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ANCHORED ANTI-HLA CLASS I MONOCLONAL ANTIBODY FAILS TO INDUCE INHIBITION OF PHA- ACTIVATED LYMPHOCYTES PROLIFERATION

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SUMMARY: It is known that anti-HLA Class I antibodies inhibit the proliferative response of PHA-activated T-lymphocytes. We found that plastic- or sepharose-linked anti-HLA Class I monoclonal antibody 01.65 does not inhibit either {^{3}H}Thymidine incorporation or recruitment in the cell cycle, nor does it reduce the expression of c-myc mRNA and the membrane expression of Interleukin-2 Receptor and Transferrin Receptor. Furthermore, particulate Protein Kinase C is not affected by anchored anti-HLA Class I monoclonal antibody 01.65. We suggest that anti-HLA Class I monoclonal antibody may act through crosslinking or internalization of HLA Class I antigens.

The main documented role of HLA Class I molecules is the presentation of a foreign antigen to T lymphocytes. HLA Class I molecules take up peptides from proteins synthesized by antigen-presenting cells at endoplasmic reticulum (ER) level, and follow the constitutive secretory pathway from ER, through Golgi to the cell surface (1). A number of studies, however, have suggested that they also have non-immunological functions. There is evidence that HLA Class I glycoproteins may play a regulatory role in the proliferative response of human T-lymphocytes to intracellular Ca^{2+}-mobilizing mitogens such as lectins, agonistic antigens or monoclonal antibodies (mAbs) recognizing CD2 or CD3 molecules (2-6). In fact, anti-HLA Class I mAbs inhibit the proliferation induced by agonistic antigens, lectins or agents acting through the CD3 (7) or CD2 pathways (8-9). In phytohemagglutinin (PHA) -activated lymphocytes, anti-HLA Class I mAb 01.65 does not affect either the increase in cytosolic free Ca^{2+} concentration or inositol phosphate generation (6). Several reports indicate that Protein Kinase C (PKC) is involved in the inhibitory

ABBREVIATIONS: BrdU, 5-Bromo-2-deoxyuridine; ER, Endoplasmic Reticulum; IL-2R, Interleukin-2 Receptor; IDMEM, Iscove's Modified Minimal Essential Medium; mAb, monoclonal Antibody; PBMC, Peripheral Blood Mononuclear Cells; PHA, Phytohemagglutinin; PKC, Protein Kinase C; RF, Recruitment Fraction in the cell cycle; TfR, Transferrin Receptor.

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function of anti-HLA Class I mAbs: corpuscolete PKC is completely depleted in PHA-stimulated lymphocytes upon treatment with anti-HLA Class I mAb 01.65 (10-12). In phorbol esters-treated lymphocytes, anti-HLA Class I mAbs do not affect either PKC levels, or proliferative response (9,12,13). Furthermore, N-N-staurosporine, a potent PKC inhibitor, is competitive with mAb 01.65 in affecting both cellular PKC level and proliferative response (14), a fact which suggests the existence of a common target. In PHA-activated lymphocytes, mAb 01.65 treatment induces a reduction in the expression of c-myc, Interleukin-2 Receptor (IL-2R), cdc2 and Thymidine kinase mRNAs (15, 16), a reduction of the percentage of cell recruited in the cell cycle (RF) (17) and an extension of the cell cycle's duration (18). In this paper we report that in PHA-activated lymphocytes plastic-coated 01.65 neither inhibit tritiated Thymidine ({^{3}H}dT) incorporation nor modifies proliferation parameters such as the recruitment in the cell cycle and the expression of IL-2R and Transferrin receptor (TfR). Furthermore, no inhibition of particulate PKC or alteration of c-myc mRNA expression is observed.

