboratories, Grand Island, NY), supplemented with anti-biotics, L-glutamine, 1% fresh mouse serum, and 5 × 10^{-5} M 2-ME in a 96-well flat-bottomed tissue culture plate (Falcon Labware, Lincoln Park, NJ). At time 0, antisense oligonucleotides at a concentration of 7.0 μM in PBS or PBS alone were added to cell cultures. 5 or 50 μg of FGG or 50 μg of purified protein derivative of tuberculin (PPD; Staten Serum Institute, Copenhagen, Denmark) were added to cultures 0, 24, or 48 h after addition of oligonucleotides. Cultures were incubated for 72 h after the addition of antigen. Cell proliferation was assayed by the addition of 1.0 μCi [^{3}H]thymidine (6.7 Ci/mmol; New England Nuclear, Boston, MA) during the last 12–16 h of culture. Cells were then harvested and processed for liquid scintillation measurement of radioactivity.

Stimulation of the D10.G4.1 T Cell Clone. D10.G4.1 T cells (a conalbumin plus I-A^k-specific T cell clone) were added to culture at a concentration of 2 × 10^4 cells/well in 100 μl of RPMI-FCS media. Antisense oligonucleotides at a concentration of 7.0 μM in PBS or PBS alone were then added to these cell cultures. 24 h later, D10 cells were stimulated with either 10 ng of the anticlonotypic mAb 3D3 + 10^{-7} M rIL-1, 50 μg/ml conalbumin + 10^5 mitomycin C–treated AKR spleen cells (which serve as APC), 1.5 μg/ml Con A + 10^{-7} M rIL-1, or a mixture of 1.0 μg/ml of PMA and 10.0 μg/ml of ionomycin. Cultures were incubated for 72 h after the addition of antigen, antibody, or pharmacological agents. Cell proliferation was assayed as described above.

Lymphokine-induced Proliferation of Preactivated D10.G4.1 Cells. D10.G4.1 T cells were activated with 1.5 μg Con A + 10^{-7} M IL-1 for 72 h to make these cells receptive to IL-2- and IL-4-mediated proliferation (14). Activated D10 cells were then harvested, washed, and added to culture at a concentration of 2 × 10^4 cells/well in 100 μl of RPMI-FCS media. Antisense oligonucleotides at a concentration of 7.0 μM in PBS or PBS alone were then added to these cell cultures. 24 h later, D10 cells were stimulated with either 10 ng of mAb 3D3 + 10^{-7} M rIL-1, 100 U of rIL-2, 500 U of rIL-4 (Amgen Corp., Thousand Oaks, CA) + 10^{-7} M rIL-1, or a mixture of 1.0 μg/ml of PMA and 10.0 μg/ml of ionomycin. Cultures were incubated for 72 h after the addition of antigen, antibody,

or pharmacological agents. Cell proliferation was assayed as described earlier.

In Vitro Stimulation of Naive Spleen Cells. Spleen cells from naive BALB/c mice were added to culture at a concentration of 2 × 10^5 cells/well in 100 μl of RPMI media containing 10% FCS. Antisense oligonucleotides at a concentration of 10.0 μM were added at time 0. 24 h later, cells were stimulated with either 1.5 μg/ml of Con A; 145.2C11, an anti-CD3 mAb (19); 70.94, an anti-Ly-6A mAb (1); or with a mixture of 1 μg/ml of PMA and 10 μg/ml of ionomycin. Cultures were incubated for 72 h after the addition of antigen, antibody, or pharmacological agents. Cell proliferation was assayed as described earlier.

Flow Cytometry. 10^6 viable cells were resuspended in 100 μl PBS + 0.1% sodium azide, and stained with biotin-labeled 70.94 (anti-Ly6A), biotin-labeled J1j (mAb anti-Thy-1), FITC-RL-172 (rat mAb anti–mouse CD4), RM2-2 (rat mAb anti–mouse CD2), and 2C11 (hamster mAb anti–mouse CD3). FITC-streptavidin (Zymed Laboratories, South San Francisco, CA), FITC-F(ab′)$_2$ mouse anti–rat Ig (Jackson ImmunoResearch, West Grove, PA), or FITC-IgG fraction of goat anti–hamster Ig (Cappel Laboratories) were used as the second stage reagents. Fluorescence analysis was carried out on an EPICS Profile Analyzer (Coulter Immunology, Hialeah, FL).

