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Enzymes and Protein Markers

Increased alkaline phosphatase isoforms in autoimmune diseases

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We found significant increases in ALP and ALP isoform band 10 in the serum of patients with early insulin-dependent diabetes, rheumatoid arthritis, and in those with multiple sclerosis during periods of disease exacerbation as compared with healthy controls. The ALP isoforms were assayed by isoelectric focusing. Our data suggest that the increase in ALP and ALP-10 closely reflects the abnormal activation of T lymphocytes that is common in autoimmune diseases, and that the source of the ALP-10 is activated T lymphocytes. ALP-10 is a sensitive but nonspecific marker of an active autoimmune process and appears to have the ability to detect abnormal T-cell activation. ALP-10 may be a useful test in the screening for autoimmune disorders.

Alkaline phosphatase (ALP, EC 3.1.3.1) is a membrane-bound enzyme that is expressed in all tissues and consists of a group of isoenzymes that are encoded by at least four gene loci: tissue nonspecific, intestinal, placental, and germ cell ALP *[1–5]*.[3] The tissue nonspecific locus is on the short arm of chromosome 1, whereas intestinal, placental, and germ cell loci are located on the long arm of chromosome 2 *[6–8]*. Isoforms of ALP isoenzymes are posttranslational modifications and differ primarily in their degree of sialidation; at least 15 isoforms or ALP "bands" have been described, and they are readily separated by isoelectric focusing (IEF) on agarose *[9, 10]*.

ALP band 10, sometimes called the "early placental band," is believed to be formed by the placenta or activated T lymphocytes *[11]*; the latter are considered to be responsible for causing abnormal activation and autoantibody production by B cells, leading to initiation, progression, and injury of tissues as occurs in autoimmune diseases *[12, 13]*. The injured tissues probably leak ALP into the circulation with a consequent increase in the total ALP activity in serum. Autoimmune processes may lead to hyperactivation of T lymphocytes with increased ALP production by these cells.

Although there are clinically significant variations between autoimmune diseases, one common thread among all of them is the abnormal activation of T lymphocytes. Autoimmune diseases such as insulin-dependent diabetes mellitus (IDDM), ulcerative colitis, and sarcoidosis are associated with high activities of ALP-10 in serum and may occur because of abnormal activation of T lymphocytes *[11]*. The autoantibodies that are involved in or are byproducts of the pathogenesis of IDDM could be related to abnormal activation of T lymphocytes by viral proteins sharing an amino acid sequence with beta cells *[14, 15]*.

Rheumatoid arthritis (RA) is a progressive and destructive disease characterized by synovial hyperplasia, inflammation, and abnormally high T-lymphocyte activity *[16, 17]*. In systemic lupus erythematosus (SLE), T lymphocytes containing the *hprt* mutations either directly or indirectly stimulate autologous B cells to produce anti-DNA antibodies. B cells may also aid in producing T lymphocytes that are active in the absence of antigenic stimulation *[18–20]*. Multiple sclerosis (MS) is characterized by central nervous system (CNS) demyelination and has an undefined etiology. Evidence suggests that MS is triggered, at least in part, by a viral infection in genetically susceptible individuals *[21–24]*. Here, the CNS demyelination involves primarily T lymphocyte abnormalities, including an increase in the $CD4^+/CD8^+$ ratio, selective loss of the suppressor–inducer T-cell subsets, $CD4^+$ and $D45R^+$, and increased expression of T-lymphocyte activation markers such as interleukin-2 (IL-2), IL-2R receptor (CD25), and CD26 *[25–29]*. The T-lymphocyte abnormal-

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[3] Nonstandard abbreviations: ALP, alkaline phosphatase; IEF, isoelectric focusing; IDDM, insulin-dependent diabetes mellitus; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; MS, multiple sclerosis; IL, interleukin; CNS, central nervous system; CMV, cytomegalovirus; EC, endothelial cell; RF, rheumatoid factor; HUVEC, human umbilical vein endothelial cells; PBMC, peripheral blood mononuclear cells; FITC, fluorescein isothiocyanate; IFN-γ, interferon-γ; and TNF-α, tumor necrosis factor-α.

