Immobilized anti-CD3 mAb induces anergy in murine naive and memory CD4\(^+\) T cells in vitro

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Abstract

The induction of non-responsiveness in resting murine CD4\(^+\) T cells was investigated using immobilized anti-CD3 mAb. Incubation of freshly isolated CD4\(^+\) T cells with immobilized anti-CD3 mAb led to apoptosis in 40–60% cells. The surviving cells were profoundly non-responsive to subsequent mitogenic stimulation. The non-responsive state was characterized by a lack of IL-2 production and hyper-responsiveness to added IL-2, but was not explained by further activation-induced cell death. The induction of non-responsiveness was not due to modulation of the TCR–CD3 complex, and required partial activation of the T cells in that it was accompanied by an increase in cell size and was inhibited by addition of cyclosporin A. Finally, analysis of anti-CD3-mediated responses in naive and memory CD4\(^+\) T cells, separated on the basis of CD44 expression, showed that both naive and memory T cells have similar sensitivity to immobilized anti-CD3 mAb-induced activation, apoptosis and anergy. These results demonstrate that TCR–CD3 engagement on freshly isolated resting CD4\(^+\) naive and memory T cells, in the absence of co-stimulation, as achieved by plastic-immobilized anti-CD3 mAb, induces both anergy and cell death.

Introduction

The T cell repertoire is rendered useful and safe by the processes of positive and negative selection in the thymus. Although extensive intra-thymic deletion of self-reactive T cells occurs, it is clear that cells with autoreactive potential escape into the periphery. One obvious reason for the failure to eradicate all self-reactive T cells centrally is that not all tissue-specific self proteins can be presented effectively in the thymus. For this reason mechanisms of inducing self tolerance peripherally are of considerable importance.

It is well established that TCR ligation, in the absence of co-stimulation, induces non-responsiveness in human and murine T cell clones in vitro (1–3). However, relatively little is known about the consequences of isolated TCR ligation in resting T cells. Several in vivo, double transgenic, models (4) have been developed in which the T cell repertoire is dominated by a single alloreactive, transgene-encoded, TCR and the alloantigen for which the TCR is specific is expressed on different tissues in the periphery. These experimental systems have yielded a variety of results. In some cases the T cells expressing the transgene-encoded TCR appear to have ignored the alloantigen, in others the specific T cells have been disarmed by down-regulation of either co-receptor or TCR expression, and in another situation the T cells were phenotypically normal, but unresponsive or ‘anergic’. A limited number of in vitro studies of tolerance induction in freshly isolated resting CD4\(^+\) T cells have been reported. Sagerstrom et al. (5) reported that resting CD4\(^+\) T cells from TCR transgenic mice did not become anergic after incubation with plastic-coated peptide–MHC complexes. In contrast, blocking the B7–CD28/CTLA-4 interaction has been shown to result both in inhibition of primary alloresponses and in specific tolerance to alloantigen (6,7). Chen et al. demonstrated that simultaneous blockade of B7-1 and B7-2 rendered murine resting CD4\(^+\) T cells unresponsive to subsequent anti-CD3 stimulation (8). Similarly, exposure to anti-CD3 mAb in the absence of co-stimulatory signals has been reported to induce non-responsiveness in murine and human resting T cells (9–10). However, confusion exists as to whether partial signalling of resting T cells leads to non-responsiveness due to the induction of apoptotic cell death or anergy. Furthermore, the extent to which the principles that apply to the induction of non-responsiveness in T cell clones apply to resting T cells...
remains unclear. For example, the following conclusions have been drawn from studies of tolerance in resting T cells: the induction of T cell anergy is not prevented by the presence of accessory cells (9), cyclosporin A (CsA) cannot prevent anergy induction (11) and unresponsiveness can occur following a proliferative response of T cells (9,10).

The purpose of this study was to investigate the consequences of co-stimulation-deficient CD3-transduced signalling in freshly isolated resting CD4\(^+\) murine T cells. The results show that exposure of CD4\(^+\) T cells to immobilized anti-CD3 mAb leads to non-responsiveness due to a combination of apoptosis and anergy. This phenomenon was time and dose dependent, and required partial activation of the T cells. Finally, CD44\(^{lo}\) (naive) and CD44\(^{hi}\) (memory) CD4\(^+\) T cells exhibited similar behaviour in response to immobilized anti-CD3 mAb. This system provides a useful experimental model to further study the mechanisms of T cell tolerance in vivo and in vitro.

