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Expression and function of CD5 and CD28 in patients with rheumatoid arthritis

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SUMMARY

To assess the role of CD5 and CD28 in the pathogenesis of the decreased cellular immune function in patients with rheumatoid arthritis (RA) we analysed the expression and function of these T-cell surface molecules. The expression of CD5 as well as of CD28 in synovial and peripheral blood T cells was similar to that of control T cells. Monoclonal antibodies (mAb) directed at CD28 and CD5 were able to provide an accessory signal to anti-CD3 activated T cells both from the synovial fluid and from the peripheral blood. However, the proliferation induced by anti-CD3 mAb in conjunction with anti-CD5 or anti-CD28 mAb was always higher in peripheral blood (PB) T cells compared to the paired synovial fluid T cells. After simultaneous ligation of CD5 and CD28, proliferation was induced in the PB T cells. However, when compared to control PB T cells, this proliferation was significantly lower in the RA patients. Purified normal memory ($CD45RO^+$) T cells proliferated less strongly than naive ($CD45RA^+$) T cells, but no difference was observed between rheumatoid and normal memory T-cell proliferative responses. However, enriched PB $CD45RA^+$ T cells from rheumatoid patients proliferated less vigorously to CD5 and CD28 ligation when compared to normal enriched $CD45RA^+$ T cells. Synovial fluid (SF) T cells, which are mainly of the memory cell type, did not proliferate after simultaneous ligation of CD5 and CD28. This refractory state of synovial T cells could not be explained by a difference in the surface expression of CD5 or CD28. Our data suggest that the cellular immune dysfunction in the PB from rheumatoid patients may be due to a decreased responsiveness of the naive T-cell subset to accessory signals provided by CD5 and CD28. In addition, SF T cells appear hyporesponsive to stimulating signals provided through CD5 and CD28.

INTRODUCTION

Rheumatoid arthritis (RA) is a multi-system disease associated with an impaired cellular immune function in the peripheral blood (PB) resulting in anergy to recall antigens,[1-3] a decreased responsiveness to mitogens[4,5] and an impaired autologous mixed lymphocyte reaction.[6] The function of synovial fluid (SF) T cells is even more dysregulated than that of the PB cells.[7-12] The relevance of this disturbed immune function to the pathogenesis of RA is unclear and the aetiology of the abnormal function remains unexplained. The decreased responsiveness to recall antigens is correlated to a decreased percentage of memory T cells in the PB of patients with active RA.[3] However, no correlation between abnormalities in the PB T-lymphocyte subsets and lymphocyte responsiveness to mitogens has been reported. Interactions between accessory cells and T lymphocytes are believed to play a major role in the dysregulated immune response in RA.[13,14] In view of this hypothesis, one could argue that the depressed cellular immune function is secondary to an impaired intercellular communication between the antigen-presenting cells (APC) and T cells.

Activation of T cells involves the specific recognition of antigen presented on the major histocompatibility complex molecules on the APC to the T-cell receptor (TcR)/CD3 complex expressed on the antigen-specific T cells.[15] This interaction between T cells and APC is enhanced by cell-surface receptors that regulate adherence and provide co-stimulatory signals to the T cell.[16] These T-cell surface molecules include CD2, CD4, CD5 and CD28 which bind their respective ligands (LFA3, HLA-DR, CD72 and BB1/B7) expressed on the APC.[16,17]

Most studies regarding the depressed immune function in RA patients have been performed with T cells contaminated by monocytes.[1-12] In these studies, no attempt was made to analyse the functional role of accessory signals provided by cellular interactions between T cells and APC. In the present study we

Abbreviations: APC, antigen-presenting cells; d.p.m., disintegrations per min; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; PB, peripheral blood; PE, phycoerythrin; RA, rheumatoid arthritis; SD, standard deviation; SF, synovial fluid.

