T CELL STIMULATION VIA THE ERYTHROCYTE RECEPTOR
Synergism Between Monoclonal Antibodies and Phorbol Myristate Acetate Without Changes of Free Cytoplasmic Ca++ Levels

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Successful stimulation of human resting T cells, stimulation leading to cell proliferation, classically requires two distinct signals. Signal one, delivered by antigen, lectin, or mAb directed against the antigen receptor complex, T3/Ti, alone is not sufficient unless the second, usually accessory cell derived signal is provided. With lectin (1) and CD3 antibody (2–4), but not with antigen (5) stimulation, signal two can be substituted by PMA (6).

Apart from triggering via T3/Ti, an alternative pathway of T cell activation has recently been described (7). A combination of two mAb detecting distinct epitopes on the sheep erythrocyte receptor (E-receptor, CD2 structure) can lead to vigorous T cell proliferation (7). In this system, the first antibody induces the expression of a thus-far hidden epitope on the E-receptor and makes it accessible to the second antibody, again a two-signal nature is evident.

In this paper, we show that, for stimulation via this alternative pathway, one signal may be substituted by PMA. T cells cultured in the presence of PMA can be stimulated by some but not all CD2 mAb. While T cell stimulation via either the CD2 or the CD3 structure has recently been causally linked to a concomitantly observed elevation of intracellular Ca++ levels (8–11), in our system, T cell growth occurs without an apparent change in intracellular Ca++. 

Materials and Methods

Monoclonal Antibodies. VIT3 detects an epitope on the T3 membrane structure (CD3). Immune precipitation revealed as its target a 19–29 kD protein complex. It comodulates with OKT3 (Ortho Diagnostics, Raritan, NJ), T3 (Coulter Immunology, Hialeah, FL), and anti-Leu-4 (Becton Dickinson Monoclonal Center, Mountain View, CA), and has the same cellular distribution of reactivity. The antibody was obtained after immunizing BALB/c mice with purified T cells, and was generated essentially as previously described (12).

VIT13 was obtained after immunizing with PHA blasts. It detects an epitope on the E-receptor, hidden on resting T cells but strongly exposed after in vitro stimulation (13). A detailed characterization of this antibody will be published (Stockinger, manuscript in preparation).

Other mAbs used in this study were VIP1 (transferrin receptor) (13), VDI1 (Ia/Dr) (13), OKT11 (Ortho Diagnostics), T11 (Coulter Immunology), BMA 0110 (Behring Corp., Marburg, Federal Republic of Germany), 9.6 (kindly provided by Dr. P. Martin,
Purification of T Cells. T cells were separated by rosetting with neuraminidase-treated SRBC, and by density gradient centrifugation (Ficoll-Hypaque, Pharmacia Fine Chemicals, Uppsala, Sweden). Cells from the interphase were collected and are referred to as non-T cells. After lysis of rosetted SRBCs, T cells obtained from the pellet were further depleted of accessory cells by plastic adherence, nylon wool adherence, and treatment with anti-Ia (Q5/13, kindly provided by Dr. Vito Quaranta, Scripps Clinic and Research Foundation, La Jolla, CA), and antimonocyte (VIM13) (16) mAb plus complement. The resulting T cell preparation contained <0.5% monocytes, as estimated by indirect immunofluorescence on a FACS 440 cell sorter. Unless supplemented with non-T cells, this population only marginally responded to PHA, PMA, or CD3 antibody stimulation.

Proliferative Assays. PHA (Wellcome, Beckenham, United Kingdom) was used at a final concentration of 2 μg/ml. PMA (Sigma Chemical Co., St. Louis, MO) was dissolved in ethanol at 10 mg/ml, diluted 1:10 with RPMI 1640, and stored at -30°C. Immediately before use, the material was thawed and used at a final concentration of 50 ng/ml, unless otherwise indicated. For stimulation, the mAbs were used at 10 μg/ml or a 1:100-1:400 dilution of ascitic fluid if the protein content was not available.

