

Synthetic Peptide Immunogens for the Development of a Cardiac Myosin Light Chain-1 Specific Radioimmunoassay

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To determine if the presence of cardiac light chains in blood could be used to detect acute myocardial infarction, we developed a specific light chain immunoassay. A synthetic peptide sequence specific for human cardiac ventricular myosin light chain 1 (VLC1) was synthesized and designated P348. This peptide coupled to keyhole limpet hemocyanin was used as an immunogen to obtain murine monoclonal antibodies specific for VLC1. Five monoclonal antibodies were obtained. One of these designated Mab-8E3 reacted equally well with both the synthetic peptide and VLC1. Although the 8E3 antibody is specific for VLC1, the use of HPLC purification of skeletal muscle myosin light chain 1 demonstrated that VLC1 is present in human skeletal muscle. The clinical utility of the assay was tested in 18 patients with creatine kinase (CK) and ECG documented acute myocardial infarction. VLC1 was below the limit of detection (<1 ng/ml) in sera obtained from healthy volunteers and patients without myocardial infarction or chest pain. In contrast VLC1 was elevated in the serum of all 18 patients with acute myocardial infarction. Combining the two test results at the time of admission resulted in 83% of patients having detectable serum levels of one or both markers.

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Cardiac myosin light chains (CLCs) have been detected in the serum of patients following myocardial infarction (5). Although several serum assay systems for the detection of circulating CLCs have been described, a highly sensitive cardiac specific assay has been difficult to achieve. The production of high affinity murine monoclonal antibodies that do not cross-react with skeletal muscle light chains (SLCs) has been technically difficult because of the close similarity between cardiac and skeletal muscle myosin light chains. Compounding this has been the problem of obtaining undegraded human light chains for use as a standard in these assays.

The use of synthetic peptides as immunogens offers an attractive solution to both of these problems. The nucle-

otide sequences of the entire translated region of both human skeletal and human cardiac ventricular myosin light chain 1 are now known (1-3). Comparison of the two sequences revealed approximately 80% homology, demonstrating the close similarity between the two (2). Several regions of divergence, however, can be seen particularly in the amino terminal region of the sequence. The present study examines the usefulness of synthetic peptides in developing cardiac myosin light chain-1 specific monoclonal antibodies for radioimmunoassay.

MATERIALS AND METHODS

Synthesis of Peptides

A 10 amino acid peptide corresponding to the amino terminal region of human cardiac myosin light chain-1 was chosen for synthesis and designated P348 (Fig. 1). To facilitate subsequent conjugation reactions, cysteine was added to the N-terminus of the peptide. The peptide was made with a Bioscience model 9500 peptide synthesizer using chemicals of reagent-grade quality and t-Boc amino acids purchased from Peninsula Laboratories. Following hydrogen fluoride treatment, P348 was judged suitable for use based upon its amino acid composition and its HPLC profile.

P348-KLH Conjugation

P348 was coupled to carrier KLH (Calbiochem) employing a thiol ether bond as previously described by Bernatowicz et al. (4). The efficiency of coupling and the incorporation of peptide into KLH was assessed by amino acid analysis of the conjugate. The molar incorporation of carboxymethyl cysteine into the conjugate was used as an indicator of peptide concentration for subsequent immunizations of mice. This was 0.24 mg peptide/ml.

Isolation of Human Cardiac Myosin

Human left ventricular tissue was obtained from autopsy material within 48 hr of death. Tissue was frozen in liquid nitrogen and stored at -70°C until the time of isolation. Cardiac myosin was isolated by the method of Shi et al. (8). The following modifications were made to this method to prevent proteolysis. EDTA in a concentration of 10 mM was used in place of EGTA and all solutions contained 1 mM PMSF.

Purification of human VLC1 from crude light chains was performed using affinity chromatography using Mab-8E3-sepharose prepared with CNBr-activated sepharose 4B (Pharmacia). Crude light chains, prepared as described below, at a concentration of 1 mg/ml were applied to the column in 0.01 M Tris-Cl, pH 7.4, 10 mM EDTA and 1 mM PMSF. After incubation for 1 hr at room

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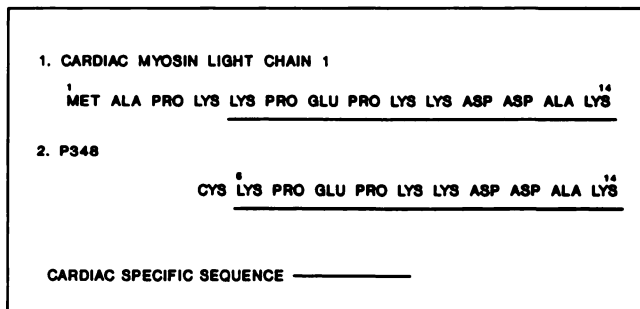