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analysed the cell-surface expression and function of the accessory T-cell surface molecules, CD5 and CD28, in PB and SF lymphocytes obtained from RA patients. First, we examined the accessory signals provided through CD5 and CD28 in T cells, activated by cross-linking CD3, in an *in vitro* model similar to previously reported studies.[18,19] Next, we examined the stimulating capacity of simultaneous cross-linking of CD5 and CD28 in purified T cells from PB and SF of RA patients, and then compared the results with those obtained from normal healthy controls.

MATERIALS AND METHODS

Patients and controls

Paired samples of PB and SF were obtained from 15 patients undergoing therapeutic joint fluid aspiration. All patients had severe, active definite or classic RA, as defined by the American Rheumatism Association criteria.[20] Blood was also donated by 11 healthy controls, of both sexes, aged between 20 and 40 years. This study was approved by the Institutional Ethics Committee.

SF and PB T-cell separation

PB and SF mononuclear cells were isolated on Ficoll–Hypaque (density 1·077) gradients. After three washes in Hanks' balanced salt solution (HBSS), the cells were further purified using a mixture of anti-natural killer cell, anti-monocyte and anti-B cell monoclonal antibodies (mAb) [RFB9 (anti-CD19; a kind gift from Professor G. Janossy, Royal Free Hospital, London, U.K.), UCHM1 (anti-CD14; a kind gift from Professor P. C. L. Beverley, Imperial Cancer Research Fund, London, U.K.) and 3G8 (anti-CD16; Medarex, Lebanon, NH)] and magnetic goat anti-mouse treated beads (Advanced Magnetics, Cambridge, MA). After incubation (20 min, 4°) the monocytes, natural killer cells and B cells bound to the magnetic beads were removed from the cell suspension. The negatively selected T-cell preparations contained more than 85% $CD3^+$, and less than 5% $CD16^+$ cells and less than 2% $CD14^+$ monocytes. Purified memory ($CD45RO^+$)/virgin ($CD45RA^+$) T cells were obtained by further incubation of the enriched T cells with mAb [SN130 (anti-CD45RA; a kind gift from D. O. Haskard, RPMS, London, U.K.) or UCHL1 (antiCD45RO; a kind gift from Professor P. C. L. Beverley)] and the cells were separated with goat anti-mouse IgG-treated beads as described above. The negatively selected memory cell subsets contained more than 85% $CD3^+$ $CD45RO^+$ cells and the negatively selected virgin cell subset contained more than 85% $CD3^+$ $CD45RA^+$ cells. The enriched T cells were incubated overnight in culture medium [RPMI-1640 (Gibco, Paisley, U.K.) supplemented with 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), gentamycin (10 mg/l) and 10% pooled normal human serum] at 37°, 5% CO_2 and washed once before use in experiments.

Monoclonal antibodies

The following mAb were used in the lymphocyte proliferation assays: anti-CD3 [OKT3, murine IgG2a; American Type Culture Collection (ATCC), Rockville, MD]; anti-CD5 (OKT1, murine IgG1; ATCC); the cells were grown in our laboratory and the hybridoma supernatant was purified on a protein A column; and anti-CD28 [mAb 9.3 (ascites), murine IgG2a; Oncogen, Bristol-Meyer and Squib Pharmaceutical Research, Seattle, WA]. The following mAb were obtained from Becton Dickinson (Mountain View, CA) for use in immunofluorescence studies: anti-Leu-4 (anti-CD3), anti-Leu-M3 (anti-CD14), anti-Leu-16 (CD19), anti-Leu-7 (CD56) and anti-Leu-1 (CD5).

Cell culture for proliferation studies

SF and PB T cells were cultured (at a concentration of $0{\cdot}25 \times 10^6$ cells/ml) in culture medium in flat-bottomed microculture plates (Costar, High Wycombe, U.K.). Culture plates were either uncoated or coated with OKT3 (10 μg/ml) or affinity-purified goat anti-mouse IgG (Sigma, St Louis, MO) as previously described.[21] All cultures were performed in quadruplicate and after 112 hr, cells were pulsed with 1 μCi of [^{3}H]thymidine (specific activity 2 Ci/mmol; Amersham International, Amersham, U.K.). Eight hours later, cells were harvested with a multiple automated sample harvesting apparatus (Skatron, Lier, Norway) and radioactivity on the filter papers was counted in a liquid scintillation counter.

