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Demonstration of CD13/Aminopeptidase N on Synovial Fluid T Cells from Patients with Different Forms of Joint Effusions

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Abstract

Cytofluorometric analysis was performed to characterize the immunophenotype of lymphocytes of the synovial fluid (SF) and the peripheral blood (PB) from patients suffering from juvenile chronic arthritis (JCA) or rheumatoid arthritis (RA). The most obvious difference could be found in expression of the surface protease aminopeptidase N (AP N/CD13). Whereas monoclonal antibodies specific to CD13 failed to reveal surface expression on lymphocytes of the PB; 63 ± 15 % of SF T cells gave positive staining for CD13 using Leu-M7. No correlation between CD13 expression and joint disease could be found in patients who had different types of inflammatory joint effusions. CD13 expression of T cells was also found in synovial tissue and inflammatory serous cavity effusions. Fixation of T cells revealed the presence of intracellular CD13 antigen already located in the PB T cells of healthy individuals. Induction of CD13 expression on PB T cells could be demonstrated after incubation with Con A/IL-2 or SF from patients with RA. Our findings suggest a role for AP N as a new activation-associated molecule of T lymphocytes.

Introduction

Although recent studies have failed to find arthritis-specific T cell phenotypes in the PB of patients with varying forms of joint diseases, the synovial lymphocytes exhibit a number of peculiarities. Besides the heavy preponderance of T cells – which are composed almost entirely of cells of the memory phenotype expressing CD45RO (1), – there is a known discrepancy between the increased expression of HLA-DR, but not of CD25 on T cells (2, 3). Synovial T cells are described as expressing specific molecules – such as UM4D4 (4) – and as representing the Th1-phenotype (5). Functional abnormalities include a reduced response to mitogenic lectins (6, 7), but comparable or increased responses to recall antigens (7, 8).

Abbreviations: *SF* = synovial fluid; *PB* = peripheral blood; *JCA* = juvenile chronic arthritis; *RA* = rheumatoid arthritis; *TA* = traumatic arthropathy; *DJD* = degenerative joint disease; *APN* = aminopeptidase N; *DP IV* = dipeptidylpeptidase IV; *FITC* = fluorescein-isothiocyanate; *PE* = phycoerythrin; *FCS* = fetal calf serum

In order to further characterize the functional state of SF T cells, we compared the immunophenotype of PB and SF lymphocytes (paired samples) of 10 patients having either RA or JCA. Particular attention was paid to the surface proteases AP N/CD13 and DP IV/CD26. In recent years, the interest in these and other proteases has increased remarkably because, among other things, peptidase inhibitors indicate roles of ectopeptidases in regulating biologically active peptides, such as cytokines and growth factors. Thus, a great number of studies have been published with respect to the participation of the constitutively expressed transmembrane protease DP IV in T cell activation and memory (9–11). However, contradictory results have been published concerning the occurrence of the ubiquitous zinc-containing exopeptidase AP N in lymphocytes. Whereas AP N-like enzymatic activities are detectable using spectrophotometric methods (9, 12–14); the available monoclonal antibodies against CD13 recognize monocytes and granulocytes, but not lymphocytes of the PB (15, 16). AP N catalyzes the removal from peptides of N-terminal, preferentially neutral, amino acid residues and is suggested to be involved in the metabolism of regulatory peptides, e.g., through small intestinal and renal tubular epithelial cells and through synaptic membranes (17).

Using two-color flow cytometry, we were able to detect the occurrence of CD26 on both PB and SF lymphocytes. Surprisingly, SF T cells – but not PB lymphocytes – showed a varying surface-labelling with different monoclonal antibodies specific for CD13. We subsequently studied the immunophenotype of SF T cells from patients with a variety of inflammatory arthropathies in order to investigate possible correlations between the expression of CD13 and the type of joint disease. In addition, lymphocytes of synovectomy tissue, spleen and serous cavity effusions were studied to see whether they displayed surface expression of CD13. Incubation experiments with SF and PB lymphocytes were carried out to obtain first insights into the regulation of CD13 on T lymphocytes.

