The N-terminal X-X-Pro sequence of the HIV-1 Tat protein is important for the inhibition of dipeptidyl peptidase IV (DP IV/CD26) and the suppression of mitogen-induced proliferation of human T cells

Sabine Wrenger[a,*], Dirk Reinhold[a], Torsten Hoffmann[b], Margot Kraft[c], Rainer Frank[c], Jürgen Faust[d], Klaus Neubert[d], Siegfried Ansorge[a]

[a] *Center for Internal Medicine, Institute of Experimental Internal Medicine, Otto-von-Guericke University Magdeburg, Leipziger Str. 44, 39120 Magdeburg, Germany*
[b] *Hans-Knöll-Institute Jena, BMFT-Group Halle, Wolfgang-Langenbeck-Str. 3, 06120 Halle (Saale), Germany*
[c] *Center for Molecular Biology, University of Heidelberg, Im Neuenheimer Feld 282, 69120 Heidelberg, Germany*
[d] *Department of Biochemistry and Biotechnology, Martin-Luther-University Halle-Wittenberg, Kurt-Motkes-Str. 3, 06120 Halle (Saale), Germany*

Received 9 February 1996

Abstract Recent data in the literature suggest that the HIV-1 Tat(1–86) protein exhibits immunosuppressive effects. Moreover, Tat was found to interact with dipeptidyl peptidase IV (DP IV), which is identical to the T cell activation marker CD26. Here we show that the N-terminal amino acid sequence of Tat is essential for the inhibition of DP IV-catalyzed IL-2(1–12) degradation. N-terminal modification of Tat with rhodamine prevented inhibition of enzymatic activity of DP IV as well as suppression of DNA synthesis of mitogen-stimulated human T cells. Moreover, natural peptides containing the X-X-Pro N-terminal motif of Tat also inhibited DP IV activity. These data suggest the existence of endogenous immunomodulatory oligopeptides which influence immune cell proliferation and differentiation via DP IV as does HIV-1 Tat.

Key words: HIV-1; Tat immunosuppressive activity; Dipeptidyl peptidase IV; CD26; Pokeweed mitogen-stimulated human T cell

1. Introduction

The 86 amino acid long Tat protein of human immunodeficiency virus type-1 (HIV-1) is a transactivator regulating the transcription of HIV genes and is essential for viral replication in vitro [1,2]. Evidence exists that Tat also occurs extracellularly. HIV-1 infected T cells release Tat into the extracellular medium [3,4]. Extracellular Tat has been shown to suppress antigen as well as anti-CD3-induced activation of human T cells [5,6].

CD26 (dipeptidyl peptidase IV, DP IV, EC 3.4.14.5) is an activation marker of T and B lymphocytes and of NK cells with peptidase activity [7–11]. It removes N-terminal dipeptides from oligopeptides with unsubstituted N-termini, if the penultimate amino acid is proline or alanine. However, peptides with an additional proline in the third position are resistant to cleavage. Data from several groups have shown a key role of DP IV in the regulation of differentiation and growth of lymphocytes. Specific inhibitors of DP IV suppressed mitogen- and alloantigen-induced T cell proliferation, cytokine production, B cell differentiation, and immunoglobulin secretion [12–17]. However, the function of DP IV in the proliferation and differentiation processes has not been resolved yet. Of special interest was the recent finding that HIV-1 Tat binds with high affinity to CD26 and inhibits the cleavage of the synthetic DP IV substrate Gly-Pro-pNA [18,19]. This suggests that the immunosuppressive effects of Tat could be mediated by DP IV.

In the current paper we demonstrate that Tat(1–86) inhibits DP IV-catalyzed cleavage of IL-2(1–12) and suppresses DNA synthesis of PWM-stimulated T cells. Our data suggest that the N-terminal sequence of Tat is essential for these effects. Since other peptides containing the N-terminal X-X-Pro sequence of Tat also inhibit DP IV we assume the existence of endogenous DP IV inhibitors regulating DP IV activity and cell proliferation.

2. Materials and methods

2.1. DP IV and synthetic DP IV inhibitor

DP IV (porcine kidney) was kindly provided by Dr. H.U. Demuth (Hans-Knöll-Institute Jena, BMFT-Group Halle, Germany). For the synthesis of the DP IV inhibitor Lys[Z(NO_2)]-thiazolidide Boc-Lys[Z(NO_2)]-OH was coupled with thiazolidine [14].