MATERIALS AND METHODS

Cell cultures. Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors by standard Ficoll/Hypaque density gradient centrifugation and cultured at 10^6 cells /ml in Iscove's Modified Dulbecco's Minimal Essential Medium (IDMEM) (Gibco) supplemented with 10% fetal calf serum (Flow Laboratories) and antibiotics (100 U/ml penicillin and 100 µg/ml gentamycin). Cells were stimulated with 1 µg/ml Phytohemagglutinin-P (Wellcome Laboratories). PBMC were cultured in 96-flat well plates. Incubations were carried out in the dark at 37°C in a 5% CO_2 humidified atmosphere.

Antibodies. The mAb 01.65 (19), which recognizes a framework determinant expressed on the heavy chain of HLA Class I antigens, was administered at 25 µg/ml. Sepharose-linked mAb 01.65 was obtained incubating 1 volume of swollen CNBr-activated Sepharose 4B (Pharmacia) with 1 volume of 5.8 mg/ml mAb 01.65 overnight at 4°C and subsequent washing in 1M NaCl, 0.05M phosphate buffered solution. As deduced by spectrophotometric analysis, 89% of mAb was linked to sepharose. Sepharose-linked mAb 01.65 was used at 50 µg/ml. Linkage of mAb 01.65 to the plastic surface was obtained by treating 96 flat-bottom wells PROBIND assay plates (Falcon) with 200 µl/well of 250 µg/ml mAb 01.65 in carbonate buffer pH 9.7 overnight at room temperature. As measured by spectrophotometric analysis of mAb concentration before and after treatment of plates, 9.6 µg of mAb per well were linked to the plastic. The release of mAb 01.65 from the plastic surface was tested by treating PBMC with IDMEM previously incubated 72 hours at 37°C in mAb 01.65-coated plates and detecting the immunofluorescence level after treatment with anti-mouse FITC linked mAb (Becton Dickinson). Flow cytometric analysis revealed that less than 1% of mAb 01.65 was released from the plastic, as deduced by comparison with immunofluorescence of PBMC treated with soluble mAb 01.65 in IDMEM.

{^{3}H}dT incorporation assay. 5 µCi/ml {^{3}H}dT (Amersham) was added 6 hours before harvesting. Results were expressed as the mean of sextuplicated cultures.

Flow cytometry. IL-2R (CD25) and Transferrin receptor (CD71) expression analyses were performed by direct immunofluorescence and flow cytometry following routine methods (17) and using a FACStar flow cytometer (Becton Dickinson) equipped with a 488 nm argon laser. For RF analysis $2x10^6$ cells per point were grown in medium containing 10 µM 5-Bromo-2-deoxyuridine

(BrdU) (Sigma). After 72 hours the cells were harvested and immunostained as previously described (17). The RF was calculated as the fraction of BrdU positive cells.

RNA analysis. RNA extraction was performed according to Sambrook et al. (20).The c-myc cDNA probe has already been described (21).

PKC analysis. 2 hours after PHA activation, PBMC were resuspended at $20x10^6$ cells/ml in 0.25M Sucrose (Sigma), 10mM Hepes pH 7.5 (Sigma), 5mM EDTA (Sigma), 10mM 2-Mercaptoethanol (Fluka), 2mM Phenyl-Methyl-Sulphonylfluoride (Sigma) and 0.01% Leupeptin (Sigma) for six times with 10-sec bursts from a MSE model MK2 sonicator. The lysate was then centrifugated at 100.000 g for 10 min, to separate the cytosolic fraction (supernatant) from the particulate fraction (pellet). The latter was resuspended in the same buffer to which 0.1% Triton x-100 (Sigma) has been added. From both cytosolic and particulate fractions, PKC was partially purified by chromatography on DEAE-cellulose column (DE-52, Whatman) equilibrated with 10mM Hepes pH 7.5, 10mM 2-Mercaptoethanol and 5mM EDTA. PKC was eluted with 0.13 M NaCl and assayed according to Melloni et al.(22). One unit of PKC activity was defined as the amount causing the incorporation of 1nM of ^{32}P into Histone IIIS under experimental conditions.

