Interaction of Immobilized Anti-Salivary Amylase Antibody with Human Macroamylases: Implications for Use in a Pancreatic Amylase Assay to Distinguish Macroamylasemia from Acute Pancreatitis

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We examined the ability of an immobilized antibody to salivary amylase (Clin Chem 1985;33:1283–8) to react with amylase in macroamylasemic sera. The antibody removed 50% (SD 23%) of the total amylase activity from 39 macroamylase sera, a percentage indistinguishable (P > 0.75) from the percentage removed from concurrently analyzed sera from healthy volunteers (49%, SD 11%). Electrophoretic analysis of 23 macroamylasemic sera revealed that the antibody removed only part of the macroamylase band(s) in 71% of the cases. We conclude that the mean isoenzyme composition of the macroamylase complexes is essentially identical to the mean isoenzyme distribution in normal sera (i.e., about half salivary and half pancreatic amylase). Further, the immobilized antibody can be used to distinguish most patients with macroamylasemia from those with acute pancreatitis, because sera from the latter contain an increased proportion (>80%) of pancreatic amylase.

When found in human circulation, a-amylase (EC 3.2.1.1) typically exists as a monomeric single-chain protein, but high-molecular-mass complexes containing amylase and other proteins have been known since 1964 (1). The prevalence of these amylase complexes, termed “macroamylases” by Berk et al. (2), has been estimated at 1% to 1.5% (3, 4). In most macroamylases, the predominant complexing protein has been identified as either A- or G-class immunoglobulin (5). By contrast, the isoenzyme composition of macroamylases is quite heterogeneous, the reported ratio of pancreatic to total amylase ranging from 0 to 1.0 (6).

Although no pathophysiological function of macroamylases is known (3), their presence can lead to clinically misleading increases in total amylase activity. A diagnostic dilemma arises when a macroamylasemic patient presents with suspected acute pancreatitis and an increased value for total serum amylase (7). If fixed-voltage electrophoresis is used to analyze a hyperamylasemic specimen that contains a macroamylase, the source of the increased amylase cannot be accurately established (8, 9). A recent survey (10) determined that about 10% of hyperamylasemic specimens contained macroamylases. Because amylase measurements are used diagnostically to identify pancreatitis (11, 12), the results in this fraction of samples are potentially misleading.

Early procedures that detected macroamylases relied upon physical-chemical means for identification, e.g., gel permeation chromatography (13), precipitation with polyethylene glycol (14), or fixed-voltage electrophoresis in agarose (15). All of these have one or more serious deficiencies that hinder their routine use in the diagnostic laboratory. In addition, some older procedures designed to detect isoamylases (e.g., fixed-voltage electrophoresis) suffered from interference by macroamylases. With specific measurement of either pancreatic or salivary amylases, by use of monoclonal antibodies (16–19), the ability to quantify an isoamylase accurately in the presence of macroamylases assumes new significance.

To determine the extent that macroamylases would influence the performance of a monoclonal-based immun assay for pancreatic amylase, we studied the interactions between a murine anti-salivary amylase antibody that has been characterized extensively (16, 20, 21) and a group of macroamylasemic sera.

Materials and Methods

Pancreatic and salivary amylase marker. This control was prepared by mixing approximately equal activities of the two purified amylase isoenzymes (22) and then adjusting the volume so the total amylase activity concentration was about 900 U/L. The preparation was pipetted into 20-μL aliquots and stored at −70 °C until needed. An aliquot was thawed, used once, and the excess was discarded.

Preparation of immobilized monoclonal antibodies (IMAB). The procedure described earlier (16) was used to immobilize anti-salivary antibodies on particles of polyvinylidene fluoride. The concentration of the immobilized antibody suspension was adjusted to 300 mL/L, and it was stored at 4 °C (16).

Normal-reference-interval serum samples. Serum samples from apparently healthy volunteers were collected at the University of Virginia and stored at −70 °C until needed. Each was thawed once, and the unused portion was then discarded.

Macroamylasemic serum samples. Two sets of macroamylasemic sera were identified at the Mayo Clinic by gel permeation chromatography (10). The sera were stored at −70 °C before use in this study. An aliquot of each of 39 macroamylasemic sera was packed in solid CO2, shipped to the University of Virginia, and stored at −70 °C until assayed. The volume of the samples in the first set was sufficient to allow their analysis by electrophoresis before and after immunoprecipitation.

Measurement of amylase activity. Total (and residual) amylase activities were quantified by using multi-point kinetics with a mixture of p-nitrophenyl-maltopentosides and -maltohexaosides ("Pantrak" Amylase; Calbiochem Behring, San Diego, CA). Enzyme activity was measured with an RA 1000 discrete analyzer (Technicon Instruments Corp., Tarrytown, NY) at 37 °C, with sample volume 18 μL, reagent volume 350 μL, delay time 5.0 min, and wave-
length setting 405 nm. The stated reference range for serum amylase was 20–110 U/L.