Methods

Mice

CBA/Ca (H-2\(^b\)) female mice were purchased from the Biological Services Unit of the RPMS and were used at 8–12 weeks of age.

mAb, cytokines, mitogens and drugs

The mAb 145-2C11 (12), specific for murine CD3 molecule, was purified from culture supernatants by affinity chromatography on Protein A–Sepharose by standard methods using 0.1 M glycine hydrochloride, 0.15 M NaCl, pH 4.0, as the elution buffer. Eluted antibody was dialysed against PBS and sterilized before use. The mAb YTS 169 (13), specific for murine CD8 molecule, was used as ascites (1:1000 dilution) (Globepharm, Esher, UK), 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 mM 2-mercaptoethanol was used as complete culture medium. Single-cell suspensions of splenic mononuclear cells were isolated by density centrifugation on Lympho-Sep gradients (Sera-lab) and treated with MACS separation according to the manufacturer’s instructions (14). Finally, CD44\(^{lo}\) (naive) and CD44\(^{hi}\) (memory) CD4\(^+\) T cells cells were collected first, the column was washed extensively and bound (CD44\(^{hi}\)) cells were eluted outside the magnetic field. After separation, CD44 expression in naive and memory T cells as well as unseparated CD4\(^+\) T cells was analysed by flow cytometry.

Purification of CD4\(^+\) T cells

Viable lymphocytes from spleen and pooled lymph nodes from normal CBA/Ca female mice were isolated from single-cell suspensions by density centrifugation on Lympho-Sep (Sera-lab, Sussex, UK). The cell preparations were then passed through nylon wool columns (Biotest, Dreieich, Germany). The enriched T lymphocytes were treated with a mAb cocktail and rabbit complement (Cedarlane, Hornby, Ontario, Canada). The mAb cocktail consisted of 10.2.16 (anti-H-2A\(^b\)), 14.4.4S (anti-H-2E\(^b\)) and YTS169 (anti-CD8). After two rounds of cytotoxic elimination, the cells recovered from these procedures were 95–96% CD3\(^+\) CD4\(^+\) CD8\(^-\) and <1% contaminating H-2A\(^b\) cells were detected by flow cytometric analysis. The purity of CD4\(^+\) T cells was also confirmed by their lack of responsiveness to the mitogen, Con A, in the absence of added accessory cells.

Induction of CD4\(^+\) T cell anergy in vitro

Purified CD4\(^+\) T cells were cultured at a density of 5×10\(^5\) cells/well for 2 days in 24-well plates (Costar, Bodenheim, Germany) previously coated with the indicated concentrations of purified anti-CD3 mAb. The T cells incubated in medium alone served as normal controls (untreated T cells). Two days later, untreated and anti-CD3-treated T cells were re-suspended, washed and added into new culture plates containing fresh culture medium. After 2 days rest, the normal and anergic T cells were re-stimulated with Con A in the presence of irradiated syngeneic accessory cells or with exogenous rhIL-2 alone.

T cell proliferation assays

RPMI 1640 medium supplemented with 10% FCS (Globepharm, Esher, UK), 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 mM 2-mercaptoethanol was used as complete culture medium. Single-cell suspensions of splenic mononuclear cells were isolated by density centrifugation on Lympho-Sep gradients (Sera-lab) and treated with 30 Gy irradiation before being used as accessory cells. The irradiated splenocytes were unable to make IL-2 when they were co-cultured with Con A due to the irradiation sensitivity of resting T cells (data not shown). This is very important point since anergic T cells were hypersensitive to exogenous IL-2. Normal or anergic T cells (1×10\(^5\) /well) were cultured in 96-well round-bottomed plates containing various concentrations of Con A (1–4 µg/ml) plus irradiated accessory cells (5×10\(^5\) /well) or exogenous rhIL-2 (50 U/ml) alone in a volume of 0.2 ml. After 2 days culture, 1 µCi of [\(^3\)H]thymidine was added into each well. Cells were harvested onto glass fiber filters after an additional 24 h. The [\(^3\)H]thymidine uptake was measured by liquid scintillation spectrometry. The results are expressed as mean c.p.m. for triplicate cultures. SEM values were routinely <10%.