RESULTS AND DISCUSSION

The effect of plastic-linked mAb 01.65 was tested by culturing PHA-activated PBMC in mAb 01.65-coated plates or treating PBMC with sepharose-linked mAb 01.65. Controls were PHA-activated PBMC treated with soluble mAb 01.65 or with no mAb. The efficiency of linking of mAb 01.65 to plastic was assayed, as reported in Matherials and Methods. While soluble mAb 01.65 strongly inhibits {^{3}H}dT incorporation (Fig. 1) and reduces the fraction of cells entering the cell cycle (Fig. 2), neither plastic-linked nor sepharose-linked mAb 01.65 significantly affect these proliferation parameters. Furthermore, we found that plastic-linked mAb 01.65 does not modify the

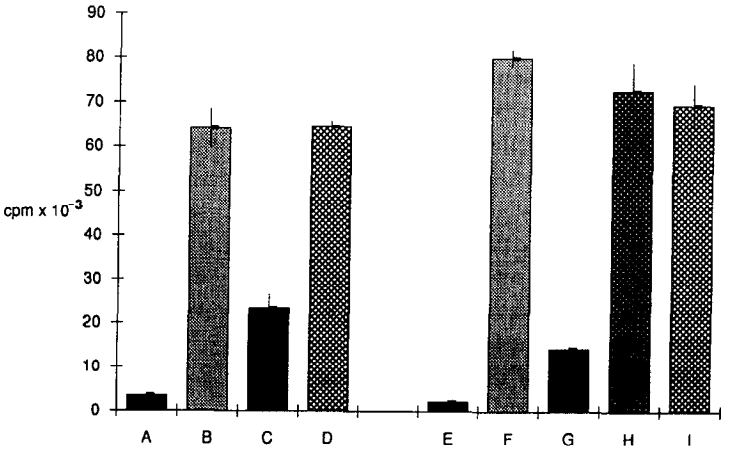

Fig. 1.
{^{3}H}dT incorporation in PBMC at 72 hours. A, E: null; B-D, F-I: PHA-activated PBMC; C, G: treated with soluble mAb 01.65. D: treated with plastic-linked mAb 01.65; H: treated with sepharose beads; I: treated with sepharose-linked mAb 01.65.

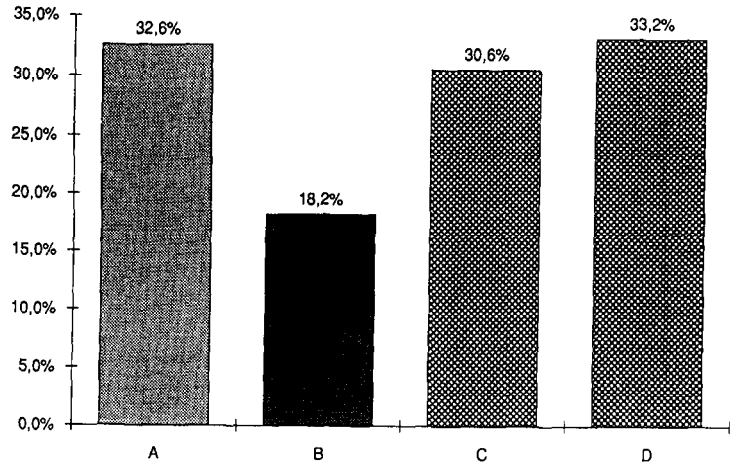

Fig. 2.
Recruitment of cells in the cell cycle. PBMC were cultured 72 hours in presence of PHA and BUdR. A: treated with no mAb. B: treated with soluble mAb 01.65. C: treated with plastic-linked mAb 01.65; D: treated with sepharose-linked mAb 01.65.

expression of IL-2R and TfR (Fig. 3 and 4). On the contrary, in cultures treated with soluble mAb 01.65, both the percentage of cells expressing IL-2R and TfR, and the mean cell density of these receptors, are reduced. It is known that in PHA-activated lymphocytes c-myc mRNA levels have an early peak 4-6 hours after activation and a late increase after 24 hours (23). Furthermore, it has been reported that only late c-myc expression is affected by mAb 01.65 treatment (15). We found