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ities are associated with a broad spectrum of cytokine abnormalities *[30–36]*.

Our goals in this study were to determine the total ALP and ALP-10 activity in the serum of patients with SLE, RA, MS, and IDDM to determine if a correlation exists between these tests and the rheumatoid factor (RF) concentration in RA, with remission or exacerbation of MS, and the duration of disease in IDDM. Because many autoimmune diseases are linked to viral infections and are associated with abnormal cytokine concentrations, we also studied the effect of cytomegalovirus (CMV)-induced activation and that of certain cytokines on the ALP and ALP-10 activity coming from T lymphocytes.

Materials and Methods

PATIENTS

We were in compliance with The Ohio State University policy on human experimentation. Blood was obtained from the patient groups as described in Table 1. SLE was diagnosed on the basis of standard criteria *[37]*. RA was diagnosed according to the American Rheumatism Association 1982 Revised Criteria. The RF titer was measured in 16 patients and was abnormally increased to >35 kIU/L in nine patients and was ≤35 kIU/L in seven patients. MS was diagnosed on the basis of magnetic resonance imaging findings and the presence of oligoclonal bands in cerebrospinal fluid; three of the patients with MS were in a state of exacerbation and six were in remission. IDDM was diagnosed on the basis of abnormal fasting glucose values or abnormal glucose tolerance tests.

SEPARATION OF ALP ISOFORMS BY IEF

To estimate ALP-10 activity, we separated ALP isoforms by IEF followed by scanning densitometry. The Resolve ALP 90 test kit, the Resolve Omega electrophoresis unit, a 2000-V power supply, and the Isoscan densitometer were all from Isolab (Akron, OH). We placed an application template 1 cm from the center of the gel, applied 15 μL of serum, and performed electrofocusing at 15 W, 1000 V, and 16 °C for 75 min. After focusing, the ALP isoforms were visualized with an Isolab kit containing α-naphthyl phosphate monosodium salt and 4-aminodiphenylamine diazonium. The gels were washed twice for 10 min in 250 mmol/L acetic acid and then twice for 10 min with distilled water. Gels were dried overnight at room temperature and then scanned with the Isoscan. The ALP-10 activity was calculated from the total ALP activity and the relative percent of total ALP activity obtained from the scanner *[38]*. Our technique was slightly different from that described elsewhere *[11]*.

Representative gel patterns of ALP isoforms in healthy and diseased individuals are shown in Fig. 1A and B. The high-M_r is not the same as ALP-10 because the high-M_r ALP does not migrate, gives an image of the application mask on the gels, and obscures the underlying ALP bands 6 to 9.

Table 1. Patient cohort in study.

Disease (no. of patients or controls)	No. of females	No. of males	Age range, years
Childhood-onset IDDM (69)	35	34	2–20
Adult-onset IDDM (6)	5	1	29–52
RA (57)	30	27	20–77
SLE (39)	28	11	22–67
MS (9)	7	2	36–87
Controls for children (18)[a]	8	10	2–17
Controls for adults (122)[b]	49	73	23–30

Control group for [a]childhood onset IDDM and [b]adult patients. The latter group was the basis of comparison for all the disorders except childhood IDDM.

ENDOTHELIAL CELL (EC) AND CMV CULTURES

Human umbilical vein ECs (HUVEC) were isolated, characterized, and propagated as detailed elsewhere *[39–41]*. The isolation and propagation of CMV strain VHL/E is described elsewhere *[40, 42, 43]*. Briefly, the CMV isolate was obtained in 1988 from duodenal ulcer biopsy material derived from a bone marrow transplant recipient with histologically verified CMV infection of the duodenum. Virus stocks were generated by serial propagation through multiple passages in HUVEC cultures, a method shown to preserve the natural endothelial cytopathogenicity of the original isolate *[43]*. Stocks of CMV-infected (or uninfected) HUVEC were generated for use as stimulators as previously described *[40]*.

