Monoclonal antibodies that distinguish between active and inactive forms of human postheparin plasma hepatic triglyceride lipase

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Abstract Hepatic triglyceride lipase (H-TGL) was purified from human postheparin plasma. Specific monoclonal antibodies (MAbs) were produced that discriminate between active (native) and inactive (denatured) forms of the enzyme. Mice immunized with native H-TGL resulted in MAbs that recognized only the native protein. The antibodies did not react with H-TGL treated with 1% sodium dodecyl sulfate or heated at 60°C. The loss of immunoreactivity with heating correlated directly with the loss of enzyme activity and there was a corresponding increase in immunoreactivity with the MAbs prepared against the denatured enzyme. Western blot analysis of postheparin plasma with the MAbs against denatured H-TGL gave a single protein band of 65 kD; preheparin plasma showed no detectable immunoreactivity with either MAb. These immunochemical studies suggest that there are no circulating active or inactive forms of H-TGL in man. Furthermore, the MAbs provide the necessary reagents for development of immunoassays for H-TGL. —**Mao, S. J. T., A. E. Rechtin, and R. L. Jackson.** Monoclonal antibodies that distinguish between active and inactive forms of human postheparin plasma hepatic triglyceride lipase. *J. Lipid Res.* 1988. **29:** 1023–1029.

Supplementary key words hepatic lipase • lipoprotein lipase • antigen-antibody • immunoassay

Human hepatic triglyceride lipase (H-TGL) is localized on the plasma membrane of liver sinusoidal (endothelial) cells and in endocytotic vesicles (1). The enzyme is released into the circulation after intravenous injection of heparin. H-TGL has both triacylglycerol hydrolase and phospholipase A_1 activity and appears to play a role in lipoprotein metabolism by catalyzing the hydrolysis of lipids of very low density lipoprotein remnants and high density lipoproteins (2, 3). Recently, the complete cDNA sequence of rat H-TGL has been reported (4). The cloned message codes for a protein of 472 amino acids; the calculated primary translation product has a molecular weight (M_r) of 53,000. On sodium dodecyl sulfate gel electrophoresis, the apparent M_r of mature H-TGL is 59,000; the higher M_r is presumably due to glycosylation (4). Rat H-TGL shows considerable amino acid sequence homology to bovine milk and human adipose tissue lipoprotein lipase (LPL) and porcine pancreatic lipase (4–7).

The most common method for measuring the activity of human H-TGL in postheparin plasma is with a triacylglycerol substrate in the presence of 1 M NaCl, a condition that inhibits LPL activity (8). However, enzymatically inactive forms of H-TGL are not detected by this assay. To develop immunoassays for the measurement of H-TGL in human plasma, we report the preparation of monoclonal antibodies (MAbs) specific for native and denatured forms of H-TGL.

MATERIALS AND METHODS

Purification of human H-TGL

H-TGL was purified from human postheparin plasma as described previously (9). Briefly, postheparin plasma (20 liters) was applied to a column of heparin-Sepharose followed by elution with 2 M NaCl, 0.05 M potassium phosphate, 20% glycerol, pH 6.8. The eluent, containing H-TGL and LPL, was diluted and loaded on a second heparin-Sepharose column; the enzymes were eluted with a linear NaCl gradient between 0.4 and 2.5 M. Two peaks with distinct triacylglycerol hydrolase activity were obtained. The first peak eluted at 0.8 M NaCl; it was active in the presence of 1 M NaCl and was defined as H-TGL. The second peak of activity, corresponding to LPL, eluted at 1.5 M NaCl; it required apolipoprotein C-II for maximal activity and was sensitive to 1 M NaCl. H-TGL was purified further by a modification of a method de-

Abbreviations: MAbs, monoclonal antibodies; H-TGL, hepatic triglyceride lipase; LPL, lipoprotein lipase; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; M_r, molecular weight; PBS, phosphate-buffered saline.

