# HUMAN T CELL ACTIVATION

## IV. T Cell Activation and Proliferation Via the Early Activation Antigen EA 1

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The tumor promoter PMA has been shown to induce accessory cell–independent T cell proliferation in conjunction with mAbs specific for CD3, CD28, and CD2 (1–6). In these systems, PMA induces IL-2-R expression while the presence of these mAbs is synergistic for the maximal expression of IL-2-R and IL-2 production.

Recently, we have identified the early activation antigen, EA 1, by the generation of mAb specific for PMA-activated T cells (7). EA 1 expression is seen as early as 30 min after the addition of PMA. EA 1 expression induced by PMA and mitogens precedes that of IL-2-R. Our further studies (8) show that protein kinase C activation is the primary pathway for the induction of EA 1 expression and that calcium-dependent pathways appear to have a secondary role. The EA 1 antigen is a series of disulfide-linked dimers (32 kD/32 kD, 32 kD/28 kD, and 28 kD/28 kD), with subunits of an identical 24-kD core protein that is phosphorylated and differentially glycosylated. Recently, EA 1 has been shown to be expressed very early by fetal thymocytes during ontogeny (Jung, L. K. L., B. F. Haynes, S. Pahwa, and S. M. Fu, manuscript submitted for publication).

In the initial study (7), mAb EA 1 was shown not to inhibit or to stimulate T or B cell proliferation. Because of its early expression during T cell ontogeny and activation and its molecular structure, the possibility that EA 1 plays an important role in either cellular interaction or interaction with a yet-to-be-defined growth factor was suggested. To explore this further, mAbs were generated against affinity-purified EA 1. In this study, a new mAb against EA 1 was shown in collaboration with PMA to induce T cells to express IL-2-R, to secrete IL-2, and to proliferate. This process appeared to be independent of monocytes. Although this mAb by itself did not induce an increase in $[Ca^{2+}]_i$ by PMA-treated T cells, crosslinking of EA 1 molecules caused a marked increase in $[Ca^{2+}]_i$.

## Materials and Methods

*Affinity Purification of EA 1 from Jurkat Cells.*    Anti-EA 1 mAb P8 (IgG2a) was prepared as previously described (7). This mAb was purified from appropriate ascitic fluids by DEAE

ion-exchange chromatography. The purified mAb P8 was coupled to Sepharose-4B beads at a ratio of 3 mg antibody per ml of packed beads.

Jurkat cells were incubated with 10 ng/ml of PMA (Sigma Chemical Co., St. Louis, MO) for 12 h and washed with PBS containing 1 mM PMSF. Cell lysates were prepared by detergent lysis at a concentration of $5 \times 10^7$ cells/ml with a lysis buffer containing 20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 7.5 mM 3-[(3-cholamidopropyl)dimethylamine]-1-propanesulfonate dihydrate (CHAPS),[1] 50 mM iodoacetamide, 1 mM PMSF, 1 U/ml aprotinin, 10 $\mu$g/ml leupeptin, 50 $\mu$g/ml trypsin inhibitor, 2 mM EDTA for 30 min at 4°C. The lysate was centrifuged at 5,000 $g$ for 30 min, then at 16,000 $g$ for 2 h to remove nuclei and debris. A detergent lysate from $10^{10}$ Jurkat cells was precleared with 10 ml of plain Sepharose-4B and Sepharose-4B conjugated with normal mouse IgG. The precleared lysate was incubated with 5 ml of mAb P8-Sepharose-4B for 2 h at 4°C. The beads were packed into a column and washed with the lysis buffer and a high-salt buffer containing 20 mM Tris-HCl, pH 7.5, 0.6 M NaCl, 5 mM CHAPS, 1 mM PMSF. The bound material was eluted with 50 mM diethylamine, pH 11. The eluate was neutralized with 1 M NaH$_2$PO$_4$, dialyzed against PBS, and concentrated. Quantitation of material recovered and assessment of purity were made by SDS-PAGE and subsequent silver staining of the gel.

*Production of mAb Against EA 1.*    BALB/c mice were immunized with affinity-purified EA 1 molecules. For the primary immunization, 1 $\mu$g of EA 1 material was emulsified with an equal volume of CFA. Further immunization was carried out with 1 $\mu$g of immunogen in IFA. Five injections were administered subcutaneously at 2–4-wk intervals. 2 mo after the last immunization, mice were given an intraperitoneal boost of 2 $\mu$g of EA 1 material. Spleens were taken 3 d later. Hybridomas were generated using myeloma cell line SP2/0 as a fusion partner as previously described (9). Hybridoma supernatants were screened by flow cytometry against PMA-activated Jurkat cells, and by ability to augment PMA-induced proliferation. The selected hybridomas were cloned twice on soft agar.

Antibody isotype was determined by ELISA with isotype-specific rabbit anti-mouse Ig antisera (Meloy Laboratories, Inc., Springfield, VA) and alkaline phosphatase-linked goat anti-mouse Ig. The new mAb against EA 1, G38, was IgG2b. This mAb was purified from ascitic fluids by DEAE ion-exchange chromatography.