T-CELL ISOLATION

Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Hypaque density gradient centrifugation *[40, 44, 45]* from fresh heparinized blood collected by venipuncture from healthy, nontransfused CMV-seropositive or seronegative individuals as classified by a passive

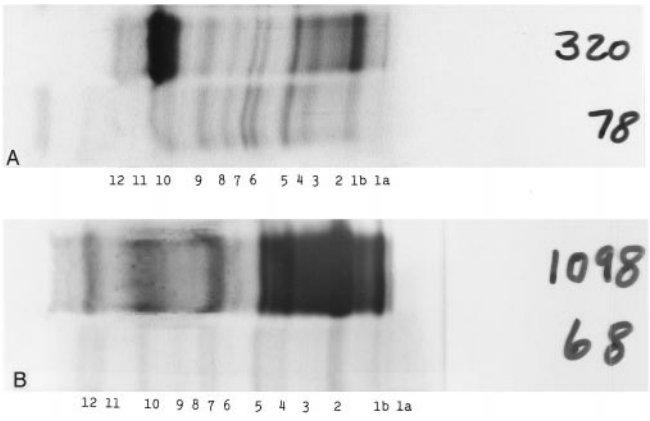

Fig. 1. ALP isoforms separated by IEF.

(*A*) Specimen 320 shows a marked increase in ALP band 10; specimen 78 is a healthy adult control. Note that the numbers are also the total ALP activity in all cases. The anode (+) is on the right. (*B*) Specimen 1098 shows marked increases in ALP bands 1–5 and 10. The smudge that overlies bands 6–9 is the high-M_r ALP and is an image of the application mask. Note that the high-M_r ALP does not obscure ALP-10. Specimen 68 is a healthy adult control.

latex agglutination test for CMV IgG/IgM (Becton Dickinson, San Jose, CA) or enzyme immunoassay (Diamedix, Miami, FL). $CD3^+$ T cells were isolated from PBMC by negative selection with a commercially available cocktail of monoclonal antibodies and complement (T-Lympho-Kwik; One Lambda, Los Angeles, CA), with the methods of Clouse et al. *[46]*. The resulting populations were washed twice with Seligman's buffered salt solution (Gibco, Gaithersburg, MD), suspended in complete culture medium (see below), kept at 4 °C, and used within 18 h.

We verified the homogeneity of purified T-cell populations by incubating the cells for 30 min at 4 °C with fluorescein-isothiocyanate-conjugated (FITC) monoclonal antibodies specific for CD3, CD4, CD8, and CD14 (Gen Trak, Plymouth Meeting, PA), or, as specificity controls, with FITC-labeled isotypically matched irrelevant antibodies, then analyzed by fluorescence flow cytometry. T-cell populations prepared in this manner routinely were 90–95% positive for $CD3^+$ with undetectable concentrations of monocyte contamination as verified by the absence of $CD14^+$ cells.

measurement of t-cell total alp

The total T-cell ALP activity was measured with an Ektachem 700 analyzer (J & J Clinical Diagnostics, Rochester, NY) with their dry-slide technology *[47]*. ALP activities of <5–10 U/L are near the lower detection limit of the Ektachem. An ALP of <5 U/L probably cannot be distinguished from a value close to zero. The ALP in low-activity specimens was determined by the technique of calibrator addition. Serum pools with >50 U/L were added to the low-activity specimens, and the ALP activity was estimated by extrapolating the concentration of the added serum pools to zero. A QC serum pool having ~80 U/L of ALP activity gave a CV of 2.45% (n = 96) on the Ektachem analyzer.

