# HUMAN T CELL ACTIVATION

## III. Rapid Induction of a Phosphorylated 28 kD/32 kD Disulfide-linked Early Activation Antigen (EA 1) by 12-*o*-Tetradecanoyl Phorbol-13-Acetate, Mitogens, and Antigens

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The tumor promoter, 12-*o*-tetradecanoyl phorbol-13-acetate (TPA)[1] has been shown (1–6) to induce human resting T cells from peripheral blood to proliferate in conjunction with mAbs specific for T3, 9.3 (Tp44) and T11. In these activation systems, monocytes or other necessary cells are not required. In particular, TPA by itself can induce a low level of IL-2-R expression hours after activation. In the presence of these mAbs, IL-2-R expression is markedly enhanced and IL-2 secretion is readily detectable.

To define further the requirements and cellular events for the induction of resting T cells to proliferate, mAbs have been generated against T cells activated either by TPA or by an IgM anti-T3 antibody (7, 8). In this study, one of them is shown to be reactive with a 28 kD/32 kD disulfide-linked and phosphorylated dimer. TPA can readily induce T cells to express this antigen within one hour of activation. Its expression precedes that of IL-2-R. Because of its rapid induction, this antigen has been termed early activation antigen 1 (EA 1).

## Materials and Methods

*Production of mAb EA 1.* CF1 mice were immunized intraperitoneally with $2 \times 10^7$ of T cells activated with TPA for 12 h and with alum as an adjuvant. 3 wk later, $2 \times 10^7$ TPA-activated T cells in PBS were injected intraperitoneally. Spleens were taken 3 d later. Hybridomas were generated using myeloma cell line SP2/0 as a fusion partner as previously described (1). Hybridoma supernatants were screened by flow cytometry against T cells and TPA-activated T cells, and those that reacted only TPA-activated T cells were selected. 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. mAb was purified from ascites using a goat anti-mouse Ig–Sepharose 4B

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[1] *Abbreviations used in this paper:* BCGF, B cell growth factor; DAG, diacylglycerol; EA 1, early activation antigen 1; pkC, protein kinase C; TPA, 12-O-tetradecanoyl phorbol-13-acetate; TT, tetanus toxoid.

column. Bound antibodies were eluted with glycine-HCl buffer, pH 2.9, dialyzed against PBS, and sterilized by filtration.

Other mAbs used were mAb Josh 524 (anti-HLA-DR, IgG1), mAb TE (anti-sheep erythrocyte receptor, IgG2a), and mAb AT-1 (anti-IL-2-R, IgG1), as described previously (1, 9). mAb H4 (anti-platelet and monocyte, IgG2a) was equivalent to that described by Burckhardt et al. (10). B1, pan-B mAb was obtained from Coulter Immunology (Hialeah, FL). As control mAb, HDP-1 (anti-DNP, IgG1), SS1 (anti-sheep erythrocyte, IgG2a), NS8.1 (anti-sheep erythrocyte, IgG2b), and NS4.1 (anti-sheep erythrocyte, IgM) were used. These antibodies were generous gifts from Dr. J. Davie (Washington University, St. Louis, MO).

*Cell Preparation.* PBMC were isolated from buffy coats or peripheral blood from normal volunteers. T cells were obtained by SRBC rosetting (11) and were usually 95% pure. These T cell preparations contained 1–2% monocytes. In certain experiments, extensive depletion of monocytes was carried out by carbonyl-iron treatment, nylon-wool column, and plastic adherence (1). Monocyte-free lymphocyte preparations were obtained by counterflow centrifugal elutriation according to a modified procedure of Wahl et al. (12). Monocytes were purified by Percoll continuous gradient centrifugation from PBMC that were isolated from defibrinated blood (13). To minimize the attachment of platelets to monocytes, monocytes were washed with PBS containing 0.5 mM EDTA. This monocyte preparation was >90% pure, as determined by nonspecific esterase staining. Granulocytes were isolated from the peripheral blood, as the pellet fraction after Ficoll-Hypaque density gradient centrifugation. Contaminating erythrocytes were removed by centrifugation on a 60, 65, and 70% Percoll discontinuous density gradient. The purity of the granulocyte fraction was >95%, as determined by Wright-Giemsa staining. Platelets were isolated from citrated platelet-rich plasma. The gated population of this platelet preparation was >95% pure, as shown by immunofluorescence staining with mAb H4. Erythrocytes were purified from the pellet fraction from peripheral blood after Ficoll-Hypaque density gradient centrifugation. B cells were isolated from tonsils by the depletion of SRBC rosetting cells (9). The resulting B cell preparation was >90% $B1^+$ and <1% $T11^+$.

*Immunofluorescence Studies.* Cells were stained with mAb at 4°C for 30 min. After extensive washing, the cells were further incubated with FITC-conjugated $F(ab')_2$ goat anti-mouse Ig antibodies at 4°C for 30 min. After further washings, the cells were analyzed with an Epics V flow cytometer (Coulter Electronics Inc., Hialeah, FL) or a Cytofluorograf IIs (Ortho Diagnostic Systems Inc., Westwood, MA). Linear integrated fluorescence of the gated population was measured and 10,000 cells were analyzed.

*DNA and Protein Synthesis Assays.* PBMC, T cells, or B cells ($2 \times 10^5$ cells) in 200 μl 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 8 h, cells were pulsed with 0.4 μCi of [$^3$H] thymidine (sp act, 75 Ci/mmol; Amersham Corp., Arlington Heights, IL) and incorporated radioactivity was determined as described (1). To measure protein synthesis, cells were incubated for 18 h in the same way and were labeled with 0.4 μCi of [$^3$H]-leucine (sp act, 128 Ci/mmol; Amersham Corp.) for the last 8 h.