*Other mAbs.*    Two mAbs against CD3, designated T3-II (IgG1) and 235 (IgM), were prepared as previously described (1). AT-1 (anti-IL-2-R, IgG1) was prepared and biotinylated as previously described (1). Purified anti-Leu-23 mAb (IgG1) was generously provided by Dr. Lanier (Becton Dickinson & Co., Mountain View, CA) (10). As control mAbs, HDP-1 (anti-DNP, IgG1), SS1 (anti–sheep erythrocyte, IgG2a), and NS8.1 (anti–sheep erythrocyte, IgG2b) were used. These mAbs were generous gifts from Dr. I. Davie (Washington University, St. Louis, MO).

*Cell Preparation.*    PBMC were isolated from buffy coats or peripheral blood from normal donors using Ficoll-Hypaque density gradient centrifugation. T cells were obtained by SBRC rosetting (11) and were usually 95% pure. These T cell preparations (E$^+$ cells) contained 1–2% monocytes by nonspecific esterase staining. To isolate monocyte-depleted T cells, T cells were further separated by nylon wool column, plastic adherence, and carbonyl-iron treatment (12–14). These T cell preparations contained <0.1% monocytes.

Cells were cultured in complete medium consisting of RPMI-1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS (HyClone Laboratories, Logan, UT), 2 mM L-glutamine, 50 U/ml penicillin, and 50 $\mu$g/ml streptomycin (Gibco Laboratories).

*Immunoprecipitation.*    Peripheral blood T cells (E$^+$ cells) activated with 10 ng/ml of PMA for 18 h were labeled with $^{125}$I (New England Nuclear, Boston, MA) by the lactoperoxidase technique (15). The labeled cells were washed with PBS containing 1 mM PMSF, 10 mM iodoacetamide, and 0.02% NaN$_3$. The cell lysates were prepared by detergent lysis at a concentration of $5 \times 10^7$ cells/ml for 15 min at 4°C in a lysis buffer containing 20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 7.5 mM CHAPS, 50 mM iodoacetamide, 1 mM PMSF, 1 U/ml aprotinin, 10 $\mu$g/ml leupeptin, 50 $\mu$g/ml trypsin inhibitor, and 2 mM EDTA. After centrifu-

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[1] *Abbreviation used in this paper:* CHAPS, 3-[(3-cholamidopropyl) dimethylamine]-1-propansulfonate dihydrate.

gation, the lysates were precleared three times with 50 $\mu$l of Sepharose-4B conjugated with goat anti-mouse Ig antibodies (3 mg/ml). The precleared lysates were added to 30 $\mu$l of Sepharose-4B conjugated with goat anti-mouse Ig antibodies that had been incubated with mAbs. The mixture was incubated for 60 min at 4°C with constant mixing, and then washed six times with a buffer containing 10 mM Tris-HCl, pH 7.8, and 0.6 M NaCl. Three additional washings with a buffer containing 10 mM Tris-HCl, pH 8.8, 0.6 M NaCl, 0.1% SDS, and 0.05% NP-40 were carried out. The absorbed proteins were released by boiling for 5 min in a sample buffer containing 0.125 M Tris-HCl, pH 6.8, 2% SDS, and 10% glycerol (with or without 5% 2-ME). SDS-PAGE was carried out on 11% polyacrylamide gels by using the discontinuous buffer system described by Laemmli (16). Gels were stained, destained, dried, and autoradiographed at $-70°C$ against X-Omat XAR-5 films.

*Proliferation Assays.* PBMC, $E^+$ cells, or monocyte-depleted T cells ($2 \times 10^5$ cells) in 200 $\mu$l of complete medium were incubated in triplicate in flat-bottomed 96-well plates at 37°C for 3 d in a 5% $CO_2$ atmosphere. During the last 9 h, cells were pulsed with 0.4 $\mu$Ci of [$^3$H]thymidine (sp act 75 Ci/mmol; Amersham Corp., Arlington Heights, IL), and incorporated radioactivity was determined as described (17).

For some experiments, PHA-P (Difco Laboratories, Inc., Detroit, MI) was used for T cell activation.

*Immunofluorescence Studies.* Cells were stained with mAb at 4°C for 30 min. After extensive washing, the cells were further incubated with FITC-conjugated goat anti-mouse F(ab')$_2$ at 4°C for 30 min. For the study on IL-2-R expression, biotinylated AT-1 was used as the first ligand, and FITC-conjugated avidin was used as the second ligand. After three washings, the cells were analyzed with a Cytofluorograf IIs cell sorter (Ortho Diagnostic Systems, Inc., Westwood, MA). Linear integrated fluorescence of the gated population was measured and $10^4$ cells were analyzed. As negative controls, the cells were stained with nonbiotinylated AT-1 and FITC-avidin.