Cultures were set up in U-bottomed microwells (Nunc, Roskilde, Denmark) in a final volume of 120 μl. The medium was RPMI 1640 (Gibco, Paisley, United Kingdom) containing 10% FBS (Flow Laboratories, Beckenham, United Kingdom), L-glutamine, and antibiotics. The cultures were incubated in a humidified atmosphere with 5% CO2 at 37°C for 96 h. 1 μCi/well [3H]thymidine (New England Nuclear, Boston, MA) was added 18 h before harvest. The incorporated radioactivity was collected on glass fiber filters and counted in a liquid scintillation counter. Values are given as cpm ± SE (× 10^-5) of triplicate cultures.

Phenotypic Analysis of Stimulated T Cells. Purified T cells, at a density of 10^6 cells/ml, were cultured in the presence of 50 ng/ml PMA for 48 h. The cells were harvested and stained with antibodies as indicated in Fig. 2. The binding of the antibodies was assessed by standard indirect immunofluorescence techniques and evaluated on a FACS 440 cell sorter (Becton Dickinson Immunocytometry Systems, Sunnyvale, CA), essentially as previously described (13).

Determination of Cytoplasmic Ca++ Levels. Purified T cells were loaded by incubation with Quin2 acetoxymethyl ester (Quin2/AM; Amersham International, Buckinghamshire, United Kingdom) following the method of Tsien et al. (8) with slight modifications. Briefly, 5 × 10^7 cells were incubated with 50 μl of a 1 mM Quin2 stock solution in 5 ml RPMI 1640 with 10% FBS for 50 min at 37°C. Subsequently, the suspension was diluted fourfold and incubated 30 min more. The cells were then spun down gently, resuspended in fresh RPMI 1640 with FBS, and kept in the dark at 37°C. Immediately before use, 1.5 × 10^7 cells were spun down, resuspended in 2.5 ml of simplified medium (145 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM CaCl_2, 0.5 mM MgSO_4, 5 mM glucose, 10 mM Hepes dissolved in double distilled water and titrated with NaOH to pH 7.40 at 37°C), and transferred to prewarmed quartz cuvettes. Fluorescence intensity was recorded on an Aminco spectrofluorometer SPF 500 (Aminco Inc., Silver Spring, MD) set on ratio mode with excitation at 359 nm and emission at 492 nm (1- and 5-nm bandwidths). The cuvette was constantly kept at 37°C and the cell suspension was continuously stirred. All reagents (Con A; Pharmacia Fine Chemicals; VIT3, 9.6, VIT13, and PMA) were prepared as 100-fold concentrated stock solution and added in 25 μl aliquots. Maximum fluorescence values (F_max) were determined by adding 10 μM of the heavy metal chelator diethylenetriaminepentaacetic acid (DTPA) (Sigma Chemical Co.) and lysing the cells with 0.1% Triton X-100 (Serva, Heidelberg, Federal Republic of Germany). Subsequently, minimum fluorescence (F_min) was determined by adding 0.5 mM MnCl_2.
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Table I
Purified T Cells Proliferate in Response to Certain Antibody and Antibody Plus PMA Combinations

| First reagent | [3H]Thymidine incorporation (cpm × 10⁻³ ± SE) in response to second reagent |
|---------------|--------------------------------------------------------------------------------|
|               | Medium | VIT13 | PMA |
| OKT11         | 0.4 ± 0.1 | 1.4 ± 0.7 | 2.1 ± 0.8 |
| T11           | 0.2 ± 0.1 | 1.0 ± 0.7 | 2.0 ± 0.7 |
| BMA 0110      | 0.4 ± 0.1 | 0.8 ± 0.4 | 1.7 ± 0.6* |
| 9.6           | 0.6 ± 0.2 | 25.4 ± 7.7* | 15.6 ± 2.9* |
| XI1           | 0.5 ± 0.1 | 36.8 ± 8.1* | 26.6 ± 6.7* |
| VIT13         | 0.3 ± 0.1 | 0.4 ± 0.1 | 28.1 ± 3.7* |
| VIT3          | 0.4 ± 0.1 | 0.4 ± 0.1 | 46.8 ± 7.7* |
| ---           | 0.3 ± 0.1 | 0.3 ± 0.3 | 2.6 ± 1.2 |

n = 3 except for *n = 5 and †n = 8.