For T cell activation, PHA (Difco Laboratories Inc., Detroit, MI), Con A (Pharmacia Fine Chemicals, Piscataway, NJ), PWM, TPA (Sigma Chemical Co., St. Louis, MO), tetanus toxoid (TT; State Laboratory Institute, Jamaica Plain, MA) and PPD (Connaught Laboratories Limited, Willowdale, Canada) were used. The reagents we used for B cell activation, were Immunobead rabbit anti-human IgM (Bio-Rad Laboratories, Richmond, CA) and B cell growth factor (BCGF; Cellular Products Inc., Buffalo, NY). Cycloheximide and Actinomycin D were from Sigma Chemical Co.

*Immunoprecipitation and Autoradiography.* TPA-activated T cells were labeled with $^{125}$I (spe act, 15.8 mCi/μg of iodine; Amersham Corp.) by the lactoperoxidase technique, or with [$^{35}$S]methionine (spe act, 1.150 Ci/mmol; Amersham Corp.) in methionine-free RPMI 1640 with dialyzed FCS. The labeled cells were solubilized in PBS containing 0.5% NP-40 and 1 mM PMSF. When gels were run under nonreduced conditions, cells were washed once with PBS containing 10 mM iodoacetamide and then were lysed with PBS containing 0.5% NP-40, 1 mM PMSF, and 100 mM iodoacetamide. After centrifugation,

supernatants were added to 30 μl of Sepharose 4B conjugated with goat anti-mouse Ig antibodies (5 mg/ml) that had been incubated with mAbs. The mixture was incubated for 60 min at 4°C with constant mixing, and then was washed three times with a buffer containing 10 mM Tris HCl (pH 7.8), 0.6 M NaCl, 0.1% SDS, and 0.05% NP-40. The absorbed proteins were released from the Sepharose by boiling the mixture for 3 min in a sample buffer containing 0.125 M Tris HCl (pH 6.8), 2% SDS, 5% 2-ME, and 10% glycerol and were subjected to electrophoresis in 9 or 12% polyacrylamide gels in the presence of 0.1% SDS. Gels were stained, destained, and dried. For $^{35}$S-labeled gels, gels were stained and treated with 0.1 M sodium salicylate before drying. Autoradiography was carried out against X-OMAT AR films at −70°C.

Phosphorylation reaction was performed as follows: T cells were washed three times with a phosphate-free buffer (10 mM Hepes [pH 7.4], 140 mM NaCl, 5.4 mM KCl, 0.4 mM $Ca(NO_3)2$, 0.4 mM $MgSO_4$). Cells were suspended in phosphate-free RPMI 1640 with 10% dialyzed FCS at $5 \times 10^6$ cells/ml and were activated with 10 ng/ml of TPA for 18 h. Then 0.1 mCi/ml of [$^{32}$P]orthophosphate (carrier-free, Amersham Corp.) was added, and incubation was continued for 3 h. The reactions were stopped with cold PBS containing 10 mM sodium pyrophosphate, 50 mM NaF, and 2 mM EDTA and cells were washed once more with this buffer. Immunoprecipitation and autoradiography were carried out as described above, except that 5 mM sodium pyrophosphate, 2 mM EDTA, and 50 mM NaF were added to the lysis buffer and washing buffers.

## Results

*Identification of EA 1 as a Phosphorylated 28 kD/32 kD Disulfide-bonded Bimolecular Complex.* CF1 mice were immunized with TPA-activated human T cells and hybridomas were generated as described in Materials and Methods. The supernatants were screened for their reactivities against TPA-activated and resting T cells by indirect immunofluorescence. One hybridoma, termed EA 1, was found to secrete antibodies reactive with TPA-activated T cells but not with resting T cells. Hybridoma EA 1 was cloned twice on soft agar and mAb EA 1 was found to be an IgG2a molecule.

TPA-activated T cells were labeled with $^{125}$I by the lactoperoxidase method. From the cell lysate, mAb EA 1 precipitated a protein of 60 kD molecular mass as determined by SDS-PAGE under nonreducing conditions (Fig. 1, lane *1*). Upon reduction, two bands of 28 kD and 32 kD were resolved (Fig 1, lane *3*). To determine whether EA 1 was a phosphoprotein, $^{32}$P-labeled TPA-activated T cells were lysed and immunoprecipitated with mAb EA 1. As shown in lane *5* of Fig. 1, both the 28-kD and 32-kD polypeptides were phosphorylated. Thus, EA 1 is a phosphorylated 28 kD/32 kD disulfide-linked bimolecular complex.

Because of the similar molecular weights between EA 1 and HLA-DR, the relationship between these two antigens was investigated further. Immunoprecipitates from $^{125}$I-labeled PHA-activated T cells by mAb EA 1 and mAb Josh 524 (anti-HLA-DR) were electrophoresed in the same gel. The H chain of the HLA-DR antigen migrated more slowly and both subunits of HLA-DR showed sharper bands as compared with EA 1. Furthermore, comparison of the V8 protease peptide-mapping patterns of EA 1 and HLA-DR molecules showed no similarity. Thus, EA 1 and HLA-DR are not related.

*Cell Distribution and Induction of EA 1 Expression.* By flow cytometry, EA 1 was found on the majority of the cryopreserved thymocytes by indirect immunofluorescence (Table I). A small number of freshly isolated T and B cells were

