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Anti-CD28 antibody- and IL-4-induced human T cell proliferation is sensitive to rapamycin

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SUMMARY

Rapamycin (RAPA) is a potent immunosuppressant. In this study we investigated the effect of RAPA on T cell proliferation triggered by various stimuli in an *in vitro* human model. The proliferation of T cells stimulated via an alternative pathway using phorbol myristate acetate (PMA) and anti-CD28 antibody (αCD28) in the absence of antigen-presenting cells (APC) was strongly inhibited by RAPA. T cell proliferation provoked via a combination of CD3/TCR and CD28 pathways using anti-CD3 antibody (αCD3) plus αCD28 was also inhibited by RAPA in the presence of APC. The mitogen (phytohaemagglutinin (PHA) or αCD3)-induced up-regulation of expression of the IL-2 receptor α chain (IL-2Rα) and the IL-4 receptor (IL-4R) was sensitive to RAPA. This suggests that RAPA's interference with the IL-2 and IL-4 autocrine loops during T cell activation might contribute to RAPA's overall immunosuppressive effect. We have further demonstrated in a two-stage culture system that RAPA strongly inhibited IL-4-stimulated proliferation of T cells, the latter being either pretreated with αCD3 in the presence of APC, or with PMA plus αCD28 in the absence of APC. The result suggests that the Ca^{++} influx during the pretreatment is not obligatory for T cells to achieve IL-4 responsiveness. The results also indicate that RAPA's antiproliferative effect on IL-4-stimulated T cells is not contingent on the various mechanisms of cell priming. Therefore, RAPA's major target is probably at the second stage after the priming. Our study has extended current knowledge about the effect of RAPA on human T cells.

Keywords rapamycin IL-4 CD28 T cell proliferation

INTRODUCTION

Rapamycin (RAPA) is a potent immunosuppressant with a chemical structure similar to that of FK506 [1–4]. RAPA is very effective in preventing allograft rejection and is 10–100-fold more potent than cyclosporin A (CsA) [2–8]. At the effective dose range (0·1–1·0 mg/kg per day) of RAPA in a rabbit heart allograft model, no significant changes in renal or liver function were noted [9]. Long-term high dose of RAPA causes hypoplasia of central lymphoid tissues in monkeys [2]. The side effect of RAPA in humans awaits the formal reports of the ongoing phase I clinical trial carried out in several centres, including ours. Our previous studies show that RAPA strongly inhibits immunoglobulin production and has an inhibitory effect on HLA-unrestricted cell killings [10–13]. RAPA is 100-fold more effective than CsA in inhibiting T cell activation and proliferation [14]. The drug not only represses the Ca^{++}-dependent TCR/CD3 pathway [14,15], but also blocks the Ca^{++}-independent CD28 pathway, which is insensitive to CsA or FK506 [15,16]. Unlike FK506 and CsA, RAPA does not reduce the expression of mRNA of many early phase activation genes such as IL-2, IL-3, IL-4, interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α), c-myc, and granulocyte-macrophage colony-stimulating factor (GM-CSF) [17]. The mRNA level of IL-2 receptor α chain (IL-2Rα) is not affected by RAPA either [17]. The antiproliferative effect of RAPA is apparent when the drug is added at the late G_1 phase of the cell cycle, while FK506 and CsA are effective only when added at the G_0 or early G_1 phase [18,19]. The prime intracellular target of RAPA is not yet known. Recent studies show that RAPA inhibits the activation (phosphorylation) of the p70 S6 kinase [20,21], which is presumably involved in regulation of protein synthesis.

In this study we further delineate the effect of RAPA on T cell activation and proliferation under conditions where the CD28 pathway and IL-4 are involved. As new findings, we document that (i) RAPA effectively inhibits the proliferation of human T cells triggered by a combination of αCD3 and αCD28 MoAb; (ii) the mitogen-induced IL-4R expression on T cells is drug-regulated; (iii) RAPA could reverse the IL-4-enhanced

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proliferation of αCD3-primed human T cells; (iv) together, αCD28 and phorbol myristate acetate (PMA) can effectively prime the T cells for the subsequent IL-4 responsiveness, which is also RAPA-sensitive. These results have extended our current knowledge about the mechanism of action of the drug.

