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Defective cAMP-dependent phosphorylation of intact T lymphocytes in active systemic lupus erythematosus

(cAMP/rheumatoid arthritis/Sjögren syndrome)

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ABSTRACT The present study was undertaken to establish whether cAMP-dependent phosphorylation of endogenous substrates is impaired in T lymphocytes from subjects with active systemic lupus erythematosus (SLE). In normal human T lymphocytes, the cell-permeable cAMP analog, N^6,$O^{2'}$-dibutyryladenosine 3',5'-cyclic monophosphate, induced phosphorylation of substrates with molecular masses of 17.5, 23/25, 33.5 kDa on one-dimensional SDS/PAGE. Maximal phosphorylation occurred at 60 min. In contrast to healthy T cells, the extent of substrate phosphorylation achieved in active SLE T cells (n = 8) was only 15% at 60 min in the 17.5-kDa substrate, 21% in the 23/25-kDa substrate, and 9% in the 33.5-kDa substrate. The rheumatic disease controls (rheumatoid arthritis; primary Sjögren syndrome; n = 8) exhibited a mean 72%, 124%, and 85%, respectively, of phosphorylation observed in healthy T cells. Because the only known mechanism by which cAMP acts is via cAMP-dependent protein kinase (protein kinase A), these data raise the possibility of a defect at the level of this kinase in SLE T lymphocytes.

In normal human T lymphocytes, the adenylate cyclase–cAMP–protein kinase A pathway conveys inhibitory signals resulting in the dampening of T-cell immune effector functions (1). Interaction of adenylate cyclase-coupled cell-surface receptors with the agonists adenosine, prostaglandin E_2, and histamine activates adenylate cyclase, enhances cAMP turnover, and results in binding of cAMP to its intracellular receptor, protein kinase A (1, 2). This binding event activates protein kinase A by dissociating the tetrameric holoenzyme into two regulatory (R) and two active catalytic (C) subunits (3). During T-cell proliferation, activation of protein kinase A leads to inhibition of the steady-state mRNA accumulation of interleukin 2 (4) and the protooncogene c-*myc* (5, 6) and appears to retard translation of the interleukin 2 receptor and the protooncogene c-*fos* mRNAs (6). These effects diminish interleukin 2 production and reduce T-cell proliferation (7). Second, activation of protein kinase A evokes a phenotypic switch in a proportion of the $CD3^+$,$CD4^+$ (helper/inducer) subset to the $CD3^+$,$CD8^+$ (suppressor) subset, resulting in enhanced *in vitro* suppressor T-cell activity (8). Finally, crosslinking cell-surface cluster of differentiation (CD) molecules activates the adenylate cyclase–cAMP–protein kinase A pathway, which mediates the directed movement of CD molecules to a pole of the cell in a process termed capping (9) and inhibits T-cell proliferation (10). However, both the capping process and the inhibition of proliferation can be prevented by H-8, an isoquinolinesulfonamide derivative {*N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide} that blocks cyclic nucleotide-dependent phosphorylation (7, 9). Thus, protein kinase A plays a pivotal role in the control of diverse T-cell functions, although the precise mechanisms remain to be elucidated.

Systemic lupus erythematosus (SLE) is a chronic autoimmune disorder characterized by protean clinical manifestations and aberrant cellular and humoral immune responses (11). Recently, particular T-cell functions that are regulated in part by the adenylate cyclase–cAMP–protein kinase A pathway have been observed to be defective in active SLE. These include the phenotypic switch from $CD3^+$,$CD4^+$ to $CD3^+$,$CD8^+$, adenosine-induced suppressor cell activity, and the capping process (12–14). The capping disorder cannot be corrected by raising intracellular cAMP levels with a cell-surface agonist, by direct activation of adenylate cyclase, by a cAMP phosphodiesterase inhibitor, or by C-6 or C-8 cell-permeable cAMP analogs (15).

This observation suggested that a defect of the cAMP pathway may exist at the level of protein kinase A. Because the only known mechanism by which cAMP acts is via protein kinase A (3), and because phosphorylation of endogenous substrate plays a major role in the regulation of cellular functions (16), we investigated cAMP-dependent phosphorylation in intact T lymphocytes of subjects with active SLE. When compared to controls, SLE T cells exhibited a significant reduction of cAMP-dependent phosphorylation of four endogenous substrates.