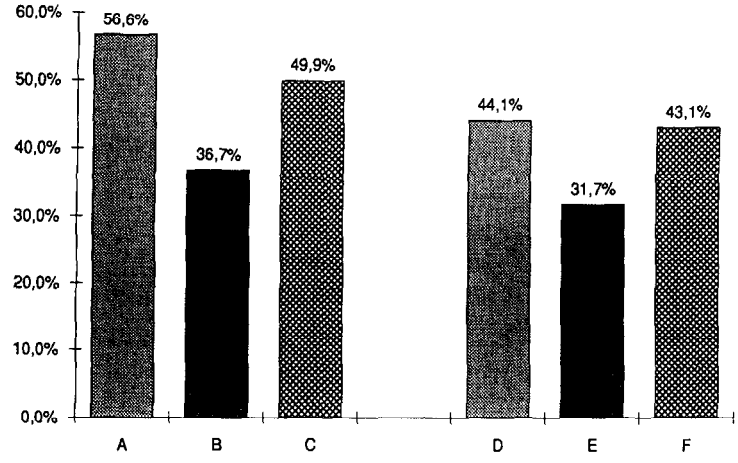

Fig. 3.
IL-2R expression (A-C) and TfR expression (D-F) in PBMC 72 hours after PHA-activation. Data are expressed as percentage of positive cells. A, D: treated with no mAb; B, E: treated with soluble mAb 01.65; C, F: treated with plastic-linked mAb 01.65.

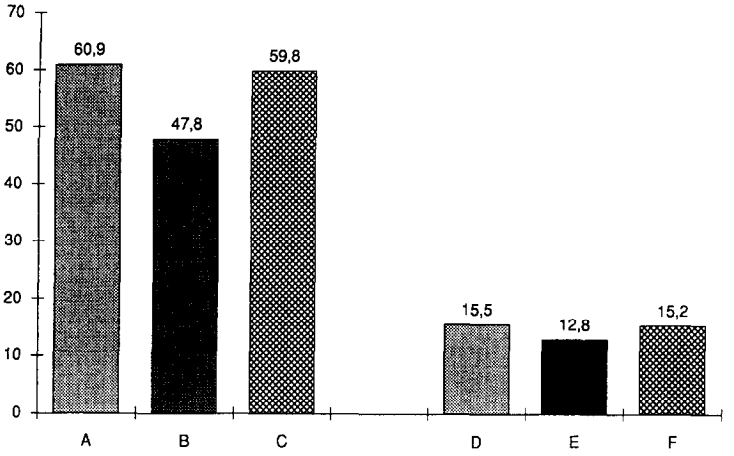

Fig. 4.
Levels of IL-2R (A-C) and TfR (D-F) expression in PHA-activated PBMC at 72 hours. A, D: treated with no mAb; B, E: treated with soluble mAb 01.65; C, F: treated with plastic-linked mAb 01.65. Fluorescence levels (linear scale) are expressed in arbitrary units.

that late c-myc expression, as measured after 48 hours, is reduced by about 50% in soluble mAb 01.65 treated PBMC but is not affected in PBMC grown in mAb 01.65-coated plates. Among the early events after PHA activation, only particulate PKC is reduced by mAb 01.65 treatment (10). 2 hours after PHA activation, while particulate PKC is reduced by 86% in soluble mAb 01.65 treated PBMC, no significant inhibition was found in PBMC treated with plastic- or sepharose-linked mAb 01.65 (Fig. 5 and 6).

Our results indicate that when PHA-activated human T-lymphocytes are treated with anchored mAb 01.65 all known inhibitory effects are no longer detectable. All the studied proliferation markers - {^{3}H}dT incorporation, RF, c-myc mRNA expression, membrane expression of IL-2R and TfR and

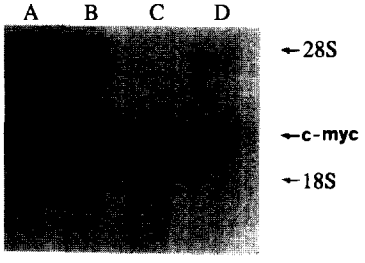

Fig. 5.
c-myc mRNA expression in PBMC culture at 72 hours incubation. A: unstimulated; B: PHA-activated PBMC; C: PHA-activated PBMC treated with soluble mAb 01.65; D: PHA-activated PBMC treated with plastic-linked mAb 01.65.