Materials and Methods

Patients and cell preparations

Patients with different types of joint effusions were studied. The diagnoses were defined by the clinicians who supplied the SF that was drawn for therapeutic reasons. The 32 arthritis patients were categorized into four clinical groups: JCA, RA, traumatic arthropathy (TA) and degenerative joint disease (DJD). Patients were treated with non-steroidal anti-inflammatory drugs and/or steroids as well as disease-modifying drugs such as sulfasalazine. None of the patients had received intra-articular steroid therapy during the last month before sampling. SF and, in ten patients with RA or JCA, paired blood samples, were collected via sterile heparinized tubes, and MNC were isolated by Ficoll-Hypaque density gradient centrifugation. Blood-stained SF specimens were excluded from the study. The cell-free SF was stored at −80 °C. Blood from healthy staff members of our laboratory served as the control group. Synovial tissues were obtained from 3 patients with JCA who were undergoing synovectomy. MNC were eluted as described by Abrahamsen (18). Human spleen tissues were kindly provided by the HLA laboratory of our Medical Faculty. MNC of the spleen tissues were

separated using Ficoll-Hypaque. During therapeutic procedures, peritoneal fluids were aspirated from 2 patients suffering from ethanolic liver cirrhosis. Pericardial fluid was obtained from ten patients undergoing heart valve replacement. Cells were collected by centrifugation and used for double-labelling after two washings.

Monoclonal antibodies

The monoclonal antibodies used were Ta1 (CD26, Coulter, Krefeld, Germany), IOT14 (CD25, Immunotech/Dianova, Hamburg, Germany), Leu-M7 (CD13, Becton Dickinson, Heidelberg, Germany), WM-47 (CD13, DAKO, Hamburg, Germany), SJ.101 (CD13, Immunotech/Dianova). All other monoclonal antibodies used were purchased from Becton Dickinson. Optimal dilutions for use in flow cytometry were established in preliminary experiments.

Immunofluorescence studies

Double-staining of MNC was performed at 20 °C for 20 minutes. After two cold washes with PBS containing 0.1 % sodium azide, the cells were fixed using 1 % paraformaldehyde. For comparison of different CD13 specific monoclonal antibodies, indirect immunofluorescence staining was used. Cells were incubated with either monoclonal antibody or purified normal mouse IgG. After two washings, cells were labelled with the PE-conjugated secondary antibody and washed and fixed as mentioned above. Cytofluorometric analyses were performed on a Becton Dickinson FACScan. Non-lymphoid cells and dead cells were excluded by the setting of appropriate forward and 90° light scatter gates. 5000 cells per sample were counted. The threshold was defined in such a manner that positive-staining included no more than 1 % of the relevant negative control.

Permeabilization of cells

In order to detect intracellular CD13 in $CD3^+$ lymphocytes, cells were gently permeabilized following the method of SCHMID (19). In brief, after blocking non-specific binding with a 1-min pre-incubation with human AB serum, cells were labelled with the first antibody (Leu-4/FITC). After two washes, the pelleted cells were fixed with 0.25 % paraformaldehyde solution at 4 °C for 1 h. After centrifugation, pelleted cells were resuspended in 0.2 % Tween 20 in PBS at room temperature, washed and centrifuged again. Staining of the internal antigen (negative control/PE or Leu-M7/PE) followed a further blocking of non-specific binding with human AB serum. After two washes in 0.2 % Tween 20 in PBS, cells could be analyzed.

Preparation of PB MNC cultures incubated with SF

Aliquots of PB MNC at 1×10^6 cells/ml were incubated for various time periods between 30 min and 18 h at 37 °C with an equal amount of autologous cell-free SF. The cells were washed twice in PBS and investigated for the presence of CD13 on $CD3^+$ cells using double-labelling.

Stimulation of PB MNC with mitogen or SF

MNC from the PB of healthy subjects were incubated at a concentration of 1×10^6 cells/ml in RPMI containing antibiotics, L-glutamine and 10 % fetal calf serum (FCS) under the following conditions: (a) with no additives or (b) with Con A (5 μg/ml) and IL-2 (10 U/ml) or (c) with SF (dilution 1:10) from patients with RA. After 1, 2 and 3 days, respectively, lymphocytes were investigated for surface molecules. In some cases, cells were incubated in the presence of cycloheximide (1.5 μg/ml).

Statistical analysis

Data are expressed as mean ± standard deviation. Student's t-test for paired differences was used to compare paired samples of blood and SF. In the other cases, statistical differences were analyzed using a t-test for unpaired data with Welch's approximation.