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scribed by Cheng et al. (10). Pooled H-TGL fractions were diluted with 5 volumes of 50 mM Tris-HCl, pH 8.6, 0.4 M NaCl and applied to a column (10 ml) of phenyl-Sepharose. The column was then washed with 100 ml of 50 mM Tris-HCl, pH 8.6, 0.4 M NaCl and H-TGL was eluted with 25 mM deoxycholate in 5 mM potassium phosphate, pH 6.8. Fractions containing H-TGL activity were diluted with 10 volumes of 5 mM potassium phosphate, pH 6.8, and applied immediately to a column (10 ml) of heparin-Sepharose. After the sample entered the resin, the column was washed with 100 ml of 5 mM potassium phosphate, pH 6.8, containing 0.4 M NaCl. H-TGL was eluted with a linear NaCl gradient from 0.4 to 2.5 M NaCl and H-TGL activity was determined. On SDS gel electrophoresis, the major protein-staining band had an apparent M_r of 65,000. The enzyme with a specific activity of 1825 μmol oleic acid released/hr · mg (20,000-fold purified) was stored in 50% glycerol at −20°C.

Dot-blot assay

The dot-blot assay was performed, in duplicate, at room temperature using a Bio-Dot apparatus (Bio-Rad). Prewashed nitrocellulose paper was inserted in the apparatus and H-TGL diluted in 100 μl of PBS (0.01 M sodium phosphate, 0.12 M NaCl, pH 7.4), was loaded into the wells to be tested. The sample was allowed to filter through the paper by gravity flow for about 30 min. Each well was washed once with PBS by applying vacuum and was then blocked with 250 μl of 1% PBS-BSA to cover any remaining binding sites. Fifty to 100 μl of MAbs of the appropriate dilution was added and allowed to filter through by gravity for 30 min. Each well was then washed twice with PBS-0.1% BSA. Peroxidase-linked goat anti-mouse IgG (Bio-Rad) diluted 1:500 in PBS-1% BSA was added and the wells were washed as described above. Finally, the nitrocellulose paper was removed from the apparatus and developed with 4-chloro-1-naphthol. The density of each dot was determined using a Bio-Rad densitometer. The rationale for using the dot-blot technique for the present study was twofold. First, the technique is similar to the Western blot procedure, thus we could screen for those denatured MAbs that would be useful on a Western blot (see Results and Discussion for more detail). Second, each nitrocellulose well can be loaded with up to 5 μg of protein allowing a greater coating capacity than the other solid supports such as microtiter plates (11) in a direct-binding assay.

Preparation of H-TGL monoclonal antibodies

For the production of native MAbs (antibodies raised against native H-TGL), animals were immunized with the native enzyme in 50% glycerol. For the denatured MAbs (antibodies raised against denatured H-TGL), glycerol was first removed with an Amicon 30 concentrator and the H-TGL was heated at 60°C for 15 min. Native or denatured H-TGL (50 μg) in 0.4 ml of 50% complete Freund's adjuvant was injected intradermally and intraperitoneally into Robertsonian mice RBF/DN (Jackson Laboratories). The mice were boosted by intraperitoneal injection with 25 μg of antigen in adjuvant at day 14. A final boost of 25 μg of antigen without adjuvant was given by tail vein injection at day 21. Mice were killed on day 24 for the fusion. Somatic cell fusion and preparation of MAbs were performed in a manner similar to that described previously (12–14) with a nonimmunoglobin-secreting myeloma cell line, FOX-NY (Hyclone).

Primary cultures seeded in 96-well microtiter plates were screened for the presence of antibodies using a Dot Apparatus (Bio-Rad) as described above (11) except that nitrocellulose paper was used as the solid support. The buffer used for all manipulations contained PBS and 1% bovine serum albumin (BSA), pH 7.4. For screening of native MAbs, native antigen (100 ng/well) was used; ten of the primary cultures (n = 980) were positive to H-TGL on dot-blot analysis. These clones were expanded and subcloned. Five of them designated as native C1, C3, C4, C5, and C7 were further characterized. These MAbs all showed some degree of inhibition (15–40%) of H-TGL catalytic activity with a substrate of trioleoylglycerol emulsified with Triton X-100 (15). For denatured MAbs, all primary cultures (n = 196) were positive to the heat-denatured enzyme. The culture media that gave the greatest immunoreactivity on a dot-blot (n = 18) were tested for their ability to recognize H-TGL on a Western blot; 16 of these clones were reactive. After limiting dilution and subcloning, four clones, designated as HL30, HL32, HL44, and HL46, reacted with denatured H-TGL on a dot-blot and showed a single band at 65 kD on a Western blot.