METHODS

Patients and Controls. Eight adult female subjects were studied who fulfilled the revised criteria of the American College of Rheumatology for the classification of SLE (17). These individuals were moderately to severely ill due to active disease when studied. The mean age was 35.1 years (range, 23–54 years). Six subjects had rashes, six had glomerulonephritis, two had central nervous system disease, and one had serositis. Four subjects were being treated daily with <20 mg of prednisone and either 400 mg of hydroxychloroquine, 100 mg of azathioprine, or no medication. Of the remaining four, one was treated daily with 400 mg of hydroxychloroquine, one with 50 mg of azathioprine, and two were untreated. Four female subjects with primary Sjögren syndrome and four females with rheumatoid arthritis [one was functional class 2; three were functional class 3 (18)] served as rheumatic disease controls (mean age, 37.7 years; range, 28–49 years). Three rheumatoid arthritis subjects were treated daily with 400 mg of hydroxychloroquine and one was treated with 12.5 mg of methotrexate per week. Prednisone and azathioprine were withheld for 24 hr, hydroxychloroquine was withheld for 4 days, and methotrexate was withheld for 7 days before study. To assess the effects of chronic steroid and immunosuppressive therapy, we studied an additional three subjects with rheumatoid arthritis, whose med-

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Abbreviations: Bt_2cAMP, N^6,$O^{2'}$-dibutyryladenosine 3',5'-cyclic monophosphate; SLE, systemic lupus erythematosus; 8-N_3-cAMP, 8-azidoadenosine 3',5'-cyclic monophosphate.

ication was not withheld prior to the study. The average daily dose of prednisone was 10 mg. Two subjects were treated with 100 mg daily of 6-mercaptopurine, and one was treated with 12.5 mg of methotrexate weekly. Three subjects with acute infections were also studied to assess the effect of nonautoimmune disease on cAMP-dependent endogenous phosphorylation. One subject had acute *Escherichia coli* pyelonephritis and two had pneumococcal pneumonia. Seven healthy women with a mean age of 32.3 years (range, 22–52 years) were used as healthy controls. All subjects abstained from foods containing methylxanthines for 24 hr before study.

Cell Cultures. Clonal sublines of S49 mouse T-lymphoma cells were grown in suspension culture (19). The wild-type subline 24.3.2 and the mutant cAMP-dependent protein kinase negative subline 24.6.1 were kindly provided by R. A. Steinberg (20).

Cell Isolation. Peripheral blood mononuclear cells were isolated from venous blood, and the monocytes and B lymphocytes were depleted as described (21). The resultant cell populations were composed of 92–98% viable T lymphocytes, as determined by staining with OKT3/fluorescein isothiocyanate anti-antibody and trypan blue. Enriched T-cell subsets utilized in some experiments were prepared as described (22) and were composed of ≥86% $CD4^+$ cells or $CD8^+$ cells.

Endogenous cAMP-Dependent Phosphorylation. T lymphocytes and S49 cells were washed three times and then incubated at 37°C for 60 min in phosphate-free buffer [157.5 mM NaCl/2.5 mM KCl/10 mM Hepes/0.2% (wt/vol) glucose/0.3% bovine serum albumin] (23). Cells were then resuspended in the buffer at 60×10^6 T lymphocytes per ml or 30×10^6 S49 cells per ml and were incubated with 0.3 mCi of $^{32}P_i$ per ml (5 mCi/ml; specific activity, 8500–9120 Ci/mmol; 1 Ci = 37 GBq; NEN) at 37°C for 45 min. After three washes in buffer without bovine serum albumin, the cells were incubated for various times in the absence or presence of $N^6,O^{2'}$-dibutyryladenosine 3′,5′-cyclic monophosphate (Bt_2cAMP). Phosphorylation was terminated by addition of stop solution (3.5% SDS/20% glycerol/0.05% bromophenol blue). 2-Mercaptoethanol was added to a final concentration of 2% and the samples were boiled for 10 min. Aliquots of each sample containing the equivalent of 3.6×10^6 T lymphocytes or 1.8×10^6 S49 cells were loaded on each lane and subjected to electrophoresis on 12.5% or 4–16% gradient SDS/polyacrylamide slab gels (24). Gels were stained with Coomassie blue, destained, and dried, and autoradiographs were prepared by exposing gels to Kodak X-Omat film. Incorporation of ^{32}P was quantified by scanning laser densitometry (LKB Ultroscan laser densitometer with the LKB 2400 Gelscan program).

