

CD3-associated heterodimeric polypeptides on suppressor hybridomas define biologically active inhibitory cells

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ABSTRACT We have investigated the relationship between CD3 expression and the suppressor T-cell function. We have isolated stable clonal cell lines of the F12.23 suppressor T-cell hybridoma that are either CD3⁺ or CD3⁻. These lines were subjected to functional assays including inhibition of *in vivo* hapten-specific delayed-type hypersensitivity responses, *in vitro* hapten-specific interleukin 2 responses, as well as hapten-specific cytotoxic T-lymphocyte assays. In all assays, the functional suppressor phenotype absolutely correlated with CD3 surface expression. Furthermore, we have immunoprecipitated heterodimeric proteins that share molecular features with some receptor polypeptides previously described. CD3 polypeptides found on the surface of suppressor T cells are phosphorylated after phorbol ester stimulation. Collectively these studies unambiguously define the suppressive supernatant function as a product of CD3⁺ receptor-bearing T cells.

Several studies have revealed that peripheral thymus-derived (T) lymphocytes express heterodimeric receptors associated with CD3 proteins (1-5). CD3-associated molecules have been found on helper and cytolytic T cells (3, 4). These receptors are involved in the recognition of foreign antigens that are presented to T cells in the context of proteins encoded by the major histocompatibility complex (6, 7). Little is known about the type of receptors found on the surface of suppressor T cells (8-11). The difficulties in unambiguous definition of the suppressor receptor proteins can be attributed to the lack of high-affinity antibodies that identify receptor proteins. Another source of ambiguity arises from the general reliance on complex *in vivo* biological assays to monitor suppressor activity (8-11). These assays usually involve intricate cellular interactions.

We have studied certain features of the immune response to the azobenzenearsonate hapten (ABA). ABA coupled to cell surface proteins has been a useful tool by which to study regulatory T cells, the general characterization of various types of cells involved in suppression, and the genetic restrictions by which they function (10). However, despite the production of several ABA-specific suppressor T-cell hybridomas (12), we have been unable to characterize the cell surface receptors present on suppressor cells until the present time.

In this report we have used monoclonal anti-CD3 antibodies to isolate clones of CD3⁺ cells from a CD5⁺ Thy-1.1⁺ Thy-1.2⁺ suppressor hybridoma cell line. We have found that only 3% of the original, supposedly clonal, hybrid cells expressed CD3 proteins. When these cells were repeatedly sorted, cloned, and subjected to biochemical and biological assays, suppressive activity absolutely segregated and correlated with the expression of CD3 molecules. Furthermore,

these cells express heterodimeric proteins on their surface that are coprecipitated with the CD3 polypeptides. This approach unambiguously identifies these suppressor T cells as CD3-expressing lymphocytes and should facilitate molecular clarification of suppressive proteins.

MATERIALS

Mice. Female A/J mice were obtained from The Jackson Laboratory. All mice used in these experiments were 8-10 weeks of age at the beginning of experiments, and each experimental group consisted of at least four animals.

T-Cell Hybrids and Culture Medium Supernatants. BW-5147, the suppressor T-cell hybridoma parental line F12.23, and all descendent cell lines were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum (FCS) at 37°C in a humidified, 10% CO₂/90% air atmosphere. Conditioned medium was collected when the cell number reached 6-10 × 10⁵ cells per ml and concentrated 10-fold for injection into mice, as described (13). For cloning, 0.3 cell per well was seeded into 96-well round-bottom (Falcon) plates and incubated in DMEM supplemented with 10% FCS and maintained as above.

Fluorescence-Activated Cell Sorter (FACS) Analysis. Hybridomas were evaluated by indirect fluorescence. Cells were adjusted to 1-2 × 10⁶ cells per sample, washed twice in FACS buffer (18), and resuspended in 150 μl of FACS buffer. Samples were then incubated with 10 μl of conditioned culture supernatants of the 145-2C11 hybridoma cell line (a hamster IgG antibody) for CD3 determination (14) or, as positive control, with purified HO13.4 (anti-Thy-1.2) (15) for 1 hr on ice. Additional negative-control primary antibodies included 7.9.5 (an anti-IgG) and 7.16.5 (an anti-IgM) (16). Both bind the *neu* oncogene-encoded protein p185 that is not expressed on murine lymphoid cells (17). Neither 7.9.5 nor 7.16.5 antibodies stained any primary hybridoma, any subclone, or any primary T-cell population. After the 1-hr incubation, the cells were washed three times and resuspended with a 1:100 dilution of secondary antisera (anti-hamster IgG or anti-mouse IgG) conjugated with fluorescein isothiocyanate for 1 hr. The cells were washed and analyzed on a Becton Dickinson FACS IV. The instrument was set at a gain of 4:1 with the 514-nm line of the laser for excitation and with barrier filters for an emission setting of 550 nm. In all cases 10,000 cells were analyzed and the percentage of positive cells was recorded above the autofluorescent threshold established by running the same clone in the presence of isotype-matched control antibody stained with the secondary reagent.