Results

Certain CD2 Antibodies Are Comitogenic with PMA. mAb against the E-receptor can have functionally adverse effects. While antibodies to the SRBC binding site of the E-receptor were found to inhibit various T cell functions (17–22), combinations (antibody pairs) of antibodies directed against determinants unrelated to the SRBC binding site can induce intense T cell proliferation (7).

When evaluating the effect of several CD2 antibodies on T cell DNA replication, we also found a clear pattern, in that several antibodies (OKT11, T11, BMA0110) could not stimulate T cells together with our antibody, VIT13, while 9.6 and XI1 served as potent costimulatory reagents (Table I).

As far as the previously observed (7) correlation between fine specificity and functional effect of these antibodies is concerned, the 9.6 antibody does not seem to follow the rules mentioned above in that it inhibits E-rosette formation (14) but nevertheless stimulates T cells in the presence of certain mAb submitted for the Second International Workshop on Human Leucocyte Antigens in Boston (23, 24), and is strongly comitogenic with VIT13 and together with PMA (Table I).

Interestingly enough, those CD2 antibodies that were comitogenic with each other (9.6, XI1, VIT13) turned out to be also comitogenic with PMA, while the CD2 antibodies that were not comitogenic with VIT13 (OKT11, T11, BMA0110) were not comitogenic with PMA either (Table I).

The kinetics of the proliferative response induced by the antibody VIT13 in the presence of PMA is shown in Fig. 1, in comparison to simultaneously measured reactions to PHA and VIT3 antibody (CD3) plus PMA. This anti-T3 antibody, although nonmitogenic with resting mononuclear cells, and even a potent inhibitor of lectin and antigen stimulation, also proved to be a potent stimulating reagent in the presence of PMA (Table I) (4). Maximum proliferation in all three systems is seen between days 4 and 5. Dilution experiments revealed that relatively high concentrations of VIT13 (0.4–10 µg/ml) are needed for stimulation, while PMA is effective at a wide range of concentrations, from 0.005 to 5 µg/ml (Table II).
FIGURE 1. Time course of proliferation of purified T cells stimulated by VIT13 plus PMA (●), VIT3 plus PMA (○), or PHA (△). For PHA stimulation, T cell cultures were supplemented with 1% non-T cells. The cultures were labelled with [3H]thymidine as described in Materials and Methods, and harvested 18 h later at time points indicated.

| Stimulants | [3H]Thymidine incorporation (cpm × 10^3 ± SE) in response to: |
|------------|-------------------------------------------------------------|
|            | PMA alone | PMA + 10 µg/ml | VIT13 + 50 ng/ml PMA |
| PMA (ng/ml) | VIT13 (µg/ml) |            |                          |
| 5,000      | 50        | 9.6 ± 0.5 | 38.1 ± 1.1 | 36.0 ± 0.6 |
| 500        | 10        | 7.3 ± 0.2 | 28.9 ± 0.2 | 25.2 ± 1.3 |
| 50         | 2         | 4.2 ± 0.6 | 22.5 ± 1.8 | 15.6 ± 1.5 |
| 5          | 0.4       | 0.6 ± 0.1 | 6.1 ± 2.8  | 11.7 ± 2.4 |
| 0.5        | 0.08      | 0.3 ± 0.1 | 1.6 ± 1.2  | 4.9 ± 0.8  |