Determination of cAMP by RIA. The intracellular concentrations of cAMP were carried out as described (25).

Statistics. Statistical significance ($P \leq 0.05$) was calculated by the one-tailed Student's t test.

RESULTS

cAMP-Dependent Phosphorylation of Normal, Intact T Lymphocytes. Incubation of normal, intact T lymphocytes with pharmacologic agents that act at the level of the A_2 (stimulatory) adenosine receptor (2-chloroadenosine), the G_s subunit of adenylate cyclase (cholera toxin), the catalytic subunit of adenylate cyclase (forskolin), or by inhibiting cAMP phosphodiesterase (RO 20-1724) stimulate the rapid turnover and/or accumulation of endogenous cAMP and phosphorylation of four substrates (data not shown). Similarly, exposure of T cells to cell-permeable C-6 (Bt_2cAMP) or C-8 [8-azidoadenosine 3′,5′-cyclic monophosphate (8-N_3-cAMP)] cAMP analogs induces phosphorylation of these four substrates (Fig. 1). The

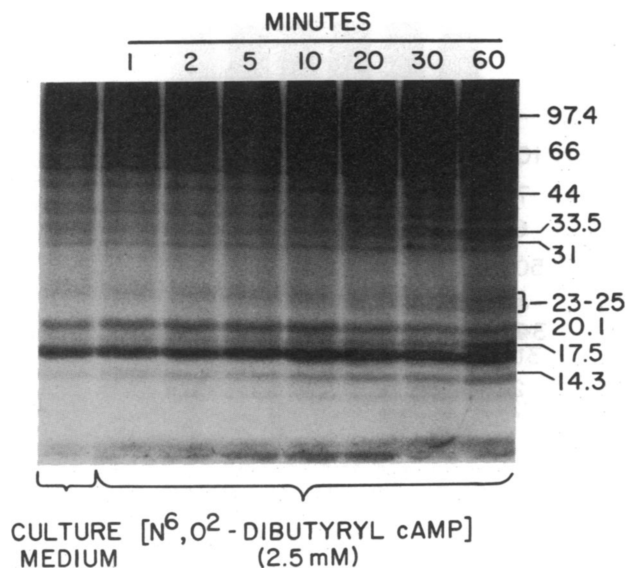

FIG. 1. Time course of endogenous cAMP-dependent phosphorylation in normal human T lymphocytes. T lymphocytes were labeled with $^{32}P_i$ for 45 min and then incubated in the absence or presence of Bt_2cAMP for the times indicated. The incubation was terminated by the addition of stop solution. Cells (3.6×10^6 per lane) were loaded and run on SDS/12.5% polyacrylamide gel. Lane 1, 0 min in medium alone; lanes 2–8, T cells incubated with 2.5 mM Bt_2cAMP for intervals of 1–60 min at 37°C. Four endogenous substrates were consistently phosphorylated in normal T lymphocytes: 17.5, 23, 25, and 33.5 kDa.

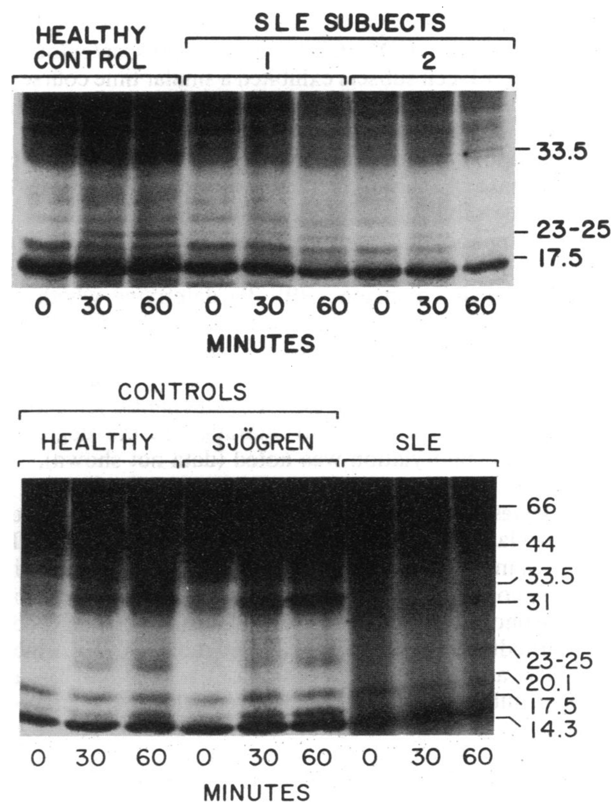

FIG. 2. Endogenous cAMP-dependent phosphorylation in T lymphocytes from a healthy control and two active SLE subjects (*Upper*); a healthy control, a disease control with Sjögren syndrome, and a subject with active SLE (*Lower*). Experimental procedures were as described in Fig. 1.