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Abbreviations: ABA, azobenzenearsonate; DTH, delayed-type hypersensitivity; FCS, fetal calf serum; FACS, fluorescence-activated cell sorter; IL-2, interleukin 2; PMA, phorbol 12-myristate 13-acetate.

Subcloning of F12.23 Cells. The F12.23 cell line has been described in detail (12). This cell line was constructed by fusing spleen cells from ABA-suppressed animals with the murine fusion partner BW5147. Suppressor clones were selected that secrete soluble factors that only inhibit ABA-specific immune reactions. Subclones were initially selected for functional activity. Our initial enrichment of F12.23 was accomplished by selecting for hybridoma cells that reacted with 145-2C11 antibody-coated plates, as described (18). Plastic Petri dishes were prepared by incubating at 37°C in 0.5 M Na₂CO₃ (pH 9.3) plus 10 µg of goat anti-hamster IgG antiserum (Cappel Laboratories, West Chester, PA). Plates were washed twice and unoccupied sites were blocked with medium plus 1% FCS for 60 min. F12.23 cells that had been incubated on ice with 25 µl of conditioned supernate from the 145-2C11 hybridoma were washed twice to remove excess antibody and added to antibody-coated plates with fresh medium containing 1% FCS. After 60 min, the plates were washed three times and adherent cells were removed by incubation on ice followed by a wash with chilled medium. The collected cells were recultured and then analyzed further.

FACS Sorting. Positive cells were sorted, reanalyzed, and immediately cloned by limiting dilution. After 2–4 weeks, growing clones were reanalyzed by flow microfluorimetry for expression of the T3 surface antigen. Seven days later, the positive cells were rescreened and clones were selected for recloning by limiting dilution (14). This cycle was repeated a minimum of five times.

Preparation of ABA. The ABA diazonium salt was prepared from *p*-arsanilic acid (Eastman Kodak). The ABA solution was activated and was conjugated to erythrocyte-free spleen cells in single cell suspensions, as described (12, 13). After being washed three times in DMEM without serum, the ABA-coupled spleen cells were utilized for priming A/J mice or for priming spleen cell cultures.

Cell-Mediated Immunity Assay. ABA-coupled spleen cells (3×10^7 cells) were injected subcutaneously in two separate sites on the dorsal flanks of the primed mice. In suppression studies, mice received five daily i.v. injections at 0.2 ml per mouse per day, as described by Bach *et al.* (13). Mice were challenged on day 5 by injecting 30 µl of a solution of 10 mM ABA diazonium salt into the left hind footpad. The footpad was measured 24 hr later and 48 hr later by using a Fowler micrometer (Schlessinger's Tools, Brooklyn, NY). The delayed-type hypersensitivity (DTH) reaction was expressed as the increment of thickness of the challenged left footpad compared with the untreated right footpad in 10^{-2} mm (mean \pm SEM).

Interleukin 2 (IL-2) Assay. Test animals were sacrificed by cervical dislocation and spleen cells from each group were collected and cultured in 24-well culture plates at 5×10^6 cells per well with 5×10^6 γ -irradiated ABA-coupled stimulator cells. After 48 hr, conditioned medium supernates were collected, clarified by centrifugation, filtered through 0.22-µm pore-size filters (Millipore), and added to 5×10^3 CTLL-2 cells, as described (18). After 24 hr, the cells were pulsed for 6 hr with tritiated thymidine, harvested, and counted. The results are expressed as the means \pm experimental error of triplicate cultures.