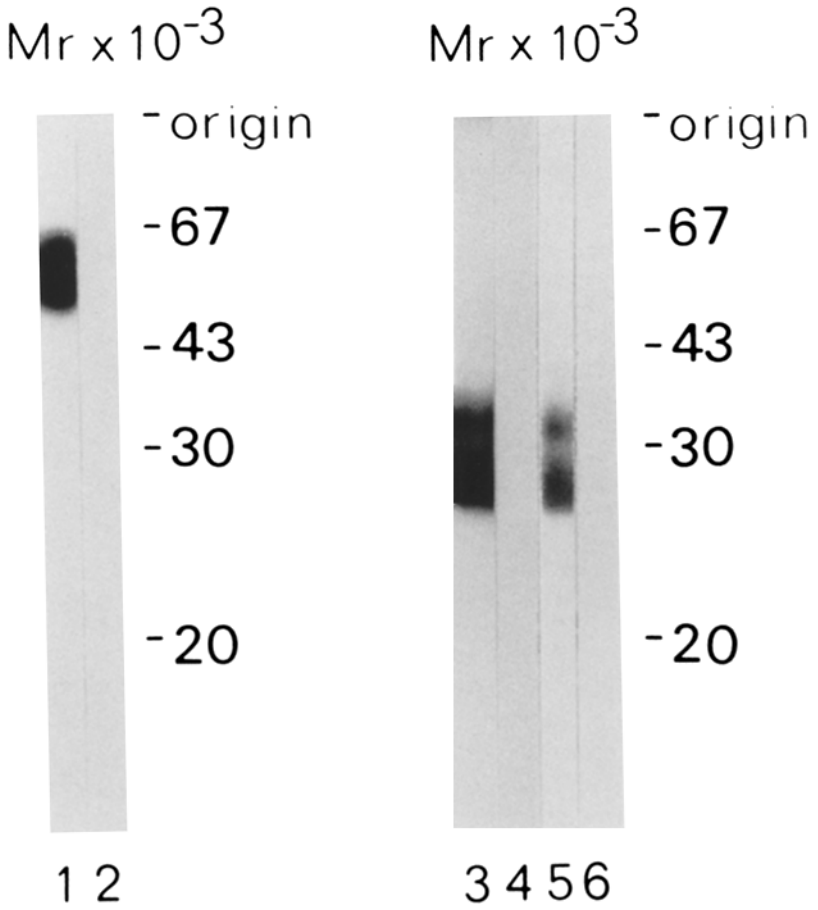


FIGURE 1. Immunoprecipitation of EA 1 from $^{125}I$ (lanes *1–4*) and $^{32}P$ (lanes *5* and *6*)-labeled TPA-activated T cells. A 28/32 kD disulfide-linked complex was precipitated and both chains were phosphorylated. T cells activated with 30 ng/ml of TPA for 18 h were labeled with $^{125}I$ by lactoperoxidase: lanes *1* and *3* (mAb EA 1) and lanes *2* and *4* (control mAb) show immunoprecipitation from $^{125}I$-labeled TPA-activated T cells, while lanes *5* (mAb EA 1) and lane *6* (control mAb) show immunoprecipitation from $^{32}P$-labeled TPA-activated T cells. Lanes *1* and *2* were run under nonreduced conditions and lanes *3–6* were under reduced conditions.

weakly stained by mAb EA 1. EA 1 was not detectable on monocytes, granulocytes, erythrocytes, or platelets.

The expression of EA 1 by T cells was readily inducible as shown in Table II. TPA induced the vast majority of the treated T cells to express EA 1 while slightly more than half of the Con A– and PHA-activated T cells stained with mAb EA 1. In the case of PWM, a lesser T cell mitogen, only 16.5% of the PWM-treated T cells expressed EA 1. In an additional experiment, PHA and Con A induced higher percentages of T cells to express EA 1. In the case of soluble antigen and alloantigen stimulation, ~20% of the T cells were induced to express EA 1. Experiments were also carried out with T cell populations depleted of monocytes. TPA induction of EA 1 expression was readily detectable.

Although mAb EA 1 was generated against TPA-treated T cells, it was readily

TABLE I

*EA 1 Was Detectable in a Major Population of Thymocytes and Weakly Expressed by a Small Number of Circulating B and T Cells*

| Cell type | Percent positive* |
|---|---|
| Thymocytes | 61.3 ± 11.4 |
| T | 7.1 ± 1.3 |
| B | 6.6 ± 5.8 |
| Monocytes | <1 |
| Granulocytes | <1 |
| Platelets | <1 |

* The percentages of positive cells were determined by flow cytometry by counting 10,000 cells. Data were presented as mean ± SD from six individuals.

TABLE II

*Activated T and B Cells Expressed EA 1*

| | Stimulating agents* | Percent positive‡ |
|---|---|---|
| T Cells | Medium | 1.0 ± 0.1 |
| | TPA | 85.9 ± 13.9 |
| | PHA | 56.5 ± 12.4 |
| | Con A | 51.6 ± 11.7 |
| | PWM | 16.5 ± 2.1 |
| | PPD | 21.5 ± 3.8 |
| | TT | 17.1 ± 1.4 |
| | MLR | 21.1 ± 1.3 |
| B Cells | Medium | 5.0 ± 3.7 |
| | TPA | 89.3 ± 16.8 |
| | Anti IgM + BCGF | 58.1 ± 14 |

* Peripheral blood T cells ($10^6$/ml) were stimulated with TPA (30 ng/ml). PHA (10 μg/ml), Con A (5 μg/ml), or PWM (0.1%) for 3 d, or with PPD (5 μg/ml), TT (40 μg/ml), or irradiated allogeneic cells ($2 \times 10^5$) for 6 d. Tonsillar B cells ($10^6$/ml) were stimulated with TPA (30 ng/ml) or anti-IgM beads (500 μg/ml) plus BCGF (5%) for 3 d. Both T and B cell preparations contained a small number of monocytes.

‡ Percentages of positive cells were determined in a flow cytometer by counting 10,000 cells. Data were presented as mean and ± SD from six individuals.

apparent that its expression was not limited to the T cell lineage. As shown in Table II, TPA induced a majority of the isolated B cells to express EA 1. Anti-μ antibodies in the presence of crude BCGF were also shown to be potent inducers.

