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Immunology

Induction of intracellular cAMP by a synthetic retroviral envelope peptide: A possible mechanism of immunopathogenesis in retroviral infections

(immunodeficiency/CKS-17/cAMP)

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ABSTRACT **A synthetic heptadecapeptide, CKS-17, represents the highly conserved amino acid sequences occurring within the transmembrane envelope protein of many animal and human retroviruses. CKS-17 has been demonstrated to exhibit suppressive properties for numerous immune functions. We have recently shown that CKS-17 acts as an immunomodulatory epitope causing an imbalance of human type 1 and type 2 cytokine production and suppression of cell-mediated immunities. cAMP, an intracellular second messenger, plays an important role in regulation of cytokine biosynthesis—i.e., elevation of intracellular cAMP levels selectively inhibits type 1 cytokine production but has no effect or enhances type 2 cytokine production. Here, we demonstrate that CKS-17 induces dramatic rises in the intracellular cAMP levels of a human monocyte cell line and of human peripheral blood mononuclear cells in a time- and dose-dependent manner. A peptide corresponding to the reverse sequence of CKS-17, used as control, has no effect on intracellular cAMP levels. The cAMP-inducing ability of CKS-17 is significantly blocked by SQ-22536, an inhibitor of adenylate cyclase. These results indicate that CKS-17, a highly conserved component of the transmembrane proteins of immunosuppressive retroviruses, induces increased intracellular levels of cAMP via activation of adenylate cyclase and suggest that this retroviral envelope peptide may differentially modulate type 1 and type 2 cytokine production through elevation of intracellular cAMP levels.**

Retroviral infection causes severe immunosuppression, leading to fatal disorders in a variety of species (1–3). However, the exact cause of the generalized immunodysfunction associated with retroviral infection remains enigmatic. Viral components have been shown to be associated with and produce dramatic immunosuppressive effects, strongly supporting the view that viral components are relevant to the pathology and progression of retroviral infectious diseases (2–7). A transmembrane envelope protein, p15E, of feline and murine leukemia viruses is one of the retroviral structural components that exert immunosuppressive activities even when human cells are used as target (2, 3).

We have investigated the mechanism(s) of retrovirus-induced immunodeficiency using a p15E-related synthetic peptide, CKS-17 (3, 7–14). CKS-17 is a heptadecapeptide homologous to the highly conserved amino acid sequences within the transmembrane envelope protein of several animal and human retroviruses (15–17). Recently, we demonstrated that this retroviral envelope peptide, CKS-17, differentially modulates human type 1 and type 2 cytokine expression and may act as an immunomodulatory epitope responsible for cytokine dysregulation that leads to suppression of cellular immunity (14). Our data show that CKS-17 suppresses type 1 cytokines, interleukin (IL) 2, IL-12, and interferon γ, while consistently enhancing a type 2 cytokine, IL-10. CKS-17 does not suppress type 2 cytokines such as IL-4, IL-5, IL-6, IL-10, and IL-13. Our studies further show that CKS-17 acts directly on monocytes and down-regulates transcriptional activation of the tumor necrosis factor (TNF-α) gene (13). The reduced production of IL-12 and TNF-α and enhanced IL-10 production from monocytes/macrophages may cause an imbalance in type 1 and type 2 cytokine production and influence cell-mediated immunity in a negative manner.

Well-established evidence indicates that cAMP, an important intracellular second messenger, exerts modulating effects on various immune functions (18). Recent findings using human and mouse T cells/T-cell clones, monocytes/macrophages, and peripheral blood mononuclear cells (PBMC) have shown that an increase in intracellular cAMP levels results in inhibition of IL-2, IL-12, and interferon γ production but not of IL-4, IL-5, IL-6, and IL-10 production. An increase in cAMP may be associated with an up-regulation of the latter group of cytokines (19–27, 29, 39). These results indicate that signal-transduction pathways for gene expression of type 1 and type 2 cytokines differ in sensitivity to an intracellular increase in cAMP levels. It has also been demonstrated that Th2 cell lines maintain significantly higher levels of cAMP per cell than a Th1 cell line (21) and that an increase in intracellular cAMP levels inhibits TNF-α production by T cells, monocytes, and PBMC (24, 25, 28, 29).

In an effort to understand some of the mechanism(s) by which retroviruses influence immune phenomena, the present studies were designed to analyze the influence of CKS-17 on intracellular cAMP levels of a human monocyte cell line, THP-1, and of human PBMC.