MATERIALS AND METHODS

Reagents

Human recombinant IL-4 was purchased from Genzyme Co. (Boston, MA). RAPA was obtained from Wyeth-Ayerst Research (Princeton, NJ). PMA and phytohaemagglutinin (PHA) were purchased from Sigma (St. Louis, MO). Biotinylated human IL-4 and avidin-FITC for IL-4R detection were from R & D Systems (Minneapolis, MN). Lymphoprep was purchased from Nycomed (Oslo, Norway). RPMI 1640, fetal calf serum (FCS), penicillin-streptomycin, and L-glutamine were obtained from GIBCO/BRL (Gaithersburg, MD). FITC-labelled αCD3 MoAb (clone SFCIRW2-8c8), PE-labelled αCD3 MoAb, and PE-labelled αCD25 MoAb (clone 1HT44H3) were from Coulter (Hialeah, FL). αCD28 MoAb (clone 9.3) was a generous gift from Dr Peter Linsley [18]. αCD3 MoAb OKT3 was from ATCC.

Cell culture

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood of healthy donors with Lymphoprep according to the manufacturer's instructions. Highly purified T cells were obtained by sheep erythrocyte rosetting as described previously [10], followed by two nylon wool passages [21]. This T cell fraction normally contained $< 1\%$ B cells ($CD20^+$) and $< 1\%$ monocytes ($MO2^+$) according to flow cytometry analysis.

Assays for ^{3}H-thymidine uptake

^{3}H-thymidine uptake was used as an indicator of cell proliferation. Cells were cultured in 96-well flat-bottomed plates (2×10^5 cells/200 μl per well), and were pulsed with 0·5 μCi ^{3}H-thymidine for 6 h before harvest in triplicate [10].

Flow cytometry for IL-2R and IL-4R

For αCD3 and αCD25 double staining, 5 μl of FITC-αCD3 and 5 μl of PE-αCD25 were added to 10^6 cells. After 60 min incubation at 4°C, the cells were washed and analysed with flow cytometry. For IL-4R detection, 10 μl of biotinylated IL-4 were added to 25 μl of cell suspension containing 10^6 cells. After 60 min incubation at 4°C, the cells were washed twice with a manufacturer-supplied buffer containing saline and protein. The cells were then resuspended in 100 μl wash buffer, and stained with 10 μl of avidin-FITC at 4°C for 30 min in the dark. Five microlitres of PE-αCD3 were added along with avidin-FITC in the case of CD3 and IL-4R double staining. Finally, the cells were washed again and resuspended in PBS for flow cytometry analysis.

RESULTS

CD28 delivers an essential costimulatory signal for T cell activation. Soluble αCD28 MoAb alone cannot drive resting T cells into G_1 phase, either in the presence or absence of antigen-presenting cells (APC) [22]. However, αCD28 can greatly enhance the proliferation and lymphokine production of T cells

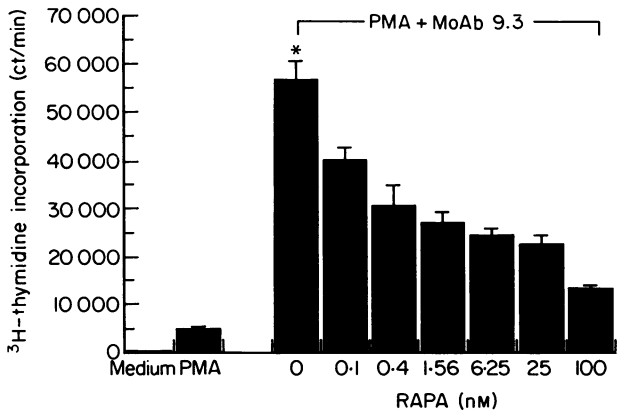

Fig. 1. Rapamycin (RAPA) inhibits T cell proliferation triggered by the CD28 pathway. Highly purified T cells were incubated with phorbol myristate acetate (PMA; 1 ng/ml), αCD28 (MoAb 9.3, 50 ng/ml), or PMA plus αCD28 in the absence or presence of RAPA (0·1–100 nM) for 3 days. The cells were pulsed with ^{3}H-thymidine for 6 h before harvesting. Consistent results were obtained in more than three experiments, and the result of a representative one is shown. Samples were in triplicate. Samples with αCD28 alone had ^{3}H-thymidine uptake of less than 1000 ct/min. Compared with the column with an asterisk, all the other values are significantly lower ($P < 0·01$, Student's *t*-test).