Repeated attempts to induce EA 1 expression by monocytes and granulocytes, as well as by fibroblasts, with TPA were unsuccessful. However, nonlymphoid hematopoietic leukemia cell lines were shown to be responsive to TPA in EA 1 induction (Table III). Most T leukemia cell lines were found to express low levels of EA 1. All six cell lines were found to be responsive to TPA by increased EA 1 staining. In the case of B cell lines, transformed by EBV, only one of the five were stained by mAb EA 1 without TPA treatment. Four of the five were

TABLE III
*EA 1 Expression by Hematopoietic Cell Lines: TPA-Induced or -Enhanced EA 1 Expression*

| Cell line | TPA | |
|---|---|---|
| | Absent | Present |
| T Cell Lines | | |
| 8042 | − | ++++* |
| Jurkat | ++ | ++++ |
| MT-1 | ++ | ++++ |
| CEM | + | ++++ |
| HSB-2 | + | ++++ |
| Molt-4 | + | ++++ |
| B Cell Lines | | |
| 32A1 | − | + |
| Daudi | − | +++ |
| Raji | − | + |
| SeD | + | +++ |
| Josh 7 | − | − |
| Non-lymphoid Lines | | |
| ML1 | + | + |
| KG1 | − | ++ |
| HL60 | − | − |
| K562 | − | +++ |
| HEL | ++ | +++ |
| U937 | + | ++++ |

* −, <5%; +, 5–15%; ++, 15–50%; +++, 50–75%; ++++, >75%. The reactivity of the cell lines were determined by indirect immunofluorescence with a flow cytometer. Three determinations were done with each cell line.

responsive and a pre–B cell line, Josh 7 (14), was not inducible. In six nonlymphoid cell lines, four were found to be responsive to TPA by an increase of EA 1 expression. These results indicate the need to explore further the possibility that nonlymphoid hematopoietic cells can express EA 1.

*Kinetics of EA 1 Expression.* The induction of EA 1 expression by T cells with TPA was studied further. As shown in Fig. 2, EA 1 induction by TPA was dose dependent. With as little as 0.3 ng/ml of TPA, a majority of T cells were induced to express EA 1. Full effect was seen with 1 ng/ml of TPA.

The induction of EA 1 expression by T cells with TPA was very rapid, as shown in Fig. 3*A*. Although not shown, as early as 30 min after the addition of TPA, a substantial number of T cells were stained by mAb EA 1. In one rare instance, EA 1 expression was seen in 15 min. After 1 h of activation, a vast majority (85–90%) of the T cells were positive. The maximal percentage of T cells expressing EA 1 was detected after 3–4 h. There was a slight increase in the fluorescence intensity until 18–24 hours. After this period, both the percentage of EA $1^+$ T cells and the intensity of staining decreased gradually. For comparison, TPA-induced EA 1 expression by B cells had less rapid kinetics, as shown in Figure 3*B*.

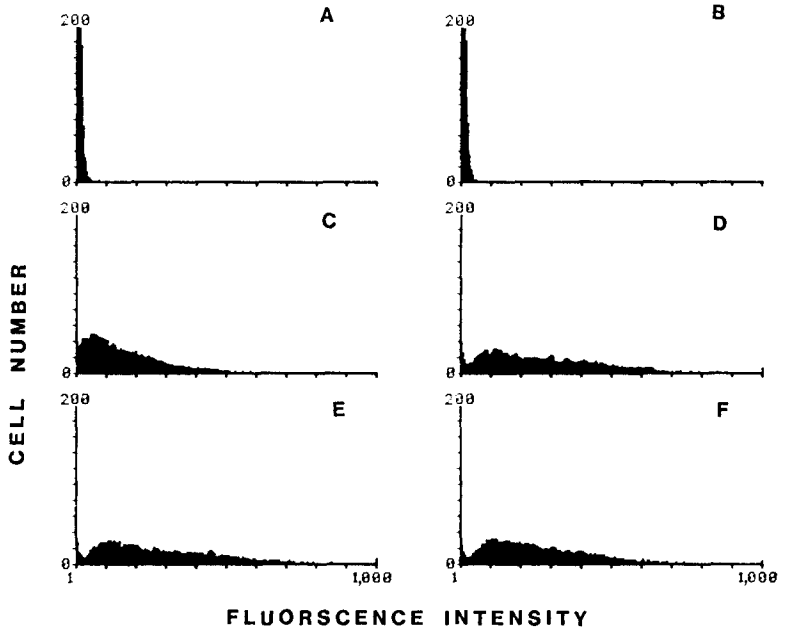


FIGURE 2. EA 1 induction by TPA is dose dependent. Isolated T cells were treated with increasing doses of TPA for 4 h and EA 1 expression was measured by indirect immunofluorescence. (*A*) Control without TPA; (*B*) 0.1 ng/ml; (*C*) 0.3 ng/ml; (*D*) 1 ng/ml; (*E*) 5 ng/ml; (*F*) 30 ng/ml.

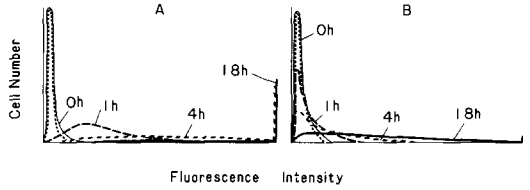


FIGURE 3. Cytofluorographic profiles of the kinetics of EA 1 expression. T cells (*A*) and B cells (*B*) activated with TPA (30 ng/ml) were taken at the indicated times and stained with mAb EA 1, followed by flow cytometer analysis. This is representative of four independent experiments.

To minimize the possibility that resting T cells are partially activated by rosetting with SRBC, a lymphocyte fraction was isolated by counterflow centrifugal elutriation at 2,000 rpm with a flow rate of 12 ml/min. This fraction was devoid of monocytes, as determined by esterase staining and surface marker analysis. It stained 92% for T11. TPA activation of this lymphocyte preparation did not induce EA 1 expression at 30 min. However, 85% of the lymphocytes were stained by mAb EA 1 after 1 h and the maximal number of EA $1^+$ T cells was seen at 3 h. An additional experiment yielded similar results.