Immunoprecipitations. Surface proteins on cells were labeled with ¹²⁵I by the lactoperoxidase method, and cells were lysed in Triton lysis buffer, as described (1, 3). Clarified lysates were precleared with anti-mouse immunoglobulin directly coupled to agarose beads. The lymph node lysates were then precipitated with 50 µl of 145-2C11 culture supernatant and the F12.23 CD3⁺ cells were precipitated with 100 µl of 4-day conditioned medium supernatants collected from 145-2C11 hybridoma cells in the presence of protein A-agarose beads. The beads were washed several times with lysis buffer (3) and bound receptor eluted by incubation of the protein A beads in NaDodSO₄ sample buffer at 68°C (1, 3).

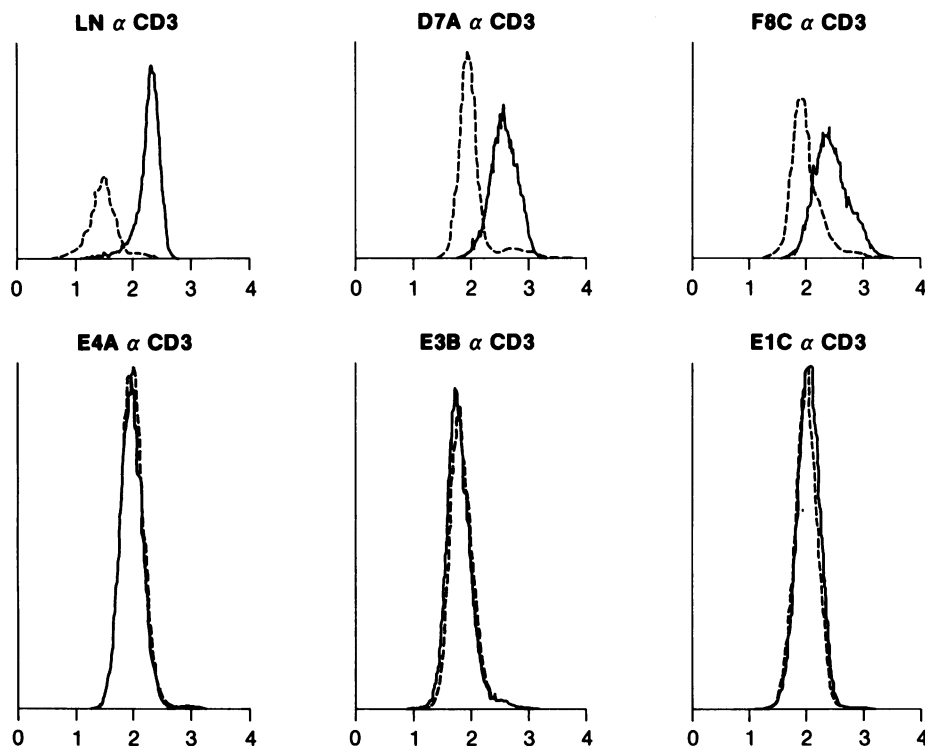


FIG. 1. FACS analysis of phenotypically cloned suppressor cells. Cells (10^6 cells) were labeled and subjected to FACS analysis. Ln, nylon-wool-purified lymph node-derived T cells; D7A and F8C, suppressor T-cell lines phenotypically cloned and scored as CD3⁺; E4A, E3B, and E1C, suppressor T-cell lines phenotypically cloned as CD3⁻ cell lines; dotted line, irrelevant primary antibody; solid line, 145-2C11 primary antibody.

Samples were analyzed by NaDodSO₄/polyacrylamide gels either under nonreducing conditions or under reducing condition after the addition of 5% (vol/vol) 2-mercaptoethanol.

CD3 Labeling. CD3 molecules were labeled by phorbol 12-myristate 13-acetate (PMA)-induced phosphorylation by incubating 5×10^6 cells for 4 hr in phosphate-free medium containing 500 μ Ci of ³²P_i (New England Nuclear) supplemented with 10% dialyzed FCS (HyClone, Logan, UT), penicillin at 10,000 μ g/ml, streptomycin at 10,000 μ g/ml, and L-glutamine at 29.23 μ g/ml. The cells were then placed on ice for 15 min and PMA (Calbiochem) was added to stimulate phosphorylation (20). Solubilization and immunoprecipitation followed by NaDodSO₄/PAGE is as described above for surface-labeled lysates.