*IL-2 Activity Assay.* $4 \times 10^3$ murine IL-2-dependent HT-2 cells in 100 $\mu$l of complete medium, containing $2.5 \times 10^{-5}$ M 2-ME were incubated with 100 $\mu$l of culture supernatants of serial twofold dilutions, in triplicate, at 37°C for 24 h. Then, 0.4 $\mu$Ci of [$^3$H]thymidine was added, and the incubation was continued for another 4 h. The incorporated radioactivity was assayed as described above. rIL-2 (Biogen, Geneva, Switzerland) was used as a standard, and IL-2 activity in culture supernatants was expressed in U/ml.

*Northern Blot Hybridization.* Methods for Northern blot analyses and sources of probes were described previously (18).

*Cytoplasmic Free Calcium Measurements.* Cytoplasmic free [$Ca^{2+}$]$_i$ was measured by a modification of the method of Rabinovitch et al. (19), as described previously (8). Briefly, cells were loaded with the acetoxymethyl ester of indo-1 (10 $\mu$M) (Molecular Probes, Eugene, OR). Indo-1-loaded cells were analyzed by flow cytometry on a Cytofluorograf IIs cell sorter. UV excitation was from an argon ion laser (Coherent Inc., Palo Alto, CA) using 40 mW at 351-364 nm. Blue (450-490 nm; Omega Optical, Inc., Brattleboro, VT) and violet (405 nm; Omega Optical, Inc.) band pass filters were used to collect into-1 fluorescence emission after separation using a 430-nm dicroic mirror (Omega Optical, Inc.). Alterations in [$Ca^{2+}$]$_i$ were measured by analyzing the shift in the indo-1 ratio of violet to blue fluorescence over a 15-min time period. UV excitation of indo-1 in the absence of calcium results in the emission of light in the blue region. The binding of calcium to indo-1 shifts the UV excitation emission of light from the blue region to the violet region. An increase in the indo-1 ratio is a measurement of the increase in [$Ca^{2+}$]$_i$. At the beginning of each experiment, the violet and blue photomultiplier tubes were adjusted to yield a basal ratio of 1.

## Results

*Identification of mAb G38 and mAb Leu-23 as anti-EA 1 Antibody.* BALB/c mice were immunized with affinity-purified EA 1 molecules and hybridomas were generated as described in Materials and Methods. Antibodies were rescreened for their reactivity with PMA-activated Jurkat cells and by their ability to augment PMA-induced

T cell proliferation. One hybridoma, termed G38, was found to fulfill both selection criteria. Hybridoma G38 was cloned twice on soft agar and mAb G38 was found to be an IgG2b molecule.

The antigen reactive with mAb G38 was determined by immunoprecipitation from a detergent lysate of ^{125}I-labeled PMA-activated T cells and SDS-PAGE. As shown in Fig. 1, the complex precipitated by mAb G38 was similar to that precipitated by mAb P8, the original anti-EA 1 mAb reported previously (8). Under reducing conditions, both precipitated two bands of 28 and 32 kD (Fig. 1 A, lanes 2 and 3). Under nonreducing conditions, the EA 1 complex was resolved into 56- and 60-kD bands with some trailing of the latter band (Fig. 1 A, lanes 5 and 6). To further prove that mAb G38 is reactive with EA 1, preclearing experiments were carried out. As shown in Fig. 1, lanes 8 and 9, preclearing a ^{125}I-labeled lysate with mAb P8 completely removed the molecules reactive with mAb P8, as well as those precipitated by mAb G38. Similar results were obtained when mAb G38 was used as the
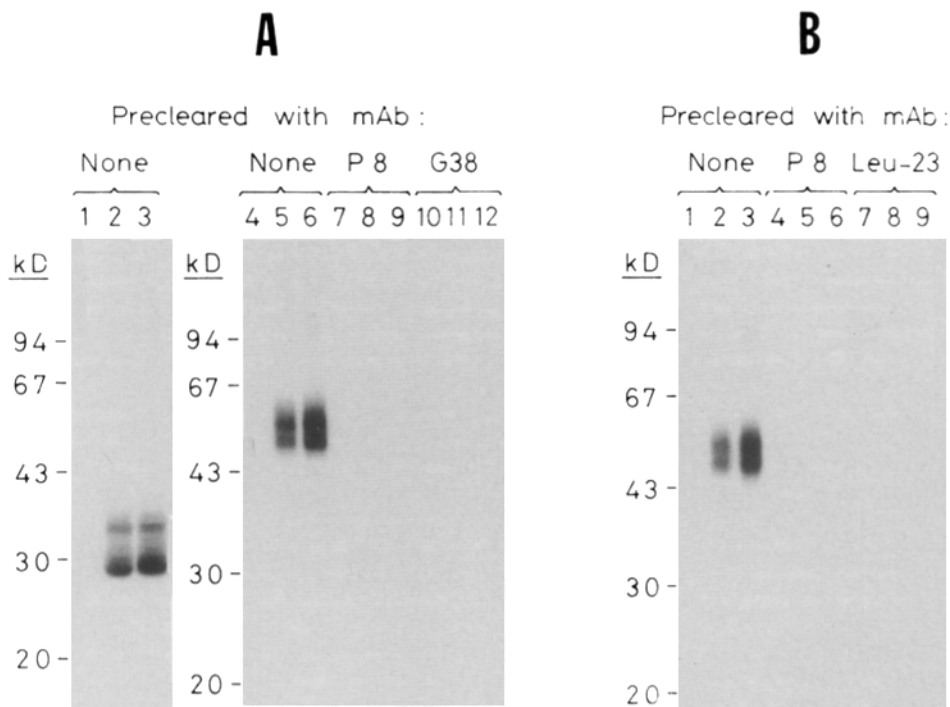