FIGURE 1. The NH₂-terminal sequence of human VLC1 and synthetic peptide P348. An amino terminal CYS residue was added to enable coupling of P348 to carrier KLH.

temperature, the column was washed with the same buffer until absorbance at 280 nm of the eluate fell to less than 0.01. The column was then washed with 1 M NaCl in PBSA until the absorbance returned to zero. VLC1 was eluted from the column with 0.1 M Glycine-HCl, pH 2.5. Pooled fractions containing protein were neutralized immediately with solid Tris base and dialyzed overnight against 6 liters of 10 mM EDTA, 1 mM PMSF, pH 7.4. Purity of the preparation was assessed by SDS-PAGE on 15% polyacrylamide gels (11).

Isolation of Skeletal Myosin Light Chains

Human skeletal muscle from autopsy material was obtained from quadriceps, iliopsoas, pectoral and deltoid muscles and frozen until the time of isolation. The isolation procedure was the same as for VLC1 (8).

Purification of Skeletal Myosin LC1 Using High Pressure Reverse-Phase Liquid Chromatography (HPLC)

Crude skeletal myosin light chains were chromatographed on a Beckman C-8 (Su, 4.6 mm × 25 cm) column. The mobile phase consisted of (B) CH₃CN, 0.1% TFA/ (A) H₂O, 0.1% TFA and light chains were eluted using a linear gradient of 40%–52% (B) over 10 min. The peaks corresponding to SLC1 were collected and lyophilized immediately.

Production of Polyclonal Antisera in A/J Mice

Female A/J mice 6–8 wk old were purchased from Jackson Laboratories. Each mouse was immunized with P348-KLH conjugate (containing 50 µg of peptide) after emulsification with complete Freund's adjuvant (Sigma chemical HR-37a strain). The group of six mice was boosted with the same dose of peptide KLH conjugate at monthly intervals in an equal volume of Freund's incomplete adjuvant (Sigma Chemical). Mice were bled at monthly intervals. Solid-phase titration was performed employing human light chain-coated (50 µg/ml) microtiter plates (Falcon) and ¹²⁵I goat antimouse Fab₂ (Cappel). Titers were determined for peptide-KLH conjugate, peptide alone and human cardiac light chains. Samples were counted in a Micromedics gamma counter.

Production of Monoclonal Antibodies Directed Against P348 and Cardiac Myosin Light Chain 1

From a panel of mice immunized with P348, the mouse with the highest solid-phase titer against VLC1 was chosen for somatic cell fusion. Myeloma cells (ATCC) were grown in DMEM media containing 20% fetal calf serum (M.A. Bioproducts). Three days prior to fusion, the mouse was hyperimmunized intravenously with 10 µg of P348 conjugated to KLH. The fusion procedure itself was as described by Kohler and Milstein (13).

Supernatants from wells with hybridoma growth were screened for the presence of anti-VLC1 antibody employing both a conventional solid-phase assay and by indirect solid-phase screening. The indirect solid-phase screening utilized microtiter plates coated with goat antimouse Fab₂ (Cappel). Hybridoma supernatants were incubated in these wells for 60 min followed by the addition of ¹²⁵I human cardiac myosin light chains (50,000 cpm/well). Wells that were positive in both screening procedures were expanded and subcloned three times by limiting dilution to ensure monoclonality. Isotyping of culture media was performed using an isotyping kit purchased from Amersham.

Production of Ascites in CAF1 Mice

Cell lines were expanded and propagated in 2,6,10,14-tetramethylpentadecane (Pristane) primed mice (14). Two weeks following priming, 1 × 10⁶ hybridoma cells were injected intraperitoneally into each mouse. Ascites fluid was harvested at the time of maximum ascites production and cells were removed by centrifugation at 1500 × g. Ascites fluid was frozen at –20°C until further use.

Purification of Monoclonal Antibodies on Protein A Sepharose

Monoclonal antibodies were purified by chromatography on Protein A Sepharose obtained from Sigma Chemical (15). Antibody purity was assessed using a Beckman Paragon Microzone electrophoresis chamber and by examining the stained gels.