Results and Discussion

It was recently shown that the presence in vitro of DNA oligonucleotides complementary to a portion of mRNA encoding a particular protein (antisense oligonucleotides) can effectively inhibit the translation of that protein in cultured cells (for review see reference 15). We first studied the effect of antisense oligonucleotides directed against the Ly-6A mRNA on secondary in vitro stimulation of in vivo primed LN cells. As depicted in Fig. 1 *A*, LN cells primed to FGG in CFA responded poorly to FGG and to PPD when restimulated in vitro in the presence of Ly-6A antisense oligonucleotides. Control cultures without oligonucleotides or with
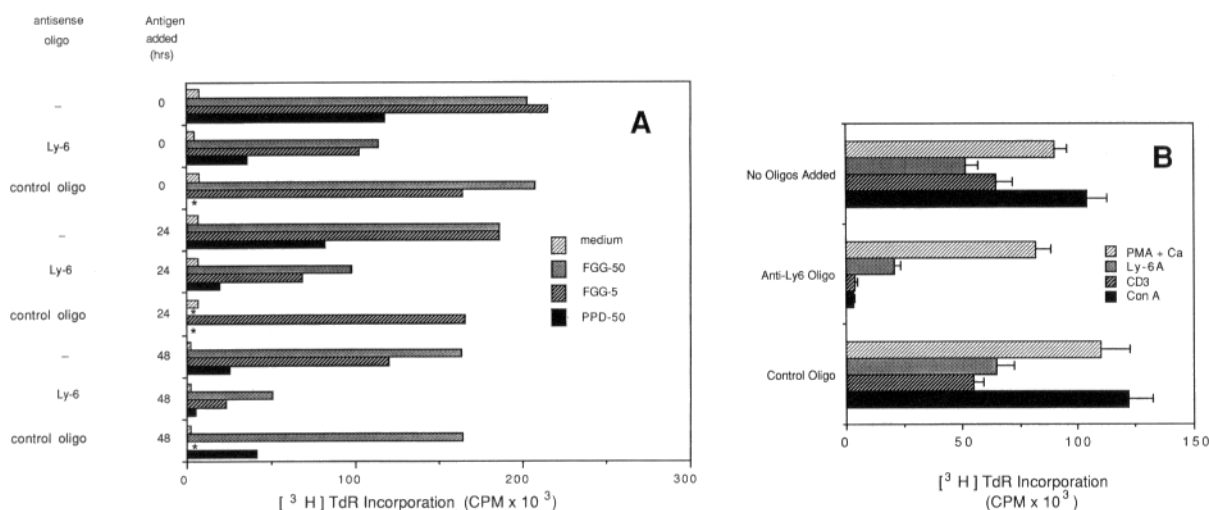
Figure 1. Inhibition of T cell proliferation by antisense oligonucleotides to the Ly-6A antigen. (*A*) FGG/CFA primed LN cells were restimulated in vitro with 5 or 50 μg/ml FGG or 50 μg/ml PPD 0, 24, or 48 h after the addition of oligonucleotides. Cell proliferation was assayed 72 h after the addition of antigen (Materials and Methods). All assays were performed in triplicate. Standard deviations were omitted for simplicity and were generally within 10–15% of the mean. (*) Not done. (*B*) Naive spleen cells were stimulated in vitro with PMA + ionomycin (PMA + Ca), mAb to Ly-6A (Ly-6A), mAb to CD3 (CD3), or Con A 24 h after the addition of oligonucleotides. Cell proliferation was assayed 72 h later. Results are presented as the mean response of triplicate cultures ± SEM.

irrelevant oligonucleotides responded very well. In this particular experiment, inhibition of proliferation was least apparent at time 0 and most apparent 48 h after the addition of antisense oligonucleotides. This was not always the case; in some experiments, the peak of inhibition was seen 24 h after the addition of the antisense oligonucleotides.