RESULTS

Cloning of CD3⁺ Cells. We employed CD3- ϵ reactive monoclonal antibodies to pan and then to repeatedly sort the F12.23 hybrid line. Sorting was followed immediately by *in vitro* limiting dilution cloning. This procedure of sorting and cloning was repeated five to seven times. By this procedure, we were able to isolate T-hybridoma clones that either did or did not express CD3 surface molecules. The criterion for stable expression of CD3 molecules was an unchanging FACS profile on three consecutive assays over a 6-week period. It was possible to isolate several stable CD3⁻ clones from the original hybridoma cell population. However, many CD3⁺ lines did not meet our criteria for maintaining stable

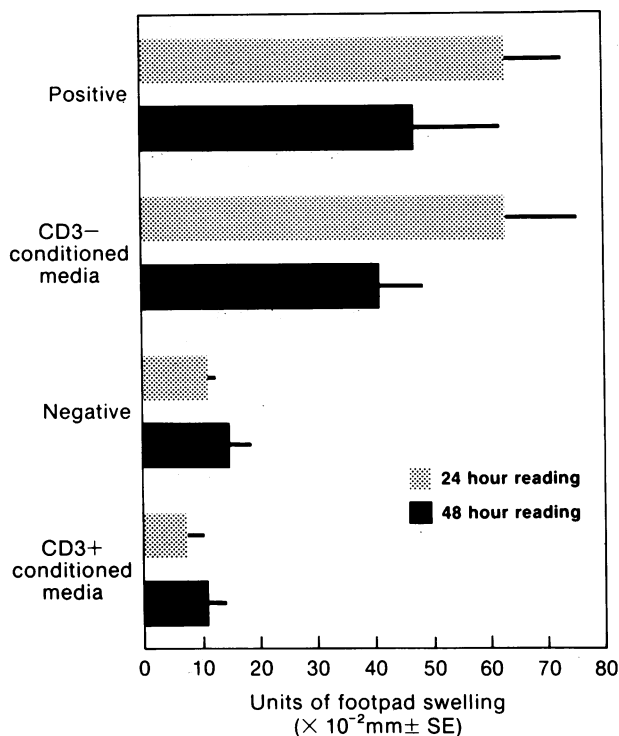


FIG. 2. Suppression of DTH responses induced by CD3⁺ cell clones and not by CD3⁻ cell clones. A/J mice were primed with ABA-coupled syngeneic spleen cells and administered as 200- μ l injections of DMEM i.v. (positive control), of conditioned medium supernatants pooled from the CD3⁺ cloned hybridoma cell lines (D7A and F8C), or of pooled supernatants from the CD3⁻ cloned hybridoma cell lines (E4A, E3B, and E1C). The negative control group was not immunized and received 200 μ l i.v. of DMEM per day for the suppressor regime. On day 5 all animals were primed by injection of 25 μ l of ABA salt solution into their left footpad. DTH responses were measured 24 hr later and 48 hr later by comparison of the difference in the size of the right and left footpads with engineer's calipers.

phenotypes over long periods of observation and were discarded. Two CD3⁺ lines were used for the assays described here. Their FACS profiles at the time of biochemical and functional evaluation are presented in Fig. 1. Conditioned medium supernatants were obtained from CD3⁺ and CD3⁻ clones. These supernatants were evaluated in several assays.

As seen in Fig. 2, only supernatants from CD3⁺ F12.23 cells had suppressive activity, whereas the conditioned medium of CD3⁻ F12.23 cells was not active. In addition to *in vivo* DTH assays, we tested the ability of CD3⁺ F12.23 and CD3⁻ F12.23 conditioned medium supernatants to affect the *in vitro* IL-2 production of syngeneic spleen cells primed with ABA antigens. Growth factor production in response to rechallenge was evaluated with the CTLL line (Fig. 3). Splenocytes from mice treated with CD3⁺ F12.23 suppressor T-cell hybrid supernatants responded poorly to subsequent rechallenge. The mice treated with CD3⁻ F12.23 supernatants responded in a similar manner to positive controls. In several independent assays—including suppression of hapten-specific *in vivo*-delayed hypersensitivity, inhibition of IL-2 synthesis by ABA-specific T lymphocytes, and suppression of *in vitro* ABA-specific cytotoxicity reactions (data not shown)—only supernatants obtained from CD3⁺ F12.23 cells were able to mediate suppression. Even when heavily concentrated (by a factor of >100) CD3⁻ F12.23 supernatants were evaluated, no suppressive activity was observed. Therefore, under the experimental conditions examined, only CD3⁺ F12.23 cells had functional suppressive activities.