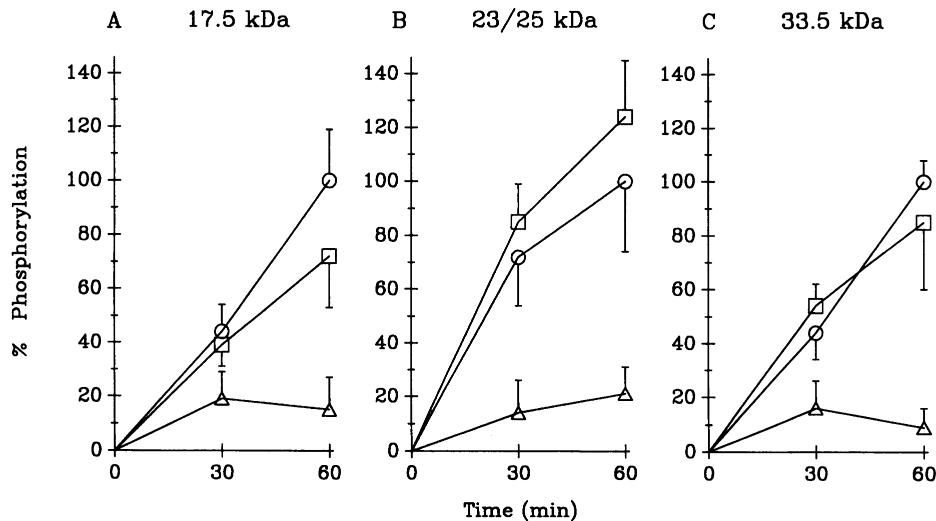

FIG. 3. Comparison of the extent of endogenous substrate phosphorylation in T lymphocytes of healthy controls (○), rheumatic disease controls (□), and subjects with SLE (△). Phosphorylation of the 17.5-, 23/25-, and 33.5-kDa bands was quantified by densitometry. The results are expressed as percent mean phosphorylation of healthy controls at 60 min and represent the net increase of band density over baseline at 0 min. Error bars indicate SEM. The differences in 17.5-kDa substrate phosphorylation were statistically significant at 60 min between healthy control and SLE groups ($P = 0.01$) and disease controls and SLE ($P = 0.015$). The differences in 23/25-kDa substrate phosphorylation at 30 min were significant between the healthy controls and SLE ($P = 0.01$) and disease controls and SLE ($P = 0.001$). At 60 min, the values were $P = 0.006$ and $P < 0.0005$, respectively. The differences in 33.5-kDa substrate phosphorylation were significant at 30 min between healthy controls and SLE ($P = 0.03$) and disease controls and SLE ($P = 0.03$). At 60 min, the respective values were $P < 0.001$ and $P = 0.001$.

apparent molecular masses of the phosphorylated substrates are 17.5, 23, 25, and 33.5 kDa. The 23- and 25-kDa substrates generally appear as a doublet on one-dimensional polyacrylamide gels. Phosphorylation was initially detectable at 2 min and became maximal at 60 min (Fig. 1). Dephosphorylation occurred over 30–60 min after removal of the stimulating agent. When T lymphocytes were separated into subsets, both the helper/inducer ($CD3^+$,$CD4^+$) and suppressor/cytotoxic ($CD3^+$,$CD8^+$) T-cell subsets exhibited a similar time course of phosphorylation of the 17.5-, 23/25-, and 33.5-kDa substrates (data not shown).

The phosphorylation of the four substrates is mediated by protein kinase A alone. Neither butyric acid nor cGMP caused discernible phosphorylation of these substrates in intact T lymphocytes. Moreover, H-8 (26) inhibited cyclic nucleotide-dependent phosphorylation of the four substrates in a dose-dependent manner (9). To determine whether a corticosteroid similar to that used for treatment of SLE would alter cAMP-dependent phosphorylation, cells were incubated with dexamethasone (1 nM to 1 μM) for 1 and 4 hr at 37°C in 5% CO_2/95% air prior to stimulation of cAMP-dependent protein kinase. No alteration in the degree of substrate phosphorylation was noted (data not shown).