SDS Polyacrylamide Gel Electrophoresis and Immunoblotting

Standard techniques were used for SDS gel electrophoresis on 15% polyacrylamide gels using a BioRad mini Protean gel apparatus (11). Separated proteins were transferred to nitrocellulose (Schleicher and Schuell) using a BioRad mini transfer apparatus (12). Nitrocellulose membranes were blocked with 10% gamma globulin free horse sera in phosphate-buffered saline (PBS) for 60 min. Following this, blots were incubated with 10⁶ cpm of ¹²⁵I 8E3 monoclonal antibody for 60 min. Blots were washed with PBS and autoradiography was performed at –70°C using Kodak AR x-ray film.

Protein Determination

Protein concentration was determined by the method of Lowry et al. (10) using bovine serum albumin (Sigma) as the standard.

Synthesis of P348 Bromoacetyl Tyrosine

Lyophilized P348 (7 mg) was dissolved in 1 ml of 0.1 M phosphate buffer pH 7.4 containing 1 mM EDTA. This solution was cooled on ice and then 3.4 mg of bromoacetylated tyrosine was added while the solution was stirring. The solution was allowed to warm to room temperature over the course of 30 min. This was followed by HPLC purification of the modified peptide as described earlier. Aliquots of the modified peptide were collected and lyophilized.

Iodination of Human Light Chains, VLC1 and P348-Tyrosine

Iodination of VLC1 and human cardiac light chains was performed using the chloramine-T method (16) followed by separation on a Sephadex G-25 column. Specific activity of VLC1 was determined by TCA precipitation of an aliquot of the reaction mixture with carrier BSA.

In the case of the P348, specific activity following iodination using chloramine-T was determined by precipitating a 10-µl ali-

quot in (from) anhydrous ether using 2 mg of a different carrier peptide. Specific activity was expressed as counts per minute of precipitable peptide prior to separation of free ^{125}I on a Sephadex G-10 spin column.

Liquid Phase Binding Studies

A conventional double antibody precipitation method was used to study the binding of monoclonal antibodies in the liquid phase. Rabbit antimouse polyclonal sera as well as goat antirabbit polyclonal sera were supplied by Dr. Robert Graham (MGH Cardiac Unit). Purified antibody, ascites and culture supernatants were titrated in liquid phase against ^{125}I P348 to determine the antibody concentration at which binding was 50% of maximal.

This concentration of antibody was then used in the liquid phase assay. Each tube contained the following: 100 μl of either normal human sera or plasma, 50 μl (5 ng) of monoclonal antibody, 330 μl of 1% BSA/H₂O 0.1% lubrol, 10 μl (16,000 cpm) of ^{125}I P348, 10 μl of serial dilutions of VLC1 (affinity purified) or P348 from 3×10^{-4} to 40 picomoles. The negative control tubes contained the same reactants except the 50 μl of a monoclonal gamma-2a kappa-designated 1B9G4, which is specific for human atrial cardiac myosin heavy chain, was substituted for the light-chain monoclonal antibody. The positive control consisted of six tubes in which 10 μl of 1% BSA were used in place of P348 or HVLC1. After addition of all reagents, tubes were incubated either overnight at 4°C or for 60 min at room temperature. A 50- μl aliquot of rabbit antimouse IgG polyclonal sera was then added and the mixture incubated at 4°C for 30 min. Then a 50- μl aliquot of goat antirabbit IgG serum was added for 30 min at 4°C. Two milliliters of normal saline containing 0.1% lubrol (w/v) were added and the tubes were centrifuged at 5000 \times g for 10 min. The supernatants were aspirated and the pellets counted. Concentrations of unknowns were read from a standard curve generated with each assay.

All measurements were performed in duplicate and s.d. and s.e.m. were calculated from six positive control tubes which contained no cold antigen. Assay tubes were counted in an LKB Compugamma 1282 gamma counter. When sera from patients with acute myocardial infarction was used, the serum was first made 1% in sodium dodecyl sulphate (SDS) and allowed to stand for 15 min. These conditions were duplicated for all standard curves at the time of determinations.

Statistical Analysis

Assay sensitivity, coefficients of variation and precision were calculated from seven different standard curves done on seven different occasions. Accuracy was calculated by adding known amounts of pure VLC1 to human sera and measuring the actual level of VLC1 in these samples utilizing the peptide-based radioimmunoassay. Results obtained for four concentrations of pure VLC1 were then read from the standard curve. The mean value obtained was then compared to the mean corresponding concentration of peptide using a two-sided t-test (26–28).