2.2. Peptides

Human IL-2(1–12), Met-IL-2(1–6), Met-IL-2(1–12), Met-IL-2(1–24), IL-1β(1–6), murine IL-6(1–12), Phe-Ala-Pro-Ala-Gly-Ala-Phe, and Val-Lys-Pro-Phe-Tyr were synthesized by solid-phase peptide synthesis with Fmoc technique using the peptide synthesizer 431A (Applied Biosystems). The HIV-1 Tat(1–86), Tat(1–9) were synthesized on polyoxyethylene-polystyrene graft resin in a continuous flow instrument constructed and operated as described by Frank and Gausepohl [20]. For rhodamine labeling the resin-bound N-terminal free Tat(1–72) and Tat(1–9) were incubated with a 3-fold excess of 5-(and-6)-carboxytetramethylrhodamine succinimidyl ester (MoBiTec, Göttingen, Germany) in the presence of PyBop (benzotriazole-1-yloxytripyrrolidinophosphonium hexafluorophosphate) and *N*-methylmorpholine. The synthetic peptides were purified by reversed-phase HPLC and characterized by mass spectrometry.

Xenopsin-related peptide II, [Lys^0,Ala^3]-bradykinin, neuromedin N, ACTH(34–39), and Ala-Arg-Pro-Ala-Lys were purchased from Saxon Biochemicals GmbH (Hannover, Germany). Met-Lys-bradykinin, human peptide YY(3–36), porcine gastrin-releasing peptide(14–27), Ala-Ala-Pro-Ala, Gly-Gly-Pro-Ala, and tuftsin were obtained from Bachem (Heidelberg, Germany).

2.3. DP IV-catalyzed hydrolysis of oligopeptides

The effects of non-substrate peptides on the enzymatic activity of DP IV were tested using human IL-2(1–12), IL-1β(1–6), and murine

*Corresponding author. Fax: (49) (391) 6713291.
E mail: sabine.wrenger@medizin.uni-magdeburg.de

Abbreviations: Boc, *t*-butyloxycarbonyl; Fmoc, 9-fluorenylmethyloxycarbonyl; PWM, pokeweed mitogen; ACTH, adrenocorticotropic hormone; GRP, gastrin-releasing peptide.

SDI S0014-5793(96)00221-9

IL-6(1–12) as substrates. After preincubation (30 min, 37°C) of 550 fkat DP IV in 1 µl 10 mM sodium phosphate assay buffer, pH 7.4, with 2 µl of the putative inhibitory peptide in assay buffer the enzymatic reaction was started by addition of 2 µl of 1 mM substrate in assay buffer. Samples were incubated for 30 min at 37°C. Thereafter, the reaction was stopped by addition of 2 µl 30 mM phosphoric acid.

Degradation of DP IV peptide substrates was measured by the method of free zone capillary electrophoresis using Biofocus 3000 system of Biorad (München, Germany). Separations were performed as described by Reinhold et al. [19].

2.4. Preparation of T cells and proliferation assay

T cells were separated from heparinized blood of healthy donors as described in [21]. Monocytes and B cells were depleted by passage through nylon wool columns (Polysciences, Warrington, USA). Purified T-lymphocytes (95% T cells, 10^5 cells/100 µl per well) were suspended in serum-free CG-medium (Vitromex, Vilshofen, Germany) and stimulated with pokeweed mitogen (PWM, 2 µg/ml; Serva, Heidelberg, Germany) in the presence of Tat(1–86), rhodamine-Tat(1–72) or Lys[Z(NO_2)]-thiazolidide. After 90 h the cultures were pulsed for an additional 6 h with [^{3}H]methylthymidine (0.2 µCi per well; Amersham, Braunschweig, Germany). The cells were harvested onto glass fiber filters, and the incorporated radioactivity was measured by scintillation counting.