Immunoprecipitation of H-TGL catalytic activity

H-TGL (200–300 ng) in 20 μl of 0.1% BSA-PBS was preincubated with 100 μl of diluted MAbs (0.2 mg/ml) or control (unrelated) MAbs at 4°C for 2 hr. This was followed by a 30-min incubation at 4°C with 50 μl of 1:50 goat anti-mouse IgG (15 μg of IgG in total, Helena Laboratories). The complex (if any) was then incubated for 5 min with 200 μl of protein A in excess (IgG sorb, The Enzyme Center, Inc.) with a maximal binding capacity of 1.58 mg IgG/ml (12). The mixture was precipitated by centrifugation at 4000 *g* for another 5 min. Two hundred μl of the supernatant fraction was assayed for H-TGL activity with a substrate of trioleoylglycerol (15).

Other procedures

Immunoglobulins of MAbs were partially purified by repeated (three times) precipitation with 50% ammonium sulfate. Control, unrelated MAbs were derived from a myeloma cell line (FOX-NY) fused with spleen cells of

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mice immunized with rat lipoxygenase. SDS-polyacrylamide gel electrophoresis (16) was carried out in 15% acrylamide gel and the gels were stained with either Coomassie blue or silver stain. All samples were diluted to a final volume of 50 μl in a buffer containing 6 M urea, 1% SDS, and 2% β-mercaptoethanol, and were heated at 60°C for 15 min prior to electrophoresis. Western blots were performed according to the procedure of Towbin, Staehelin, and Gordon (17). Affinity-column purified goat IgG against mouse IgG conjugated with horseradish peroxidase (Bio-Rad) was used for both Western and dot-blot analyses. Triacylglycerol hydrolase activity was determined with a Triton X-100-stabilized trioleoylglycerol emulsion (15). H-TGL activity was measured in 1 M NaCl, while LPL activity was determined in 0.1 M NaCl in the presence of human apolipoprotein C-II (5 μg).

RESULTS

H-TGL was purified 20,000-fold from human post-heparin plasma. Mice were then immunized with either the native or heat-denatured H-TGL. Five MAbs prepared against the native enzyme and four against the denatured enzyme were further examined. By dot-blot analysis, each MAb was capable of detecting 12.5 ng of the respective antigen. **Fig. 1** shows sensitivity and specificity of the dot-blot assay using these MAbs (details of the procedure are described in Materials and Methods). The lower limit of the assay was between 3 and 6 ng and the most sensitive detectable range was between 12 and 200 ng of each respective H-TGL antigen. These MAbs did not cross-react with human LPL, serum albumin, or antithrombin III. Little or no background, i.e., nonspecific binding, was seen when the antigen was blotted with an unrelated MAb (Fig. 1).

Fig. 2 shows the typical results of immunoprecipitation of H-TGL activity with increasing amounts of MAbs C3 and HL44. The C3 native MAb (prepared against the native enzyme) gave greater than 90% immunoprecipitation of enzyme activity. In contrast, the HL44-denatured MAb (prepared against the denatured enzyme) gave only 20% immunoprecipitation; an unrelated MAb did not immunoprecipitate H-TGL. To ascertain whether denatured MAbs reacted with the same component of the H-TGL preparation that was also recognized by native MAbs, a native MAb immunoaffinity column was prepared and enzymatically active H-TGL was applied. The bound fraction that eluted from the affinity column with PBS, pH 11.0, showed H-TGL triacylglycerol hydrolase activity and gave a single band of 65 kD on SDS gel electrophoresis. All the denatured MAbs reacted with this fraction on both Western and dot-blot analyses (data not shown).