MATERIAL AND METHODS

Preparation of Synthetic Peptide. A dimer of CKS-17, termed MN10021 [(LQNRRGLDLLFLKEGGLC)$_2$] was prepared by inclusion of the naturally occurring cysteine at the carboxyl terminus and dimerization via cysteine-disulfide linkage. The reverse peptide dimer, termed MN20050 [(LGGEKLFLLDLGRRNQLC)$_2$] was prepared similarly and used as a control. For simplicity, MN10021 and MN20050 are referred to as MN21 and MN50, respectively.

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Abbreviations: IL, interleukin; TNF-α, tumor necrosis factor α; MN21, dimer of the synthetic retroviral envelope peptide CKS-17; MN50, dimer of a reverse sequence of CKS-17; PBMC, peripheral blood mononuclear cells.

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Reagents. SQ-22536[9-(tetrahydro-2-furyl)adenine], a cell-permeable inhibitor of adenylate cyclase (30), was obtained from Biomol Research Laboratories (Plymouth Meeting, PA).

Cells. THP-1, a human acute monocytic leukemia cell line (31), was obtained from American Type Culture Collection. Cells were routinely maintained in RPMI 1640 medium/10% fetal bovine serum (HyClone), penicillin at 100 units/ml, streptomycin at 100 μg/ml, 2 mM L-glutamine at a density of 0.5–1 × 10^6 cells per ml at 37°C in a humidified 5% CO_2/95% air atmosphere. PBMC from healthy volunteers (human immunodeficiency virus-negative) were obtained by density-gradient centrifugation on Ficoll-Paque (Pharmacia).

Quantitation of cAMP Levels. THP-1 cells or human PBMC (1.25 × 10^6) were cultured in a 12-well tissue culture plate (Corning) and treated with MN21 or MN50 in 0.5 ml of Hanks' balanced salt solution (GIBCO/BRL) at 37°C in a humidified 5% CO_2/95% air incubator. The reaction was stopped by adding 100% ethanol to a final concentration of 65%. The cells were allowed to settle, and the supernatant was removed to a fresh tube. The precipitate was washed with 100 μl of 70% (vol/vol) ethanol, and the wash was added to a microcentrifuge tube. THP-1 cells or human PBMC treated with SQ-22536 were cultured in a Falcon 2059 polypropylene tube (Becton Dickinson) or a 1.5-ml microcentrifuge tube (United Laboratory Plastics, St. Louis). Cells (1.25 × 10^6) were treated with SQ-22536 in the various concentrations for 15 min at room temperature. The cells were then treated with the appropriate concentrations of MN21 for 15–20 min at 37°C in the presence of SQ-22536. The reaction was terminated, and SQ-22536 was removed from the reaction mixture so as not to crossreact with the cAMP assay. This removal was accomplished by adding 5 ml or 1 ml, respectively, of cold phosphate-buffered saline and spinning at 500 × *g* for 5 min (model J-6M, Beckman) or 5000 rpm for 2 min (Eppendorf centrifuge 5402, Brinkmann) at 4°C. One milliliter of ice-cold 70% (vol/vol) ethanol was added to the pellet, which was mixed gently; when using Falcon 2059 tubes, the mixture was then transferred to a microcentrifuge tube. All samples from both treatments were centrifuged at 2000 × *g* for 15 min at 4°C, and the supernatant was removed to a fresh tube. The extract was evaporated in a SpeedVac concentrator (Savant) at 45°C, and the resulting pellet was stored at −20°C. Just before use the pellet was resuspended in assay buffer, and cAMP levels were measured by using an ^{125}I-labeled cAMP assay system (dual range) (Amersham).

RESULTS AND DISCUSSION

CKS-17 Induces Increased Intracellular Levels of cAMP. Because previous studies showed that THP-1, a human monocyte cell line, can respond to CKS-17 (13), in the present study we investigated the influence of CKS-17 on intracellular levels of cAMP by using THP-1 cells. First, to examine whether the dimer of CKS-17, MN21, can induce increased intracellular levels of cAMP, time-course and dose–response experiments were done. In the time-course experiments THP-1 cells were treated with 30 μM of MN21 or MN50 for 5, 15, 30, 60, or 90 min. Fig. 1 shows that intracellular cAMP levels were remarkably induced by MN21 in a time-dependent manner, and the cAMP level reached a maximum between 15 and 30 min after treatment of THP-1 cells with MN21. The control peptide, MN50, had no effect on the intracellular cAMP levels. Fig. 2 shows the results of MN21 dose–response experiments. THP-1 cells were treated with various concentrations (1.875, 3.75, 7.5, 15, 30, or 60 μM) of MN21 for 15 min. As shown, MN21 increased cAMP levels in a dose-dependent manner. Thirty micromolar MN21 induced a 50-fold increase in cAMP level compared with background cAMP levels in THP-1 cells incubated in medium alone. Additional experiments showed that MN21 also induced the intracellular cAMP levels of human PBMC. Incubation of human PBMC with 7.5, 15, or 30 μM of MN21 for 20 min resulted in a 5-, 8-, or 11-fold increase in cAMP levels, respectively, compared with background cAMP levels in PBMC incubated in medium alone (data not shown).