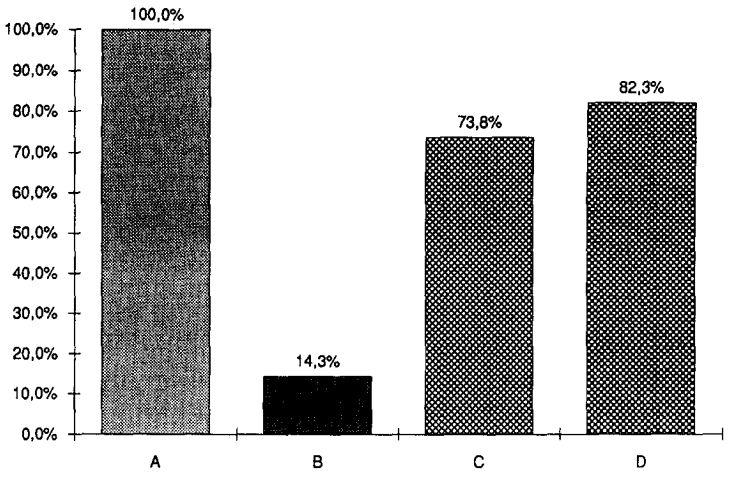

Fig. 6.
Particulate PKC activity in PBMC 2 hours after PHA stimulation. A: treated with no mAb; B: treated with soluble mAb 01.65; C: treated with plastic-linked mAb 01.65; D: treated with sepharose-linked mAb 01.65.

particulate PKC values - are not modified. The ineffectiveness of anchored mAb 01.65 indicates that the interaction between HLA Class I molecules and the specific antibody is not sufficient to inhibit lymphocyte proliferation. This may be the effect of the prevention of crosslinking of HLA Class I antigens or of interference with their internalization and recycling. It has been suggested that the degree of inhibition might be related to the degree of crosslinking of HLA Class I antigens on the cell membrane (7). When anchored antibody is used, either the number of HLA antigens involved mayt be quantitatively too low, or their mobility on the cell membrane reduced, thus preventing the aggregation of HLA Class I molecules.

Internalization of MHC Class I antigens spontaneously occurs in lectin-activated T-lymphocytes, and, to an even lower extent, in resting T-lymphocytes (24). The phoenomenon is specific, since it does not occour in B-lymphocytes even after activation, or in non-lymphoid cells. It has been reported that binding of mAb directed against MHC Class I determinants has little effect on the internalization of these molecules in activated T-lymphocytes, and does not induce MHC Class I antigen internalization in cells that do not internalize them spontaneously (25). HLA Class I molecule endocytosis follows the pattern of other cell membrane receptors involved in constitutive and ligand-induced endocytosis. Receptors cluster in clathrin-coated pits, enter the cell via clathrin-coated vescicles, and then pass to acidified endosomes where ligands and receptors are sorted. They may eventually be recycled to the cell surface, or stored intracellularly, or degraded in lysosomes (26). Amino acid sequences encoded by exon seven of HLA Class I genes apparently constitute a specific signal required for endocytosis (27). Bound anti-HLA Class I mAbs are internalized and cycled back to the cell surface with HLA Class I molecules still attached (28). We

suggest that the internalization of mAb-bound HLA Class I molecules may interfere with the co-internalization of molecules associated with HLA Class I antigens on the cell membrane. However, we do not exclude that specific signals may be released along the endocytic route, even through the liberation of inhibiting molecules, affecting the Ca^{2+} dependent-PKC activation pathway. This phenomenon may be the result of a physiological system regulationg PKC activity in lectin-activated T-lymphocytes.

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