Since it has recently been shown that H-TGL is structurally similar to LPL (4–7), we next examined the specificity of the H-TGL antibodies. **Fig. 3** shows that the denatured MAb gave a single protein by Western blot analysis with an apparent M_r of approximately 65,000 for

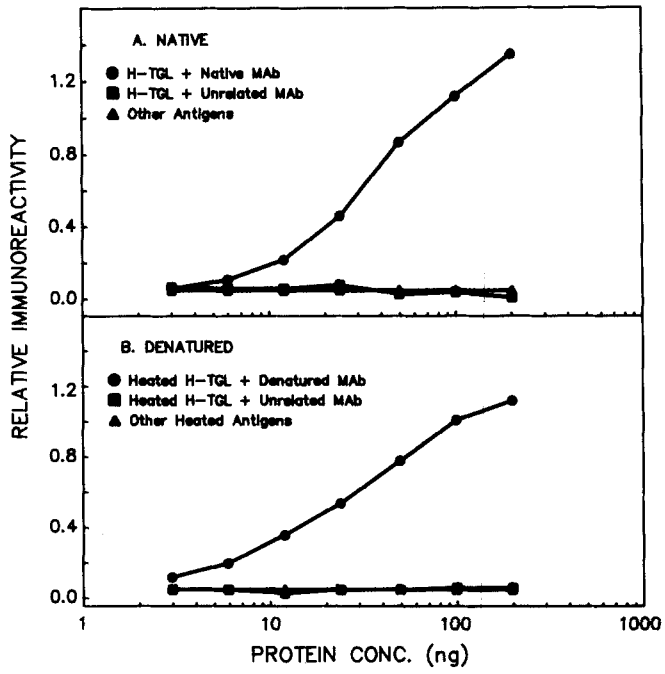

Fig. 1. Sensitivity and specificity of native MAb C3 and denatured MAb HL44 using a dot-blot technique. Native MAb (A) and denatured MAb (B) were incubated with each respective native and heated H-TGL at room temperature for 30 min. Human LPL, albumin, antithrombin III, IgG were used as other antigens (▲). An unrelated MAb (■) prepared against rat lipoxygenase was used as a control. Relative immunoreactivity was determined by measuring the area density of each dot using a Bio-Rad densitometer.

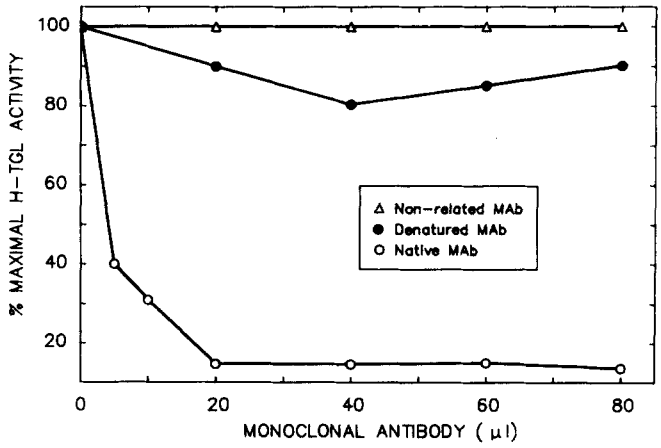

Fig. 2. Immunoprecipitation of catalytic activity of H-TGL by native MAb C3 and denatured MAb HL44. Native H-TGL was incubated with different amounts of MAb (0.2 mg/ml) for 2 hr at 4°C. The antigen–antibody complex, if any, was then precipitated by protein A in the presence of goat anti-mouse IgG as described in Materials and Methods. After centrifugation, enzyme activity remaining in the supernatant fraction was determined. An unrelated MAb prepared against rat lipoxygenase was used as a control.

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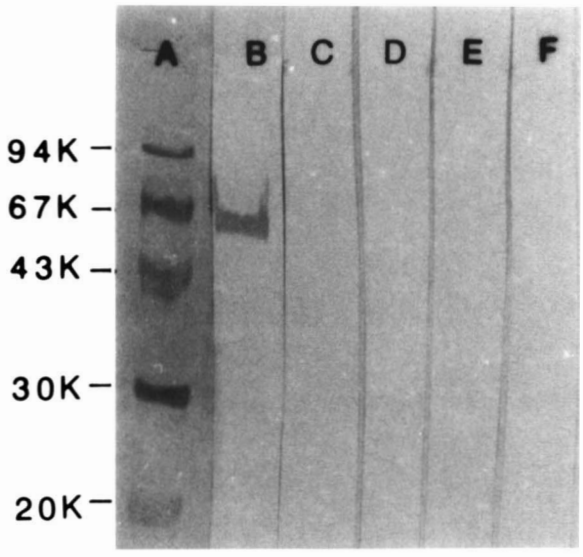