*EA 1 Expression Preceded that of IL-2-R.* We compared the kinetics of expression of EA 1 and selected activation antigens on activated T and B cells (Fig. 4). In the case of TPA-induced T cell activation, as shown in Fig. 4*A*, >85% of T cells were positive for EA 1 within 1 h. By 4 h, >95% were positive. The expression of IL-2-R was clearly delayed in comparison with that of EA 1. In

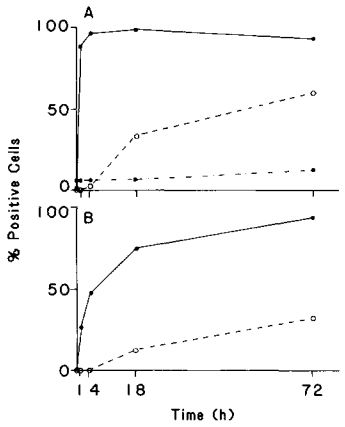


FIGURE 4. Kinetics of expression of EA 1 and selected activation antigens on activated T and B cells. EA 1 expression preceded that of IL-2-R. A summary of four activation experiments is shown: (*A*) EA 1, IL-2-R and HLA-DR on T cells activated with TPA (30 ng/ml); and (*B*) EA 1 and IL-2-R on B cells activated with TPA (30 ng/ml). 10,000 cells were analyzed with a flow cytometer. (—●—) EA 1, (--○--) IL-2-R, (-●-) HLA-DR.

this system, HLA-DR was not induced significantly by 72 h. In the case of B cells, TPA-induced EA 1 expression was less rapid, and 18–36 h were required for its maximal expression (Fig. 4*B*). However, it is apparent that in the B cells, EA 1 expression also preceded that of IL-2-R.

The earlier expression of EA 1 in comparison with IL-2-R expression was also documented by biosynthetic labeling experiments. EA 1 was readily precipitated by mAb EA 1 from T cells treated with TPA and labeled with [$^{35}$S]methionine for 4 h (Fig. 5, lane *5*). In contrast, mAb AT-1 (9), reactive with the IL-2-R, failed to precipitate any $^{35}$S-labeled peptide (Fig. 5, lane *6*). Both antibodies did not precipitate $^{35}$S-labeled peptides from unstimulated T cells (Fig. 6, lanes *2* and *3*).

In addition to TPA, polyclonal mitogen stimulation of T cells also induced EA 1 earlier than IL-2-R expression (Table IV). By 4 h, EA 1 expression was readily detectable. In another experiment, PHA induced 78.8, 94.0, and 56.5% of EA 1$^+$ cells at 4 h, 18 h, and 72 h, respectively. Similarly, 68.5, 69.8, and 51.6% of EA 1$^+$ cells appeared after 4 h, 18 h, and 72 h, of activation with Con A, respectively. Although EA 1 expression was readily detectable on T cells stimulated by soluble antigens and alloantigens, its expression was not clearly shown to precede that of IL-2-R. Studies at more time points between 84 and 108 h would yield more definitive results.

*Both Cycloheximide and Actinomycin D Inhibited EA 1 Expression.* Because of the rapid appearance of EA 1, the possibility that EA 1 was not newly synthesized was considered and ruled out. As shown in Fig. 6, cycloheximide inhibited EA 1 expression in a dose-dependent manner. In both T and B cells, significant inhibition of EA 1 expression was seen at 50 μM of cycloheximide. At 500 μM, at which no leucine incorporation was detected, EA 1 expression was completely inhibited. This inhibition was confirmed by experiments involving [$^{35}$S]methionine labeling and immunoprecipitation (Fig. 7).

EA 1 expression was further shown to require newly transcribed mRNA. At 1

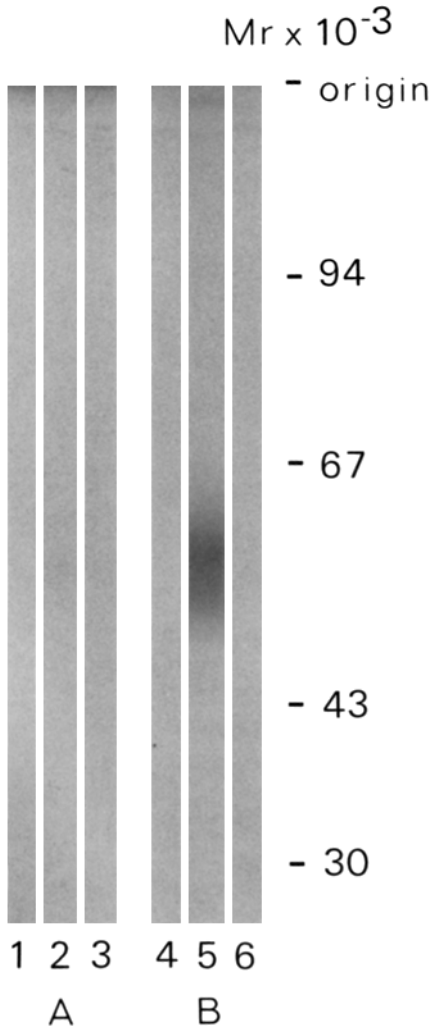


FIGURE 5. EA 1 synthesis preceded that IL-2-R. T cells ($10^7$/ml) labeled with [$^{35}$S]methionine for 4 h in the absence (*A*) or presence (*B*) of 30 ng/ml of TPA. Lanes *1* and *4*, control mAb; lanes *2* and *5*, mAb EA 1; lanes *3* and *6*, anti-IL-2-R mAb AT 1 under nonreducing conditions.

$\mu$g/ml, actinomycin D significantly inhibited EA 1 expression, as shown in Fig. 8*C*. When TPA and actinomycin D were added simultaneously, even at 10 $\mu$g/ml of the RNA synthesis inhibitor, a low level of EA 1 expression was seen, with a small shoulder in the histogram (Figure 8*E*). Complete inhibition was seen when the T cells were pretreated for 15 min before the addition of TPA (Fig. 8*F*). Although measurements were made at 4 h after the addition of TPA in the presented experiments, similar results were obtained when EA 1 expression was determined 1 h after TPA activation.