IL-2 and IL-4 bioassays

Culture supernatants harvested from the primary and re-stimulation cultures were tested for IL-2 and IL-4 activity using CTLL-2 (TIB-214; ATCC) and CT-4S (16) cells as indicator cells in the presence of neutralizing mAb 11B11 specific for
Anti-CD3 mAb-mediated anergy in resting murine CD4\(^+\) T cells

IL-4 or S4B6 specific for IL-2 respectively. The growth of CTLL-2 or CT-4S cells was measured by \[^{3}H\]thymidine uptake as described in T cell proliferation assays.

Flow cytometric analysis of cell purity, cell size, DNA content and TCR–CD3 expression

The purity of CD4\(^+\) T cells was examined by staining with anti-CD3, anti-CD4 and anti-CD8 mAb directly conjugated to FITC (Sigma F-7275, F-7400 and F-7525 respectively) or isotype-matched controls. Cell preparations were also stained with 10.2.16 mAb plus sheep anti-mouse IgG-FITC conjugate (Sigma F-3008) to determine the level of contamination with MHC class II\(^\text{a}\) cells before use in functional assays. After the rest period, normal and anergic T cells were stained again with anti-CD3–FITC or control mAb (Sigma F-7275 and F-6647) to detect TCR–CD3 modulation. To examine the changes in cell size during the cultures, freshly isolated CD4\(^+\) T cells were gated on forward and side scatter before the primary cultures, and compared with cells recovered from the primary and rest cultures at various time points.

Apoptosis was detected utilizing the method developed by Nicoletti et al. (17) with some modifications. Briefly, the cells harvested from the primary or re-stimulation cultures were centrifuged, the cell pellet was fixed in 0.5 ml cold 70% ethanol at 4°C for 1 h. Then the cells were centrifuged again and resuspended in 0.5 ml RNase (1 mg/ml) and incubated at room temperature for 20 min. Finally, 30 \(\mu\)l PI (0.1 mg/ml) was added into each sample and incubated for another 10 min in the dark at room temperature before cell cycle was analysed by flow cytometry. The percentage of cells in apoptosis was determined from pre-G\(_1\) peak on the PI (FL3) and TCR–CD3 expression histogram. This method has been shown to yield results similar to classic DNA fragmentation assays. All flow cytometric analyses were carried out using an EPICS Excel flow cytometer (Coulter Electronics, Luton, UK).

Results

Exposure of resting CD4\(^+\) T cells to immobilized anti-CD3 mAb results in apoptosis in a fraction of cells

Previous studies have suggested that resting CD4\(^+\) T cells undergo apoptosis following cross-linking the TCR complex. To further address this possibility in our system, the DNA from untreated and anti-CD3-treated T cells was labelled with PI at 24, 48 and 96 h of the primary and rest cultures, and cell cycle was analysed by flow cytometry (Fig. 1). Apoptosis was determined by an increase in PI staining within a region to the left of the G\(_1\) peak, corresponding to hypodiploid cells. More hypodiploid cells were detected in the anti-CD3-treated T cells (E: 12%, M: 28% and Q: 44% after 24, 48 and 96 h exposure to anti-CD3 mAb respectively) than in the untreated
Anti-CD3 mAb-mediated anergy in resting murine CD4\(^+\) T cells