Analysis of Serum VLC1 in Patients with Acute Myocardial Infarction

Serum samples were collected in duplicate from 18 patients presenting to the emergency department at The Wellensley Hospital with acute chest pain of suspected cardiac origin. Patients were included in the study only if they met all three of the following conditions: 30 min or more of anginal type chest pain, 1.5 mV of ST segment elevation in three leads on ECG recordings and documented rises in serum CK and CK-MB. Patients with renal

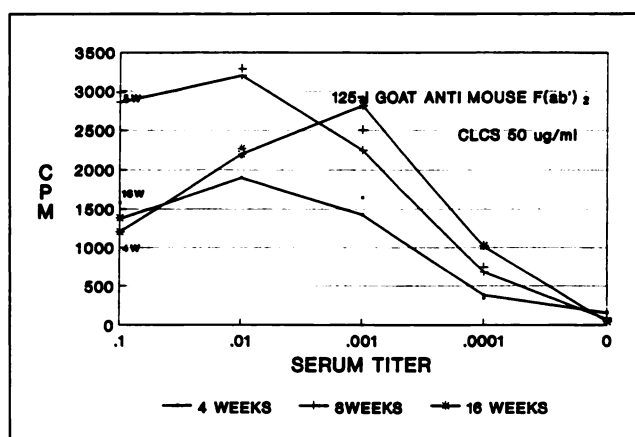


FIGURE 2. Polyclonal serum titers of a mouse immunized with P348-KLH. Titers were performed at 4 wk (4W), 8 wk (8W) and 16 wk (16W) postprimary immunization. Fusion was performed at 16 wk. Results are from solid-phase screening using microtiter plates coated with 50 $\mu\text{g}/\text{ml}$ of cardiac myosin light chains (CLCs).

failure, recent surgery, cardiopulmonary resuscitation within the past 24 hr and more than 6 hr of pain prior to presentation were excluded. Samples were collected at 2-hr intervals for the first 8 hr and thereafter at 8-hr intervals for the first 24 hr. Daily serum samples were obtained following this for 7 days total. Serum samples for VLC1 analysis were frozen at -20°C until the time of the assay. CK, MB, SGOT and LDH determinations were performed on the day of collection in the routine clinical lab.

RESULTS

Production of Polyclonal Antisera in A/J Mice

Figure 2 shows the antibody response of P348 immunization in an A/J mouse with time. All mice immunized with peptide-KLH complexes responded by producing antibodies to P348 and KLH and human cardiac light chains in solid-phase screening. The titers of cross-reactive sera rose consistently following each successive booster immunization.

Hybridomas and Monoclonal Antibodies

The spleen from one mouse immunized with P348 was selected for fusion for generation of monoclonal antibodies based on the results of serum titers. The antiserum from this mouse at 16 wk demonstrated 50% of maximum binding to antigen-coated plates at a serum dilution of 1/7,500 (Fig. 2). The hybridoma growth frequency in the fusion was 95%. Of these, five clones were selected which reacted with human cardiac myosin light chains and P348 in both solid-phase and indirect solid-phase screening procedures. Ascites from these four cell lines was subjected to microzone electrophoresis to confirm the presence of antibody. One antibody designated 8E3B6D10 (Mab-8E3) was chosen for further study. Figure 3 demonstrates the electrophoretic patterns of both the 8E3 ascites and the Protein-A purified Mab-8E3 antibody.

Iodine-125-Labeled P348-TYR

HPLC was used to follow the reaction of P348 with bromacetyl-tyrosine. HPLC profiles before and after the