To determine the effect of cytokine stimulation on intracellular or secreted T-cell ALP activity, we cultured the cells with one added cytokine for 6 days in round-bottom 96-well microtiter plates (1×10^5 cells/well in 200 μL; Linbro/Flow, McLean, VA) in the presence of IL-2 at concentrations of 3.12, 6.25, 12.5, and 25 kIU/L; interferon-γ (IFN-γ; Chemicon, Temecula, CA), or tumor necrosis factor-α (TNF-α; Genzyme, Cambridge, MA) at concentrations of 25, 50, 100, and 200 kIU/L. A particular experimental condition was carried out in three or four wells. To determine the effect of viral-induced activation, T cells were cocultured in triplicate with CMV-infected (1×10^3 cells/well) or uninfected (1×10^4 cells/well) HUVEC. Four types of control wells included culture medium alone, T cells cultured alone, uninfected HUVEC, or CMV-infected HUVEC alone. The culture medium consisted of Dulbecco's minimal essential medium (Gibco) supplemented with 100 mL/L human serum (Sigma Chemical Co., St. Louis, MO), 25 mmol/L HEPES buffer, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, 0.67 mmol/L L-arginine, 0.27 mmol/L L-asparagine, 0.014 mmol/L folic acid, 0.05 μmol/L β-mercaptoethanol, 100 kU/L penicillin, and 100 mg/L streptomycin *[40]*. To suppress viral reproduction and prevent the possibility of lymphocyte infection, the culture medium was supplemented with 300 μmol/L phosphonoformic acid, a specific inhibitor of CMV DNA polymerase *[48]*. After a 6-day incubation, the T lymphocytes were ruptured by three cycles of freezing and thawing, followed by sonication.

t-cell proliferation assay

We determined the T-cell activation state induced by each of the culture conditions described above; microtiter cultures identical to those described were incubated for 6 days, including a terminal pulse of a 15-h exposure to [^{3}H]thymidine in the culture medium *[40, 44]*. Cells were harvested onto glass wool filters, and the incorporated radiolabel was measured by β-scintillation spectrometry. The means and SEs were derived from the three to four replicate wells.

statistical analysis

The statistical differences between ALP or ALP-10 for each autoimmune disease and the corresponding control group were estimated by a two-tailed Student's *t*-test. The differences between the effect of various cytokines on T-cell activation were estimated by two-way ANOVA with Scheffé's posthoc test.

Results

total alp and alp-10 activities in patients and controls

The total ALP activity in all the groups listed in Table 2 was significantly higher ($P < 0.05$) when the diseased group was compared with the appropriate control group. With the exception of the MS patients, this observation held for ALP-10 as well. The ALP-10 activity was in-

Table 2. Comparison of total ALP and ALP-10 (U/L, mean ± SE) in autoimmune diseases.

Disease (no. of patients)	Total ALP	ALP-10
Adult controls (122 healthy adults)	57.4 ± 1.51	10.0 ± 0.69
Childhood controls (18 healthy children)	160.0 ± 11.7	10.13 ± 1.93
IDDM (6 adults)	298.0 ± 10.5[a]	6.61 ± 1.59
IDDM (69 children)	224.1 ± 10.6[a]	28.2 ± 3.30[a]
RA (57 adults)	118.9 ± 12.6[a]	15.72 ± 2.33[a]
SLE (39 adults)	110.7 ± 12.4[a]	12.62 ± 2.57[a]
MS (9 adults)	78.0 ± 8.54[a]	5.74 ± 1.11

We obtained blood specimens from patients with the above autoimmune diseases and from healthy controls. With the exception of children with IDDM, the adult control values were used for all the groups (adult IDDM, RA, SLE, and MS) in calculating statistical significance; children without metabolic diseases served as controls for children with IDDM only.

[a] $P < 0.05$ as compared with appropriate controls.

creased in IDDM patients having the disorder for 8–13 years after diagnosis, and then it decreased to the control value (Fig. 2). The ages of the patients with various durations of IDDM are shown in Table 3. Our ALP-10 findings in healthy children differ from those of Murthy et al., who found no ALP-10 in healthy children [*49*]. This difference is difficult to explain; we have consistently found ALP-10 in healthy children, although the ALP-10 was on occasion faint. The differences found by Murthy et al. and us may be due to a difference in sensitivity of the ALP isoform assay.