*mAb EA 1 Did Not Block T or B Cell Proliferation.* To investigate whether or not mAb EA 1 has any effect on T cell or B cell proliferation, mAb EA 1 was purified from ascites by Sepharose 4B coupled with goat anti–mouse Ig. As

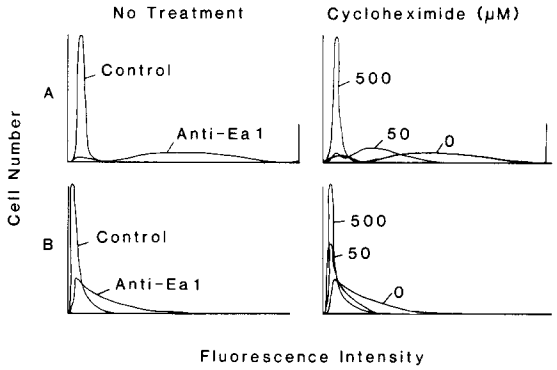


FIGURE 6. EA 1 expression by T cells activated with TPA is inhibited by cycloheximide, a protein synthesis inhibitor. T cells (*A*) and B cells (*B*) were treated with increasing doses of cycloheximide, and EA 1 expression with measured by indirect immunofluorescence.

TABLE IV

*Kinetics of EA 1 Expression by T Cells Activated by Mitogens, Soluble Antigens or Alloantigens*

| Stimulating agents* | Percent positive cells after:‡ | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 4 h | 18 h | 60 h | 84 h | 108 h | 132 h | 156 h | 180 h |
| Expression of Ea 1 | | | | | | | | |
| PHA | 28.1§ | 49.2 | 51.7 | —‖ | — | — | — | — |
| Con A | 43.6 | 55.4 | 40.3 | — | — | — | — | — |
| PWM | 9.1 | 12.5 | 15.7 | — | — | — | — | — |
| PPD | — | — | 3.6 | 3.8 | 15.7 | 16.8 | 21.5 | 17.9 |
| TT | — | — | 1.5 | 0.8 | 11.5 | 10.1 | 14.2 | 17.1 |
| MLC | — | — | 3.4 | 3.5 | 20.1 | 16.1 | 21.1 | 17.4 |
| Expression of IL-2-R | | | | | | | | |
| PHA | 1.3 | 15.7 | 28.9 | — | — | — | — | — |
| Con A | 1.4 | 38.1 | 28.8 | — | — | — | — | — |
| PWM | 1.3 | 9.6 | 10.9 | — | — | — | — | — |
| PPD | — | — | 3.7 | 4.1 | 11.4 | 10.1 | 18.8 | 23.5 |
| TT | — | — | 3.1 | 3.1 | 8.6 | 5.7 | 13.4 | 12.4 |
| MLC | — | — | 4.9 | 3.7 | 13.4 | 13.7 | 25.8 | 28.9 |

* See Table II.

‡ The percentages of positive cells were determined by indirect fluorescence with mAb EA 1 and a flow cytometer. <5% of mitogen-stimulated T cells stained weakly with control mAb of appropriate isotypes. In the case of T cells in cultures with antigens (PPD, TT, and MLC), <2% were weakly positive. These controls were done at all time points.

§ The averages of three experiments were presented.

‖ —, not done.

shown in Table V, mAb EA 1 had no effect on T cell proliferation induced by TPA or PHA. B cell proliferation induced by TPA or anti-IgM plus BCGF was not inhibited either. In addition, no significant inhibition by mAb EA 1 in Con A–, PPD-, or allogeneic cell–induced proliferation or in IL-2-dependent proliferation of T blasts was detected. mAb EA 1 was not mitogenic for either PBMC or T or B cells.

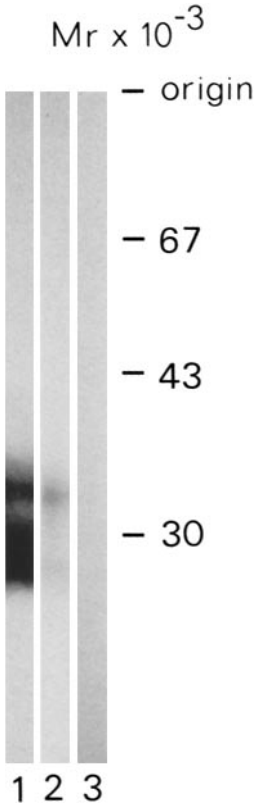


FIGURE 7. EA 1 synthesis by T cells activated with TPA is inhibited by cycloheximide. TPA activated T cells were labeled with [$^{35}$S]methionine in the absence of cycloheximide (lane *1*) and in the presence of 50 $\mu$M cycloheximide (lane *2*) and 500 $\mu$M cycloheximide (lane *3*).

## Discussion

Activation of human lymphocytes is associated with the expression of new surface antigens. These antigens include IL-2-R (reviewed in reference 15), transferrin receptor (16, 17), insulin receptor (18), vitamin B 12 receptor (19, 20), enzymes (21–24), HLA-DR (25), as well as functionally yet undetermined molecules (26–38). Among these, IL-2-R, transferrin receptor, 4F2, and TLiSA1 are early activation antigens. However, these are only marginally detectable several hours after activation and their maximal version occurs 24–72 h after activation. In this study, EA 1 has been shown to be different from these reported activation antigens by its molecular weight, cell distribution, and rapid induction within 1 h of activation.