3. Results

3.1. Inhibition of DP IV-catalyzed IL-2(1–12) degradation by HIV-1 Tat(1–86)

In the classical DP IV assay the cleavage of small synthetic chromogenic substrates (e.g. Gly-Pro-pNA) was spectrophotometrically measured. Gutheil et al. observed that HIV-1 Tat(1–86) inhibits DP IV-catalyzed cleavage of Ala-Pro-pNA [18]. Based on the method of capillary electrophoresis we established an alternative DP IV assay, which allows the use of oligopeptides analogues to N-terminal X-Pro sequences of potential physiologic substrates [22]. In Fig. 1(A,B) the DP IV-catalyzed degradation of IL-2(1–12) is shown. The addition of 40 µM HIV-1 Tat(1–86), a 10-fold lower concentration compared to the substrate, markedly inhibited IL-2(1–12) cleavage (Fig. 1C,D). In seven independent experiments, Tat inhibited 61 ± 12% of IL-2(1–12) degradation in sodium phosphate buffer without NaCl. Similar results were obtained with the DP IV substrates IL-1β(1–6) and murine IL-6(1–12) (data not shown). The same Tat concentration also inhibited DP IV-catalyzed degradation of IL-2(1–12) in the presence of 40 and 140 mM NaCl (41 ± 8 and 21 ± 10% inhibition, respectively).

3.2. Importance of the N-terminus of Tat for the inhibition of DP IV

Interestingly, N-terminal modified rhodamine-Tat(1–72) had no effect on IL-2(1–12) cleavage by DP IV (Fig. 2). The short N-terminal part, Tat(1–9), in a 10-fold higher concentration than Tat(1–86), inhibited DP IV, whereas rhodamine-Tat(1–9) again had no effect.

3.3. Effects of Tat(1–86) and N-terminal modified rhodamine-Tat(1–72) on proliferation of PWM-stimulated T cells

To investigate whether the N-terminus is also crucial for the immunosuppressive action of the Tat protein, we tested the effects of the natural Tat(1–86) and the N-terminal modified rhodamine-Tat(1–72) on the proliferation of purified human T cells after stimulation with PWM. We observed a decrease in the DNA synthesis down to 58 ± 5% of the control with 1 µM Tat, the highest concentration used (Fig. 3). Lys[Z(NO_2)]-thiazolidide, a highly specific inhibitor of DP IV (IC_{50} = 2.7 ± 0.3 µM [14]), exerts similar effects in the same concentration range. As expected, the N-terminal modified rhodamine-Tat(1–72) had no effect on the DNA synthesis of PWM-stimulated T cells. Similar effects were observed with PWM-stimulated human peripheral blood mononuclear cells (data not shown).

3.4. Inhibition of DP IV catalyzed IL-2(1–12) cleavage by oligopeptides containing N-terminal X-X-Pro motif

During the search for putative endogenous DP IV inhibitors we focused on peptides with similarities to Tat(1–9). In-

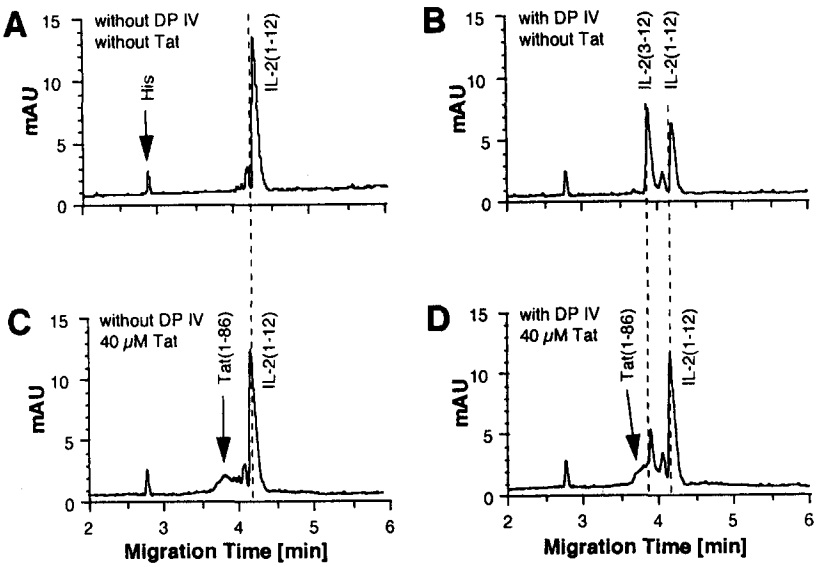

Fig. 1. Influence of HIV-1 Tat(1–86) on DP IV-catalyzed IL-2(1–12) degradation. Samples were applied to free zone capillary electrophoresis. Absorbance was detected at 200 nm. In (A,B) the electropherograms show IL-2(1–12) and its cleavage products after 30 min incubation of 400 µM IL-2(1–12) in the absence and presence of DP IV. 200 µm His was added as internal standard. In (C,D) incubations were performed in the presence of 40 µM Tat(1–86).