Fig. 2. Induction of anergy in CD4\(^+\) T cells by immobilized anti-CD3 mAb. (A) Exposure of resting CD4\(^+\) T cells to anti-CD3 mAb resulted in proliferative unresponsiveness upon re-stimulation with Con A. CD4\(^+\) T cells (5\(\times\)10\(^5\)/well) were incubated for 2 days in 24-well plates coated with 10 \(\mu\)g/ml anti-CD3 mAb (2C11) or medium (medium), washed and rested for 2 days in medium alone. The untreated or anti-CD3-treated T cells (1\(\times\)10\(^5\)/well) were then re-stimulated with the indicated concentration of Con A plus irradiated (30 Gy) accessory cells (5\(\times\)10\(^5\)/well) in 96-well round-bottomed plates for 3 days. The mean c.p.m. of untreated and anti-CD3-treated cells in response to exogenous rhIL-2 (50 U/ml) were 503 and 208,487. (B) Unresponsiveness correlated with a defect in IL-2 production. After 48 h of re-stimulation, supernatants were harvested and assayed for IL-2 with CTLL-2 cells (3\(\times\)10\(^3\)/well) at a final supernatant concentration of 25% in the presence of anti-IL-2 mAb (11B11) to determine IL-2 activity. Proliferation of the CTLL-2 cells was measured at day 2. (C) Unresponsiveness correlated with a defect in IL-4 production. After 48 h of re-stimulation, supernatants were harvested and assayed with CT-4S cells (1\(\times\)10\(^4\)/well) at a final supernatant concentration of 25% in the presence of anti-IL-4 mAb (S4B6) to determine IL-4 activity. Proliferation of the CT-4S cells was measured at day 2. (D) Dose response of anti-CD3 mAb in the induction of anergy. CD4\(^+\) T cells (5\(\times\)10\(^5\)/well) were incubated for 2 days in 24-well plates coated with 0.1, 1 and 10 \(\mu\)g/ml anti-CD3 mAb or with medium alone, washed and rested for 2 days in medium. The untreated and anti-CD3-treated T cells (1\(\times\)10\(^5\)/well) were then re-stimulated with the indicated concentration of Con A plus irradiated (30 Gy) accessory cells (5\(\times\)10\(^5\)/well) for 3 days. Proliferative responses of untreated and anti-CD3-treated T cells to rhIL-2 (50 U/ml) were 113 (medium), 31,112 (0.1 \(\mu\)g/ml), 120,173 (1 \(\mu\)g/ml) and 128,520 (10 \(\mu\)g/ml) respectively when the proliferation was measured at day 3.

Immunodized anti-CD3 mAb induces anergy in resting CD4\(^+\) T cells

Although apoptosis was induced in response to immobilized anti-CD3 antibody, this only applied to between 40 and 60% cells. In order to determine the reactivity of the surviving cells, viable cells were recovered following 2 days incubation with immobilized anti-CD3 mAb and a further 2 days rest. These cells were then re-stimulated with Con A in the presence of syngeneic accessory cells or IL-2 alone. Culture supernatants from the re-stimulation cultures were also harvested and tested for IL-2 and IL-4. As shown in Fig. 2(A), pre-incubation of CD4\(^+\) T cells with immobilized anti-CD3 mAb for 2 days resulted in proliferative unresponsiveness upon re-stimulation with Con A. The anti-CD3-treated T cells were hyper-responsive to exogenous IL-2 (see figure legends), suggesting that this non-responsive state was not explained by cell death. Measurement of cytokine production by the T cells revealed that the defect following exposure to immobilized anti-CD3 mAb was an inability to secrete IL-2 and IL-4, as shown in Fig. 2(B and C). In contrast, substantial levels of IL-2 and IL-4 were detected in the supernatants from the T cells that had been cultured with medium alone for 4 days. The induction of non-responsiveness was dose-dependent, as illustrated in Fig. 2(D). T cells cultured in wells coated with 1 or 10 \(\mu\)g/ml anti-CD3 mAb were profoundly non-responsive, however, no inhibitory effect was seen when the anti-CD3 concentration was reduced to 0.1 \(\mu\)g/ml.
In order to exclude the possibility that exposure to anti-CD3 mAb altered the kinetics of the response to subsequent re-challenge, proliferation to Con A was measured after 1, 2 and 3 days in the second culture. No proliferation was seen at any of these time points (data not shown). The possibility that the lack of response to mitogen was due to TCR-CD3 down-modulation was examined by flow cytometric analysis. The T cells that had been exposed to anti-CD3 mAb and rested for 2 days expressed comparable levels of the TCR-CD3 complex to T cells which had been cultured with medium alone for 4 days (data not shown).

These findings demonstrate that the resting T cells that do not undergo apoptotic cell death in response to immobilized anti-CD3 mAb are unable to secrete IL-2 and IL-4, but are hyper-responsive to exogenous IL-2. These features are consistent with the induction of a state of non-responsiveness commonly referred to as anergy.