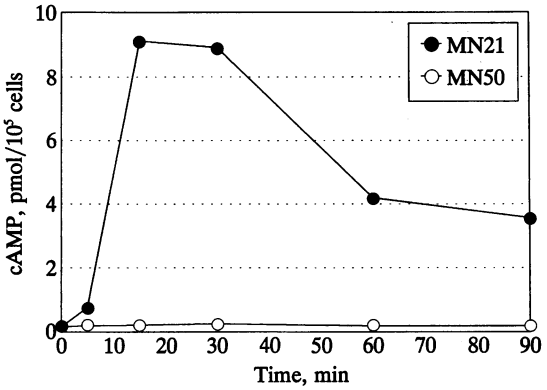

FIG. 1. Time course curve of MN21 intracellular cAMP inducing activity. THP-1 cells were treated with 30 μM MN21 (-●-) or MN50 (-○-) for 5, 15, 30, 60, or 90 min. Cell extracts were monitored for cAMP. Each point represents the mean from seven experiments. The background intracellular level of cAMP in THP-1 cells was 0.163 pmol per 10^5 cells.

CKS-17 Induces Increased Intracellular cAMP via Activation of Adenylate Cyclase. The levels of intracellular cAMP depend on two factors—i.e., activation of adenylate cyclase and/or inhibition of cyclic nucleotide phosphodiesterase. Adenylate cyclase is the membrane-bound catalytic enzyme that converts ATP to cAMP. Cyclic nucleotide phosphodiesterase is the enzyme that breaks down cAMP to 5′-AMP. It is improbable that MN21 significantly elevates intracellular cAMP levels above background merely by inhibiting phosphodiesterase because MN21 induces significant increases in cAMP levels in the absence of any other stimulant. Therefore, experiments were designed to determine whether MN21 induces intracellular cAMP through activation of adenylate cyclase. In these experiments, THP-1 cells were pretreated with 10, 100, 250, 500, or 1000 μM SQ-22536, a weak but specific inhibitor of adenylate cyclase (30), for 15 min and then treated with 30 μM MN21 for another 15 min. As shown in Fig. 3, pretreatment with SQ-22536 significantly blocked the induction of intracellular cAMP by MN21 in a dose-dependent manner. The treatment of THP-1 cells with 1000 μM SQ-22536 in Falcon tubes reduced the cAMP inducing activity of 30 μM MN21 by 70%. Percentage inhibition was calculated

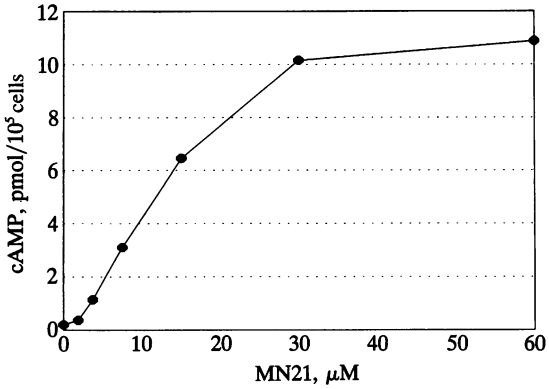

FIG. 2. Dose–response curve of MN21 intracellular cAMP-inducing activity. THP-1 cells were treated with 1.875, 3.75, 7.5, 15, 30, or 60 μM MN21 for 15 min. Cell extracts were monitored for cAMP. Each point represents the mean from four experiments. The intracellular level of cAMP in THP-1 cells incubated without MN21 was 0.204 pmol per 10^5 cells.

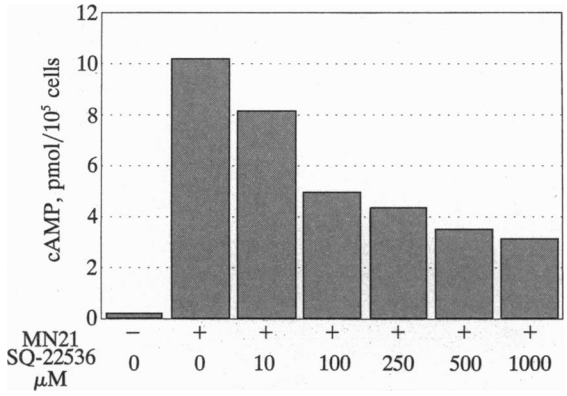

FIG. 3. Inhibition of MN21 cAMP-inducing activity by SQ-22536, an inhibitor of adenylate cyclase. THP-1 cells in Falcon tubes were pretreated with 0, 10, 100, 250, 500, or 1000 μM SQ-22536 for 15 min and then treated with 30 μM MN21 for 15 min. After washing the cells, cell extracts were prepared and monitored for cAMP. Each point represents the mean from three experiments. The intracellular level of cAMP in THP-1 cells incubated without MN21 and SQ-22536 was 0.220 pmol per 10^5 cells.