Immunofluorescence analysis of lymphocytes

After overnight incubation, the T cells were washed and resuspended in 100 μl of phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA), and immediately used for immunofluorescent staining. The cell suspension was incubated for 30 min at 4° with 5 μl of fluorescein isothiocyanate (FITC)-conjugated OKT3, anti-Leu-1 and mAb 9.3 and/or with phycoerythrin (PE)-conjugated anti-Leu-2a, anti-Leu-3a, anti-Leu-4, anti-Leu-16, anti-Leu-7 and anti-Leu-M3. Controls included cells incubated with PE- and FITC-conjugated mouse IgG. After washing, the cells were fixed in 1% paraformaldehyde. Analysis was performed on a FACScan (Becton Dickinson). Forward and side scatter gating were set to include lymphocytes and lymphoblasts.

RESULTS

CD28 can provide the necessary second signal in anti-CD3-stimulated PB and SF T cells

We first examined the functional capacity of CD28 in SF and PB T cells from RA patients. To examine whether soluble mAb 9.3 was able to deliver an accessory signal to anti-CD3-stimulated cells, we cultured the SF and PB T cells with or without soluble mAb 9.3 on OKT3-coated plates; control cultures were performed on non-coated plates. Soluble mAb 9.3 alone did not induce any proliferation in the SF or PB T cells (Fig. 1). Suboptimal concentrations of solid phase bound OKT3 induced minimal proliferation. However, this proliferation was greatly enhanced by addition of mAb 9.3 to both the SF and PB T-cell cultures (Fig. 1). In all the RA patients studied, we observed that PB T cells proliferated to a greater extent than SF T cells, when both OKT3 and mAb 9.3 were added to the cultures. Parallel cultures were performed with PB T cells obtained from healthy controls (Fig. 1). In healthy controls, mAb 9.3 significantly enhanced the OKT3-induced T-cell proliferation, in a manner similar to the results obtained from the RA PB T cells. Identical proliferative results were observed when the cultures were performed with solid phase bound mAb 9.3 (cultures performed on GAM-coated plates) (data not shown).

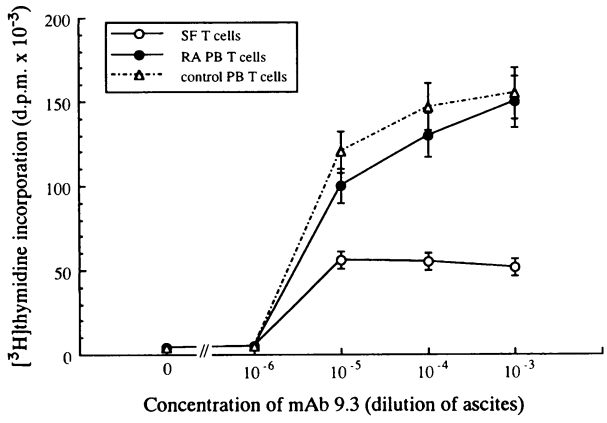

Figure 1. Soluble mAb 9.3 can provide a second signal to OKT3-stimulated SF and PB T cells from rheumatoid patients. SF and PB T cells from a representative patient with RA and control PB T cells ($0{\cdot}25 \times 10^6$/ml) were incubated for 5 days on OKT3 (10 μg/ml)-coated plates with or without mAb 9.3 (concentrations ranging between $1/10^3$ and $1/10^6$ dilution of ascites). Proliferation was measured as [^{3}H]thymidine incorporation (d.p.m.). Values represent the means $\pm$ SD of quadruplicate cultures.