PMA Induces Expression of the VIT13 Determinant. The fact that VIT13 does not seem to react with resting T cells but nevertheless induces T cell proliferation in the presence of PMA raised the question of whether PMA induces expression of the VIT13 determinant. As shown in Fig. 2, this is indeed the case. After 48 h of culture, 75% of PMA-stimulated T cells are VIT13+, in contrast to 6% of cells cultured in medium alone. Staining by 9.6 antibody, clearly visible on resting cells, was strongly enhanced, indicating that the number of binding sites for this antibody increased considerably. As estimated by forward light scatter during FACS analysis, the cell sizes also increased in response to PMA treatment (data not shown). It is therefore possible that the enhancement of 9.6 fluorescence may not necessarily reflect a higher antigen density (per square micrometer of cell surface), but possibly a net increase correlating to blast formation. No exposure of another activation antigen, the transferrin receptor, as seen by the VIP1 antibody, was observed. Our observations confirmed previous reports (25) that the T3 structure is downregulated by PMA (Fig. 2).

Anti-Tac mAb Inhibits T Cell Proliferation Induced by CD2 Antibodies Plus PMA. Table III shows that the proliferation induced by VIT13 plus PMA, as
Effect of PMA on the expression of T cell surface markers. After 48 h of culture in the presence of 50 ng/ml PMA (P) or medium alone (M), the cells were harvested and stained with either VIT13 (CD2), 9.6 (CD2), VIP1 (transferrin receptor), or VIT3 (CD3) antibodies. The graphs displayed were obtained by logarithmic amplification. The smoothed line represents the negative control curve.

### Table III

**Anti-Tac Antibody Inhibits Proliferation of Costimulated T Cells**

| Stimulus       | [³H]Thymidine incorporation (cpm × 10⁻³ ± SE) in response to added antibody |
|----------------|--------------------------------------------------------------------------|
|                | None                      | Anti-Tac                  | Control (VID1) |
| PHA*           | 44.4 ± 1.4                | 33.8 ± 2.6                | 47.9 ± 3.5     |
| VIT3 + PMA     | 42.4 ± 4.7                | 27.4 ± 2.6                | 45.5 ± 2.5     |
| VIT13 + PMA    | 29.0 ± 0.7                | 4.9 ± 1.2                 | 27.0 ± 3.2     |
| Anti-Tac       | 0.9 ± 0.1                 | 1.5 ± 0.8                 | 2.1 ± 1.3      |

* Supplemented with 1% non-T cells

well as that of PHA and of VIT3 plus PMA–stimulated T cells is substantially inhibited in the presence of the IL-2-R antibody anti-Tac. Under these conditions, the DNA synthesis of T cells stimulated by VIT13 plus PMA is reduced by 80%. The addition of high concentrations of an unrelated control antibody (VID1) has no effect. Therefore, the cell growth seen after VIT13 antibody plus PMA stimulation seems to follow the classical IL-2-dependent pathway of T cell activation (26).

**CD2 Antibodies Plus PMA Stimulate T Cell Proliferation Without Measurable Changes of Intracellular Ca++ Levels.** It has recently been reported that stimulation of resting T cells with lectins (8), CD3 antibodies (27), and certain CD2 antibody combinations (9) is followed by a rapid elevation of intracellular Ca++ levels. Our studies confirm these observations (Fig. 3, Table IV). Con A, at a dose optimal for mitogenesis (10 µg/ml) changes Ca++ levels from 112 ± 12 nM to 406 ± 59 nM (mean increase, 3.5 ± 0.3–fold, n = 5) within 2 min after being added to the cell suspension. A similar reaction is observed with the nonmitogenic CD3 antibody VIT3. There, the Ca++ levels rise from 151 ± 11 nM to 304 ± 28 nM (mean increase 1.9 ± 0.1–fold). In addition, the combination of the CD2 antibodies 9.6 or X11 plus VIT13 leads to an increase of Ca++ levels from 142 ± 16 nM to 534 ± 158 nM (mean increase 3.7 ± 0.9–fold, n = 4) (Fig. 3, Table IV).