EA 1 is a 28 kD/32 kD disulfide-linked dimer. Its molecular mass is very similar to that of HLA-DR. In addition to the presented data, TPA-activated T cells have been shown not to stain with both mouse monoclonal and rabbit heterologous antibodies specific for HLA-DR (data not shown). HLA-DR is readily detectable on resting B cells and monocytes, while EA 1 is not expressed by these cells. This is conclusive evidence that EA 1 is not related to HLA-DR. The 60 kD molecular mass of EA 1 under nonreducing conditions is also similar

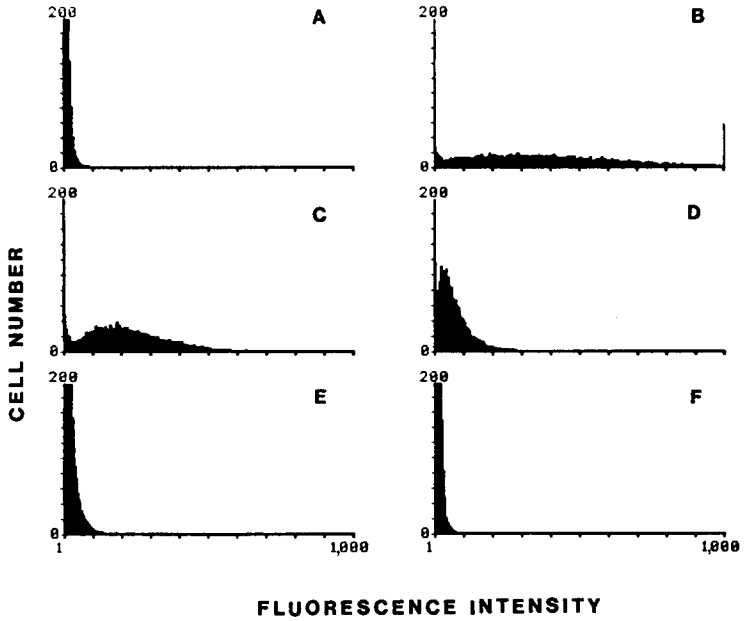


FIGURE 8. EA 1 expression by T cells activated with TPA requires mRNA transcription. T cells were activated with TPA (30 ng/ml) in the presence of actinomycin D (Act D). EA 1 expression was measured 4 h after treatment. TPA and Act D were added at the same time in experiments shown in *C–E*. (*A*) Control without TPA and Act D; (*B*) no Act D; (*C*) 1 μg/ml Act D; (*D*) 5 μg/ml Act D; (*E*) 10 μg/ml Act D. (*F*) Pretreatment of T cells for 15 min with 10 μg/ml Act D before the addition of TPA completely blocked EA 1 expression.

to pp $56^{LSTRA}$, a murine lymphocyte-specific, protein-tyrosine kinase, initially identified in the T cell lymphoma LSTRA (39). However, EA 1 is a biomolecular complex, whereas pp $56^{LSTRA}$ is a single peptide. In addition, preliminary experiments indicate that threonine and serine are primarily phosphorylated in EA 1. Thus EA 1 is not likely to be analogous to $pp56^{LSTRA}$. Udey et al. (40, 41) have studied early changes in protein synthesis in human T lymphocytes exposed to mitogens with [$^{35}$S]methionine labeling and two-dimensional gel electrophoresis. A membrane protein with a molecular mass of 28 kD was shown to be synthesized rapidly upon activation. Whether or not this protein is identical to the smaller subunit of EA 1 remains to be determined.

Although EA 1 is not easily detectable on peripheral blood lymphocytes, its presence on thymocytes is readily shown. The thymocytes used in the reported experiments were cryopreserved and recovered. In general, the surviving thymocytes are the more immature cells. It is likely that EA 1 is expressed on immature thymocytes and it disappears as these thymocytes mature further. In situ analysis with thymic tissue sections and other T cell surface markers defining various stages of T cell differentiation will provide supportive evidence for this thesis.

The induction of EA 1 expression is not limited to T cells. B cells are readily inducible although kinetics of EA 1 expression is slower. Repeated attempts to induce monocytes and granulocytes to express EA 1 have not been successful. However, the finding that nonlymphoid cell lines can express EA 1 suggests that

TABLE V

*mAb EA 1 Did Not Block T and B Cell Proliferation*

| mAb | μg/ml | [$^3$H]Thymidine incorporation (cpm) | | | |
|---|---|---|---|---|---|
| | | TPA* | PHA* | TPA‡ | Anti-IgM + BCGF‡ |
| EA 1 | 0.0 | 25,932 | 64,951 | 20,842 | 11,209 |
| | 0.1 | 25,259 | 64,220 | 19,041 | 10,914 |
| | 0.3 | 23,926 | 61,308 | 19,195 | 11,049 |
| | 1.0 | 23,332 | 61,215 | 19,608 | 11,932 |
| | 3.0 | 21,687 | 60,896 | 20,985 | 10,717 |
| | 10.0 | 19,899 | 61,374 | 19,599 | 10,655 |
| SS1 (IgG2a anti-SRBC) | 0.1 | 23,563 | 61,592 | 21,322 | 11,943 |
| | 0.3 | 24,105 | 63,455 | 21,971 | 10,110 |
| | 1.0 | 24,800 | 61,422 | 22,225 | 11,382 |
| | 3.0 | 22,946 | 62,571 | 19,099 | 12,175 |
| | 10.0 | 21,205 | 60,333 | 21,632 | 11,299 |
| Without mitogen§ | | 392 | 962 | 205 | 183 |

* T cells ($2 \times 10^5$) were stimulated with TPA (30 ng/ml) or PHA (10 μg/ml).

‡ B cells ($2 \times 10^5$) were stimulated with TPA (30 ng/ml) or anti-IgM beads (500 μg/ml) plus 5% BCGF. [$^3$H]Thymidine incorporation was measured after 3 d of incubation. Standard deviations of each mean value were within 15%. Both T and B cells had 1–2% monocytes.

§ mAb EA 1 was not added to these cultures.

EA 1 has a broader cell distribution. It would be of considerable interest to determine that progenitor cells of nonlymphoid hematopoietic lineages constitutively express EA 1. If this is not the case, the question whether EA 1 is inducible needs to be entertained.