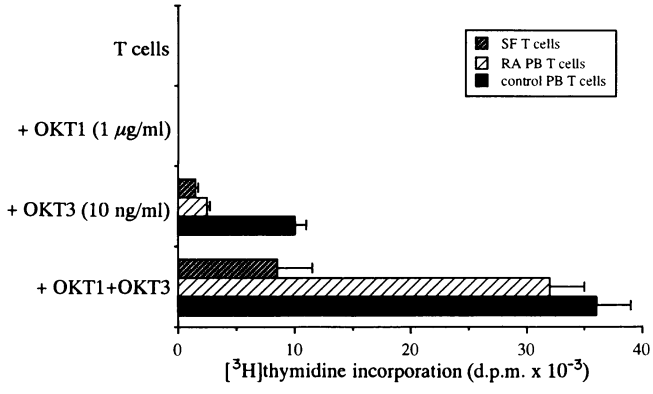

Figure 2. Solid phase bound OKT1 can provide an accessory signal in SF and PB T cells from patients with RA. SF and PB T cells from a representative patient with RA and control PB T cells ($0{\cdot}25 \times 10^6$/ml) were incubated for 5 days with or without OKT3 (10 ng/ml) with or without OKT1 (1 μg/ml) on goat anti-mouse IgG-coated plates. Proliferation was measured as [^{3}H]thymidine incorporation (d.p.m.). Values are the means $\pm$ SD of quadruplicate cultures.

Solid phase bound anti-CD5 mAb is able to provide an accessory signal to anti-CD3-stimulated PB and SF T cells

To examine whether the T-cell surface antigen CD5 was able to provide a second signal to purified OKT3-stimulated T cells, we performed the next set of experiments. Paired samples of PB and SF T cells were cultured on goat anti-mouse IgG-coated plates with or without OKT3 and/or OKT1. As shown in Fig. 2, OKT1 alone induced no proliferation in either PB or SF T cells. Addition of OKT3 to the cultures induced minimal proliferation and this was strongly enhanced when OKT1 was added to the cultures. Similar to the observations made with mAb 9.3, the proliferation induced by OKT1 and OKT3 was more pronounced in the PB T cells than in the SF T cells. Soluble OKT1, however, was not able to provide an accessory signal in either PB or SF cells (data not shown). Similar results were observed in the normal controls (Fig. 2) and the proliferative response was of the same order as that for the RA PB T cells.

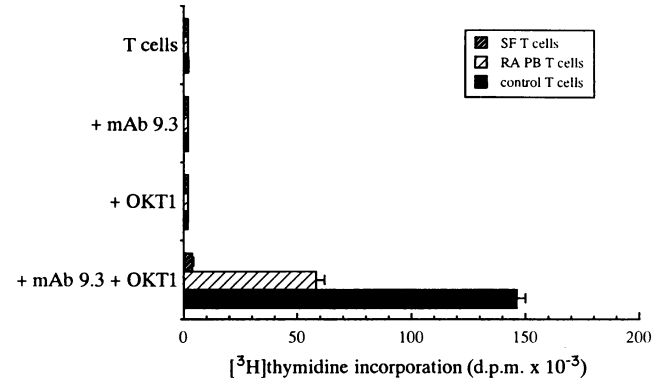

Figure 3. Simultaneous cross-linking of CD5 and CD28 induces proliferation in PB T cells from patients with RA and healthy controls but not in SF T cells. SF and PB T cells from a representative patient with RA and control PB T cells ($0{\cdot}25 \times 10^6$/ml) were incubated for 5 days with or without OKT1 (1 μg/ml) with or without mAb 9.3 ($1/10^4$ dilution of ascites) on goat anti-mouse IgG-coated plates. Proliferation was measured as [^{3}H]thymidine incorporation (d.p.m.). Values are the means $\pm$ SD of quadruplicate cultures.