Results

Immunophenotyping of paired samples of SF and PB T cells of patients with RA or JCA

Figure 1 compares the expression of five selected surface molecules on $CD3^+$ lymphocytes from patients suffering from RA or JCA (paired samples of SF and PB). We found a significant preponderance of the expression of HLA-DR and CD45RO on SF versus PB $CD3^+$ cells (76 ± 15 % versus 7 ± 3 %, and 90 ± 7 % versus 42 ± 15 %, respectively) as already described by several authors (1–4). No clear difference could be demonstrated in the case of CD25 expression (14 ± 9 % versus 16 ± 13 %). Surprisingly, the most obvious difference between surface molecule expression in SF and PB was found for CD13. As in the PB of healthy controls (data not shown), only a negligible amount of surface expression was demonstrable on $CD3^+$ cells in the PB of all the patients studied. By contrast, 63 ± 16 % of SF $CD3^+$ cells were $CD13^+$, using the monoclonal antibody Leu-M7. Expression of CD13 was not an all-or-none phenomenon. Instead, the fluorescence histograms were typically rather broad (Fig. 2). Both $CD4^+$ and $CD8^+$ SF T cells expressed CD13, as shown in Figure 3. Furthermore, a slightly – but significantly – decreased expression of the exopeptidase CD26 was demonstrable on SF versus PB $CD3^+$ cells (45 ± 13 % versus 59 ± 13 %) (Fig. 1).

Comparison of different monoclonal antibodies specific to CD13

Using the PE-conjugated Leu-M7, the FITC-conjugated WM-47 and the FITC-conjugated SJ.1D1 in one sample of SF cells, we observed a staining

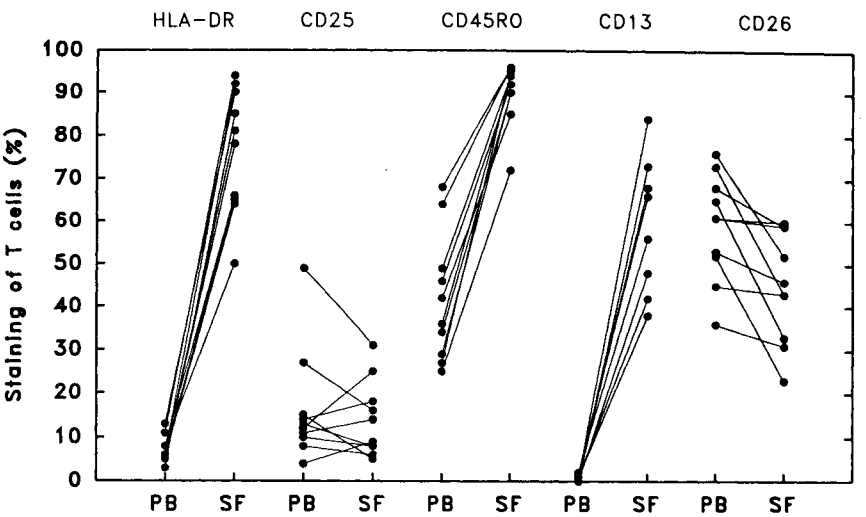

Figure 1. Comparison between PB and SF T cells of 10 patients suffering from JCA or RA (paired samples) using two-color flow cytometry. Results are given as a percentage of staining of $CD3^+$ cells.

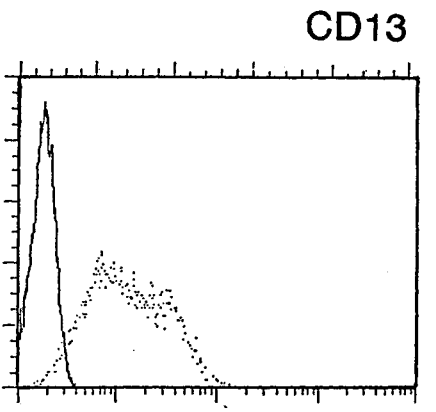

Figure 2. Fluorescence histogram of $CD3^+$ T cells from SF stained with Leu-M7 (PE). The vertical axis shows the number of T cells, and the horizontal axis the log intensity of fluorescence.

of cells in the ratio: 6:2:1. We found no obvious difference in staining when comparing the 3 above-mentioned antibodies in a non-conjugated form and using an indirect method with a PE-conjugated second antibody (data not shown). In the following experiments, PE-conjugated Leu-M7 was used (unless otherwise indicated) for the demonstration of CD13.