FIGURE 1.    mAb G38 and mAb Leu-23 immunoprecipitated EA 1 from PMA-activated T cells. Peripheral blood T cells (5 × 10⁶ cells/ml) incubated with 10 ng/ml of PMA for 18 h were labeled with ^{125}I by lactoperoxidase. The lysate was precleared with Sepharose-4B beads conjugated with goat anti–mouse Ig antibodies. Immunoprecipitation was carried out in these precleared samples. Samples were analysed by SDS-PAGE under reducing (R) and nonreducing (NR) conditions. (A) Sequential immunoprecipitation with mAb P8 and mAb G38. The precleared samples were immunoprecipitated with pooled control mAbs (SS1 and NS8.1) (lanes 1, 4, 7, and 10), mAb P8 (lanes 2, 5, 8, and 11), and mAb G38 (lanes 3, 6, 9, and 12). (B) Sequential immunoprecipitation with mAb P8 and mAb Leu-23. The precleared samples were immunoprecipitated with pooled control mAbs (SS1 and HDP-1) (lanes 1, 4, and 7), mAb P8 (lanes 2, 5, and 8), and mAb Leu-23 (lanes 3, 6, and 9).

preclearing Ab (Fig. 1, lanes *11* and *12*). It is of note that the radioactivity of mAb G38 immunoprecipitates was higher than that of mAb P8 immunoprecipitates. This was observed in an additional experiment.

To determine whether mAb G38 was reactive with the same epitope as mAb P8, biotinylated mAb P8 was used with FITC-labeled avidin as the detecting agent. mAb G38 did not block the staining of mAb P8. By two-color immunofluorescence analysis, both mAbs stained the same population of thymocytes and PMA-activated T cells. These results indicate that mAb G38 recognizes a different epitope of EA 1.

Recently, Lanier et al. (10) and Chen et al. (20) reported the identification of an activation antigen, Leu-23, that is rapidly induced and phosphorylated after IL-2 stimulation of NK cells. Since Leu-23 antigen has structural properties and a cellular distribution similar to EA 1, a sequential immunoprecipitation experiment was carried out to determine whether mAb P8 and mAb Leu-23 were reactive with identical molecules. As shown in Fig. 1 *B*, both mAb P8 and mAb Leu-23 were reactive with a similar molecular complex. Preclearing of $^{125}$I-labeled lysate of PMA-activated T cells with mAb P8 completely removed molecules reactive with mAb Leu-23 and vice versa (Fig. 1 *B*, lanes *6* and *8*). These results indicate that mAb Leu-23 also recognize EA 1. Again, it is of note that mAb Leu-23 appeared to be a better precipitating antibody.

*Augmentation of PMA-induced PBMC Proliferation by mAb G38.* PBMC were stimulated with 1:100 diluted ascitic fluids or 1:4 diluted culture supernatants of hybridomas G38 and P8 in the presence of varying doses of PMA. At all doses of PMA, both G38 ascitic fluid and supernatant augmented PMA-induced PBMC proliferation significantly with more than doubling the amounts of [$^3$H]thymidine incorporation. A representative experiment is summarized in Table I. Two additional experiments yielded similar results. In contrast, the effects of P8 ascitic fluid and supernatant at the dilutions used were weak or not detectable.

EA 1 molecules are also induced on cell surface with PHA or anti-CD3 mAb stimulation as described previously (7). We examined whether or not mAb G38 could augment PBMC proliferation induced by suboptimal doses of these mitogens. In these experiments, a trend of augmentation was detected. However, these effects were only marginal at 20–30% augmentation (data not shown). In addition, augmentation was not detected in one additional experiment.

*T Cell Proliferation Induced by Anti-EA 1 mAb in the Presence of PMA.* PBMC, E$^+$ cells with 1% contaminating monocytes, and T cells extensively depleted of monocytes were cultured with various doses of PMA and 1:100 diluted G38 ascites. As shown in Table I, mAb G38 augmented PMA-induced PBMC proliferation considerably. In addition, it induced E$^+$ cells and extensively purified T cells to proliferate in the presence of PMA. The purified T cells were depleted of monocytes to <0.1% by nylon wool column, plastic adherence, and carbonyl iron treatment. This T cell population was nonresponsive to PHA. These results indicate that the effect of mAb G38 is monocyte independent in this system.