Simultaneous cross-linking of CD5 and CD28 induces proliferation in PB but not in SF T cells

We have recently observed that simultaneous ligation of CD5 and CD28 induces T-cell proliferation, independent of CD3 ligation in purified resting T cells.[22] In this set of experiments, optimal concentrations of anti-CD5 (OKT1, 1 μg/ml) and anti-CD28 (mAb 9.3, $1/10^4$ dilution of ascites) were used to induce T-cell proliferation. As shown in Fig. 3, simultaneous cross-linking of CD5 and CD28 induced PB T-cell proliferation. However, no proliferation was induced in the SF T cells after simultaneous ligation of CD5 and CD28 (Fig. 4). Different concentrations of OKT1 and mAb 9.3 induced lower proliferative responses in the PB T cells, but no proliferation was induced in SF T cells. Furthermore, anti-CD5 and anti-CD28 mAb alone did not induce proliferation in any of the different T-cell populations tested (Fig. 4). The proliferation induced in the RA T cells upon simultaneous cross-linking of CD5 and CD28 was significantly lower in the RA population when compared to healthy controls (Fig. 4).

CD3, CD5 and CD28 expression is identical on SF and PB T cells

In an attempt to explain the different proliferative results obtained between normal and RA PB T cells and between RA SF and PB T cells, the following experiments were performed. First, the surface expression of CD3, CD5 and CD28 in SF T cells and PB T cells from RA patients were determined using immunofluorescence staining. We did not observe any difference between the SF and PB T cells either in the percentage or in the intensity of expression of CD3, CD5 and CD28 (Fig. 5).

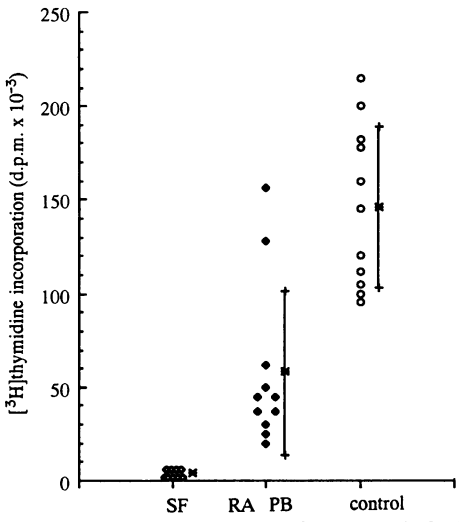

Figure 4. Simultaneous cross-linking of CD5 and CD28 induces proliferation in PB T cells from RA patients and healthy controls, but not in SF. $0{\cdot}25 \times 10^6$/ml SF and RA PB T cells from 11 RA patients and PB T cells from 11 healthy donors (control) were incubated for 5 days with OKT1 (1 μg/ml) and mAb 9.3 ($1/10^4$ dilution of ascites) on GAM-coated plates. Proliferation was measured as [^{3}H]thymidine incorporation (d.p.m.). The stars represent the means of the proliferation induced in both groups and the bars represent the SD.

Accessory function of CD5 and CD28 in enriched memory and virgin T cells

Next, we analysed whether the observed differences in T-cell proliferation, induced by simultaneous ligation of CD5 and CD28, was secondary to differences in T-cell subsets in the PB T cells between normal and RA PB. We determined the CD4/CD8 and the CD45RA/CD45RO ratio in PB and SF of three RA patients and compared these with normal controls. Both the CD4/CD8 ratio and the CD45RA/CD45RO ratio were higher in the RA population (Table 1). Next, we isolated naive and memory T-cell subsets from PB of three RA patients and from three normal volunteers. Using these enriched T-cell populations we found that simultaneous cross-linking of CD5 and CD28 induced similar T-cell proliferation in the memory cell subset from RA patients and normal volunteers (Fig. 6). The activation of naive T cells, however, was significantly lower in the patients' naive T cells compared to the healthy donors (Fig. 6).

DISCUSSION

The series of experiments reported in this study demonstrate that: (1) naive RA PB T cells proliferate to a lesser degree than normal naive PB T cells after simultaneous ligation of CD5 and CD28, and (2) SF T cells from RA patients are hyporesponsive to the stimulatory signals provided through CD5 and CD28. In discussing the results of our study it is best to focus first on the observed abnormalities in the PB T lymphocytes and then to interpret our observations with regard to the abnormalities of the synovial fluid.