Immunophenotyping of SF T cells of patients with different forms of joint diseases

To investigate a possible correlation between the type of arthritis and a special pattern of expression on SF T cells of activation molecules and CD13 and CD26, double-staining experiments were performed with SF

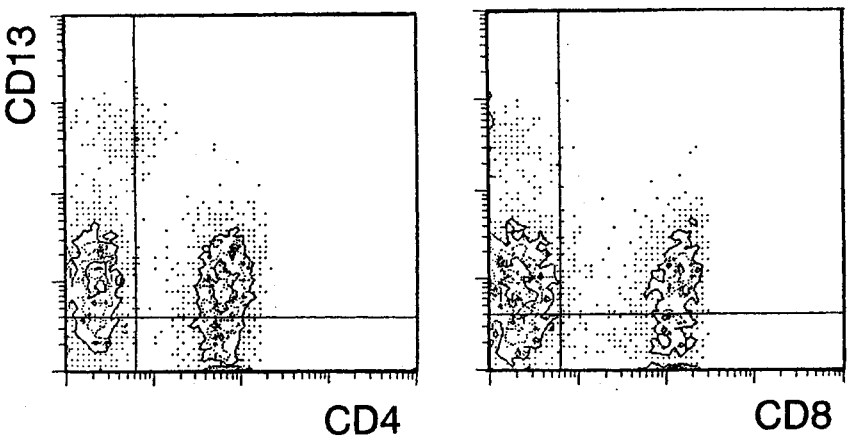

Figure. 3. Two-color immunofluorescence analysis of SF T cells from a representative patient with JCA.

Table 1. Expression of five selected surface molecules on SF T cells of patients with varying joint diseases. Results are given as a percentage of $CD3^+$ cells; values represent mean ± SD from n experiments

Diagnosis	n	HLA-DR	CD45RO	CD25	CD13 (Leu-M7)	CD26
JCA	6	80 ± 12	91 ± 4	12 ± 8	68 ± 16	51 ± 12
RA	6	73 ± 14	95 ± 3	20 ± 13	50 ± 15	40 ± 6
TA	6	69 ± 11	93 ± 5	11 ± 6	42 ± 16	55 ± 21
DJD	6	82 ± 8	95 ± 4	9 ± 4	44 ± 7	43 ± 13

cells of different inflammatory joint effusions. We could find no significant difference in the expression on $CD3^+$ cells of all the surface molecules studied, although CD13 seemed to be increased in patients with a highly-active JCA (Table 1).

Expression of CD13 on human T cells from different sources

To answer the question whether CD13 occurs on T cells exclusively within SF, we investigated the immunophenotype of lymphocytes from different sources. On T cells prepared from synovectomy tissue of 3 patients with JCA, we were able to demonstrate a CD13 expression comparable to SF lymphocytes (32, 52 and 67% of $CD3^+$ cells, respectively). Next, spleen tissue of 3 kidney donors was investigated. The expression of CD13 on lymphocytes was as negligible as in PB. T cells prepared from non-inflammatory peritoneal fluid were also $CD13^-$. In contrast, T cells from the pericardial fluid of ten patients undergoing heart valve replacement showed varying levels of CD13 expression (27 ± 14% of $CD3^+$ cells).

Detection of CD13 in fixed lymphocytes

Immunophenotyping of blast cells from acute myeloid leukaemia has shown that the CD13 antigen may be detected in the cytoplasm of myeloblasts before its membrane expression (20). For this reason, we

Table 2. Enhanced binding of Leu-M7 (PE) after permeabilization of SF T cells by paraformaldehyde and Tween 20. Results are given as a percentage of staining of $CD3^+$ cells

		CD13 staining	
	(n)	native cells	permeabilized cells
1. Healthy persons, PB lymphocytes	(8)	0–2	4–14
2. JCA patients, PB lymphocytes	(5)	0–3	8–22
3. JCA patients, SF lymphocytes	(5)	42–84	56–92

studied CD13 expression of fixed $CD3^+$ cells (i) from the PB of healthy controls, (ii) from the blood of patients suffering from JCA or RA and (iii) from the SF of those patients (Table 2). Interestingly, as many as 4–14 % of PB T cells from healthy persons were stained positively for CD13. 8–22 % of fixed PB lymphocytes of JCA patients stained $CD13^+$, a result that needs further investigation. Permeabilized SF cells also exhibited a greater amount of CD13-expression than native SF cells.

Incubation of PB MNC with SF

In order to examine the effect of autologous SF on the immunophenotype of PB MNC, we incubated MNC of the PB of 5 patients with JCA at 37 °C between 30 min and 18 h in the presence of an equal amount of cell-free SF. We observed negligible staining for CD13 after an incubation of lymphocytes ranging from 30 min to 4 h (2–5 % of $CD3^+$ cells were $CD13^+$). Only an 18-h incubation resulted in a distinct expression of CD13 on PB T cells (6–14 % of $CD3^+$ cells became $CD13^+$). This can be taken as an argument against CD13 expression only occurring as a result of an alteration of the surface of lymphocytes by synovial enzymes.