stimulated by TCR/CD3 occupation [23,24]. Phorbol esters of submitogenic concentrations can replace the signals via TCR/CD3, presumably by activation of protein kinase C (PKC), and render pure T cells competent to αCD28 stimulation [22]. The signalling via CD28 does not involve Ca^{++} influx and is insensitive to CsA [22,24,25]. We demonstrated in this study that proliferation of pure T cells triggered by αCD28 plus a submitogenic concentration of PMA was inhibitable by RAPA in a dose-dependent fashion (Fig. 1). This result is consistent with that reported by Bierer *et al.* [16] in a similar system. We also showed that RAPA strongly repressed T cell proliferation triggered by a combination of αCD3 and αCD28 in the presence of APC (Fig. 2). At 10 nM of RAPA, the proliferation was reduced to a level significantly below that triggered by αCD3 alone ($P < 0·01$, Student's *t*-test). Such a result indicates that RAPA can effectively inhibit the activation triggered by the synergistic stimuli involving both Ca^{++}-dependent and Ca^{++}-independent processes.

During T cell activation, the activated T cells up-regulate the expression of their IL-2R and IL-4R, directly or indirectly under the influence of IL-2, IL-4 or other lymphokines [26–30]. Hence, autocrine loops are formed to enhance T cell growth. We wondered whether RAPA would inhibit the expression of IL-2R or IL-4R, and interfere with these autocrine loops.

With two-colour flow cytometry it was shown that IL-2Rα expression was up-regulated on $CD3^+$ T cells of PBMC 2 days after PHA stimulation (Fig. 3). $CD3^+CD25^+$ cells increased from 2·3% to 49·5% among total PBMC (Fig. 3a, region 2), and from 2·3% to 48·4% among $CD3^+$ cells (Fig. 3b). The up-regulation was accompanied by significant increase of mean fluorescence intensity (MFI) from 8·8 to 15·7 (Fig. 3b). In the presence of 10 nM of RAPA, up-regulation was partially inhibited. $CD3^+CD25^+$ cells decreased from 49·5% to 39·9% among PBMC, and from 48·4% to 39·6% among $CD3^+$ cells. The decrease of MFI was more obvious (from 15·7 to 9·9, Fig.

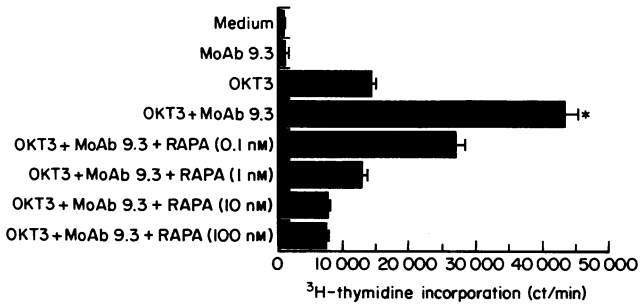

Fig. 2. Rapamycin (RAPA) inhibits T cell proliferation triggered by a combination of CD3 and CD28 pathways. Peripheral blood mononuclear cells (PBMC) were incubated with αCD28 (clone 9.3, 50 ng/ml), αCD3 (OKT3, 50 ng/ml) or a combination of both for 3 days in the absence or presence of RAPA (0·1–100 nM). The cells were pulsed with ^{3}H-thymidine for 6 h before harvest. The result of a representative experiment is shown, and similar results were obtained from two other experiments. Compared with the column with an asterisk, all the other values are significantly lower ($P<0·01$, Student's t-test). Further, the two bottom columns were significantly lower than the column of OKT3 alone ($P<0·01$, Student's t-test).

3b). These results have extended the observation made by R. Morris [2].