The total ALP and ALP-10 activities in RA patients who had an abnormal RF test (>35 kIU/L) was significantly higher ($P < 0.05$) than in RA patients who had a normal RF test (Fig. 3). The ALP-10 activity was significantly higher ($P < 0.05$) in MS patients with active disease than in MS patients who were in remission; ALP-10 activities were 9.11 ± 1.20 vs 4.47 ± 1.98 U/L, respectively.

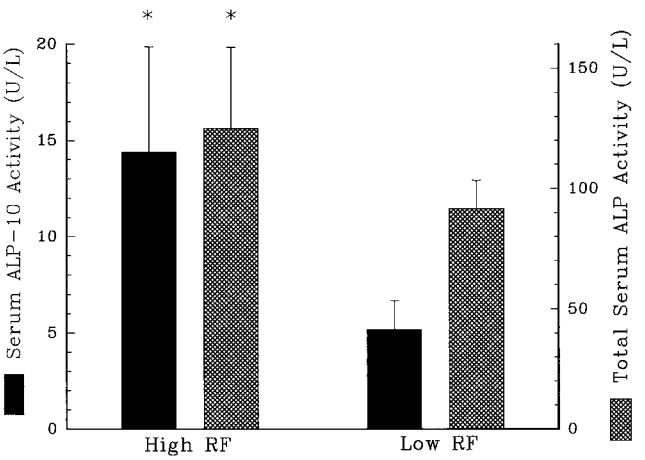

Fig. 3. RF vs ALP-10 and total ALP activities in serum.

Blood specimens were collected from RA patients (n = 9) with abnormal RF titers (>35 kIU/L) and from RA patients (n = 7) with normal RF titers (≤35 kIU/L). Both ALP-10 and total ALP activities in RA patients with an abnormal RF were significantly higher than those with low RF titer ($P < 0.05$). Results are expressed as mean + SE.

Effect of cytokine and viral infection on ALP-10

To determine the effect of cytokines and virally induced activation on T-cell ALP expression, purified T cells isolated from healthy volunteers were incubated in vitro with IL-2, IFN-γ, TNF-α, or CMV-infected ECs. Our results show that the cytokines IL-2, IFN-γ, and TNF-α stimulated T cells to release or produce ALP in a concentration-dependent manner (Fig. 4). The concentration of cytokines (in kIU/L) that produced a 50% increase in the activity of ALP-10 were 10.3 ± 1.3 for IL-2, 35 ± 4.08 for IFN-γ, and 29 ± 5.35 for TNF-α. IL-2 was significantly more potent ($P < 0.05$) than either IFN-γ or TNF-α in stimulating the production of ALP by T lymphocytes. The ALP produced by CMV-seropositive donor T lymphocytes that had been incubated with CMV-infected ECs was significantly higher ($P < 0.05$) than that produced by incubating T lymphocytes with noninfected ECs. The CMV-induced increase in ALP was not observed in T cells isolated from two of three seronegative individuals; the third subject showed only a slight increase in ALP (see Fig. 5). We also performed ALP isoform analysis of the T-cell homogenates and found faint bands at positions 8, 9, 10, and 11. Thus the activated T lymphocytes contained ALP-10 and other ALP isoforms at low activity.

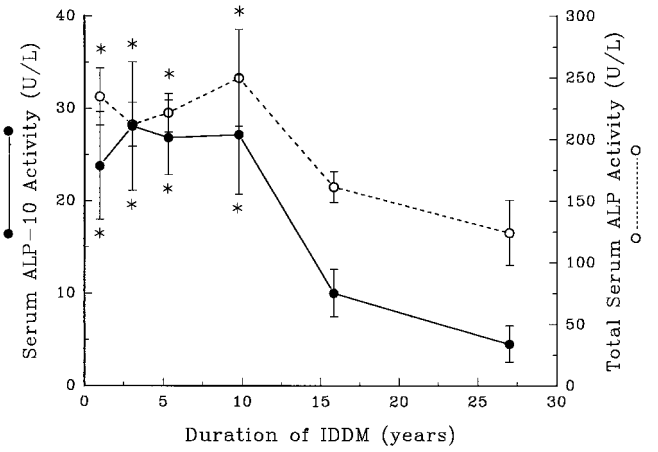

Fig. 2. Activity of ALP-10 and total ALP in serum vs duration of IDDM.