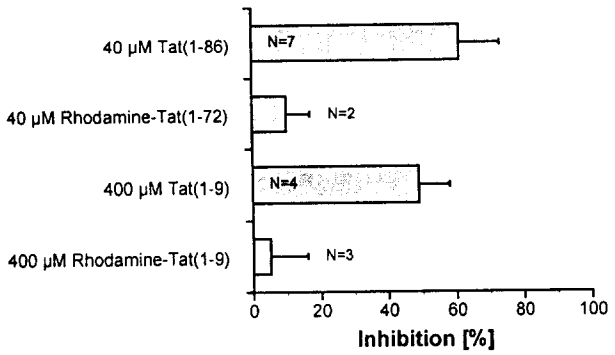

Fig. 2. Effects of Tat(1–9) and of the N-terminal modified Tat peptides rhodamine-Tat(1–72) and rhodamine-Tat(1–9) on the inhibition of DP IV catalyzed IL-2(1–12) degradation. The enzymatic activity was measured as described in section 2. The inhibition is equal to the difference of the residual activity (in per cent of the control) from 100. *N* indicates the number of independent experiments, each being carried out in triplicate. Data are expressed as mean inhibition ± S.D.

deed, other peptides with the N-terminal X-X-Pro-motif of Tat are also capable of inhibiting DP IV catalyzed IL-2(1–12) degradation (Fig. 4). The non X-X-Pro-peptide Met-Lys-bradykinin had no effect on IL-2(1–12) degradation. All inhibitory X-X-Pro-peptides found are greater than 6 amino acids in length. The tetra-, penta- and hexa-X-X-Pro-peptides used did not inhibit DP IV. X-X-Pro-peptides with 7 up to 34 amino acids applied in equimolar concentration to the substrate (400 μM) exerted 30–70% inhibition of IL-2(1–12) degradation. Tat consisting of 86 amino acids was the most potent, since a 10-fold lower concentration inhibited 61 ± 12% of IL-2(1–12) degradation.

4 Discussion

Even early in infection, the lymphocytes of HIV-1 positive individuals exhibit a defect in their response to soluble antigens [23]. Recently, it was shown that the Tat protein of HIV-1 formerly known as a transcription activator, also exerts immunosuppressive effects. In cell culture, it suppressed antigen-induced proliferation of human T lymphocytes [5]. Interestingly, Gutheil et al. have shown that Tat binds to CD26, an activation marker of T lymphocytes identical with dipeptidyl peptidase IV (DP IV) [18]. With the small synthetic Gly-Pro-pNA substrate for DP IV they observed that Tat is a strong inhibitor of DP IV, at least at low salt concentrations. Curiously, the inhibition of the Gly-Pro-pNA degradation by Tat is salt-dependent. With the data at 0 and 40 mM NaCl, Gutheil et al. proposed that Tat does not inhibit the activity of DP IV at physiological salt concentrations.

We established a DP IV assay, which exploits the sensitivity of capillary electrophoresis and allows the use of N-terminal oligopeptides of putative physiological DP IV substrates, e.g. IL-2(1–12). At low salt concentrations, which are preferred for capillary electrophoresis, 40 μM Tat inhibits 61 ± 12% of IL-2(1–12) degradation. In accordance with Gutheil et al. we observed a decreased inhibitory effect of Tat at 40 mM NaCl [18]. However, at 140 mM NaCl, we still observed inhibition of 21 ± 10% of IL-2(1–12) degradation with the same Tat concentration. This suggests that Tat binds to DP IV at physiological salt conditions and that it is a natural inhibitor of DP IV.