Separation of amylase isoenzymes by agarose electrophoresis. Amylase isoenzymes were separated on Mylar-supported 10 g/L agarose films ("Special Purpose" Agarose; Corning Diagnostics, Palo Alto, CA). An earlier procedure was used (16) except that two 1.2-μL aliquots of each sample were applied to the same sample well. After constant-voltage electrophoresis (90 V dc) for 2 h, amylase activity was made visible by pouring a suspension of dyed-starch particles (pre-warmed to 37 °C) onto the agarose plate and incubating it at 45 °C for 90 min in a sealed, moist chamber. After incubation, the dyed particle suspension was rinsed from the plate with distilled water, immersed in 50 mL/L acetate acid for 5 min, and then dried at 56 °C for 20 to 30 min.

Immunochromosomal assay. The IMAB suspension was gently resuspended at room temperature by magnetic stirring (20 min) before pipetting 75 μL of serum sample into each of two 0.5-mL Eppendorf tubes, labeled "TOTAL" and "PANCREATI," respectively. We delivered 75 μL of diluent buffer (pH 7.5, containing, per liter, 20 mmol of Tris and 15 mmol of sodium azide) to each "TOTAL" tube, vortex-mixed, and incubated at room temperature for 15 min. To each "PANCREATIC" tube, 75 μL of well-mixed IMAB suspension was added, vortex-mixed, and allowed to sit for 15 min. The contents of each "PANCREATIC" tube were resuspended twice, by vortex-mixing, during incubation. All tubes were centrifuged (12 000 × g, for 4 min, room temperature) and then the amylase activity in each supernate was measured. We multiplied the amylase activity from each "TOTAL" tube by 2.0 to correct for dilution. To obtain true pancreatic amylase activity, we corrected the observed amylase activity from each "PANCREATIC" tube, as described earlier, for volume displacement by the solid-phase particle (20). The fraction of amylase activity (as per cent pancreatic) was obtained by dividing the corresponding corrected PANCREATIC activity by the TOTAL amylase activity and multiplying by 100. Observed amylase activities were also corrected for reagent drift.

Statistics. Data are presented as means and SD. The significance of differences between means were determined by unpaired t-tests.

Results

Use of immobilized anti-salivary amylase antibody (IMAB). Thirteen normal sera were assayed and the values for total and percent pancreatic fraction are shown in Figure 1, parts a and c. The former ranged from 47 to 116 U/L (mean = 62, SD 31 U/L), the latter from 24% to 67% (mean 49%, SD 11%), in good agreement with mean values (49–52%) published earlier (16, 20).

Immunochromosomal analysis of macroamylasemic sera. Two sets of macroamylasemic sera were analyzed by use of IMAB. The total and fractionated amylase results from both sets are shown in Figure 1, parts b and d. For Set 1 (n = 23), values for total amylase ranged from 99 to 1989 U/L (mean 407, SD 419 U/L) while per cent pancreatic values ranged between 15% and 97%, the mean being 53% (SD 23%), similar to the value obtained from analysis of normal sera (16). For Set 2 (n = 16), total amylase ranged from 158 to 1083 U/L (mean 396, SD 235 U/L), while per cent pancreatic values ranged between 9% and 95%. The mean per cent pancreatic amylase, 47% (SD 22%), and agreed well with the value obtained from analysis of normal sera. Results for the two sets of samples were statistically indistinguishable. For both sets of macroamylasemic sera, the mean per cent pancreatic amylase did not differ significantly (P > 0.15) from the mean per cent pancreatic amylase for the reference samples.

Electrophoresis of macroamylase samples. Aliquots of sera from Set 1 (n = 23) were subjected to fixed-voltage electrophoresis in agarose and then made visible as described. The electrophoretic patterns were then compared with the fraction of pancreatic amylase (Figure 2). We saw no correlation between a sample's electophoretic pattern and its amylase composition. Whereas most electrophoretic patterns consisted of the well-known "smear," only sharp distinct bands were observed for one sample after electrophoresis, one of which co-migrated with salivary amylase; the others had different mobilities. This pattern of distinct bands after electrophoresis has also been observed (10, 23) for other macroamylase samples.

When the supernates of the TOTAL and PANCREATIC tubes from Set 1 were analyzed by agarose electrophoresis, interesting patterns of amylase activity (i.e., residual vs removed) were observed. Figure 3 shows six sample pairs that illustrate typical results for this analysis. Although some amylase activity obviously was removed after treatment with anti-salivary IMAB, a portion of activity remained in the "smear" form.

In 71% (17 of 23) of the macroamylasemic sera we studied using immunosorption and electrophoresis, the macroamylase appeared to contain both salivary and pan-
creatic amylase as judged by the partial removal of the macroamylase by the monoclonal antibody. In 4% (1 of 23) a distinct salivary amylase band also was observed, whereas in 65% (15 of 23) a separate, distinct pancreatic amylase band was observed. This difference (4% vs 65%) may reflect the fact that many macroamylase "smears" simply overlapped the location of free salivary amylase but not that of free pancreatic amylase. We determined the identity of individual fractions by comparing the residual activity with mobilities of purified markers and the ability of this monoclonal antibody to bind quantitatively and remove purified salivary amylase (15). For 8% (2 of 23) of the samples, we noted that the "blurred" amylase activity pattern extended completely across the gel, with no interruptions.