Results are expressed as mean ± SE. *$P < 0.05$ vs 122 adult controls. See Table 3 for age ranges at each duration-of-disease time.

T-cell proliferation

The cytokines IFN-γ, TNF-α, and noninfected ECs did not stimulate an increase in T-cell proliferation as assessed by the [^{3}H]thymidine incorporation assay. IL-2, by contrast, stimulated T-cell proliferation. CMV-infected ECs stimulated the proliferation of T cells from CMV-positive donors but not from CMV-negative donors (see Fig. 6).

Table 3. Ages of patients and duration of IDDM.

No. of patients	Range of ages, years	Age ± SD, years	Range of years with IDDM	Years with IDDM ± SD
16	2–19	9.4 ± 5.1	0.1–1.9	0.98 ± 0.52
20	6–20	11.7 ± 3.8	2.1–4.0	3.2 ± 0.64
19	5–16	11.4 ± 3.9	4.3–7.9	5.3 ± 1.0
12	12–17	15.8 ± 6.3	8.5–11.3	9.9 ± 0.90
5	13–52	33 ± 18.7	13–18	15.8 ± 2.1
3	29–42	33 ± 7.5	25–28	27 ± 1.7

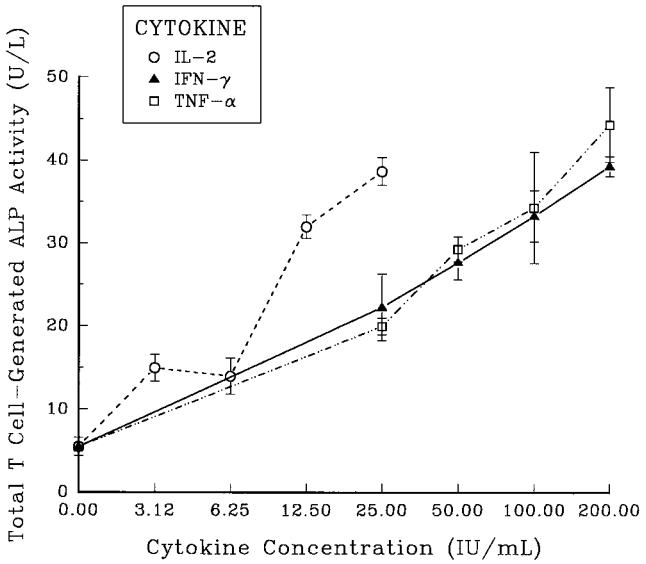

Fig. 4. Effect of the cytokines IL-2, IFN-γ, and TNF-α on ALP production by T cells.

T cells were incubated with or without various concentrations of cytokines. After incubation, the cells were ruptured by sonication. Controls without cytokines showed no increase in ALP activity. Results are expressed as mean ± SE, n = 3 for each cytokine and concentration.

Discussion

T lymphocytes are implicated in the pathogenesis of autoimmune diseases and may produce and (or) secrete ALP-10 and other ALP isoforms. In agreement with the work of others *[11]*, our results show that the total serum ALP activity is significantly higher in patients with autoimmune diseases when compared with the appropriate control groups. This could be due to leakage of ALP from cells that were killed or injured by the autoimmune processes and (or) by abnormal cell activation. In either case, the ALP activity in blood was increased. The ALP-10 activity in childhood IDDM, and in adults with RA or SLE, was significantly higher than that in the corresponding control group; this may be due to an abnormal T-cell activation in these diseases.