Fig. 3. Western blots of denatured MAb HL44 tested against human plasma H-TGL, LPL, antithrombin III, and albumin. Lane A, molecular weight standards blotted on nitrocellulose paper and stained with amido-black; lane B, immunoblot of H-TGL; lanes C–E, immunoblots of human plasma LPL, antithrombin, and albumin, respectively; lane F, H-TGL blotted with native MAb C3. Ten micrograms of each protein were used for electrophoresis.

purified H-TGL. The denatured MAb did not bind to human LPL or human serum albumin and antithrombin III, proteins with *M*, values similar to H-TGL.

The native MAb did not react with H-TGL by Western blot (Fig. 3, lane F) suggesting that these antibodies recognize epitopes that are destroyed upon heating the protein in 6 M urea and 1% SDS, conditions used for gel electrophoresis. To obtain direct evidence for this possibility, native H-TGL was treated with various reagents and immunoreactivity was analyzed by dot-blot. **Fig. 4** shows that heating the enzyme at 60°C for 15 min or incubating with 1% SDS completely abolished the immunoreactivity to the background level with this native MAb, but not by the denatured MAb. Urea (3 M) or 2% mercaptoethanol only partially decreased the immunoreactivity by native MAb and may be explained by a reversible conformational change of H-TGL that occurs on the nitrocellulose paper following the PBS buffer wash (see Materials and Methods). In addition, denatured MAb still reacted with unheated H-TGL to some extent on the dot-blot (Fig. 4). This result is in contrast to that obtained from the immunoprecipitation data (Fig. 2). One possible explanation is that some of the native H-TGL was denatured during the dot-blotting procedure.

The results shown above suggest that the antibodies prepared against H-TGL might discriminate between active and inactive forms of the enzyme. To test this possibility, native H-TGL was heated at 45°C and triacylglycerol hydrolase activity and immunoreactivity were monitored as a function of time. **Fig. 5** shows that both enzyme activity and immunoreactivity with the native MAb C3 decreased in parallel with heat treatment; the immunoreactivity with denatured MAb HL44 increased. Furthermore, reagents that reduce disulfide bonds also caused a decrease in enzyme activity and immunoreactivity. As demonstrated in **Table 1,** dithiothreitol, cysteine, and glutathione at 5 mM reduced enzyme activity to 22% or less; such decreases tended to be associated with the loss of immunoreactivity detected by native MAb.

The data presented above are consistent with the view that the conformation of H-TGL is important in maintaining the antigenic structure (for native MAb) and enzyme activity. The results in **Table 2** show that phenyl boronic acid, a site-specific inhibitor that completely inhibits triacylglycerol hydrolase, did not affect H-TGL