CD26 expression on T and B lymphocytes and on NK cells was shown to be dependent on their activation status. Interestingly, specific inhibitors of DP IV suppressed mitogen-stimulated T cell proliferation and modulated cytokine production [21]. In cultures of the histiocytic U937-H cell line, which express high levels of DP IV, DP IV inhibitors also decreased DNA synthesis and modulated cytokine production, whereas they had no effect on U937-L cells, which express low levels of DP IV [24]. Recently, we demonstrated that Tat also suppressed DNA synthesis of U937-H cells and influenced cytokine production in the same manner as that of synthetic DP IV inhibitors [19]. These data provide evidence that DP IV plays a crucial role in the regulation of activation and proliferation, not only of T lymphocytes, but also of other cells and that DP IV is involved in the mediation of Tat's immunosuppressive activity.

Several investigators established anti-proliferative effects of Tat on antigen-induced lymphocyte proliferation [5,6], but also reported that Tat had no effect on phytohemagglutinin-induced T cell proliferation. In contrast, synthetic DP IV inhibitors suppressed antigen-induced as well as mitogen-induced T cell proliferation. To test our hypothesis that Tat acts via DP IV inhibition, we examined effects of Tat on PWM-stimulated human peripheral blood mononuclear cells (PBMC) and on human T lymphocytes. In both cell systems we observed decreased DNA synthesis in the presence of Tat. Tat was as potent as the synthetic DP IV inhibitor Lys-[$Z(NO_2)$]-thiazolidide (IC_{50} = 2.7 ± 0.3 μM [14]). These results confirm that Tat's immunosuppressive effects on T lymphocytes could be mediated by an interaction with DP IV. The existence of a viral immunomodulatory oligopeptide implies the existence of an endogenous counterpart. In further studies, we looked for the part of the Tat protein which mediates the inhibitory action on DP IV. N-terminal modified rhodamine-Tat(1–72) has lost the ability to inhibit DP IV-catalyzed IL-2(1–12)degradation as well as to suppress mitogen-induced

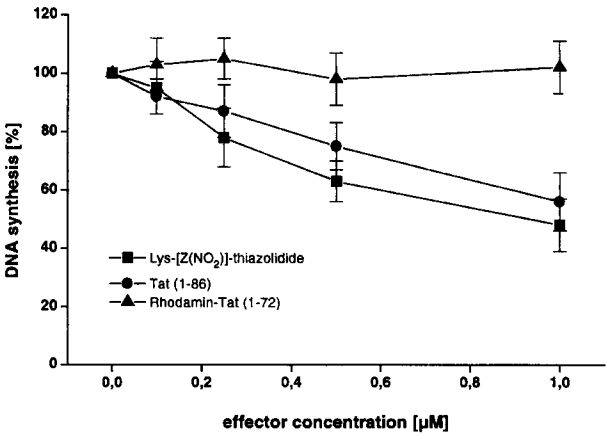

Fig. 3. Influence of Tat(1–86) (●), rhodamine-Tat(1–72) (▲), and Lys[$Z(NO_2)$]-thiazolidide (■) on DNA synthesis of PWM stimulated purified T cells. Purified T cells (95%, 10^6 cells/ml) were incubated with PWM (2 μg/ml) in presence of Tat(1–86), rhodamine-Tat (1–72), or Lys[$Z(NO_2)$]-thiazolidide in the indicated concentrations. After 90 h, the cultures were pulsed with [^{3}H]methylthymidine ([^{3}H]TdR) for an additional 6 h. [^{3}H]TdR incorporations in cpm are indicated as mean ± S.D. from 4 different experiments. The values are expressed as % [^{3}H]TdR incorporated related to control cultures without effectors ([^{3}H]TdR incoporation in control cultures: 65 600 ± 8400 cpm).