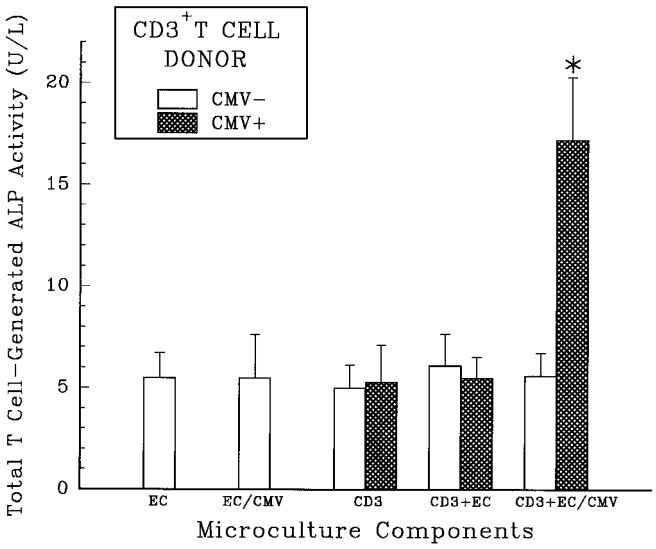

Fig. 5. Effect of CMV on ALP production by T cells and controls.

The sequence of bars, left to right, is: EC, noninfected ECs (a control with no T cells); EC/CMV, CMV-infected ECs (a control with no T cells); CD3, T cells isolated from CMV-seronegative (*open bars*) or CMV-seropositive donors (*hatched bars*); CD3+EC, T cells isolated from CMV-seronegative or CMV-seropositive donors incubated with uninfected ECs; CD3+EC/CMV, T cells from CMV-seronegative or CMV-seropositive donors incubated with CMV-infected ECs. *P <0.05 vs all other bars. Results are expressed as mean + SE, n = 3 for each condition.

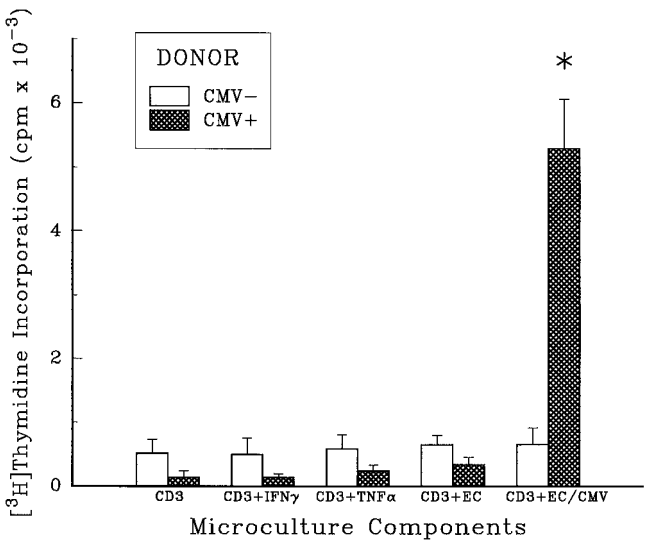

Fig. 6. Effect of cytokine treatment and CMV stimulation on T-cell proliferation.

The sequence of bars, left to right, is: CD3, CD3 T cells isolated from CMV-seronegative (*open bars*) or -seropositive donors (*hatched bars*) incubated alone for 6 days (a control); CD3 plus the previous in the presence of IFN-γ, 200 kIU/L; TNF-α, 200 kIU/L, cocultured with irradiated uninfected EC; or cocultured with irradiated CMV-infected (EC/CMV) ECs. T-cell proliferation was determined by a terminal 15-h [^{3}H]thymidine pulse. Little or no proliferation was detected in T cells cultured with either cytokine or with uninfected EC regardless of the donors' serum status. Although the response of CMV-seropositive donor-derived T cells to CMV-infected EC (*) was significantly greater (P <0.05) than their response to uninfected EC or to cytokines, no such increase was observed in the CMV-seronegative population. Results are plotted as mean radiolabel incorporation, counts/min × 10^{-3} + SE, n = 3 for each condition.