Anti-CD3-induced unresponsiveness is not a consequence of cell death during the re-stimulation cultures

An alternative explanation for the non-responsiveness of the surviving anti-CD3-treated T cells was that they were induced to undergo apoptosis upon re-stimulation, perhaps due to the expression of FasL. In order to address this possibility, the DNA from normal and anergic T cells was labelled with PI at 24 and 48 h of the re-stimulation cultures, and apoptosis was analysed by flow cytometry. As shown in Table 1, 32.9 and 32.4% cells fell into the sub-diploid peak after 24 and 48 h re-stimulation of anergic T cells with Con A and irradiated splenocytes. Given that ~18% irradiated spleen cells fell into the sub-diploid peak, this implies that ~15% anti-CD3-treated T cells underwent apoptosis upon re-stimulation. These data demonstrate that the induction of further cell death did not account for the hyporesponsiveness observed in the anti-CD3-treated T cells. The anergic T cells were, however, more sensitive to IL-2 withdrawal than normal T cells in that more apoptotic cells were seen in anti-CD3-treated T cells if they were cultured in medium alone (Fig. 1F). Similar results were also observed when normal and anergic T cells were re-stimulated by co-immobilized anti-CD3-pulsed anti-CD28 mAb rather than Con A-pulsed irradiated accessory cells (data not shown). Therefore, the unresponsive state induced by co-stimulation deficient CD3-transduced signalling was due to the induction of T cell anergy, rather than the induction of activation-induced cell death.

Anti-CD3-mediated anergy induction requires T cell activation

The extent to which the induction of T cell anergy requires prior T cell activation is unclear based on previous reports (2,9,10,18). In order to determine whether the non-responsive ness observed here was accompanied by T cell activation, changes in cell size, proliferation and IL-2 production were measured. As shown in Fig. 3, CD4+ T cells were small cells with high viability after 24, 48 and 96 h incubation with medium alone (left hand panels). In contrast, after 24 h incubation with anti-CD3 mAb, the volume of CD4+ T cells began to increase and small numbers of dead cells appeared; after 48 h the anti-CD3-treated cells were large blast cells and the number of dead cells also increased; after 48 h rest, the anergic T cells were reduced in size and more dead cells were observed (right hand panels). The increase in cell size was paralleled by measurable proliferation after 28 h which was more marked after 44 h (Fig. 4A). IL-2 was detectable in the supernatants of anti-CD3-stimulated cells at 7 h and reached a maximum at 62 h (Fig. 4B). These results demonstrate that the induction of anergy in resting CD4+ T cells by exposure to immobilized anti-CD3 mAb occurs despite the features of full T cell activation.

Although activation of T cells could be measured by cell enlargement, proliferation and IL-2 production, the number of live cells after 44 h culture in anti-CD3-coated wells of a 96-well plate did not increase (Fig. 4A). When CD4+ T cells were cultured in anti-CD3-coated 24-well plates, the number of live cells recovered from 24–48 h of the primary culture never exceeded the input cell number (data not shown). These results reflect the fact that T cell proliferation was accompanied by cell death, as shown in Fig. 1.

Table 1. Apoptosis and proliferation during the rechallenge

<table>
<thead>
<tr>
<th>Cells</th>
<th>Day</th>
<th>Medium</th>
<th>Irradiated spleen</th>
<th>Spleen + Con A</th>
<th>Proliferation (c.p.m.)</th>
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</thead>
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<tr>
<td>Irradiated spleen</td>
<td>0</td>
<td>0.2</td>
<td>ND^</td>
<td>13.8</td>
<td>0 ND^</td>
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<tr>
<td></td>
<td>1</td>
<td>17.3</td>
<td>ND</td>
<td>15.5</td>
<td>1 ND^</td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
<td>ND</td>
<td>18.3</td>
<td>16.5</td>
<td>2 ND^</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>ND</td>
<td>18.3</td>
<td>18.1</td>
<td>0 ND^</td>
</tr>
<tr>
<td>Anti-CD3</td>
<td>0</td>
<td>ND</td>
<td>32.9</td>
<td>26.9</td>
<td>0 ND^</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>ND</td>
<td>32.4</td>
<td>28.2</td>
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<tr>
<td>Normal</td>
<td>3</td>
<td>284</td>
<td>18,746</td>
<td>188</td>
<td>3 299</td>
</tr>
<tr>
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<td>3</td>
<td>399</td>
<td>3624</td>
<td>225,507</td>
<td>3 399</td>
</tr>
</tbody>
</table>

^ND, not done.