Defective cAMP-Dependent Phosphorylation in Intact T Lymphocytes from Active SLE Subjects. cAMP-dependent phosphorylation of the 17.5-, the 23/25-, and the 33.5-kDa substrates in intact T lymphocytes from subjects with active SLE was compared to that of healthy and disease controls. The phosphorylation of these substrates in intact SLE T cells was markedly reduced after 30 and 60 min (Fig. 2). A mean 15%, 21%, and 9% phosphorylation of the 17.5-, 23/25-, and 33.5-kDa substrates, respectively, was achieved when compared to the average phosphorylation of healthy controls at 60 min (Fig. 3). In contrast, rheumatic disease controls exhibited a mean 72%, 124%, and 85% of phosphorylation of the 17.5-, 23/25-, and 33.5-kDa substrates, respectively, in healthy controls. To assess the effects of chronic corticosteroid, an immunosuppressive therapy, on cAMP-dependent phosphorylation in intact T lymphocytes, three additional subjects with rheumatoid arthritis were studied; there was no alteration of substrate phosphorylation when compared with the other rheumatic disease controls (data not shown). Phosphorylation of the four substrates in the subjects with acute infection was the same as in healthy controls.

When compared with either healthy or disease controls, the reductions in SLE T-cell phosphorylation of the 17.5-,

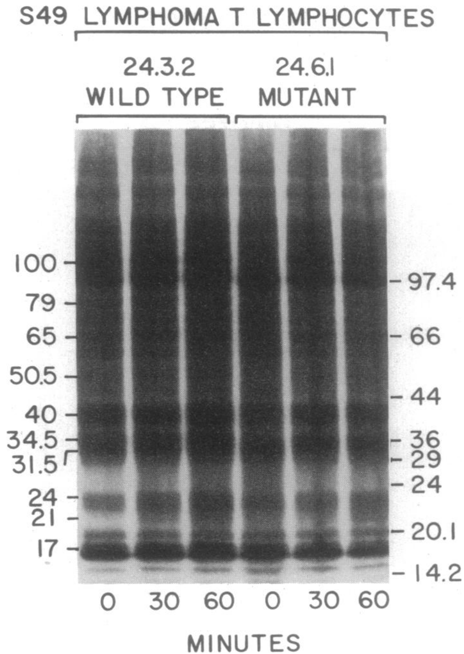

FIG. 4. Endogenous cAMP-dependent phosphorylation in S49 T-lymphoma cells. Cells were labeled with $^{32}P_i$ for 45 min and then incubated in the presence of 2.0 mM Bt_2cAMP. At the times indicated, incubations were terminated by the addition of stop solution. Cells (1.8×10^6 per lane) were loaded and electrophoresis was performed on 4–15% linear gradient SDS/polyacrylamide gel. The relative molecular masses (kDa) of the substrates from the wild-type subline 24.3.2. phosphorylated in response to Bt_2cAMP are indicated on the left. Note the absence of cAMP-dependent phosphorylation in the mutant cell subline 24.6.1, which lacks protein kinase A, compared to the wild-type cell line.

23/25-, and 33.5-kDa substrates at 60 min were statistically significant (Fig. 3). This impaired phosphorylation of SLE T cells could not be accounted for by failure of Bt_2cAMP to enter the cell effectively, for exposure of cells to 1 mM Bt_2cAMP for 10 min at 37°C caused an increase in cAMP from 138 ± 18 fmol per 10^6 lymphocytes to 753 ± 101 fmol per 10^6 lymphocytes ($n = 4$) and complete inhibition of $[^3H]8\text{-}N_3$-cAMP photoaffinity labeling (15). Similarly, healthy control T cells incubated with 1 mM Bt_2cAMP for 10 min at 37°C exhibited an increase in cAMP from 142 ± 20 fmol per 10^6 lymphocytes to 658 ± 108 fmol per 10^6 lymphocytes and complete inhibition of $[^3H]8\text{-}N_3$-cAMP photoaffinity labeling (21). Moreover, neither substitution of other cell-permeable C-6 or C-8 cAMP analogs nor addition of 100 μM RO 20-1724, a cAMP phosphodiesterase inhibitor, augmented phosphorylation in SLE T cells.