IL-4R expression in αCD3- or PHA-stimulated PBMC (Fig. 4a and b, respectively) after 3 days of culture was also up-regulated, and IL-4R$^+$ cells increased from 3·9% to 14·7%, and from 3·9% to 22·4%, respectively. MFI of the stimulated cells decreased in both cases, due to the fact that most of the emerging IL-4R$^+$ cells were of low fluorescence intensity. RAPA (10 nM) suppressed the up-regulation of IL-4R expression (Fig. 4a, b, right panels). The percentage of IL-4R$^+$ cells was decreased from 14·7% to 9·3% and from 22·4% to 16·8% in αCD3 and PHA-stimulated PBMC, respectively. The diminishing of the low intensity IL-4R$^+$ cells caused the relative increase of MFI in the RAPA-treated cells compared with the samples with mitogens alone. IL-4R expression was further analysed by two-colour staining with PE-αCD3 and biotin-IL-4/avidin-FITC. As shown in Fig. 5a, PHA induced IL-4R expression on both CD3$^+$ and CD3$^-$ subpopulations (regions 2 and 1, respectively, of the middle panel), and RAPA inhibited up-regulation in both (regions 2 and 1 of the third panel). The inhibition of IL-4R on T cells was better illustrated when CD3$^+$ cells were gated as shown in Fig. 5b. In all the experiments described above, cell viability was not affected by RAPA according to both trypan blue staining and light scatter analysis with flow cytometry (data not shown). These results suggest that RAPA might interfere with the autocrine loops by diminishing the IL-2R and IL-4R expression.

IL-4-promoted T cell growth represents a type of CsA-insensitive process [26]. We wondered whether this process was RAPA-sensitive. Resting T cells do not respond to IL-4, and IL-4 responsiveness can be achieved by priming the pure T cells with PHA or immobilized αCD3 along with IL-4 in a one-stage culture system [26]. However, in a two-stage culture system, TCR occupancy alone at the first stage of culture by antigen, soluble αCD3 or concanavalin A (Con A) is not sufficient to induce pure resting T cells to respond to IL-4 at the second stage, unless additional costimulation by APC at the first stage is provided [31]. We stimulated human PBMC with soluble αCD3 in the first stage culture, and this represented TCR occupancy in the presence of APC costimulation. The T cells thus stimulated underwent proliferation, the peak being around day 3 (the day when the culture started was referred to as day 1) (data not shown). If αCD3 was washed out on day 3, the proliferation quickly subsided within the next 1–2 days (data not shown). We demonstrated that addition of IL-4 to these cells in the second-stage culture could induce vigorous proliferation, and the latter was inhibited in a dose-dependent fashion by RAPA when

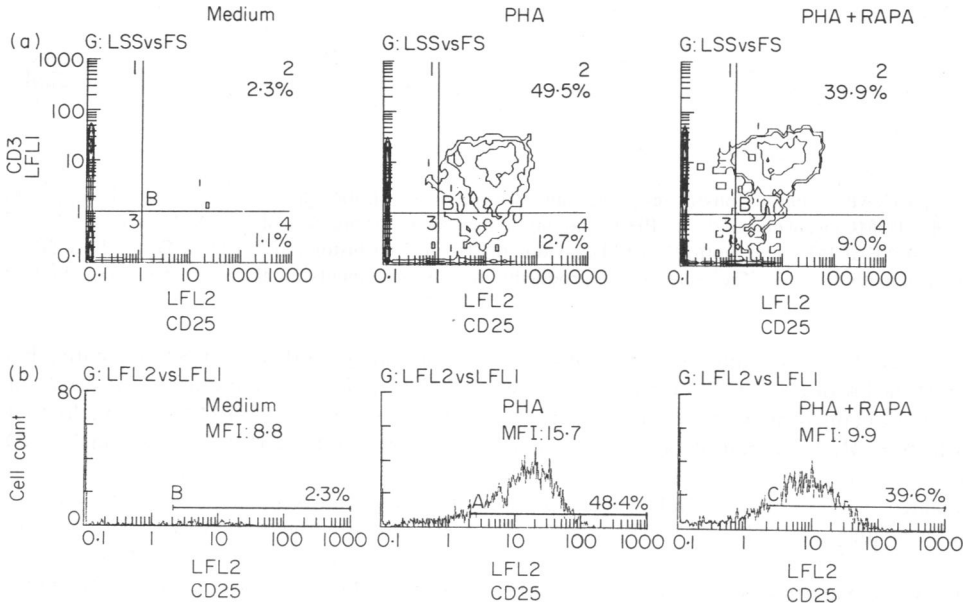

Fig. 3. Rapamycin (RAPA) inhibits phytohaemagglutinin (PHA)-induced IL-2Rα expression of CD3$^+$T cells. Peripheral blood mononuclear cells (PBMC) were incubated with PHA (2 μg/ml) for 2 days in the absence or presence of RAPA (10 nM). The cells were then double-stained with FITC-αCD3. The result of a representative experiment is shown, and similar results were obtained in two other experiments. (a) Two-colour histogram. (b) The CD3$^+$ T cells in (a) were gated and their expression of IL-2Rα (CD25) is illustrated.