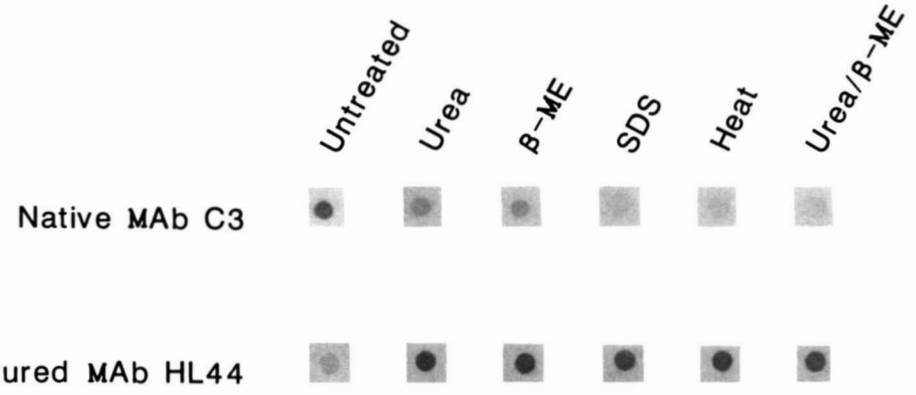

Fig. 4. Dot-blots of native MAb C3 and denatured MAb HL44 tested against chemically and physically treated H-TGL. The heated sample was prepared by incubating H-TGL at 60°C for 15 min. Treated or native H-TGL (100 ng) was applied to nitrocellulose paper and then washed with 500 μl of PBS and 500 μl of BSA-PBS. After 2 hr incubation with MAb at 24°C, the samples were incubated with peroxidase-conjugated goat anti-mouse IgG for another 2 hr. The entire assay was accomplished within about 5 hr. Denaturation did not affect the binding of H-TGL on the paper as evidenced by staining with amido-black.

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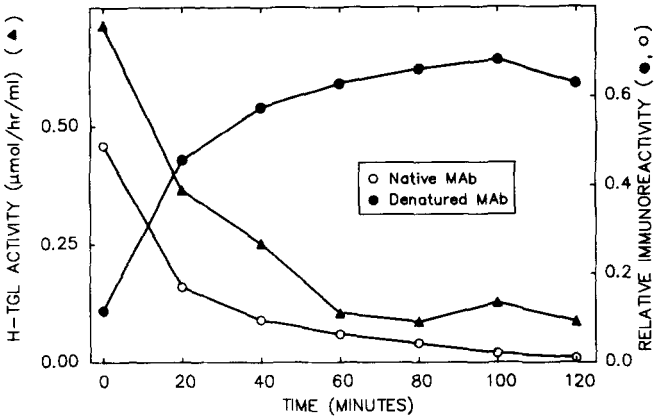

Fig. 5. Effect of thermal denaturation on catalytic- and immunoreactivity of H-TGL. Native H-TGL (300 ng) was incubated at 45°C for 0–120 min. Equal aliquots of each sample were used for analyses of triacylglycerol hydrolase activity and immunoreactivity. Relative immunoreactivity was determined using a reflex densitometer (Bio-Rad) on dot-blot with MAbs C3 and HL44, and is expressed as relative light density.

immunoreactivity for both antibodies. Thus, the inhibitor does not appear to induce any gross conformational change in H-TGL, and native MAbs do not appear to react with the active site of the enzyme.

It is generally thought that human H-TGL does not circulate but is immobilized on the sinusoidal surfaces of the liver and is released with heparin (18). This conclusion is based, in part, on the fact that there is no H-TGL enzyme activity in the circulation. However, it is not known whether an enzymatically inactive circulating form is present. In the next experiment, we tested this possibility by using both native and denatured MAbs. Two normal male subjects were given a single, intravenous injection of heparin (100 units/kg of body weight). Blood was drawn every 5 min over a period of 20 min and H-TGL triacylglycerol hydrolase activity was determined; enzyme activity was maximal after 5 min. Pre- and postheparin plasma (10 ml) were applied to heparin-Sepharose and the bound proteins were eluted with 1.0 M NaCl. **Fig. 6A** shows that

TABLE 1. Effect of sulfhydryl reagents on H-TGL triacylglycerol hydrolase activity and immunoreactivity

Reagents	Concentration	H-TGL Activity	Relative Immunoreactivity with Native MAb[a]
	mM		
Control	0	100%	2.2[b] (100%)
Dithiothreitol	5.0	17%	0.84 (38%)
Cysteine	5.0	5%	0.04 (2%)
Glutathione	5.0	22%	0.40 (15%)

[a]No decrease in immunoreactivity assayed with denatured MAbs.
[b]Immunoreactivity was determined by a dot-blot analysis. Each value (mean of duplicates) represents the area-density measured by a Bio-Rad densitometer.