The S49 murine T-lymphoma cell wild-type subline 24.3.2 possesses a functional cAMP-dependent protein kinase, which is known to autophosphorylate the type I regulatory subunit (20). In contrast, a mutant S49 subline, 24.6.1, lacks cAMP-dependent protein kinase activity (20). We found that the wild-type subline phosphorylated 10 substrates in response to Bt_2cAMP, whereas the mutant subline failed to phosphorylate any of these substrates (Fig. 4). Thus, the S49 wild-type and mutant sublines provided appropriate positive and negative controls for cAMP-dependent phosphorylation of T cells from both healthy and active SLE subjects.

DISCUSSION

The T lymphocytes of seven subjects with active SLE exhibited a profound disorder of cAMP-dependent phosphorylation of the 17.5-, 23/25-, and 33.5-kDa substrates. Neither substitution of other cell-permeable C-6 or C-8 cAMP analogs nor addition of a cAMP phosphodiesterase inhibitor augmented substrate phosphorylation. Moreover, impaired T-cell cAMP-dependent phosphorylation in active SLE did not appear to be a secondary effect of corticosteroid and/or immunosuppressive therapy, for neither chronic treatment with these agents in rheumatic disease controls nor *in vitro* exposure of normal T cells to dexamethasone altered effective substrate phosphorylation. However, the defect in T-cell phosphorylation was not uniformly observed. A single SLE subject who was asymptomatic, but exhibited marked hypocomplementemia, a high titer of anti-DNA autoantibody, and biopsy-proven acute diffuse proliferative glomerulonephritis did not show defective phosphorylation.

In contrast to SLE, T cells from both healthy and rheumatic disease controls demonstrated time-dependent phosphorylation of the four substrates in response to C-6 or C-8 cAMP analogs. Neither activation of normal, resting T cells *in vitro* for 24 hr with anti-CD3 monoclonal antibody nor incubation of normal T cells in the presence of active SLE sera for 24 hr impaired cAMP-dependent substrate phosphorylation (P.H. and G.M.K., unpublished results). Finally, acute nonautoimmune illness due to bacterial infections did not depress endogenous cAMP-dependent phosphorylation of T cells.

The only known mechanism by which cAMP can effect endogenous phosphorylation is via activation of protein kinase A (3). Failure of cell-permeable cAMP analogs to traverse the cell or to bind to the R subunits of protein kinase A could account for the altered phosphorylation in SLE T cells. However, the demonstration of equivalent intracellular cAMP levels and inhibition of $[^3H]8\text{-}N_3$-cAMP photoaffinity labeling in SLE and control T cells (15, 21) excludes impermeability of SLE T cells to cAMP or an apparent defect of cAMP binding to the R subunits. Taken together, our data raise the possibility of a defect in the cAMP pathway at the level of protein kinase A or distally as a mechanism for the reduced phosphorylation.

Potentially, several defects of protein kinase A could account for the impaired phosphorylation of SLE T cells. Altered binding affinities of the R subunits for cAMP (27) could reduce the efficiency with which C subunits dissociate from R subunits, impairing activation of the enzyme. Similarly, a decreased affinity of the C subunit for ATP could hamper substrate phosphorylation. A mutant C subunit with decreased affinity for ATP resulting in diminished substrate phosphorylation has been described in a subline of Chinese hamster ovary cells (28). A third potential disorder is a deficiency of the A kinase protein such as that found in the mutant S49 murine T-lymphoma subline 24.6.1 (20). Finally, the presence of abnormal cytosolic and/or membrane-bound inhibitors of the C subunit could block substrate phosphorylation.

Alternatively, the reduced cAMP-dependent phosphorylation in active SLE T cells could be explained on the basis of deficient amounts of substrate proteins. This may be the result of an intrinsic deficiency of the substrate or of failure to induce the synthesis of the substrates via a cAMP-dependent pathway.

At present, it remains uncertain whether impaired cAMP-dependent phosphorylation reflects a global T-cell disorder or one restricted to a particular subset. Notwithstanding, the inability to phosphorylate particular cytosolic, nuclear, and/or membrane-bound substrates may contribute to the aberrant immune T-cell effector functions in SLE. Moreover, a defective A kinase activity could also lead to other cellular dysfunctions, including altered mRNA transcription or post-transcriptional events (29).

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