Stimulation of PB MNC of healthy persons with mitogen or SF

Due to the fact that several studies describing an increase of aminopeptidase activities after mitogen stimulation of lymphocytes already exist (13, 14), we investigated CD13 expression on $CD3^+$ cells after stimulation of PB MNC in control subjects with Con A/IL-2. In addition, the cells were cultivated with SF from a patient with RA. Figure 4 illustrates the course of expression of CD13, CD25 and HLA-DR in one representive experiment. Incubation of cells in RPMI + 10 % FCS caused a slight increase of both $CD13^+$ and $HLA\text{-}DR^+$ T cells. This fact could be interpreted as being a result of the developing autologous mixed leukocyte

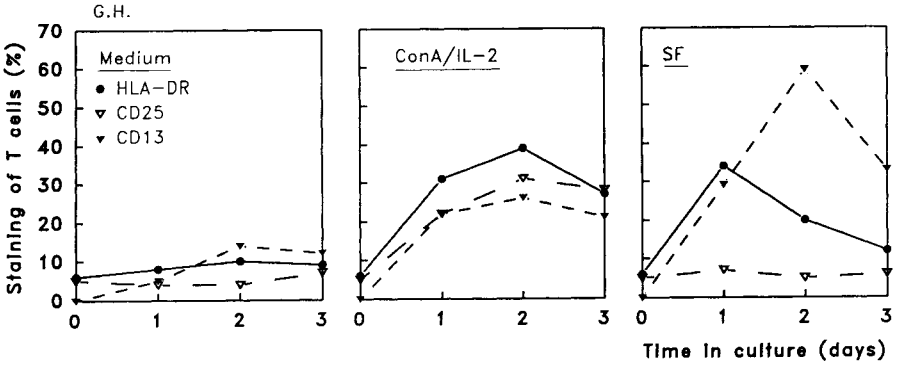

Figure 4. Expression of HLA-DR, CD25 and CD13 on PB T cells after cultivation in RPMI (10 % FCS), in RPMI with Con A/IL-2 or with SF (1:10) from a patient with RA after 1, 2 and 3 days. One representative experiment out of five done is demonstrated.

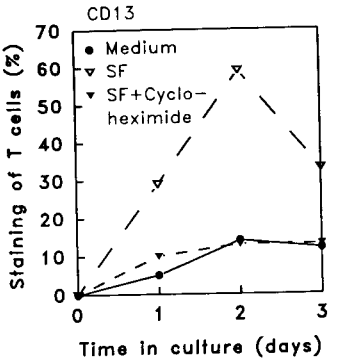

Figure 5. Expression of CD13 on PB T cells (control group) after a 1- to 3-days cultivation in RPMI with 10 % FCS and SF with – or without – cycloheximide (1.5 μg/ml).

reaction. Furthermore, we observed a tight co-expression of all 3 surface molecules studied after stimulation with Con A/IL-2, though there were differences over the course of time and between individuals. On the other hand, incubation of MNC with SF from a patient with RA resulted in a clear increase of CD13 and of HLA-DR, but in a slight decrease of CD25. After addition of cycloheximide to SF, only approximately 10 % of $CD3^+$ cells stained positively for CD13 (Fig. 5). This could be the result of the distribution of internal «storage places», because staining for CD13 of fixed PB lymphocytes also revealed approximately 10 % $CD13^+$ T cells (Table 2). Possible conclusions are as follows: (1) CD13 expression on PB lymphocytes is inducible by mitogens – though not to the extent reached in SF; (2) CD25 and CD13 are regulated independently of each other; (3) the cell-free SF contains CD13- and HLA-DR- but not CD25-augmenting mediators and (4) expression of CD13 on more than approximately 10 % of $CD3^+$ cells needs pathways that may be inhibited by cycloheximide. The effects of SF on PB lymphocytes have not been uniformly discussed in literature. CROUT (21) reported that the addition of autologous cell-free SF resulted in an increased incorporation of tritiated thymidine by PB lymphocytes. On the other hand, HAIN (22) demonstrated that SF by itself had no significant stimulatory effect on PB lymphocytes. Further experiments are necessary to obtain a clearer picture of the influence of cell-free SF on lymphocytes.