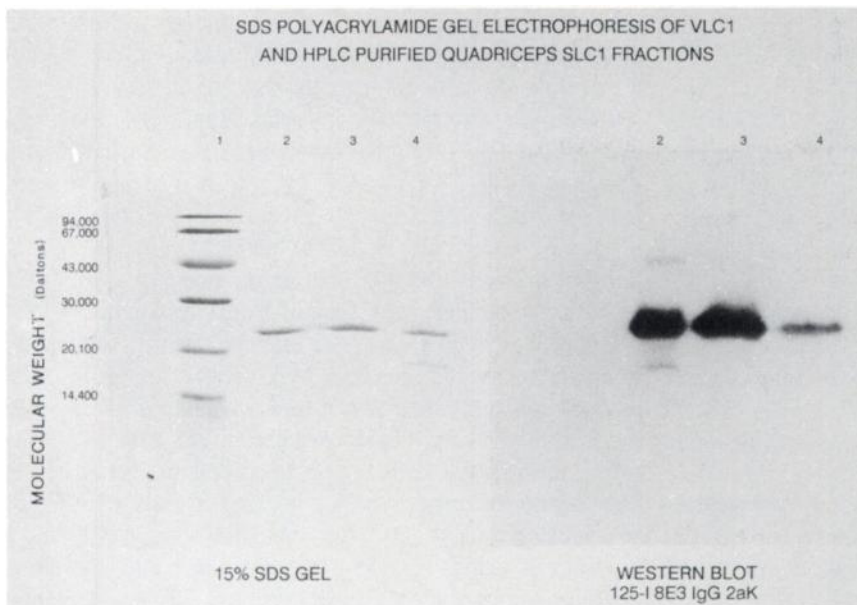


FIGURE 6. Coomassie blue-stained 15% PAGE-SDS gels of: (1) Bio-Rad standards; (2) affinity-purified VLC1; (3) HPLC-purified quadriceps LC1 F1; (4) HPLC-purified quadriceps LC1 F2. Corresponding western blot using ^{125}I -labeled Mab-8E3 is shown on the right.

normal control patients without evidence of angina or myocardial infarction, VLC1 levels were below the limit of detection of the assay (<1 ng/ml). In all 18 patients with acute myocardial infarction, serum VLC1 levels were elevated. Table 3 summarizes the clinical characteristics of these patients. The mean time to appearance of VLC1 (6.66 hr) and CK (6.11 hr) was similar (Table 4). Time to peak CK (21.6 hr) and time to peak VLC1 (52.4 hr) were significantly different. No correlation was obtained between peak CK and peak VLC1 levels. The mean peak VLC1 serum level was 44.7 ng/ml for the entire group (Table 4). Unlike CK time to peak, VLC1 was independent of the use of thrombolytic therapy. The overall sensitivity of VLC1 was 100% detecting CK-confirmed myocardial infarction.

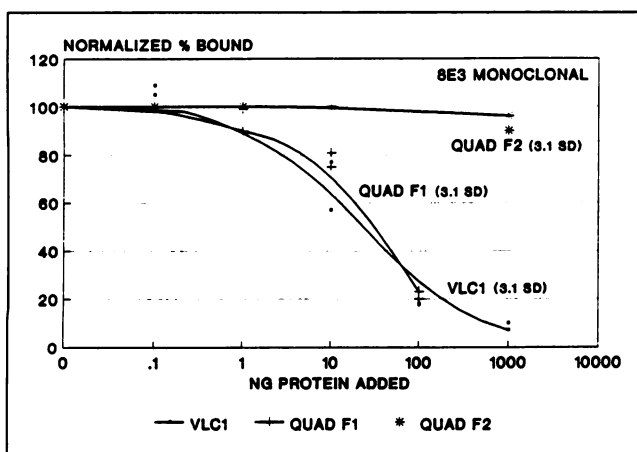


FIGURE 7. Liquid-phase binding studies using ^{125}I -P348 and HPLC fractions F1 or F2 from human quadriceps muscle. VLC1 is shown for comparison. The HPLC separated quadriceps F1 (VLC1) closely mimics the curve for true VLC1. The quadriceps F2 (SLC1) does not inhibit the binding of P348 to the 8E3 antibody over the concentration range tested.

Interestingly, at the time of admission (2.17 hr from onset of chest pain), 55% of patients had measurable levels of VLC1 compared to 38% for CK (Table 5). Utilization of both tests at the time of admission showed 83% of patients positive for one or both of the two markers.

DISCUSSION

Previous radioimmunoassays for detection of cardiac myosin light chains have employed polyclonal antisera or monoclonal antibodies produced by immunizing animals with native cardiac myosin light chains or VLC1 (17,19). The two monoclonal antibody sandwich assay techniques increased the specificity of the assay for VLC1 but suffered from low sensitivity (5). To date, single monoclonal antibodies with specificity for VLC1 and of high enough affinity to be useful alone in these assays have not been reported.