The observations made in our study may provide further insight into the pathogenesis of the depressed cellular immune function observed in the PB of RA patients. Despite the multitude of reports focusing on the immune function in RA, the pathogenesis of the immune alterations remains to be elucidated. Numerous tests of PB T-cell function have been undertaken as a further means of defining immune system status in patients with RA. Patients with active disease have a diminished responsiveness to recall antigens,[1] and this is correlated with a decreased number in the PB memory T-cell subset.[3] The function of naive T cells in the PB of RA T cells may also be abnormal, as suggested by the abnormal autologous mixed lymphocyte reaction.[6] Several explanations have been put forward for the depressed cellular function in the PB of RA patients. Mechanisms involving monocytes have been proposed.[23-25] To avoid interference of monocytes in our current study we attempted to remove all contaminating monocytes. This resulted in minimal proliferation after stimulation with suboptimal concentrations of anti-CD3 mAb. The second signal necessary to induce proliferation in anti-CD3-stimulated T cells, could be provided by ligation of CD28 or CD5. We observed no difference in proliferative responses between normal PB and RA T cells in these cultures, indicating that the CD5 and CD28 function normally in anti-CD3-stimulated cells.

We have recently described a novel T-cell activation pathway involving CD5 and CD28.[22] Upon simultaneous ligation of these molecules, T cells are activated in the absence of CD3 occupancy.[22] The resulting T-cell proliferation proceeds by autocrine production and action of interleukin-2 (IL-2).[22] Using this novel T-cell-activating stimulus, we further examined the function of CD5 and CD28 in RA PB T cells. We observed a much less intense proliferation in RA PB T cells after this trigger when compared to normal controls. This difference could not be explained by an abnormal expression of CD5 or CD28 on RA T cells. One likely explanation for this difference is an abnormality of the PB lymphocyte subsets in RA patients. Indeed, many abnormalities of the lymphocyte subsets in the PB of RA patients have been described.[1-3] In accordance with these previously published reports, the CD4/CD8 and the CD45RA/CD45RO ratio were increased in our patients. To interpret the strikingly different proliferative results between RA patients and normal controls, we purified CD45RA$^+$ and CD45RO$^+$ T cells from the PB of RA patients and controls. Purified memory T cells from normal controls proliferated less to simultaneous ligation of CD5 and CD28 than did purified naive T cells. We have previously reported that, upon simultaneous ligation of CD5 and CD28, the expression of activation antigens (CD25, CD69) occurs mainly on naive T cells.[22] Accordingly, the observed differences between normal memory and naive T cells are not surprising. Also in the RA patients, the proliferation induced in the memory T cells was lower than the proliferation in the naive cells. Of interest is the observation that memory T cells of patients and of controls proliferated in a similar manner. However, the enriched naive T cells from RA patients proliferated far less vigorously than normal naive T cells.

As the molecular mechanisms involved in the novel T-cell activation pathway remain to be elucidated, we can only speculate on the possible causes for the differences observed between normal and RA naive T cells. As both anti-CD5 and anti-CD28 mAb enhanced anti-CD3-induced T-cell proliferation in the PB T cells, it would appear likely that, in RA patients, as in normal controls, cross-linking of these surface molecules induces a rise in intracellular calcium.[26] We are currently examining the effect of CD5 and CD28 ligation on calcium