with the following formula: 100 × {(MN21 − Med) − [(MN21 and SQ-22536) − Med]}/(MN21 − Med), where Med is cAMP levels of THP-1 cells incubated in medium alone. Because of crossreactivity of SQ-22536 with the cAMP assay system and difficulty removing wash, microcentrifuge tubes were preferred. Table 1 shows that 1000 μM SQ-22536 in microcentrifuge tubes reduced the cAMP inducing ability of 15 μM MN21 on THP-1 cells by 99.6%. Percentage inhibition was calculated with the following formula: 100 × {(MN21 − Med) − [(MN21 and SQ-22536) − SQ-22536]}/(MN21 − Med). These results clearly show that MN21 induces increased intracellular levels of cAMP through adenylate cyclase activation.

Discussion and Concluding Remarks. It is well known that membrane-associated heterotrimeric guanine nucleotide-binding proteins (G proteins) control the turnover of cAMP by regulating adenylate cyclase activity (32). Possible interpretation of the present observations is that MN21 activates adenylate cyclase through a receptor that couples it to G proteins. Although p15E has been shown to directly bind to surfaces of human cells (33), a receptor for CKS-17 has not yet been demonstrated. Another mechanism would be that internalized MN21 activates adenylate cyclase directly.

Our prior studies (12–14) have shown that CKS-17 suppresses TNF-α and IL-12 expression while enhancing IL-10 expression and that CKS-17-induced inhibition of IL-12 p40 heavy-chain mRNA expression is independent of enhancement of IL-10 production by CKS-17. Because it has been shown that cAMP-elevating agents inhibit TNF-α (24, 29) and IL-12 (39) production but enhance IL-10 production in monocytes/macrophages (29) or whole blood (39), the present results suggest that CKS-17 mediates its effects on the TNF-α and IL-10 genes by elevating intracellular cAMP levels. The gene expression of IL-12 p40 heavy chain as well as TNF-α may be regulated by a cAMP-sensitive signal-transduction pathway, whereas the signaling pathway(s) for IL-10 gene expression may be positively regulated by cAMP.

Table 1. Inhibition of MN21 cAMP-inducing activity by SQ-22536, an inhibitor of adenylate cyclase

MN21, μM	SQ-22536, μM	cAMP, fmol/10^5 cells
0	0	176
15	0	4708
15	250	1498
15	1000	320
0	1000	301

THP-1 cells in microcentrifuge tubes were pretreated with SQ-22536 for 15 min and then treated with MN21 for 20 min. After washing the cells, cell extracts were prepared and monitored for cAMP.

Understanding the exact molecular mechanism(s) whereby CKS-17 exhibits such differential effects on TNF-α, IL-12 p40 heavy chain, and IL-10 mRNA expression is a further subject of interest. Recently, it has been shown that cAMP-dependent pathways may antagonize calcineurin-regulated cascades for IL-2 gene transcription (34). Calcineurin, a Ca^{2+}/calmodulin-dependent serine/threonine protein phosphatase, has been found to stimulate a nuclear transcription factor, NF-κB, by enhancing inactivation of IκB-α/MAD3, an inhibitor of NF-κB (35). It has also been demonstrated that calcineurin participates in the induction of TNF-α gene transcription in T and B cells (36) and that consensus DNA-binding motifs for NF-κB proteins exist in the regulatory region of the TNF-α gene (37). In the light of these reports one simple interpretation of our findings may be that elevated levels of intracellular cAMP by CKS-17 antagonizes calcineurin, which then suppresses NF-κB induction and leads to inhibition of TNF-α gene transcription.

In conclusion, the present study shows that this highly conserved retroviral octadecapeptide induces remarkable levels of intracellular cAMP via stimulation of adenylate cyclase in a human monocyte cell line and in human PBMC. cAMP controls physiologic cellular responses, such as cell growth and differentiation, as well as immune effector functions (18). Of interest in this context, human immunodeficiency virus proteins have been recently shown to impair lymphocyte proliferation by induction of intracellular cAMP levels (38). Hence, CKS-17, highly conserved among various retroviral envelope components, could affect several physiologic cellular functions, as well as cytokine gene expression, by increasing intracellular levels of cAMP. Our observations may thus be relevant to understanding the molecular mechanism(s) by which retrovirus infections influence cellular functions, including immune functions.

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