Our results demonstrate that very high affinity monoclonal antibodies directed against VLC1 can be produced using a synthetic peptide as antigen. The apparent affinity of 8E3 is 1.5×10^{10} L/M (23). The 8E3 antibody is capable of reacting equally well with both the synthetic peptide and the native protein in liquid-phase binding studies. Attempts to iodinate affinity-purified VLC1 by conventional means resulted in low specific activity of the light chain. This was

TABLE 3
Patient Characteristics

Number	18
Sex	12 male 6 female
Age	60 ± 12.1 yr
Duration of pain at arrival	2.17 ± 2.2 hr
MI Type	10 anterior 8 inferior

TABLE 4
Serum CK and VLC1 in Acute Myocardial Infarction Patients

	CK (U/liter)	VLC1 (ng/ml)	
Peak	2526 (± 1704)	44.7 (± 32.9)	
Range	222–6800	5.5–115	
Time to appearance	6.11 hr (± 3.8)	6.66 hr (± 7.23)	NS
Time to peak	21.6 hr (± 14.5)	52.4 hr (± 27.3)	

independent of the iodination procedure. The low specific activity of VLC1 probe necessitated the use of much higher concentrations of the probe and therefore the antibody being used, which shifted the binding curve to the right and rendered the assay less sensitive.

Liquid-phase competition curves of labeled P348 against unlabeled VLC1 and SLC1 demonstrated that the antibody is specific for VLC1. Apparent cross-reactivity with SLC1 is present with all skeletal muscles tested.

When HPLC purified preparations of SLC1 are used, this cross-reactivity appears to be due to the presence of true VLC1 in all muscles tested. The ratio of VLC1:SLC1 increases from 2% in quadriceps muscle to 30% in deltoid muscle. This finding is in agreement with the cDNA hybridization results of Kurabayashi et al. (1). They have reported that human iliopsoas muscle expresses exclusively cardiac myosin VLC1. Previous reports have shown similarities between VLC1 and slow skeletal muscle LC1 (25). It is not surprising then to find VLC1 is present in human skeletal muscle. Other HPLC methods for the purification of VLC1 have not attempted to separate the cardiac and skeletal isoforms from each other. The method we have used is capable of doing this.

Traditionally screening for VLC1 specific antibodies has been based upon demonstration of a lack of cross-reactivity with SLC1. It is now clear that using such a screening procedure would tend to select low-affinity antibodies only. High-affinity antibodies would be discarded under the premise that they were not cardiac-specific because they would bind to the small quantities of cardiac myosin VLC1 present in skeletal muscle. All monoclonal antibodies thus far reported in the literature exhibit some degree of cross-reactivity with skeletal light chains. Most of the monoclonal antibodies with low cross-reactivity do not bind well in liquid phase. This is due to the low affinity of the antibodies. It occurs because the act of measuring an antigen-

TABLE 5
Clinical Utility of Serum CK and VLC1 on Admission

Serum test	Sensitivity
At time of admission (2.17 hr)	
VLC1	55%
CK	38%
CK + VLC1	83%
During hospitalization	
VLC1	100%

antibody complex utilizing a second antibody increases the dissociation rate constant of the first antibody thereby leading to difficulty in measuring the antigen (29).

In 18 patients with acute myocardial infarction, the sensitivity for detecting CK-documented myocardial infarction was 100%. Our results agree with other previously published data with respect to the time course and quantity of VLC1 released into serum following myocardial infarction (17,19,21). Although the time to appearance of VLC1 and CK are similar, in this small group of patients, VLC1 was elevated in 55% of patients at the time of admission. CK was elevated in only 38% of patients at this point. The use of both tests resulted in 83% of patients with acute myocardial infarction being detected at the time of admission. Thus, the two tests appear to complement each other. The reason for this is not clear. VLC1 has been shown to be elevated in patients with unstable angina who have normal CK values (21). It is therefore possible that those patients who were VLC1-positive and CK-negative had unstable angina immediately prior to acute myocardial infarction, giving rise to small elevations in VLC1 prior to the acute event. This study is too small to fully evaluate this possibility.

The 8E3 antibody appears to be very attractive for use in clinical radioimmunoassay to quantitate serum VLC1. The fact that it binds to both the peptide and native protein equally well allows the use of the synthetic peptide to generate standard curves in place of the native protein. Thus, the human protein which is difficult to obtain is no longer required. This is an effective approach which has been used in other systems as well (30,31). Reaction between antibody and peptide is also virtually complete within 60 min, thus allowing very short incubation times. A large variety of covalent modifications of the peptide are possible as specific amino acids can be added to allow coupling of different enzymes or proteins to the peptide. This could increase the potential applications of both the peptide and antibody. Finally, the initial patient results demonstrate the assay to be superior to and complementary with CK determinations in the early biochemical detection of MI. The ultimate clinical diagnostic utility of this assay, however, must await further investigations.

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