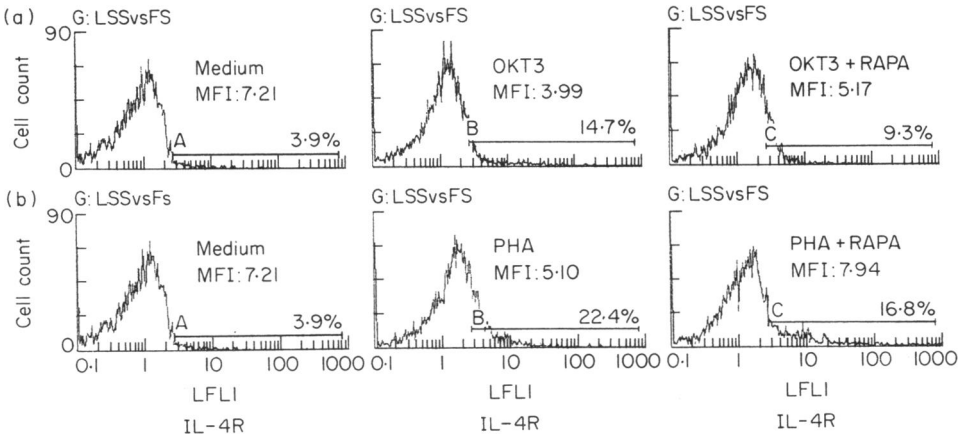

Fig. 4. Rapamycin (RAPA) inhibits mitogen-induced IL-4R expression on peripheral blood mononuclear cells (PBMC). PBMC were cultured with αCD3 (50 ng/ml (a)) or phytohaemagglutinin (PHA; 2 μg/ml (b)) for 3 days in the absence or presence of RAPA (10 nM). The cells were stained with biotin-IL-4 followed by FITC-avidin. A representative experiment is shown, and similar results were obtained in two other experiments. The left panels of a and b represent samples cultured in plain medium.