TABLE 2. Effect of a site-specific inhibitor, phenyl boronic acid, on H-TGL triacylglycerol hydrolase activity and immunoreactivity

Inhibitor	H-TGL Activity	Relative Immunoreactivity[a] with Native MAb	Relative Immunoreactivity[a] with Denatured MAb
M	%		
0	100	2.24	2.20
8.2×10^{-7}	71	2.13	2.18
8.2×10^{-6}	34	2.18	2.13
8.2×10^{-5}	7	2.13	2.16
8.2×10^{-4}	0	2.17	2.16
8.2×10^{-3}	0	2.03	2.15

[a]Immunoreactivity was determined by a Bio-Rad densitometer on a dot-blot as described in Table 1.

neither immunoreactivity (determined by native or denatured MAbs using heat-treated and untreated fractions eluted from the column) nor enzyme activity was detectable in the preheparin plasma. However, the immunoreactivity (determined by the same technique described above) and triacylglycerol hydrolytic activity were superimposed in the postheparin sample (Fig. 6B). In addition, when nine different MAbs, including native and denatured MAbs, were used for the heparin-binding fractions (heated and unheated) of preheparin plasma, the immunoreactivity was not detectable. Western blot analysis with the denatured MAb gave a single protein component (65 kD) in the postheparin plasma (Fig. 6B), but not in the preheparin sample (Fig. 6).

DISCUSSION

Monoclonal antibodies have been utilized extensively for probing the structure and function of proteins. They have also served as specific reagents for detecting subtle structural changes of antigenic sites (12, 19). Two types of antigenic determinants on proteins have been identified (19). The first, a sequence-dependent determinant, consists of five to seven consecutive amino acids in the polypeptide chain; the antibodies recognize this determinant regardless of the overall conformation of the protein (19). The second type, a conformation-dependent determinant, consists of discontinuous amino acids that are formed from polypeptide folding (19). For this type of determinant, a conformational change leads to loss of immunoreactivity. It is conceivable that we have developed H-TGL monoclonal antibodies for both types of antigenic determinants in the present study. Native H-TGL has antigenic determinants that are conformation-dependent and are limited in number. However, heat denaturation of H-TGL exposes more antigenic epitopes that are presumably buried in the native enzyme and these sequence-dependent regions are now recognized by denatured MAbs.

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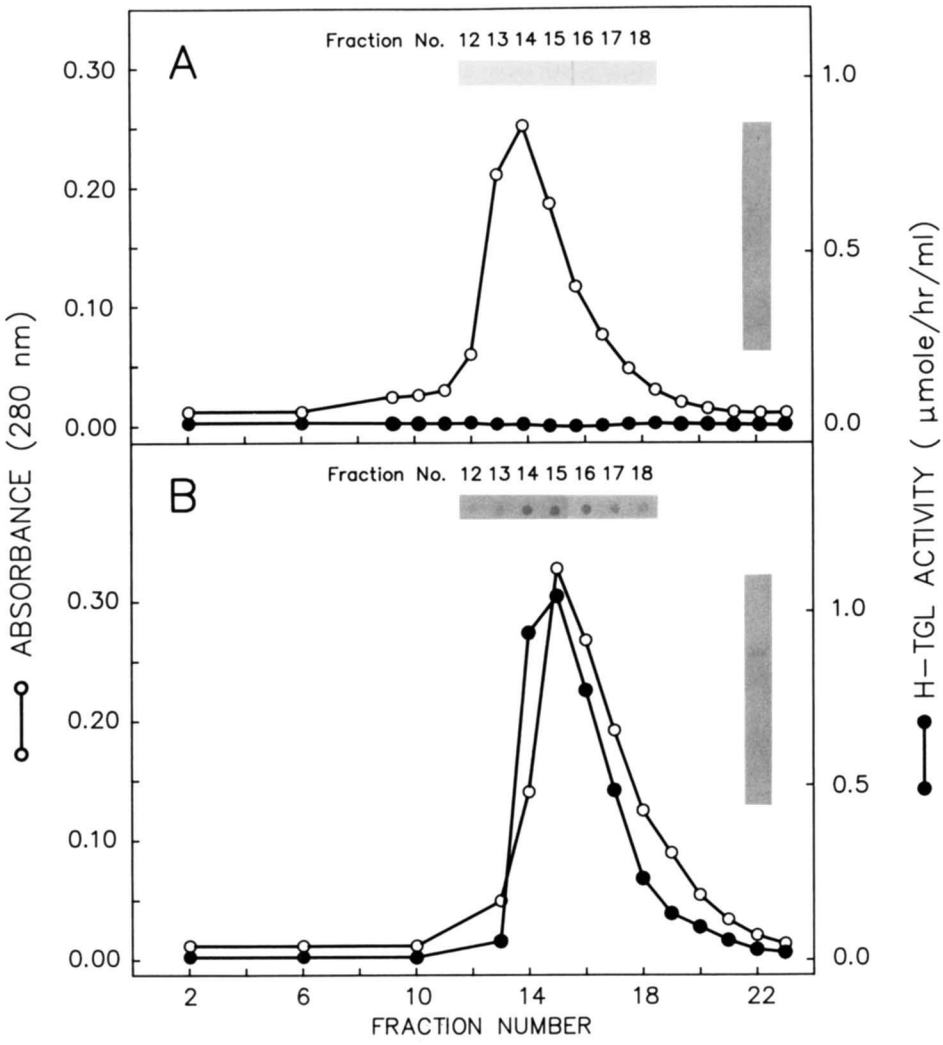