To demonstrate that T cell proliferation induced by mAb G38 in the presence of PMA was dose dependent, purified mAb was used. As shown in Table II, purified mAb G38 induced T cell proliferation in the presence of PMA at a wide range of mAb concentrations (0.5–100 $\mu$g/ml) in a dose-dependent manner. This is more apparent when 3 ng/ml of PMA was used. In this experiment, purified mAb Leu-23

TABLE I

*Effects of Anti-EA 1 mAb G38 on PMA-induced PBMC
and T Cell Proliferation*

| Cell population | Stimulating agents | [³H]Thymidine incorporation in the presence of: | |
| --- | --- | --- | --- |
| | | Medium | mAb G38* |
| PBMC | Medium | 1,085 | 2,397 |
| | PHA | 72,651 | 80,969 |
| | PMA (10 ng/ml) | 11,001 | 76,311 |
| | PMA (3 ng/ml) | 9,129 | 10,422 |
| | PMA (1 ng/ml) | 3,280 | 5,684 |
| E⁺ Cells | Medium | 356 | 398 |
| | PHA | 52,354 | 56,723 |
| | PMA (10 ng/ml) | 4,342 | 63,413 |
| | PMA (3 ng/ml) | 2,949 | 77,837 |
| | PMA (1 ng/ml) | 2,004 | 50,803 |
| Monocyte-free | Medium | 130 | 363 |
| T cells | PHA | 1,367 | 1,161 |
| | PMA (10 ng/ml) | 2,608 | 50,883 |
| | PMA (3 ng/ml) | 2,351 | 65,463 |
| | PMA (1 ng/ml) | 1,182 | 9,359 |

mAb G38 augmented PMA-induced PBMC and T cell proliferation. T cell
proliferation induced by PMA and mAb G38 was monocyte-independent.
PBMC, E⁺ cells (1% contaminating monocytes), monocyte-free T cells (2 ×
10⁵) were stimulated with mAb G38 in the presence of 10 μg/ml of PHA or
various concentrations of PMA. [³H]Thymidine incorporation was measured
in triplicate after 3 d. SD of each value were within 15%.

* G38 ascites were used at final dilution of 1:100.

was included and found to be very potent in this assay. mAb P8 was much less effec-
tive as a mitogen. Significant proliferation was seen only at doses of 50 and 100 μg/ml.
These results were in agreement with those in Table I. P8 ascites contained 2 mg/ml,
and at 1:100 dilution, mAb P8 was at 20 μg/ml, a dose expected to be slightly mito-
genic in the presence of PMA.

*IL-2-R Expression and IL-2 Production.*    T cell activation with anti-EA 1 mAb G38
in the presence of PMA was associated with a marked increase in IL-2-R expression
as detectable by our mAb AT-1 (Fig. 2). Because the cells were cultured in the pres-
ence of mouse mAb, biotinylated AT-1 was used with FITC-avidin. With medium
or mAb G38 alone, 6–8% of the T cells weakly expressed IL-2-R at all time points
studied (data not shown). Although PMA did not induce a high level of prolifera-
tion, a significant number of T cells (34%) were positive for IL-2-R by 72 h. In
the presence of mAb G38 and PMA, 80% of T cells were positive by 24 h, and 90%
became strongly positive by 72 h. For comparison, IL-2-R expression induced by
PMA and anti-CD3 mAb T3-II was included in Fig. 2.

IL-2 production by T cells stimulated with mAb G38 in the presence of PMA
was detected 24 h after the initiation of cultures (Table III). Significant proliferation
of HT-2 cells was detected even when the supernatants were diluted to 1:32, although
the IL-2 activity was less than that in supernatants from cultures with mAb T3-II

*T Cell Proliferation Induced by Purified Anti-EA 1 mAbs
in the Presence of PMA Was Dose Dependent*

| Antibodies | | [³H]Thymidine incorporation at PMA concentration (ng/ml) of: | | |
| --- | --- | --- | --- | --- |
| | | 0 | 3 | 10 |
| | *μg/ml* | | *cpm* | |
| Medium | | 274 | 10,983 | 18,162 |
| T3-II | 10 | 1,027 | 80,780 | 81,767 |
| G38 | 100 | 364 | 51,842 | 56,728 |
| | 50 | 364 | 46,105 | 48,966 |
| | 10 | 491 | 36,531 | 43,259 |
| | 5 | 404 | 31,080 | 45,299 |
| | 1 | 299 | 16,130 | 24,876 |
| Leu-23 | 10 | 228 | 76,404 | 74,698 |
| | 1 | 435 | 45,164 | 52,435 |
| P8 | 100 | 253 | 39,348 | 37,197 |
| | 50 | 472 | 27,193 | 33,959 |
| | 10 | 230 | 15,527 | 24,500 |
| | 5 | 547 | 15,941 | 20,765 |
| | 1 | 597 | 14,029 | 15,362 |
| | 0.5 | 372 | 11,718 | 17,352 |

T cells ($E^+$ cells) ($2 \times 10^5$) were stimulated with various concentrations of purified mAbs in the presence of PMA. [³H]Thymidine incorporation was measured in triplicate after 3 d. SD of each value were within 15%.

and PMA. The supernatant of T cells with PMA showed marginal level of IL-2 activities.