Biochemical Analysis of CD3⁺ F12.23 Cells. We next evaluated whether the CD3⁺ molecules were associated with conventional heterodimeric receptor-type proteins (1-4, 20-24). As shown in Fig. 4, anti-CD3 antibody immunoprecipitated conventional CD3 proteins (Note: longer exposure of the gels permitted clearer visualization of the CD3 polypeptides) as well as two other proteins at 35 kDa and 45 kDa. These proteins are not disulfide-linked as deduced from the similar gel patterns obtained under reducing and nonreducing

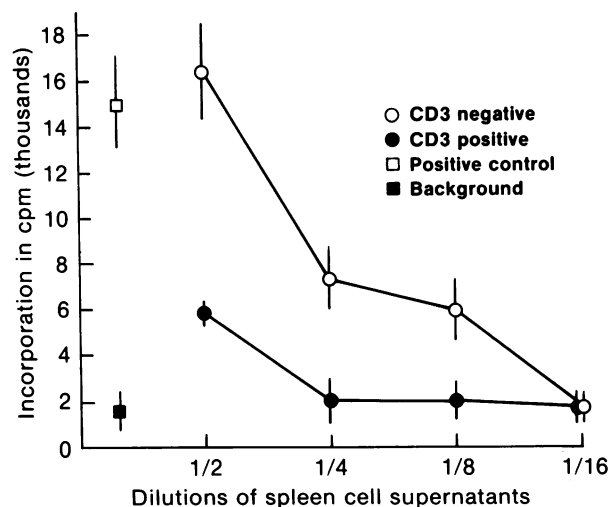


FIG. 3. IL-2 production by conditioned medium supernatant-treated spleen cells. Animals were subjected to the suppressor regime. On day 5 their spleens were harvested and pooled by test group. Spleen cells separated by "teasing" cells apart with dissecting needles were incubated with irradiated feeder layers of ABA-coupled syngeneic spleen cells. After 48 hr, conditioned medium supernatant was collected and added to 96-well plates containing an IL-2-dependent cell line (18). Twenty-four hours later the cells were pulse-labeled for 6 hr with tritiated thymidine and harvested. Open square, spleen cells from positive control group at a 1:2 dilution; solid square, background incorporation of an IL-2-dependent line; open circles, CD3⁻ cells; closed circles, CD3⁺ cells.