Fig. 6. Elution profiles of pre-(A) and post-(B) heparin plasma on heparin affinity columns. Ten ml of pre- and postheparin plasma were collected 20 min before and after intravenous administration of heparin (100 units/kg body weight) in a final EDTA concentration of 0.1%. Freshly prepared plasma was applied to a heparin-Sepharose column equilibrated with 0.4 M NaCl, 0.01 M phosphate, pH 7.4; bound protein was then eluted with 2 M NaCl in the same buffer. Catalytic activity was determined in the presence of 1 M NaCl. Immunoreactivity of all fractions (as indicated in panels A and B) was determined by dot-blots using both native (not shown) and denatured MAb HL44 (as shown). Immunoreactivity was not found in preheparin plasma. Western blots (inserts) showed that MAb HL44 reacted specifically with a protein component corresponding to the M_r of H-TGL. Immunoreactivity was not detectable in unbound column fraction using either antibody.

The present study also offers an explanation for the recent report by Goldberg et al. (20) indicating that MAbs specific to LPL did not react with the enzyme on a Western blot (consistent with our results for the native MAb). In addition, Cheng et al. (10) reported that the MAb specific to H-TGL reacted with the enzyme only after denaturation with detergents, results that are consistent with the present study using denatured MAbs.

Several lines of evidence show that the denatured MAbs were indeed made against H-TGL. First, the denatured MAbs reacted with purified H-TGL and partially purified H-TGL from postheparin plasma on Western blot; they did not react with preheparin plasma. Furthermore, a single immunoreactive band of 65 kD was identified that is in close agreement with the molecular weight of H-TGL reported by Cheng et al. (10). Second, MAb HL44 reacted with a CNBr fragment of human H-TGL (21) that has a sequence homology with rat H-TGL (4). Third, H-TGL purified on an immunoaffinity column immobilized with native MAbs reacted with denatured MAbs on a Western blot.

Peterson, Bengtsson-Olivecrona, and Olivecrona (22) recently showed that a significant amount of H-TGL activity is present in plasma of mice. No H-TGL activity is detectable in human preheparin plasma. However, this does not rule out the possibility that there is an inactive

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form of the enzyme. Since the denatured MAbs react with the inactive form of H-TGL, it was possible to determine the presence of H-TGL. The present study shows that, unlike mouse H-TGL, this enzyme is not detectable in human preheparin plasma in either an active or inactive form. We speculate that human H-TGL has a higher affinity than mouse H-TGL for the receptor molecules on endothelial cells and the enzyme is therefore not released under normal conditions. This is consistent with the observation of Peterson et al. (22) who demonstrated that when human H-TGL was injected into mice, it rapidly disappeared from plasma; the human lipase localized to the mouse liver and was released back into circulation by injection of heparin.

In summary, our results show that the characteristics and properties of H-TGL MAbs are dependent on the structure of the immunizing antigen. For the laboratories that are currently developing H-TGL immunoassays using MAbs, the properties of antibodies should be carefully evaluated.

We are indebted to Dr. Steven Busch for his helpful suggestions and to Mrs. Rita Hoeper and Susan Treadway for the preparation of the manuscript.

Manuscript received 9 October 1987 and in revised form 4 February 1988.

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