*Expression of IL-2 and IL-2-R mRNA in T Cells Stimulated with mAb G38 and PMA.* The observation that T cell activation with mAb G38 and PMA was associated with an increase in IL-2-R expression and IL-2 secretion was substantiated at the mRNA level. T cells were stimulated with mAb G38 and PMA for 24 h and the induction of IL-2 and IL-2-R mRNA synthesis was examined by Northern blot analysis. As shown in Fig. 3, very little IL-2 mRNA accumulation was seen when PMA was used alone. Although the accumulation of IL-2-R mRNA was barely observed as presented
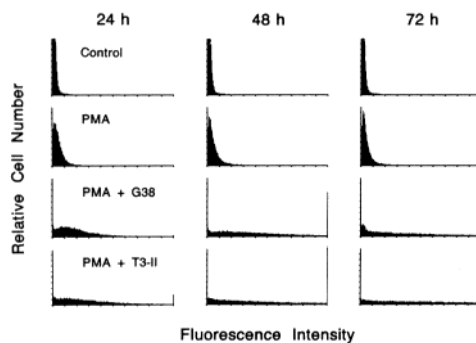


FIGURE 2. Time course of expression of IL-2-R on T cells stimulated with anti-EA 1 mAb in the presence of PMA. T cells ($E^+$ cells) were cultured with medium, PMA (10 ng/ml) alone, PMA and mAb G38 (1:100 diluted ascites), or PMA and mAb T3-II (1 μg/ml). IL-2-R$^+$ cells were determined by biotinylated AT-1 and FITC-avidin at the indicated times, as described in Materials and Methods. Linear integrated fluorescence of the gated population was measured in these experiments.

TABLE III

*IL-2 Production by T Cells Stimulated with Anti-EA 1 mAb in the presence of PMA*

| Culture conditions | [³H]Thymidine incorporation at final dilutions of: | | | | IL-2 activity |
| --- | --- | --- | --- | --- | --- |
| | 1:2 | 1:8 | 1:32 | 1:128 | |
| | | | | | U/ml |
| Medium | 117 | 204 | 162 | 174 | <0.5 |
| TPA | 2,701 | 656 | 358 | 261 | <0.5 |
| TPA + G38 | 43,061 | 19,954 | 4,903 | 751 | 7.2 |
| TPA + T3-II | 47,389 | 56,995 | 38,368 | 10,592 | 71.0 |

T cells (E⁺ cells) (10⁶ cells/ml) were cultured in the presence of stimulator(s). 10 ng/ml of PMA and 1 μg/ml of T3-II were used. G38 ascites were used at a final dilution of 1:100. After 24 h, supernatants were harvested and their ability to stimulate [³H]thymidine incorporation into murine IL-2-dependent HT-2 cells was determined. SD of each value were within 15%.

in Fig. 3, lane *4*, the induction of IL-2-R mRNA by PMA alone was apparent where autoradiography was carried out for a longer period of time. In contrast, mAb G38 and PMA in combination induced IL-2 mRNA synthesis to a readily detectable level (Fig. 3, lane *5*). In addition, mAb G38 increased PMA-induced IL-2-R mRNA accumulation by ~10-fold. These mRNA levels were comparable with those induced by anti-CD3 mAb and PMA (Fig. 3, lane *2*). Although not shown, mAb G38 alone did not induce any detectable IL-2 or IL-2-R mRNA synthesis.

*Induction of [Ca²⁺]ᵢ Increase by mAb G38 after Crosslinking by Anti–mouse Ig Antibodies.* Changes in [Ca²⁺]ᵢ are shown to be an early event of T cell activation. To determine if mAb G38 could induce an increase in [Ca²⁺]ᵢ, monocyte-depleted T cells were activated with 10 ng/ml of PMA for 18 h to induce EA 1 on cell surface before loading with indo-1. As a positive control, anti-CD3 mAb 235 (IgM) which is very potent
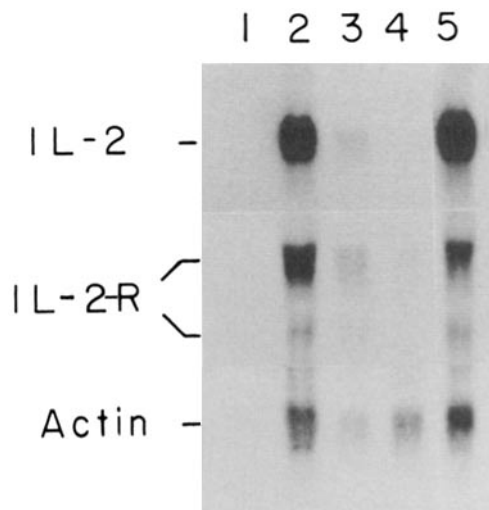