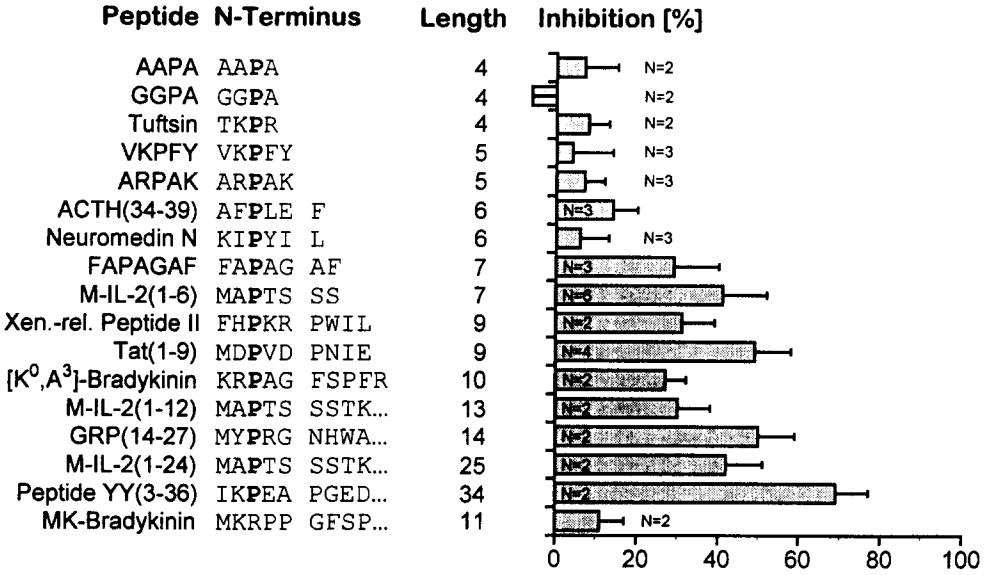

Fig. 4. Influence of X-X-Pro-peptides on DP IV catalysed IL-2(1–12) degradation. 400 μM IL-2(1–12) was incubated with DP IV for 30 min in the presence of 400 μM X-X-Pro-peptide. 400 μM MK-bradykinin was used as control. The N-terminal amino acid sequences are written in single-letter code. The error bars indicate the standard deviation of *N* different experiments, each performed in triplicate. ACTH, adrenocorticotropic hormone; Xen.-rel. peptide II, xenopsin-related peptide II; GRP, gastrin-releasing peptide.

PBMC or T cell proliferation. Since the short N-terminal part Tat(1–9) also inhibited DP IV, whereas N-terminal modified rhodamine-Tat(1–9) had no effect, the N-terminal structure of the Tat protein seemed to be important for the inhibition of DP IV. Interestingly, X-X-Pro-peptides other than Tat(1–9) also inhibited DP IV. The extent of inhibition depends on the length of these peptides: All inhibitory X-X-Pro-peptides found are longer than 6 amino acids and Tat consisting of 86 amino acids was the most potent. Two of the inhibitory X-X-Pro-peptides are peptide YY(3–36), which results from the claevage of peptide YY by DP IV, and gastrin-releasing peptide (14–27). The consequences of our findings for the gastrointestinal system remain to be investigated.

Interestingly, some cytokines and cytokine precursors contain many prolines near the N-terminus [25]. Erythropoietin, IL-6 and TGF-β1-prepropeptide are X-X-Pro-peptides and G-CSF, IL-1α-precursor, IL-1β-precursor, LIF, lymphotoxin-precursor and IL-13 are putative substrates for DP IV that release X-X-Pro-peptides after cleavage of the N-terminal X-Pro- or X-Ala-dipeptide. Our results raise the possibility that DP IV-mediated effects on the proliferation and differentiation of activated T-lymphocytes and of other cells expressing DP IV, e. g. U937-H cells, could be regulated by endogenous peptides. Furthermore, we obtained evidence that the immunosuppressive effects of the HIV-1 Tat protein were mediated by CD26/DP IV. Thus, CD26-Tat interactions may play a crucial role in the development and progression of HIV mediated AIDS disease.

Acknowledgements: This work was supported by the Deutsche Forschungsgesellschaft, SFB 387-A4.