In Fig. 1 B, normal spleen cells were stimulated with the T cell mitogen Con A, anti-CD3 mAb, anti-Ly-6A mAb, or the combination of PMA and ionomycin, a calcium ionophore. In the presence of Ly-6A antisense oligonucleotides T cell activation was always inhibited except when the pharmacological agents PMA and ionomycin were used to induce cellular proliferation (Fig. 1 B). It is important to note that all the activation signals that were inhibited by Ly-6A antisense oligonucleotides have been previously shown to be delivered through the CD3/TCR complex (11, 14, 16).

We then tested whether Ly-6A antisense oligonucleotides could inhibit the proliferation of a cloned CD4+, antigen-specific T cell line. We used the T cell clone D10.G4.1 (D10), a conalbumin plus I-A^k–specific Th2 T cell clone (17). D10 cells were stimulated with either antigen plus I-A^{k+} APCs, Con A + IL-1, 3D3 (an anticlonotypic mAb, 18) + IL-1, or PMA + ionomycin. The results illustrated in Fig. 2 show once again that activation with anti-TCR antibodies, mitogen, and antigen was inhibited in the presence of Ly-6A antisense oligonucleotides but not in the presence of an irrelevant oligonucleotide (Fig. 2). Again, activation by PMA + ionomycin in the presence of Ly-6A antisense oligonucleotides was unaffected.

The activation pathway of the D10 clone allowed us to further define the stage of activation where the Ly-6A an-

tigen is required. It was previously shown that the D10 clone first requires an activation signal such as Con A + IL-1 in order to be receptive to stimulation by other lymphokines such as IL-2 or IL-4 + IL-1 (14). In the experiments shown in Fig. 3, D10 cells were first activated with Con A + IL-1 for 3 d, washed, and restimulated with IL-2, IL-4 + IL-1, 3D3 + IL-1, or PMA + ionomycin. In this case, the presence of Ly-6A antisense oligonucleotides had no effect on the secondary activation by IL-2, IL-4 + IL-1, or PMA + ionomycin. Only the physiological activation by the clonotypic antibody 3D3 + IL-1 was greatly reduced, since a preactivation step is not required. It seems therefore that once D10 cells receive the first activation signal by Con A + IL-1, Ly-6A is no longer required for activation by other lymphokines.

The inhibitory effects of Ly-6A antisense oligonucleotides on T cell activation appear to be due to the inhibition of Ly-6A expression during activation. Flow cytometric analysis of LN cells stimulated with FGG in the presence of Ly-6A antisense oligonucleotides showed that the expression of Ly-6A membrane antigens was specifically reduced by 60–80% as compared with cells stimulated in the absence of oligonucleotides or in the presence of irrelevant oligonucleotides (Fig. 4). The expression of Thy-1 (another PI-linked protein), CD2, CD3, and CD4, all of which were shown to be involved in T cell activation, was unaffected (Fig. 4). Similar selective reduction of Ly-6A antigen expression by Ly-6A antisense oligonucleotides was also seen on D10 cells stimulated by Con A + IL-1 (data not shown).