TPA-induced EA 1 expression by T cells appears to be in a dose-dependent manner. It is independent of monocytes. This induction is readily demonstrable on lymphocytes not treated with SRBC. The slight delay in EA 1 expression would indicate that a degree of T cell activation is induced by rosetting with SRBC. The degree of EA 1 induction by mitogens is more variable. This may relate to the varying numbers of monocytes and accessory cells in the cell preparations. Further studies with T cell preparations that are extensively depleted of monocytes will provide conclusive evidence whether mitogen-induced EA 1 expression is independent of monocytes.

The speed of EA 1 induction is surprisingly rapid. In both TPA and mitogen systems, EA 1 induction precedes that of IL-2-R in both T and B cells. In TPA-activated T cells, EA 1 expression is detectable within 30–60 min, a time interval at which little IL-2 mRNA induction has been demonstrated (42, 43). The complete inhibition of EA 1 expression by cycloheximide and actinomycin D indicates that EA 1 expression requires new mRNA and protein synthesis. It also provides evidence for the need of gene activation for EA 1 expression. The rapid nature of EA 1 induction would make it a desirable model for the studies of gene activation.

Accumulative evidence indicates that sequential gene activation occurs during the induction of resting T cells to proliferate (1, 4–6, 15, 42–44). Different

inductive stimuli are required for various gene activation. The signals involving activation via the T3/Ti complex have been studied in some detail (reviewed in reference 44). The increase in free cytoplasmic $Ca^{2+}$ concentration and the activation of protein kinase C(pkC) are identified as early events leading to IL-2 synthesis and proliferation. The synergism between these two signals is demonstrated. In using TPA as a probe, it is postulated that many of the TPA-induced cellular effects may be explained by its ability to activate pkC (reviewed in reference 45). This has been supported by the studies (reviewed in reference 46) using synthetic diacylglycerols (DAG), which may serve as a more physiological signal for pkC activation. Recently, we have shown (47) that both L-oleoyl-2-acetylglycerol and 1,2-dioctanoylglycerol could induce EA 1 expression. In addition, the protein kinase inhibitor H-7, the most potent pkC inhibitor (48) inhibits EA 1 expression completely (Bjorndahl, J. M., L. K. L. Jung, and S. M. Fu, manuscript in preparation). Anti–T3 antibodies are shown to induce a marked increase in free cytoplasmic $Ca^{2+}$. This stimulus does not induce EA 1 but provides a synergizing signal to DAG-induced EA 1 expression. These studies provide further evidence for the role of pkC activation in lymphocyte activation. With the development of a cDNA probe for EA 1, it will be possible to determine whether anti-T3 antibodies exert their synergism by increasing the rate of either mRNA or protein synthesis. Although DAG can replace TPA in EA 1 induction, we have obtained data indicating that IL-2-R expression is not induced either by DAG, alone, or in the presence of anti-T3 antibodies. Thus, the possibility that TPA has additional physiological effects in addition to pkC activation needs to be considered. These studies also provide evidence to identify EA 1 induction as a distinct stage of T cell activation.

The physiological signal for EA 1 induction and the function of EA 1 remain to be determined. Because of its molecular structure, it is not likely that EA 1 may serve as the IL-1-R (49). The inability of mAb EA 1 to block lymphocyte proliferate is not sufficient to preclude EA 1 as a receptor for a yet-to-be-identified growth factor or its role in cell–cell interaction. The availability of mAbs to differing epitopes of EA 1 would be helpful in further experiments. The rapid induction kinetics may facilitate the search for a physiological ligand for EA 1.

## Summary

With human T cells activated by 12-*o*-tetradecanoyl phorbol-13-acetate (TPA) as immunogen, an IgG2a mAb, early activation antigen 1 (EA 1), was generated against a 60-kD protein with disulfide-linked 28-kD and 32-kD subunits. Both subunits were phosphorylated. The antigen, EA 1, was readily detected on ~60% of isolated and cryopreserved thymocytes, as determined by indirect immunofluorescence. A low level of EA 1 expression was detectable on 6–7% of blood lymphocytes. TPA-activated T cells expressed EA 1 as early as 30 min after activation. By 1 h, 85–90% of the T cells stained with mAb EA 1. By 3–4 h, the expression of EA 1 was detected in >95% of the T cells. Although the percentages of EA $1^+$ T cells did not change, the intensity of staining increased slightly. After 18–24 h, both the percentage of EA $1^+$ cells and the intensity of staining decreased gradually. TPA-induced EA 1 expression was independent of mono-

cytes. EA 1 expression was slightly delayed in T cells that were isolated without the rosette selection and treated with TPA. Nevertheless, >85% of these T cells expressed EA 1 within 1 h, and the maximal number of EA $1^+$ T cells was also detected at 3–4 h. In T cell populations with 1–2% monocytes, about 50–90% of the PHA- or Con A–activated T cells expressed EA 1 with a slower kinetics. EA 1 expression preceded that of IL-2-R in these activation processes. Similarly, T cells activated by soluble antigens (tetanus toxoid and PPD) and alloantigens in MLR also expressed EA 1 after a longer incubation. ~20% of the T cells stained for EA 1 at day 6. EA 1 expression was not limited to activated T cells. B cells activated by TPA or anti-IgM antibody plus B cell growth factor expressed EA 1. The kinetics of EA 1 expression was markedly slower and the staining was less intense. Repeated attempts to detect EA 1 on resting and TPA-activated monocytes and granulocytes have not been successful. However, the detection of EA 1 in nonlymphoid cell lines would indicate that EA 1 may have a broader cell distribution. EA 1 expression was due to de novo synthesis, as the induction of EA 1 was blocked by cycloheximide and actinomycin D. Thus, EA 1 is the earliest activation antigen detectable to date, and it would serve as a useful marker for early T cell activation. Although mAb EA 1 has been shown not to block T or B cell proliferation, the possibility that EA 1 is a receptor for a yet-to-be-defined growth factor or that it plays an important function in cellular interaction needs further consideration.

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