The pathogenesis of IDDM is related to an autoimmune process in genetically susceptible individuals. Abnormally activated T lymphocytes have been postulated to play a central role in the pathogenesis of IDDM, with subsequent formation of autoantibodies against islet cells, resulting in their destruction *[35]*. Our results confirm the abnormal activity of T lymphocytes as indicated by a significant increase in the serum ALP-10 activity. This isoform was increased in IDDM patients for ~8–13 years after diagnosis, suggesting that the autoimmune process with abnormal T-lymphocyte activity continues for years, a finding that may be useful in designing a clinical trial to test the effect of T-lymphocyte suppression on the course and prognosis of childhood IDDM.

ALP-10 activity was significantly higher in RA patients with a positive RF than in those with a negative RF test, indicating a correlation between ALP-10 activity and RF activity. RF is an IgG or IgA isotype with evidence of

somatic mutations that requires T-lymphocyte activation *[50]*. Because ALP-10 is probably secreted by T lymphocytes, the magnitude of ALP-10 activity most likely depends on activation of T lymphocytes. We believe that both ALP-10 activity and the concentration of RF depend on T-cell activation. The correlation between ALP-10 and RF is probably related to the abnormal activity of T lymphocytes.

SLE is associated with abnormal T-lymphocyte activity that may cause abnormal activation of B lymphocytes to produce an array of SLE-related autoantibodies *[51–54]*. Our results show that ALP-10 activity in SLE patients was significantly higher than that in the control group, an observation that most likely is due to the presence of abnormally active T lymphocytes in patients with SLE.

In contrast to the above autoimmune diseases, the ALP-10 activity in MS patients was not significantly different ($P > 0.05$) from the control group of healthy adults, an observation that could be attributed to the administration of corticosteroids that suppress T lymphocytes, with a subsequent decrease of ALP-10. This is supported by the evidence that cyclosporin A and cortisone, both of which suppress T lymphocytes, dramatically decrease ALP-10 activity in patients with poststreptococcal glomerular nephritis *[10, 55]*. MS patients who had active disease had significantly higher ($P < 0.05$) ALP-10 activities compared with MS patients who were in remission, probably because disease exacerbation is associated with abnormal T-lymphocyte activity.

Autoimmune diseases are associated with abnormal concentrations of different cytokines that modulate the activity of T lymphocytes *[56]*. For example, IFN-γ has been shown to induce T-cell activation, leading to demyelination in MS *[32]*; TNF-α stimulates synovial lining cells to synthesize hyaluronan in RA patients *[33]*; and the enhanced $CD3^+$ proliferation response in SLE patients is possibly related to the IL-2/IL-2R interactions *[47]*. A finding that is consistent with abnormal activation of T lymphocytes by cytokines is that IL-2, IFN-γ, and TNF-α induced the release and (or) production of ALP by T lymphocytes in a concentration-dependent manner. How cytokines induce T lymphocytes to produce ALP is unknown; it could be due to an increase in the transcription of the ALP gene and (or) increased translation of ALP mRNA.

Autoimmune diseases are strongly linked to viral infections. The molecular mimicry theory suggests that T cells and antibodies directed against a viral gene product might cross-react with self proteins and trigger an autoimmune process. Others believe that the persistent viral infection is more important in initiating an autoimmune process. Viruses can activate autoantigen-reactive T-cell clones, a possible mechanism in both MS and IDDM *[57–59]*. Further investigation is required to clarify the role of viral infections in ALP production.

We conclude from our results that the increase of ALP and ALP-10 in autoimmune diseases closely reflects the abnormal activation of T lymphocytes; the mechanism of increased ALP-10 could be related to viral infection and (or) abnormal cytokine concentrations. Measurements of ALP-10 may be useful in the clinical assessment of various autoimmune diseases. The test is sensitive but nonspecific and appears to have the capability to detect abnormal T-cell activation. The test is technically simple to perform, and multiple assays can be performed on one plate. The lack of specificity could be an advantage in screening for T-cell activation of any cause.

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