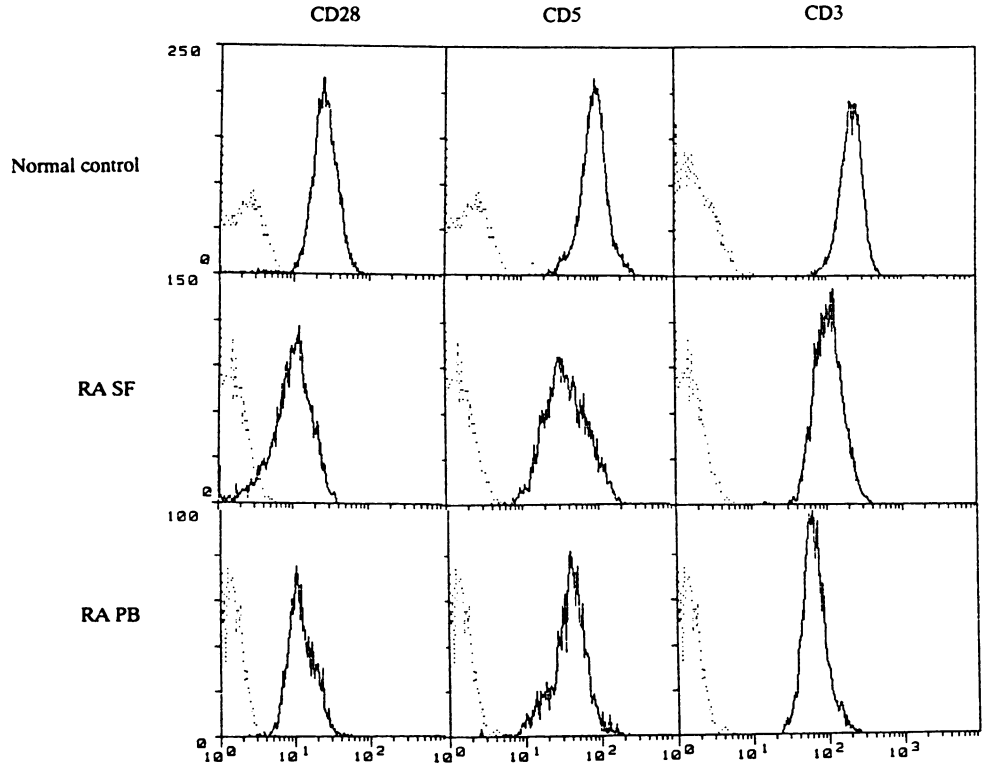

Figure 5. CD3, CD5 and CD28 expression is identical on SF and PB T cells. PB T cells from a normal control (upper panel) and SF T cells (middle panel) and PB (lower panel) of a representative patient with RA were incubated with FITC-labelled mAb 9.3 (left column), Leu-1 (middle column) and OKT3 (right column) and control mouse IgG (·····) and analysed by flow cytometry.

Table 1. Lymphocyte subsets in blood and synovial fluid from RA patients and from healthy controls

Cell ratio	Normal control*	RA PB†	RA SF
CD4/CD8	1·56	1·81	1·05
CD45RA/CD45RO	0·46	0·68	0·05

* Mean of three healthy controls.
† Mean of three RA patients.

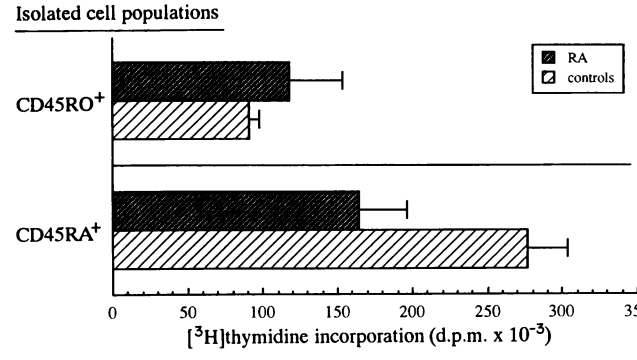

Figure 6. Simultaneous cross-linking of CD5 and CD28 induces proliferation in enriched memory ($CD45RO^+$) and naive ($CD45RA^+$) PB T cells from patients with RA and healthy controls. $0{\cdot}25 \times 10^6$/ml $CD45RA^+$ and $CD45RO^+$ T cells from three patients with RA and three healthy controls were incubated for 5 days with OKT1 (1 μg/ml) and mAb 9.3 ($1/10^4$ dilution of ascites) on goat anti-mouse IgG-coated plates. Proliferation was measured as [^{3}H]thymidine incorporation (d.p.m.). Values are represented as the mean proliferation and the bars represent the SE of the mean.

mobilization in RA T cells. The proliferation induced by simultaneous cross-linking of CD5 and CD28 depends on protein kinase C (PKC) activation, as this proliferation is inhibited by staurosporine and H7.[22] In PB T cells from patients with RA the PKC pathway is normal.[2] Accordingly, differences in proliferative responses after simultaneous ligation of CD5 and CD28 may be either due to abnormalities in other signal transducing events or to post-signal tranduction intranuclear events. Such an abnormality might well contribute to the general immunosuppressed state observed in RA patients.