CD4+ T cells (5 × 10^5/well) were incubated for 2 days in 24-well plates pre-coated with 10 µg/ml anti-CD3 mAb or medium, washed and rested for 1 day in medium alone. The viable T cells in normal or anti-CD3-treated population were isolated by density centrifugation on lympho-sep. Normal and anti-CD3-treated T cells (1 × 10^5/well) were then re-stimulated with 4 µg/ml Con A plus irradiated (30 G7) splenocytes (5 × 10^5/well) or rhIL-2 (50 U/ml) in 96-well round-bottomed plates for 3 days. Apoptosis was tested at day 1 and day 2, and proliferation was measured at day 3 according to the procedures described in detail in Methods.
Naive and memory CD4⁺ T cells exhibit similar sensitivity to anti-CD3 mAb-induced activation, apoptosis and anergy

One possible explanation for the finding that 40–60% resting T cells underwent apoptotic cell death, and the remainder became anergic was memory and naive T cells have different sensitivities to activation-induced cell death. To address this possibility, freshly isolated CD4⁺ T cells were further divided into naive and memory T cells according to CD44 expression using biotinylated anti-CD44 and streptavidin-conjugated magnetic beads (Fig. 5A and B). Both populations were highly enriched for CD3⁺ CD4⁺ cells (95–98%) and did not contain any MHC class II⁺ accessory cells. After exposure to immobilized anti-CD3 mAb, both naive (CD44lo) and memory (CD44hi) T cells increased in size, expressed increased levels of IL-2R, when the proliferation was measured at day 3.
Anti-CD3 mAb-mediated anergy in resting murine CD4⁺ T cells

Fig. 5. (A and B) The expression levels of CD44 in naive and memory CD4⁺ T cells after magnetic bead sorting on the MACS. CD4⁺ T cells were stained with biotinylated anti-CD44 mAb (I42/5–biotin), following by streptavidin magnetic beads and FITC–avidin, separated on the MACS, and analyzed by flow cytometry. After separation, 90% of memory T cells expressed high levels of CD44 (B) while only 4% of naive T cells expressed the same level of CD44 (A). (C–F) Naive and memory T cells undergo apoptosis after exposure to immobilized anti-CD3 mAb. T cell anergy was induced as indicated in the legend to Fig. 2. At 24 (C and D) and 48 (E and F) h of pre-incubation with immobilized anti-CD3 mAb (30 µg/ml), apoptosis was detected in both anti-CD3-treated naive and memory T cells as described for Fig. 1. Panels (C) and (E) show 12% (A in panel C) and 15% (I in panel E) of anti-CD3-treated naive T cells underwent apoptosis after 24 and 48 h of exposure to immobilized anti-CD3, while panels (D) and (F) show 13% (E in panel D) and 14% (M in panel F) of anti-CD3-treated memory T cells died due to apoptosis after 24 and 48 h of the primary culture.

produced IL-2 (data not shown) and proliferated (Fig. 6A and B), suggesting that both naive and memory T cell populations were activated by immobilized anti-CD3 mAb during the primary culture. This was accompanied by the induction of apoptosis in comparable fractions of both cell populations (Fig. 5C–F).

After the primary culture, anti-CD3-treated naive and memory T cells were rested for 2 days to allow recovery of normal levels of TCR expression before rechallenge with Con A and syngeneic accessory cells. As shown in Fig. 6(C and D), pre-incubation of naive and memory T cells with immobilized anti-CD3 mAb for 2 days resulted in proliferative unresponsiveness upon re-stimulation with Con A, although the dose of anti-CD3 mAb required to induce proliferative non-responsiveness was significantly lower for the memory than for the naive T cells. This non-responsive state was not due to cell death or TCR down-modulation, since anti-CD3-treated naive and memory T cells still showed an enhanced response to exogenous rhIL-2 (see figure legends) and after 2 days rest anti-CD3-treated T cells re-expressed comparable levels of TCR–CD3 to T cells which had been cultured with medium alone for 4 days (data not shown). Measurement of cytokine production by anti-CD3-treated naive and memory T cells in the secondary culture (rechallenge) revealed that unresponsiveness correlated with an inability to secrete IL-2 (data not shown).