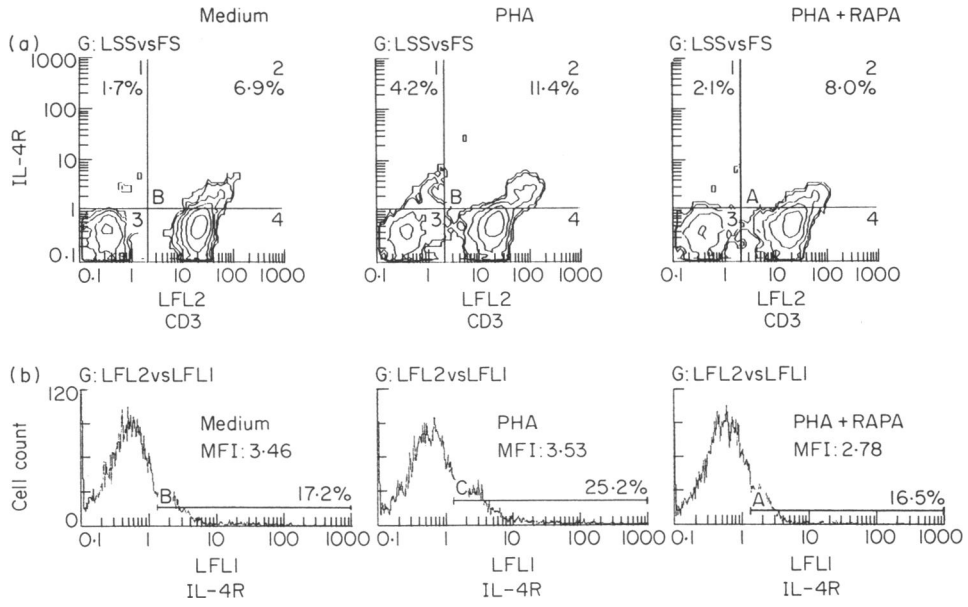

Fig. 5. Rapamycin (RAPA) inhibits phytohaemagglutinin (PHA)-induced IL-4R expression on $CD3^+$ T cells. Peripheral blood mononuclear cells (PBMC) were cultured with PHA (2 μg/ml) in the absence or presence of RAPA (10 nM) for 3 days. The cells were double-stained with PE-αCD3 *versus* biotin-IL-4/FITC-avidin. (a) Two-colour histogram. (b) The $CD3^+$ cells in (a) were gated and their IL-4R expression is illustrated. The results were reproducible in three experiments and the result of a representative plot is shown.

added along with IL-4 (Fig. 6). This indicates that the IL-4-driven proliferation of the previously primed T cells is sensitive to the drug. Our results are compatible with a previous study in a murine model, in which the IL-4-driven proliferation of several mouse T cell lines was inhibited by RAPA [14].

We next investigated whether other stimuli could replace signals from TCR occupancy and APC at the first stage of culture, and render pure T cells responsive to IL-4 at the second stage. If so, we would also explore whether such IL-4 responsiveness was RAPA-sensitive. For this purpose, αCD28 (50 ng/ml) and a submitogenic concentration (1 ng/ml) of PMA were used to pretreat the pure T cells for 3 days. The stimulants were washed out and the cells were placed in plain medium for 24 h followed by another wash. Subsequently, the cells were cultured for an additional 2 days in the absence or presence of IL-4. In this system, αCD28 or PMA individually could not induce T cell response to IL-4 (Fig. 7). Although αCD28 and PMA in combination could drive pure T cells into proliferation in the first-stage culture (Fig. 1 and [23]), the proliferation quickly subsided after the stimulants were washed out (data not shown), and the cells did not proliferate in the second-stage culture without IL-4 (Fig. 7). However, in the presence of IL-4, the T cells pretreated with αCD28 plus PMA underwent vigorous proliferation (Fig. 7). It has been reported that signals via CD28 can provide T cells with necessary costimulation normally furnished by APC [23]. It is therefore likely that in our system

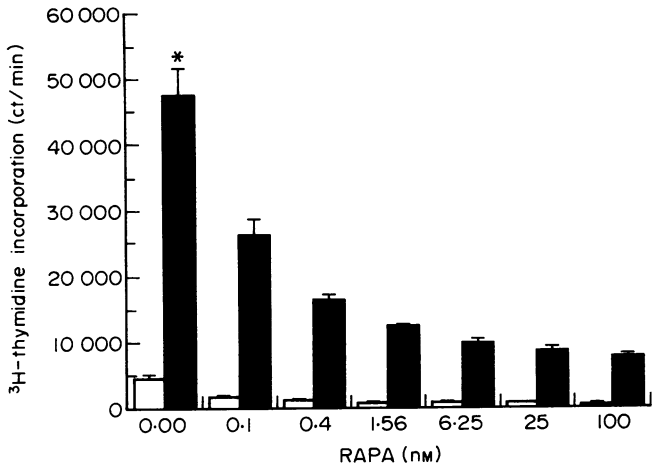

Fig. 6. IL-4-augmented proliferation of αCD3-primed peripheral blood mononuclear cells (PBMC) is rapamycin (RAPA)-sensitive. PBMC were stimulated with αCD3 (OKT3, 50 ng/ml (■)) or culture in plain medium (□) for 3 days. The cells were then washed and recultured in the presence of IL-4 (15 ng/ml) for an additional 3 days. RAPA of different concentrations was added to the second-stage culture. The cells were pulsed for 6 h before harvest. Consistent results were obtained in more than three experiments, and the result of a representative experiment is shown. Without IL-4 in the second stage, the ^{3}H-thymidine uptake was less than 1000 ct/min for the αCD3- or medium-preincubated cells. Compared with the column with an asterisk, all the other values are significantly lower ($P < 0{\cdot}01$, Student's *t*-test).