In discussing the abnormalities in SF in our study, it is pertinent to make reference to a number of previously reported and important studies. Indeed, the dysregulated function of the RA SF T cells has been studied much more thoroughly than the PB T-cell dysfunction. Early reports attempting to elucidate the SF T-cell hyporesponsiveness described the production of cytokine inhibitors (IL-1 inhibitor[27] and IL-2 inhibitors[28]) by the RA SF monocytes. These inhibitors may explain the depressed *in vitro* mitogen responsiveness of unpurified SF mononuclear cells. However, purified SF T cells are also hyporesponsive to mitogens, and this defect cannot be reversed by addition of the PKC activator phorbol 12-myristate 13-acetate (PMA).[11,29] Jahn *et al.*[11] have proposed that the hyporesponsiveness of SF T cells to mitogenic stimuli is secondary to down-modulation of

the CD3 molecule. However, we observed no difference in the CD3 molecule expression on the SF T cells when compared to the PB T cells from patients and controls. The apparent discrepancies between our observations and those of Jahn *et al.*[11] may be due to differences in purification techniques. We obtained T cells by negative selection, whereas Jahn and colleagues used sheep red blood cell rosetting to isolate T cells.[11] Indeed, ligation of CD2 to lymphocyte function-associated antigen-3 (LFA-3) on sheep red blood cells activates resting T cells.[30] Accordingly, the previously reported CD3 down-modulation could be an *in vitro* phenomenon secondary to T-cell activation by CD2 perturbation during the isolation procedure.[11].

In our enriched SF T cells, suboptimal concentrations of anti-CD3 mAb alone did not induce T-cell proliferation. Ligation of CD5 as well as CD28 could induce T cells to proliferate after CD3 cross-linking. However, SF T cells proliferated much less vigorously than the paired RA PB T cells. In addition, none of the SF T cells could be induced to proliferate after simultaneous ligation of CD5 and CD28. In accordance with previous published reports, in our study the T cells in the SF were mainly of the memory cell type.[31,32] However, purified memory T cells proliferate, albeit less strongly, than purified naive T cells, after simultaneous ligation of CD5 and CD28. Accordingly, the complete absence of proliferation seen in SF T cells can not solely be explained by the CD54RO cell type. Moreover, the absence of proliferation could not be explained by an abnormality in cell-surface expression of CD5 and CD28. Interestingly, anti-CD5 mAb can inhibit synovial fluid lymphocyte proliferation induced by IL-2.[23] These observations suggest that CD5 can not only enhance anti-CD3-induced T-cell activation, but can also deliver a negative signal to SF T cells. The molecular mechanism for these seemingly contradictory observations is unknown. We are, however, currently examining the effects of anti-CD5, anti-CD28 and anti-CD3 mAb on intracellular calcium concentrations and inositol phosphate production in SF T cells. However, we are still unable to provide a satisfactory explanation for the hyporesponsiveness of synovial T cells to signals provided through CD5 and CD28. This partially refractory state of SF T cells to new signals may well be secondary to their *in vivo* pre-activated state.

In summary, our data indicate that SF T cells are relatively insensitive to second signalling through the cell-surface antigens CD5 and CD28. In addition, naive PB T-cell proliferation induced by simultaneous cross-linking of CD5 and CD28 is abnormal in RA patients. Elucidation of the molecular mechanisms involved in this novel pathway of T-cell activation may shed new light on the abnormal T-cell function in RA.

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