These findings demonstrate that exposure of naive and memory T cells to immobilized anti-CD3 mAb induces an unresponsive state characterized by an inability to secrete IL-2, commonly referred to as anergy.
Fig. 6. (A and B) Pre-incubation of naive and memory CD4$^+$ T cells with immobilized anti-CD3 mAb resulted in cell proliferation. Naive (A) and memory (B) CD4$^+$ T cells ($5 \times 10^4$/well) were cultured in duplicate 96-well flat-bottomed plates pre-coated with 1, 3, 10 or 30 µg/ml anti-CD3 mAb for 2–4.5 days. (C and D) Induction of anergy in naive and memory CD4$^+$ T cells by immobilized anti-CD3 mAb. Naive (C) and Memory (D) CD4$^+$ T cells ($5 \times 10^5$/well) were incubated for 2 days in 24-well plates pre-coated with 1, 3, 10 or 30 µg/ml anti-CD3 mAb (2C11) or medium (medium), washed, and rested for 2 days in medium alone. The untreated or anti-CD3-treated naive (C) or memory (D) T cells ($1 \times 10^5$/well) were then re-stimulated with the indicated concentration of Con A plus irradiated (30 Gy) accessory cells ($5 \times 10^5$/well) in 96-well round-bottomed plates for 3 days. Mean c.p.m. of untreated and anti-CD3-treated naive T cells in response to exogenous IL-2 (50 U/ml) were 134 (N Medium), 47,224 (N 1 µg/ml), 59,407 (N 3 µg/ml), 51,954 (N 10 µg/ml) and 42,674 (N 30 µg/ml). Mean c.p.m. of untreated and anti-CD3-treated memory T cells in response to exogenous IL-2 (50 U/ml) were 248 (M Medium), 84,599 (M 1 µg/ml), 83,731 (M 3 µg/ml), 92,414 (M 10 µg/ml) and 106,117 (M 30 µg/ml).

Discussion

The results presented in this study demonstrate that exposure of resting, purified, CD4$^+$ T cells to immobilized anti-CD3 mAb induces apoptotic cell death and anergy. Approximately 50% cells underwent apoptosis and the remaining cells were hyper-responsive to exogenous IL-2, while unable to secrete IL-2 or IL-4 upon re-stimulation. The unresponsiveness to re-stimulation was not explained by further cell death since the percentage of surviving cells that underwent apoptosis did not increase significantly during the rechallenge culture. Furthermore, the induction of non-responsiveness was not due to modulation of the TCR-CD3 complex and required activation of the T cells, in that it was accompanied by an increase in cell size and was inhibited by addition of CsA. Finally, naive and memory CD4$^+$ T cells showed similar sensitivity to immobilized anti-CD3-mediated activation, apoptosis and anergy.

The phenomenon of anergy has been extensively studied in established human and murine T cell clones, particularly of the T_h1 type (1–3,18,22–23). The anergic state is characterised by an inability of the affected T cells to secrete IL-2 and to undergo autocrine proliferation (21). However, the anergic T cell exhibits an exaggerated proliferative response to added exogenous IL-2 (24). The ability to induce anergy in resting T cells has been less thoroughly studied. In terms of self tolerance, this is an important issue, in that the deletion of T cells with autoreactive potential in the thymus is inevitably incomplete.

Three circumstances have been defined in vitro that lead to an anergic state in T cell clones. The first involves the receipt of TCR-CD3-transduced signals, in the relative or complete absence of co-stimulatory signals that are usually delivered through the B7–CD28 interaction (21,25). This kind of partial signalling usually fails to induce IL-2 secretion or proliferation by the T cell clone. The second results from the transduction of altered signals through the TCR-CD3 complex...
due to the recognition of a subtly altered cognate ligand (26).
This is uninfluenced by the presence of full co-stimulation. The third set of conditions that has been shown to lead to T cell anergy is the delivery of full signals to the T cell in the absence of cell division (20). The response of resting T cells to immobilized anti-CD3, that ultimately leads to anergy, does not fully correspond to any of these three circumstances, leaving a number of unanswered questions, as outlined below.