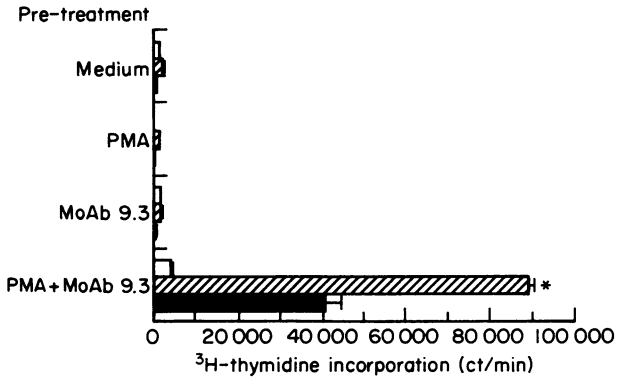

Fig. 7. IL-4 induces proliferation of pure T cells pretreated with phorbol myristate acetate (PMA) plus αCD28 and the proliferation is rapamycin (RAPA)-sensitive. Highly purified T cells were incubated with PMA (1 ng/ml), αCD28 (clone 9.3, 50 ng/ml), PMA plus αCD28, or plain medium for 3 days. After washing, the cells were placed in medium (□) for 24 h and then washed again. These cells were then stimulated with IL-4 (15 ng/ml) for an additional 2 days in the absence (■) or presence (■) of RAPA (10 nM). The culture was pulsed with ^{3}H-thymidine for 6 h before harvest. Consistent results were obtained in three experiments, and the result of a representative experiment is shown. Compared with the column with an asterisk, all the other values are significantly lower ($P < 0{\cdot}01$, Student's *t*-test).

αCD28 has played such a role. We also demonstrated that RAPA strongly inhibited IL-4-driven T cell proliferation when the drug was added at the second stage (Fig. 7). This suggests that there exists a major target(s) for RAPA at a later stage during IL-4 stimulation after the cells are primed.

DISCUSSION

In this study we investigated the effect of RAPA on T cell proliferation triggered by αCD28 with or without IL-4 in an *in vitro* human model.

In a one-stage culture system, RAPA was shown to strongly inhibit T cell proliferation triggered via the CD28 pathway, or via a combination of the CD3/TCR and CD28 pathways. Inasmuch as the CD28 pathway is not sensitive to CsA but is to RAPA, and the targets of the two drugs are different, the two drugs might have synergistic effects in preventing allograft rejection. Indeed, this has been the case in animal models [32].

Mitogen (αCD3 or PHA)-up-regulated IL-2R or IL-4R expression on T cells was repressed by RAPA. It is worth mentioning that since the inhibition was not complete for both receptors, such effects might only partially account for the overall effect of RAPA on T cell activation and proliferation.

In our two-stage culture system, RAPA was shown to be strongly inhibitory on IL-4-stimulated T cell proliferation, whether these cells were primed with αCD3 or with PMA plus αCD28. Previous reports show that TCR occupancy which triggers both PKC translocation and Ca^{++} influx [33] is necessary for the subsequent IL-4 responsiveness [31]. Our result suggests that Ca^{++} influx is not an obligatory event in inducing IL-4 responsiveness of the T cells, since neither αCD28 nor PMA, individually or in combination, are able to trigger Ca^{++} influx [24,25].

It has been reported that IL-4 can induce IL-4R expression of mitogen-stimulated T or B cell in one-stage culture models [30]. We wondered in our two-stage culture system whether IL-4 at the second stage could also induce IL-4R and whether RAPA could inhibit the up-regulation. According to flow cytometry, IL-4R on the pretreated T cells was not further up-regulated by IL-4, and RAPA added at the second stage had no effect on the levels of the existing IL-4R (data not shown). This suggests that the effect of IL-4 added in the second stage of culture does not depend on further augmentation of IL-4R expression, and that the down-regulation of IL-4R is not a mechanism for the inhibitory effect of RAPA in the second stage.

The ability of RAPA to inhibit lymphokine-induced proliferation of previously primed T cells as shown in various models [10,16,19], including our own, indicates that RAPA could restrict the expansion of specific T cells in an immune response, even when the drug is administered after the priming stage. This is consistent with our recent findings that RAPA effectively alleviates the ongoing allograft rejection *in vivo*, when the drug is given 4 days after transplantation [34]. Our present study has provided additional information about the effect of RAPA on T cell activation and proliferation triggered by CD28 pathway and IL-4, and will contribute to our eventual understanding of the mechanism of action of the drug.

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