References

[1] Sodroski, J.G., Rosen, C.R., Wong-Staal, F., Popovic, M., Arya, S., Gallo, R.C. and Haseltine, W.A. (1985) Science 227, 171–173.
[2] Dayton, A.I., Sodroski, J.G., Rosen, C.A., Goh, W.C. and Haseltine, W.A. (1986) Cell 44, 941–947.
[3] Ensoli, B., Barillari, G., Salahuddin, S.Z., Gallo, R.C. and Wong-Staal, F. (1990) Nature 345, 84–86.
[4] Ensoli, B., Buonaguro, L., Barillari, G., Fiorelli, V., Gendelman, R., Morgan, R.A., Wingfield, P. and Gallo, R.C. (1993) J. Virol. 67, 277–287.
[5] Viscidi, R.P., Mayur, K., Lederman, H.M. and Frankel, A.D. (1989) Science 246, 1606–1608.
[6] Subramanyam, M., Gutheil, W.G., Bachovchin, W.W. and Huber, B.T. (1993) J. Immunol. 150, 2544–2553.
[7] Ansorge, S., Schön, E. and Kunz, D. (1991) Biomed. Biochim. Acta 50, 799–807.
[8] Tanaka, T., Camerini, D., Seed, B., Torimoto, Y., Dang, N.H., Kameoka, J., Dahlberg, H.N., Schlossman, S.F. and Morimoto, C. (1992) J. Immunol. 149, 481–486.
[9] Fleischer, B. (1994) Immunol. Today 15, 180–184.
[10] Bühling, F., Junker, U., Reinhold, D., Neubert, K., Jäger, L. and Ansorge, S. (1995) Immunol. Lett. 45, 47.
[11] Bühling, F., Kunz, D., Reinhold, D., Ulmer, A.J., Ernst, M., Flad, H.D. and Ansorge, S. (1994) Nat. Immun. 13, 270.
[12] Schön, E., Jahn, S., Kiessig, S.T., Demuth, H.U., Neubert, K., Barth, A., Von Baehr, R. and Ansorge, S. (1987) Eur. J. Immunol. 17, 1821–1826.
[13] Schön, E., Demuth, H.U., Eichmann, E., Horst, H.J., Körner, I.J., Kopp, J., Mattern, T., Neubert, K., Noll, F., Ulmer, A.J., Barth, A. and Ansorge, S. (1989) Scand. J. Immunol. 29, 127–132.
[14] Schön, E., Born, I., Demuth, H.U., Faust, J., Neubert, K., Steinmetzer, T., Barth, A. and Ansorge, S. (1991) Biol. Chem. Hoppe-Seyler 372, 305–311.
[15] Dang, N.H., Torimoto, Y., Shimamura, K., Tanaka, T., Daley, J.F., Schlossman, S.F. and Morimoto, C. (1991) J. Immunol. 147, 2825–2832.
[16] Flentke, G.R., Munoz, E., Huber, B.T., Plaut, A.G., Kettner, C.A. and Bachovchin, W.W. (1991) Proc. Natl. Acad. Sci. USA 88, 1556–1559.
[17] Torimoto, Y., Dang, N.H., Vivier, E., Tanaka, T., Schlossman, S.F. and Morimoto, C. (1991) J. Immunol. 147, 2514–2517.
[18] Gutheil, W.G., Subramanyam, M., Flentke, G.R., Sanford, D.G., Munoz, E., Huber, B.T. and Bachovchin, W.W. (1994) Proc. Natl. Acad. Sci. USA 91, 6594–6598.
[19] Reinhold, D., Wrenger, S., Bank, U., Bühling, F., Hoffmann, T., Neubert, K., Kraft, M., Frank, R. and Ansorge, S. (1995) Immunobiology (in press).
[20] Frank, R. and Gausepohl, H. (1988) p. 42 (Tschesche, H. Ed.) De Gruyter, Berlin.

[21] Reinhold, D., Bank, U., Bühling, F., Neubert, K., Mattern, T., Ulmer, A.J., Flad, H.D. and Ansorge, S. (1993) Immunobiology 188, 403–414.
[22] Hoffmann, T., Reinhold, D., Kähne, T., Faust, J., Neubert, K., Frank, R. and Ansorge, S. (1995) J. Chromatogr. A 716, 355–362.
[23] Lane, C., Depper, J.M., Greene, W.C., Whalen, G., Waldmann, T.A. and Fauci, A.S. (1985) N. Engl. J. Med. 131, 79–84.
[24] Reinhold, D., Bank, U., Bühling, F., Kähne, T., Kunt, D., Faust, J., Neubert, K. and Ansorge, S. (1994) Immunobiology 192, 121–136.
[25] Vanhoof, G., Goossens, F., De Meester, I., Hendriks, D. and Scharpé, S. (1995) FASEB J. 9, 736–744.