The most trivial mechanism to account for the effects of anti-CD3 mAb was that the T cells were rendered 'blind' due to modulation of the TCR--CD3 complex. This was clearly not the case, in that, after the 2 day rest period, the levels of CD3 expression were the same as on control T cells. In studies of this kind, it is important to include a rest phase for this reason, in order to exclude the possibility that non-responsiveness merely reflects the inability of the T cells to receive TCR--CD3-transduced signals. A second phenomenon that can confound studies of T cell non-responsiveness is that of premature re-stimulation. It has been documented that signalling the T cell through its antigen receptor twice in a short space of time can effectively paralyse the cell (27). This form of non-responsiveness is characterized by an inability to proliferate even to added IL-2 and is therefore distinct from the state of anergy, as described here. This was a further reason to rest the cells after anti-CD3 stimulation. A third alternative mechanism that could account for non-responsiveness induced following exposure to immobilized anti-CD3 mAb is activation-induced cell death. The appearance of apoptotic cells after anti-CD3 stimulation was detected by staining with PI. This revealed that ~12 and 28% of the T cells were apoptotic after 24 and 48 h stimulation by immobilized anti-CD3, and a further fraction (44% and 58%) of the T cells underwent apoptosis during a prolonged primary culture comprising 2 days treatment with anti-CD3 and 2 days rest. Importantly, however, upon re-stimulation of the surviving cells, less than one-third underwent apoptosis. This was only 10% more than was observed with T cells that had not undergone prior activation. These findings suggest that anergy and apoptosis are two closely related, but distinct, outcomes of co-stimulation deficient T cell stimulation.

One of the interesting features of the non-responsiveness induced by immobilized anti-CD3 mAb was that it occurred despite clear evidence of T cell activation, including the production of IL-2 and a proliferative response. At face value this is markedly different from the circumstances that accompany the induction of anergy in T cell clones (3,18). However, similarities do exist in that the provision of co-stimulation, in the form of splenic accessory cells, protected from the induction of anergy, and incubation of the T cells with the anti-CD3 mAb for a period of 3 and 4 days did not lead to non-responsiveness (Chai and Lechler, unpublished). The 3 and 4 day culture period was accompanied by significant proliferation. These findings may be best interpreted by suggesting that the key factors that were instrumental in mediating these effects were inadequate co-stimulation and, as a consequence, inadequate T cell proliferation to prevent the induction of anergy. This interpretation is predicated on the concept that T cell activation is always accompanied by the production of negative regulators of IL-2 gene transcription that have been loosely referred to as 'anergy proteins' (28, 29). Provided that a T cell undergoes sufficient rounds of cell division, the intracellular concentration of these negative regulatory proteins falls and the T cell remains in a responsive state. If, on the other hand, insufficient cell division occurs, the negative regulators of the IL-2 gene transcription render the T cell refractory or anergic.

When these studies were expanded from whole CD4+ T cells into naive and memory T cells, we found that both populations had a similar sensitivity to anti-CD3-mediated activation, apoptosis and anergy. These results differ from those described by Luqman and Bottomly (30) who observed that pretreatment of naive T cells with immobilized anti-CD3 antibody did not induce cell proliferation, cell death or unresponsiveness to subsequent stimulation by anti-CD3 in the presence of antigen-presenting cells. One possible contributory factor to these different outcomes is that in the earlier experiments naive T cells (CD45RBhi) were prepared by positive, rather than negative, selection. Recently, it has been demonstrated that CD45 is a signalling molecule (31); it is conceivable, therefore, that ligation of CD45 altered the reactivity of the CD45hi cells. In our experiments, naive T cells were purified by negative selection on the MACS and there is no evidence to suggest that CD44, a major cell surface receptor for hyaluronate (32), is involved in T cell signalling.

This system for inducing anergy in resting T cells offers opportunities to explore in more detail the events that may be responsible for the induction of peripheral tolerance.

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Abbreviations

References


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