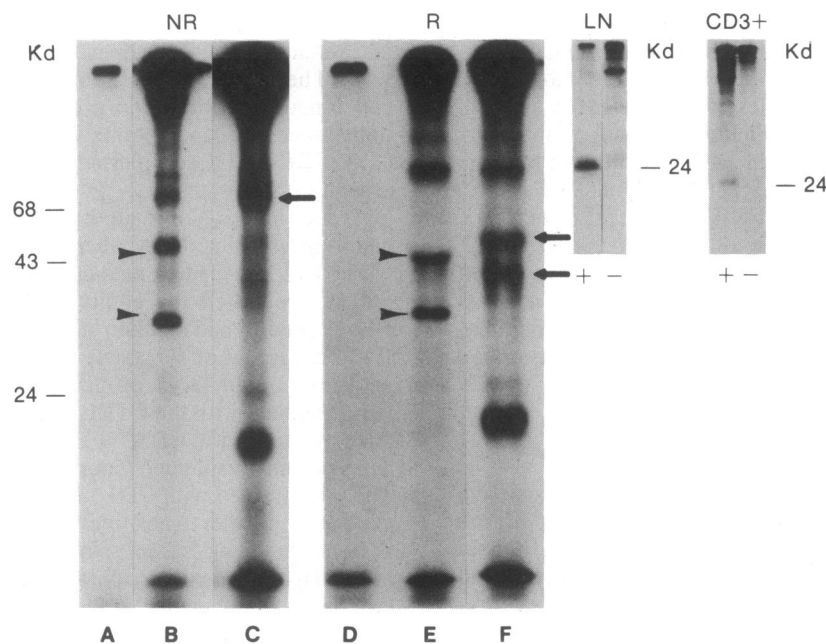


FIG. 4. Biochemical characterization of CD3-associated proteins. ^{125}I -surface-labeled cells were immunoprecipitated with anti-T3 reagents, as described (3, 14). CD3 clones had identical patterns whereas no immunoprecipitation of labeled CD3-associated polypeptides was observed in CD3⁻ lines. Lanes: A and D, CD3⁻ line; B and E, CD3⁺ D7A line; C and F, nylon-wool-purified T cells from A/J lymph nodes. Arrowhead, suppressor clone CD3-associated polypeptides; arrow, A/J lymph node CD3-associated polypeptides. There is a change in the molecular mass of the 45-kDa chain under reducing conditions due to the migration of reduced heavy chains of the precipitating antiserum at the same molecular mass. Lanes LN, phosphorylation of CD3 polypeptide in the presence (+) or absence (-) of PMA; CD3⁺, phosphorylation of CD3 polypeptides in the presence (+) or absence (-) of PMA.

conditions. Additionally, these proteins migrated on the diagonal in off-the-diagonal gels (3) (data not shown).

Further conformation of the CD3 phenotype of the receptor-associated complex was undertaken by phosphorylation studies (1, 4). Phosphorylation of CD3 polypeptides has been shown to occur with perturbation of the heterodimeric T-cell receptor (1, 4). We evaluated whether CD3 T-cell receptor-associated polypeptides found on our suppressor clones respond to activation with PMA in a similar manner to the phosphorylation pattern observed in the A/J mouse peripheral T-cell population. Addition of 10 ng of PMA induced phosphorylation of polypeptides at 20–25 kDa in nylon-wool-purified A/J lymph node T cells as well as CD3⁺ F12.23 cells. Phosphorylation of the CD3 polypeptides was not observed in the absence of PMA stimulation.

DISCUSSION

Suppression may be mediated by many diverse cell types (8–11, 25) and by a variety of soluble products (10, 25, 26). We have now shown that ABA-specific suppressive F12.23 suppressor T cells express CD3⁺-associated heterodimeric proteins and that clones that lack these cell-surface structures are not able to mediate discernible suppressive functions. These observations unambiguously identify CD3⁺ CD5⁺ F12.23 cells as the producer of suppressive activity.

Some types of receptor proteins have been reported on suppressor T cells (27, 28). In other cases heterodimeric polypeptides have not been identified (29, 30). In general, biochemical analysis of receptors or suppressor hybridomas has not been convincing nor generally reproducible. We were able to precipitate heterodimeric polypeptides from the cell surface of CD3⁺ F12.23 cells but not from CD3⁻ F12.23 cells. Similarly, CD3 polypeptides were readily identified only on the CD3⁺ T cells. Of interest was the apparent absence of disulfide bonding of the polypeptides and the molecular masses of the two chains. The polypeptides resolved with molecular masses of 35 kDa and 45 kDa. In

addition to precipitating CD3 molecules from the cell surface, we were able to show that PMA predictably induces their phosphorylation.

It is interesting to speculate that the receptor polypeptides have similar molecular masses to murine γ - δ heterodimeric proteins (21–24). Additionally, the weaker extent of CD3 surface labeling would support this speculation. However, differences such as the lack of disulfide linkage between these chains clearly exist. Clearly further biochemical and molecular characterization is necessary to clarify the identity of the CD3-associated polypeptides and their relationship to the A/J genome.

Our primary analysis of the parental F12.23 line revealed that only 3% of the cells of the suppressor line was CD3⁺. The clonal analysis presented here demonstrates that all suppressive activity segregates with the CD3⁺ population. Therefore, it is apparent that only a minimum of the cell population needs to have suppressive functions for a cell line to be considered inhibitory. This observation agrees with the potent actions reported for suppressor cell products in variety of systems (8–11, 26, 31). When molecular analyses are performed on these cells, genetic features ascribed to the bulk population, which for the most part is CD3⁻, will be obtained. Through the use of CD3⁺ F12.23 clones, it may now be possible to identify whether released receptor proteins mediate suppression. It is possible that suppressor molecules are shed or secreted forms of specific receptor proteins. Since one ambiguity in the studies of murine suppression relates to the I-J epitope (32–34), the cells described herein may be useful to resolve whether I-J is a unique structure or a conformation found on T-cell receptor polypeptides.

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