Discussion

To the best of our knowledge, in this paper we are discussing the occurrence of CD13 on human SF T lymphocytes using different monoclonal antibodies specific to this antigen for the first time. The same antibodies could not detect CD13 on the surface of PB lymphocytes of healthy persons and patients with RA and JCA, but permeabilization of

T cells revealed the presence of intracellular CD13. Our observations confirm results of enzymatic evidence of AP N in lymphocytes (9, 12, 13). It seems unlikely that T cells acquire the surface aminopeptidase as a consequence of the migration process through a tissue compartment, since lymphocytes prepared from spleen tissue show no detectable expression of CD13 by use of different monoclonal antibodies. In addition, also T cells of the peritoneal fluid of patients who had ethanolic liver cirrhosis displayed no detectable staining for CD13. We lean towards the assumption that T cells could acquire this phenotype within the synovium.

It is known that CD13 antibodies can be subgrouped according to their ability to bind at least 3 different epitopes of the AP N glycoprotein (23). Furthermore, it has been suggested that at least five subpopulations of AP N exist, owing to differential utilization of glycosylation sites or to subtle differences in oligosaccharide composition (24). This raises the question as to whether an unmasking or alteration of certain CD13 epitopes by synovial enzymes is the reason for the binding of monoclonal antibodies to SF lymphocytes. This seems not to be the case, because an incubation of PB lymphocytes of patients with RA in autologous SF lasting 4 h at 37 °C induces no subsequent labelling of lymphocytes with CD13 specific antibodies. Furthermore, in the presence of Con A/IL-2, a 3-day incubation of PB MNC from control persons not only induced an increase on T cells of the activation markers HLA-DR and CD25, but also of CD13 (Fig. 4). This data strongly supports the hypothesis that CD13 is a new activation-associated antigen of lymphocytes.

Results from literature concerning the function of CD13 on other blood cells are not uniform. GRIFFIN (25) investigated myeloid precursor cells with the CD13 specific monoclonal antibody anti-MY7. He suggested that MY7-antigen, though not recognized by the antibody, could be expressed at low antigen-density throughout myeloid differentiation. Detectable MY7-expression identified an actively proliferating fraction of myeloid colony forming cells (25). While investigating CD13 on monocytes and rheumatoid synovial tissue macrophages using the monoclonal antibody 3D8, KOCH (26) observed an augmentation of CD13 when monocytes were exposed to interferon-gamma and lipopolysaccharide. The CD13 antigen increased parallel with the MHC class II antigen expression and also when monocytes in culture were maturing to macrophages. The author suggested that as monocytes enter the synovial tissue from the blood, they rapidly migrate to the synovial lining layer, where they bear both MHC class II and some activation antigens, such as CD13 (26). WERFEL (27) observed variable enhancement of CD13 expression on monocytes and granulocytes within minutes after these cells had been stimulated with C5a. Though these examples are not simply transferable to the situation in lymphocytes, they show the complexity of involvement of CD13 in the activation processes of blood cells. The rare occurrence of CD13 on lymphatic leukaemia cells – interpreted as myeloic co-expression with a worse prognosis (28) – is a further example of the scarcely understood integration of CD13 in prolifer-

ation or differentiation processes. In addition, the observation that CD13 represents a receptor for coronaviruses could be of major pathological importance (29, 30).

As a membrane protease that cleaves N-terminal amino acid residues from small polypeptides, AP N seems suitable for regulation of biologically active peptide hormones. This has been already shown for enkephalins and endorphins, degraded by AP N in synaptic membranes of the central nervous system (31). In this case, the enzyme is accompanied by another zinc-dependent membrane protease, the endopeptidase 24.11 (CALLA/CD10). We were unable to detect this differentiation antigen of lymphoid precursor cells on synovial lymphocytes (< 3 %, data not shown). However, the majority of T cells expressed CD13 together with CD26, a membrane protease expressed on activated T cells irrespective of cell cycle (32). This result makes it tempting to speculate about a functional cooperation of both the peptidases.

In summary, we have described the occurrence of CD13 as a new activation-associated molecule on synovial T cells of patients with different forms of joint effusions. The estimation of $CD13^+$ T cells is not useful in establishing a diagnosis in patients with different forms of arthritis, as we had expected it to be. However, experiments are in preparation to investigate a possible association between inflammatory activity and CD13 expression on T cells, not only from SF, but also from other inflammatory effusion fluid.

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