These results indicate that the expression of the Ly-6A protein on the cell surface is required for TCR-mediated activation of CD4+ T cells. Thus, activation by antigen + MHC, mitogen, and anti-TCR antibodies was inhibited by Ly-6A-
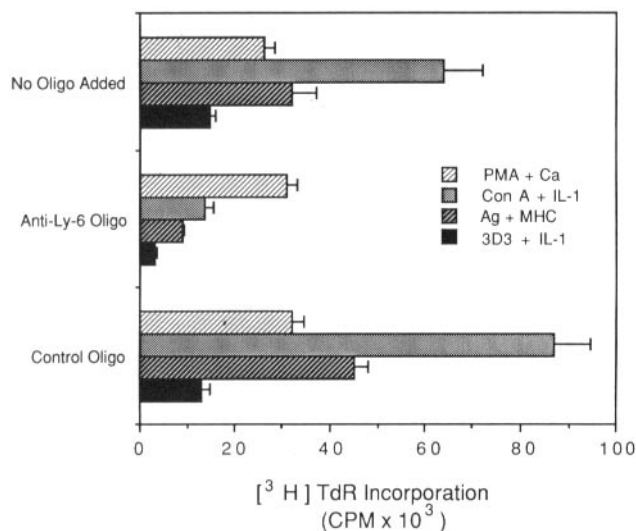
Figure 2. Inhibition of proliferation of the antigen-specific T cell clone D10.G4.1 by antisense oligonucleotides to the Ly-6A antigen. 2 × 10⁴ D10 cells were cultured for 24 h with antisense or control oligonucleotides and then stimulated with PMA + ionomycin (PMA + Ca), Con A + IL-1, conalbumin + APCs (Ag + MHC), or the mAb 3D3 + IL-1. Cell proliferation was assayed 72 h later. Results are presented as the mean response of triplicate cultures ± SEM.
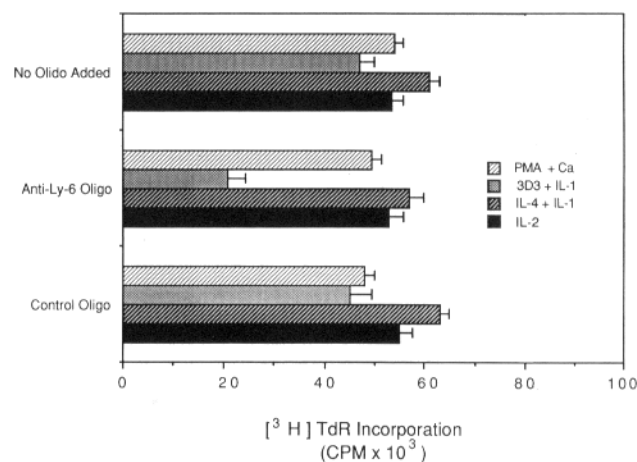
Figure 3. Antisense oligonucleotides to Ly-6A do not inhibit lymphokine-induced proliferation of preactivated D10.G4.1 cells. D10 cells were first activated with Con A + IL-1 for 72 h. 2 × 10⁴ activated D10 cells were then cultured for 24 h with antisense or control oligonucleotides followed by stimulation with PMA + ionomycin (PMA + Ca), the mAb 3D3 + IL-1 (3D3 + IL-1), IL-4 + IL-1, or IL-2. Cell proliferation was assayed 72 h later. Results are presented as the mean response of triplicate cultures ± SEM.
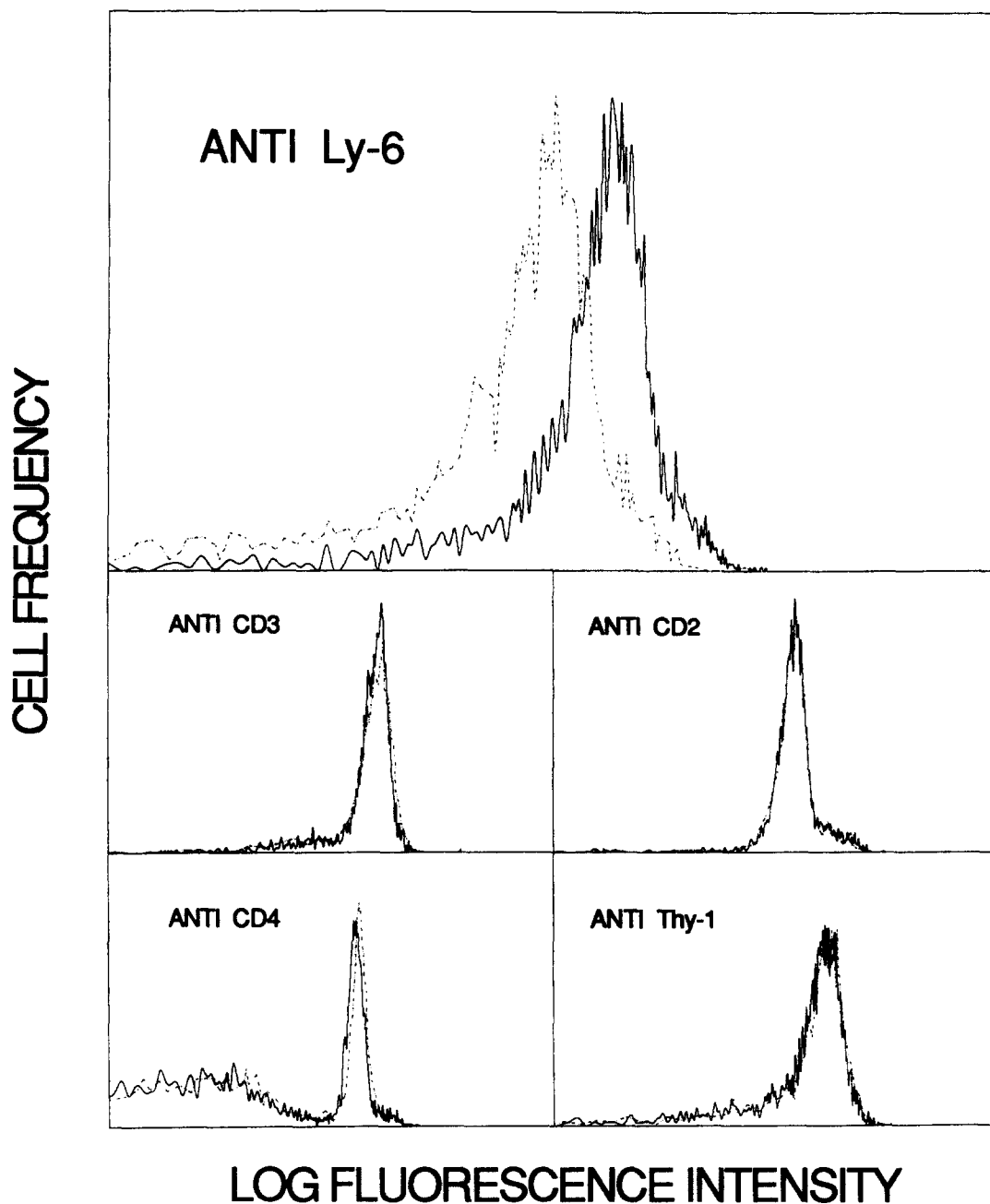
Figure 4. Inhibition of membrane Ly-6A antigen expression on cells treated with antisense oligonucleotides to Ly-6A. Primed LN T cells (J11d + complement–treated LN cells) were cultured for 24 h with antisense or control oligonucleotides and then stimulated with 50 μg/ml of antigen. 24 h later cells were harvested and stained with anti-Ly-6A, anti-CD2, anti-CD3, anti-CD4, or anti-Thy-1. (*Broken lines*) cells from cultures containing Ly-6A antisense oligonucleotides. (*Solid lines*) cells from cultures containing control oligonucleotides.