None of the investigated CD2 antibodies alone, however, be they stimulatory
Measurement of free cytoplasmic Ca$^{++}$. Purified T cells were loaded with Quin2 and their fluorescence intensity was recorded on an Amino spectrofluorometer SPF 500 in response to Con A (10 µg/ml), VIT3, 9.6, and VIT13 antibodies (10 µg/ml) and PMA (50 ng/ml).

**Figure 3.** Measurement of free cytoplasmic Ca$^{++}$. Purified T cells were loaded with Quin2 and their fluorescence intensity was recorded on an Amino spectrofluorometer SPF 500 in response to Con A (10 µg/ml), VIT3, 9.6, and VIT13 antibodies (10 µg/ml) and PMA (50 ng/ml).

**Table IV**

| Mitogen Increase (10 µg/ml) | Increase | n |
|-----------------------------|----------|---|
| Con A 3.5 ± 0.3             | 5        |
| VIT3 1.9 ± 0.1              | 5        |
| 9.6/X11 + VIT13 3.7 ± 0.9   | 4        |

Values are expressed as x-fold increase measured 3 min after mitogen addition. Resting Ca$^{++}$ levels were determined to be 132 nM ± 6 (n = 14).

with PMA or not, leads to changes in Ca$^{++}$ as measured by Quin2. No increase was observed when these antibodies were used in the presence of even high concentrations (50–5,000 ng/ml) of PMA (Fig. 3). Similarly, the addition of 9.6 or VIT13 to T cells, preincubated with PMA for 4 or 24 h, caused no apparent change in Ca$^{++}$ levels (data not shown). As evident from these data, PMA alone did not raise Ca$^{++}$ levels either, which is also in agreement with previous reports (8, 28). On the other hand, it did not lead to unresponsiveness of T cells in this system, since Con A (Fig. 3) and the combination of VIT13 plus 9.6 or X11 (data not shown) still induced an influx of Ca$^{++}$ ions in T cells pretreated with PMA.
Discussion

In the course of our studies, we found that certain CD2 antibodies can induce proliferation of resting T cells in the presence of PMA, while other CD2 antibodies fail to have such an effect. One CD2 antibody that is mitogenic in the presence of PMA is the classical E-receptor antibody, 9.6. This antibody sees a determinant near the SRBC binding site, and inhibits E-rosette formation (14). Similar data were obtained also with X11, another T111 antibody. Three other antibodies of the T111-like reaction type (7), i.e. the antibodies BMA 0110, OKT11, and T11, are not mitogenic in the presence of PMA, however (Table I).

The third CD2 antibody found to be mitogenic in the presence of PMA is the VIT13 antibody, directed against a determinant of the E-receptor only displayed by activated but not by resting T cells. Interestingly, the expression of this epitope or its surface accessibility to VIT13 is not only induced after lectin activation (15) but also by PMA treatment (Fig. 2).

Simultaneous triggering of the 9.6 or X11, and VIT13 epitopes by the respective antibodies also induces T cells to proliferate in the absence of PMA (Table I). Measurements of cytoplasmic Ca++ levels indicated, however, that the mechanisms are different for these two systems of T cell stimulation via the CD2 structure.

When using the Quin2 fluorescence indicator, we observed, like others, that Con A rapidly elevates intracellular Ca++ levels (Fig. 3, Table IV). We showed, to our knowledge for the first time, that a nonmitogenic CD3 mAb (VIT3) (4) very efficiently elevates free cytoplasmic Ca++ in blood T lymphocytes.

In contrast to that, none of the CD2 antibodies under study led to detectable changes of intracellular Ca++ levels. More important, this held even true when they were added to T cells together with PMA, in spite of the fact that three of them were comitogenic with PMA.

CD2 antibody-induced increases of intracellular Ca++ levels were, however, detectable after the combined addition of 9.6 or X11 plus VIT13 (Fig. 3, Table IV). Under these conditions, resting T cells were stimulated to proliferate in the absence of PMA as well.

The mitogenic effect and the Ca++ increase induced by the combination of the two CD2 antibodies 9.6 plus VIT13 resembles the observations of Weiss et al. (9) with antibody combinations of T112 plus T113 type.