FIGURE 3. Expression of IL-2 and IL-2-R mRNA in T cells stimulated with anti-EA 1 mAb in the presence of PMA. T cells (E⁺ cells) were cultured with medium (lane *1*), PMA (10 ng/ml) and mAb T3-II (1 μg/ml) (lane *2*), PHA (10 μg/ml) (lane *3*), PMA alone (lane *4*), or PMA and mAb G38 (1:100 diluted ascites) (lane *5*) for 24 h. RNA was extracted from samples of 5 × 10⁷ lymphocytes. Northern blot hybridization to IL-2, IL-2-R, and β actin probes were performed as described in Materials and Methods.
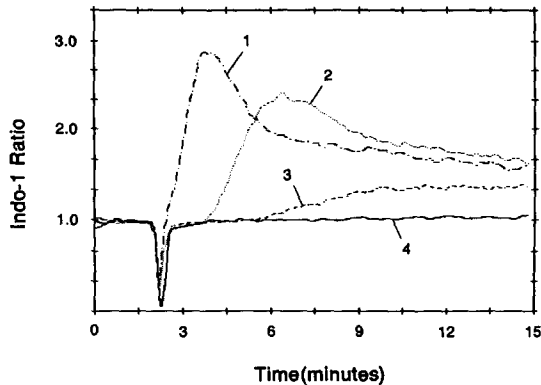
in the induction of a rise in $[Ca^{2+}]_i$, as described previously (8), was used. As shown in Fig. 4, mAb 235 at 1 μg/ml induced a marked rise in $[Ca^{2+}]_i$, although TCR/ CD3 complexes were downregulated with PMA (Fig. 4, curve $1$). In contrast, both mAb G38 and mAb P8 by themselves did not induce a detectable change in $[Ca^{2+}]_i$. However, the addition of goat anti-mouse Ig antibodies as the second antibodies induced a significant rise in $[Ca^{2+}]_i$ by mAb G38 (Fig. 4, curve $2$). Although the increase in $[Ca^{2+}]_i$ was much smaller, mAb P8 at 10 μg/ml after crosslinking was also effective in this assay (Fig. 4, curve $3$). Both anti-EA 1 mAbs and goat anti-mouse Ig antibodies had no effect on $[Ca^{2+}]_i$ when monocyte-depleted T cells were used without PMA activation.

## Discussion

EA 1, an early activation antigen, was originally identified by our mAb P8 (7). The antigen is a complex of phosphorylated disulfide-linked dimers with a core polypeptide of 24 kD that is differentially glycosylated to generate 28- and 32-kD glycoproteins (8). The inductive signal requirement for EA 1 expression appears to be the activation of protein kinase C, although its maximal expression required the participation of $Ca^{2+}$-mediated pathways as well. The induction of EA 1 is detectable within 30 min after the addition of PMA. It is the earliest T cell activation antigen induced by PMA and other polyclonal mitogens, the expression of which requires de novo RNA and protein synthesis. Recently, the expression of EA 1 has been documented by fetal thymocytes as early as 8–9 wk after inception before thymic cortico-medullary organization occurs (Jung, L. K. L., B. F. Haynes, S. Pahwa, and S. M. Fu, manuscript submitted for publication). Its expression follows shortly after the expression of CD7 and CD2. After cortico-medullary organization, the majority of the medullary thymocytes express EA 1. These findings indicate that EA 1 may play an important role in T cell ontogeny and T cell activation.

In the present study, mAb G38, a new anti-EA 1 mAb, was shown to augment PMA-induced PBMC proliferation. With T cell preparations extensively depleted

of monocytes, mAb G38 was capable to induce T cell proliferation in collaboration with PMA in a dose-dependent manner. In certain experiments, the magnitude of T cell proliferation induced by mAb G38 and PMA was comparable with that induced by anti-CD3 mAb and PMA. Antibodies specific for hormone or growth factor receptors have been shown to mimic the functional effects of their ligands (21–24). Thus, the collaboration between anti-EA 1 mAb and PMA to induce T cell proliferation provides direct evidence that EA 1 has a functional role in T cell activation. However, the identification of the physiological ligand for EA 1, which may be either a growth factor or a surface molecule involved in cell-cell interaction, remains to be accomplished.

T cell proliferation induced by anti-EA 1 mAb and PMA was shown to be accompanied by IL-2-R expression and IL-2 secretion. This was demonstrated at both protein and mRNA levels. mAb G38 potentiated the expression of IL-2-R induced by PMA and collaborated with PMA to induce IL-2 synthesis and secretion. These findings together with the observation that the activation of protein kinase C is sufficient for EA 1 expression indicate that EA 1 may participate in T cell proliferation in an autocrine system after the initial activation events.