specific antisense oligonucleotides, while lymphokine and pharmacological activation were unaffected. The fact that antisense oligonucleotides can inhibit APC-dependent activation (mitogen, antigen + MHC, and anti-Ly6A mAb [9, 19]) and APC-independent responses (anti-CD3 and anticlonotypic mAb + IL-1) precludes the possibility that the antisense oligonucleotides inhibit T cell activation by inhibiting the expression of Ly-6 antigens only on APC (20). Furthermore,

flow cytometric analysis confirmed the fact that Ly-6A antisense oligonucleotides specifically inhibited Ly-6A expression on T cells and T cell clones and did not effect expression of CD2, CD3, and CD4, all of which are surface proteins important in T cell activation. It was previously shown that T cell hybridomas lacking the expression of Ly-6A, or cells treated with phosphatidylinositol phospholipase C (PI-PLC) to remove all PI-linked proteins (including all Ly-6 proteins),

show markedly decreased responses to activation signals transduced via the TCR (13). It was also shown that crosslinking of the Ly-6 surface proteins by mAbs resulted in T cell activation, which in the case of Ly-6A antigens, was dependent upon the presence of the CD3/TCR complex (11) since T cells that did not express the CD3-TCR complex were refractory to stimulation by crosslinking Ly-6A antigens (11, 12). These as well as other studies (9–13) strongly suggested that Ly-6-encoded proteins are involved in TCR-mediated signal transduction. Our studies indicate that Ly-6A-encoded proteins are an absolute requirement for optimal activation induced by this type of signal transduction.

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