Discussion

This study examined the ability of a monoclonal antibody to recognize its epitope when its antigen was complexed with endogenous antibody(ies). For comparison purposes, use of this monoclonal antibody to salivary amylase allowed us to accurately estimate pancreatic amylase activity in reference sera. The values for per cent pancreatic fraction agreed with values previously published by others (18) and from this laboratory (16, 20).

![Fig. 2. Representation of the mobilities of 23 macroamylases in agarose gel electrophoresis](image)

Mobilities of concurrently analyzed purified pancreatic amylase (V) and salivary amylase (V) markers are denoted for each sample. Sample application site is indicated by the dotted line. The cathode is on the left. Total amylase activity (U/L) and fraction of pancreatic amylase (%) for each sample are listed on the right.

![Fig. 3. Comparison of electrophoretic separation of total and residual pancreatic amylase activities in six selected macroamylasemic sera](image)

Analysis of two sets of macroamylasemic sera with anti-salivary IMAB suggested that the mean per cent pancreatic values did not differ significantly (P > 0.75) from the values for the reference sera or from previous data. We interpret this as evidence that this antibody to salivary amylase recognizes an epitope different from the one(s) bound by the endogenous (complexing) antibodies. This finding also suggests that analysis of sera (normal or macroamylasemic) with this monoclonal antibody should prove accurate results when amylase isoenzymes are separately estimated.

A few macroamylasemic samples (Figure 1) contained an increased proportion of the pancreatic isoenzyme. Possibly the salivary amylase epitope in these macroamylase complexes was not accessible to the murine antibody. Alternatively, we feel that this finding is compatible with the source of the sera, i.e., patients with suspected acute pancreatitis. Acute pancreatitis and macroamylasemia are known to coexist in some patients. In fact, the incidence of increased per cent pancreatic amylase in our study (5 of 39, Figure 1) is similar to the 10% (11 of 108) prevalence of acute pancreatitis among 108 macroamylasemic sera reviewed by Klomoff (4). Not all murine monoclonal antibodies to isoamylase appear to bind both complexed (macro) and uncomplexed isoamylases. The apparent low pancreatic isoamylase composition of six macroamylase samples that was reported recently (19) may well reflect an overlap between the respective epitopes of the endogenous antibody(s) and the murine anti-pancreatic monoclonal antibodies used in the assay (19).

The extent of the heterogeneity of most macroamylase complexes is not entirely clear. Our results and those of others (6, 24) indicate that most macroamylasemic sera contain both salivary and pancreatic amylases, thus producing some heterogeneity of the macroamylase complexes. Although the immunoglobulin content of some macroamylases reportedly is not monoclonal (e.g., based upon the mixed composition of heavy and light chain types), several investigators have published evidence that the immunoglobulin portion of many macroamylases is monoclonal. Evidence of monoclonality includes results of
Scatchard binding studies (25) and typing of the immunoglobulin heavy and light chains (23, 26). On the basis of these findings, we believe most macroamylases appear to be complexes formed between salivary and pancreatic amylases and a monoclonal antibody.

The available molecular data do not suggest, however, a molecular heterogeneity that could explain the preponderance of "smeared" patterns that we (Figure 3) and others (8-10, 23) see. We favor the interpretation that the "smear" on electrophoresis and the presence of (unbound) amylase isoenzyme bands on electrophoresis reflect dissociation of macroamylase complexes during electrophoresis. First, a smear similar to that obtained from natural macroamylases (10) occurred after monoclonal antisalivary antibody was mixed with purified amylase and electrophoresed (27). Secondly, one of us (R.W.F.) has found that in all but a few of several hundred macroamylases studied by use of column chromatography, the amylase activity was eluted completely in the high-molecular-mass fractions (i.e., >200 000 Da). This occurred even with specimens that appeared to have substantial "normally migrating" bands upon electrophoresis. Moreover, after purified pancreatic and salivary amylases were added to macroamylasic sera, the amylase activity was still eluted in the high-molecular-mass region (unpublished data, R.W.F.). Thus amylase appears to be bound entirely in most macroamylasic sera and, indeed, the sera have an additional capacity to bind amylase, a capacity that can be titered (25, 28).

The epitope recognized by this murine monoclonal apparently differs from the complexing epitope involved in the formation of most naturally occurring macroamylase complexes. This permits binding (and subsequent removal) of salivary amylase by the murine antibody. The present studies also suggest that the antibodies in most human macroamylases, like most mouse antibodies (16), are directed at epitopes that do not distinguish salivary from pancreatic amylase.

The antibody used in this study has been licensed by the University of Virginia to Hoffmann-La Roche (Roche Diagnostic Systems). The present studies were undertaken independently of that licensing and without financial support from Hoffmann-La Roche.

References

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