The transduction signals delivered by anti-EA 1 mAbs to PMA-activated T cells were investigated. Although anti-EA 1 mAbs did not induce an appreciable increase in $[Ca^{2+}]_i$ by themselves, the addition of second goat anti–mouse Ig antibodies induced marked influx of $Ca^{2+}$. Recently, Ledbetter et al. (25) reported mAbs specific for CD2, CD4, CD5, CD6, CD7, CD8, and CD28 cause an increase in $[Ca^{2+}]_i$ after these antigens are crosslinked on the cell surface. In their system, the increase in $[Ca^{2+}]_i$ after crosslinking of CD2 and CD28 appears to be associated with expression of IL-2-R. mAbs to these two antigens are capable of inducing T cells to proliferate in the presence of PMA. The exact signal transduction mechanisms used by CD2 and CD28 have not been clearly defined. While CD2 pathway is linked to the TCR/CD3 pathway, CD28 pathway is independent of the TCR/CD3 pathway. Whether the EA 1 pathway utilized a similar mechanism as the CD28 pathway deserves further exploration. In view of the recent observation that the CD28 pathway is relatively insensitive to cyclosporin A (26), it would be of interest to determine if the EA 1 pathway is also resistant to this immunosuppressive agent.

The new anti-EA 1 mAb G38 was shown to react with an epitope different from that with the initial anti-EA mAb P8. mAb G38 appears to have a higher binding affinity. In a dose-response experiment, it was demonstrated that a several-fold higher concentration of mAb P8 was required to induce detectable T cell proliferation. This indicates that the reactive epitopes, as well as binding affinity of the mAbs, are important in determining their mitogenic potential. In addition, the finding offers an explanation for our initial unsuccessful attempt to demonstrate mitogenic effects of mAb P8.

There are two mAbs in the literature that are very similar to our anti-EA 1 antibodies. One of them is mAb Leu-23 (12). Leu-23 is rapidly induced on lymphocytes by mitogen. The kinetics of its expression is similar to EA 1. Leu-23 is also induced after IL-2 stimulation of NK cells. Appearance of Leu-23 on NK cells closely parallel IL-2-induced cytotoxicity. Our data showed that the antigen reactive with mAb Leu-23 was identical with EA-1. In addition, mAb Leu-23 was also shown to be mitogenic for T cells in collaboration with PMA. Thus, the demonstration of EA 1 on

NK cells shows a broader cellular distribution. It adds support to the hypothesis that EA 1 may have an important role in the general process involved with cellular activation.

Cosulich et al. (27) also reported an early activation antigen identified by mAb MLR 3 that is structurally similar to EA 1. It is of interest that mAb MLR3 inhibits IL-1-dependent T cell or thymocyte proliferation. Although mAb P8 does not have inhibitory effects on similar systems (unpublished data), effects of mAb G38 have not been examined. Furthermore, Delia et al. (28) reported that mAb MLR3 alters the thymidine uptake of mitogen-treated lymphocytes; augmentation is detected when cells are stimulated with PMA and ionomycin, and reduction is detected when stimulated with PHA, respectively. Moreover, they are observable only when the cells are depleted of monocytes. Unlike EA 1 and Leu-23, MLR3 is not expressed on unstimulated thymocytes (27), but is induced on activated fetal thymocytes (28). MLR3 is modulated on Jurkat cells cultured with IL-2, but EA 1 is not (unpublished data). These differences suggest MLR3 is a distinct molecule. However, it is possible that these mAbs recognize different epitopes on the same molecule. A direct comparison is needed to resolve these differences in functional assay.

## Summary

A new mAb G38 was generated against purified EA 1, an early activation antigen. In immunoprecipitation, it was reactive with the same complex precipitated by the initial anti-EA 1 mAb P8. mAb G38 augemented PMA-induced proliferation of PBMC. It was shown to be mitogenic for purified T cells in collaboration with PMA in a dose-dependent manner. This effect was independent of monocytes and other accessory cells. mAb G38 augmented PMA-induced IL-2-R expression. In conjunction with PMA, it induced IL-2 synthesis and secretion. Its effects on IL-2-R and IL-2 expression were documented at both protein and mRNA levels. Both anti-EA 1 mAbs did not induce $Ca^{2+}$ influx by themselves in PMA-treated T cells. However, the addition of second anti-mouse Ig antibodies induced readily detectable increases in $[Ca^{2+}]_i$. $Ca^{2+}$-mediated pathways may be utilized as the transduction signal mechanisms. mAb Leu-23 was shown to be reactive with EA 1. mAb Leu-23 was also mitogenic for T cells in the presence of PMA. These findings provide evidence for a functional role for EA 1 in T cell activation and proliferation.

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