The mitogenic effect of certain CD2 antibodies in the presence of PMA recalls the findings (2–4) with soluble, not surface-presented CD3 antibodies, which are not mitogenic per se but can activate T cells together with PMA.

Using OKT3 and PMA, other authors (29–31) recently distinguished two distinct steps in the activation of Jurkat cells. They showed that the addition of OKT3 leads to the generation of inositol triphosphate, followed by a rapid increase of cytoplasmic Ca++. For further activation and the induction of IL-2 and IFN-γ production, a second signal was needed, which they could trigger by adding PMA.

On the basis of these results, and in view of the fact that PMA directly activates the protein kinase C (32, 33), a highly Ca++-dependent enzyme, Imboden et al.
(10) suggested that the increase in Ca++ plays a crucial role in antigen-receptor-mediated activation.

For the activation of T cells via the CD2 structure, the so-called alternative pathway of T cell activation, the situation seems to be more complex. It can occur concomitant with (two CD2 antibodies), or in the absence of (one CD2 antibody plus PMA) detectable Ca++ responses.

We cannot formally exclude the possibility that 9.6 or VIT13 antibodies alone still lead to a Ca++ influx too small to be detected by our method. In view of the readily observed Ca++ responses to the other agents, however, we consider it to be unlikely.

The other explanation for the observed induction of proliferation in the absence of Ca++ influx could be the fact that activation of the C kinase (e.g. by PMA) results in a dramatic increase of its affinity for Ca++ ions (34). Thereby the enzyme, also under physiologic conditions, may not be dependent on additional Ca++, either from intracellular stores or from the extracellular compartment (35). While PMA may contribute to the induction of DNA replication via this mechanism, it is obviously not sufficient per se. The mAbs against the CD2 structure used must deliver some kind of additional activation signal, which seems, however, to be unrelated to Ca++ homeostasis. The underlying mechanisms may be similar to those involved in the recently reported (28) induction of T cell proliferation by protein A in the absence of measurable Ca++ increases. Regardless of the mechanism by which T cells triggered via CD2 are finally committed to proliferation, our data clearly show that a single CD2 antibody already significantly alters the activation stage of a given resting T cell.

On the other hand, there is now some evidence that the combination of Ca++ elevation and protein C kinase activation does not necessarily lead to the initiation of lymphocyte proliferation. We have, for instance, seen that wheat germ agglutinin, which causes a pronounced elevation of intracellular Ca++ (36 and Fischer, unpublished observations), is not comitogenic with PMA (data not shown).

Summary

We observed that certain E-receptor antibodies (CD2 antibodies) can induce proliferation of resting human T cells in the presence of PMA, while other CD2 antibodies fail to have such an effect. The same CD2 antibodies that were mitogenic in the presence of PMA (9.6, X11, VIT13), but not the nonreactive ones, were also able to induce T cell proliferation via the so-called alternative pathway of T cell activation, i.e., when added pairwise in certain combinations to T cells in the absence of PMA.

While the simultaneous addition of two comitogenic CD2 antibodies (9.6 or X11 plus VIT13) or the addition of a single nonmitogenic CD3 antibody (VIT3) led to a clearcut elevation of intracellular Ca++ levels, no such effect could be observed after the addition of one CD2 antibody alone. Even in the presence of PMA, one comitogenic CD2 antibody alone was unable to trigger a significant Ca++ response, although this combination induced a proliferative response.

These data indicate that, distinguishable by their influence on free cytoplasmic Ca++, there are two different mechanisms of T cell activation via CD2. While
simultaneous triggering with two antibodies leads to cell proliferation preceded by an increase of Ca++ levels, stimulation with one antibody plus PMA results in proliferation without a measurable early Ca++ response. We conclude that T cells treated by certain CD2 antibodies alone already recognize an activation signal probably unrelated to Ca++ homeostasis, a signal that can further be developed by PMA to result in a completely developed proliferative response.

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