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1 [Review]

2 Laboratory and Clinical Features of Abnormal Macroeozymes Found in Human Sera

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5 **Key words:** Macroenzyme; Macro aspartate aminotransferase (AST); Macroamylase;
6 Macro lactate dehydrogenase (LD); Macro creatine kinase (CK).

7

8 **Abbreviations:**

9 EC, Enzyme Commission; AST, Aspartate aminotransferase; sAST, Supernatant
10 aspartate aminotransferase; mAST, Mitochondrial AST; Camy, Amylase clearance; Ccr,
11 Creatinine clearance; ALT, Alanine aminotransferase; LD, Lactate dehydrogenase; ALP,
12 Alkaline phosphatase; GGT, gamma-Glutamyltransferase; LAP, Leucine
13 aminotransferase; IgA, Immunoglobulin A; IgG, Immunoglobulin G, IgM,
14 Immunoglobulin M; Fab, Fab fragment of immunoglobulin molecule; Fc, F(ab')₂,
15 F(ab')₂ fragment of immunoglobulin molecule; Fc fragment of immunoglobulin
16 molecule; HPLC, High performance liquid chromatography.

1

2 **Abstract**

3 We report the analysis of unusual macroenzymes, performed in our laboratory,
4 and review the relevant literature. In particular, we focused on macro AST,
5 macroamylase, macro LD and macro CK. Macroenzymes are seen in healthy subjects,
6 but can also be related to disease; thus, accurate detection is useful in day-to-day
7 clinical practice. The macroenzyme is thought to be a specific antigen-antibody
8 complex from following findings: (1) the complex could be dissociated under acidic pH
9 levels; (2) binding specificity of immunoglobulin in the complex was observed; (3) the
10 binding site of immunoglobulin in the complex was Fab portion; and (4) the maternal
11 IgG involved with macroenzyme was transferred to her children.

12

13 **Introduction**

14 Numerous patient serum samples are tested in biochemical laboratories every day.
15 Measurement of serum enzyme activity is one of the most common tests, and is
16 performed using computerized, automated analyzers. Although automated analyzers

1 have contributed greatly to mass analysis in clinical laboratories, there is an increasing
2 tendency to depend on them. Consequently, clinicians often fail to evaluate the
3 individual data printed out by these analyzers.

4 So-called spurious or factitious data are certainly present among the enormous
5 amounts of raw data obtained by analyzers, and these data are not derived from
6 mechanical and/or systematic errors. A typical example of these results is abnormal
7 high-mass molecular enzyme, so-called macroenzymes, found in human sera.
8 Macroenzymes are formed by association with serum immunoglobulins. At present,
9 numerous macroenzymes are known, for example, macro aspartate aminotransferase
10 [EC 2.6.1.1; AST] [1, 2], macroamylase [EC 3.2.1.1] [3, 4], macro lactate
11 dehydrogenase [EC 1.1.1.27; LD] [5, 6] and macro creatine kinase [EC 2.7.3.2; CK] [7,
12 8]. The existence of these macroenzymes has long been known, but they have only
13 recently become a problem in clinical practice. Here, we describe our analyses of
14 abnormal macroenzymes in our laboratory, and review the available data from previous
15 macroenzyme studies. It is important for clinical laboratories to change the
16 characterization of macroenzymes and their laboratory detection methods in order to

avoid errors.

1. History of Macroenzymes

In 1967, Berk et al. [3] reported the existence of macromolecular amylase in sera from patients with persistently elevated serum amylase. In these patients, there was no apparent cause for hyperamylasemia, and their renal function was essentially normal. This was the first description of a so-called “macroenzyme”. In 1968, Levitt and Cooperband [9] confirmed IgA as a binding protein in the abnormal macromolecular amylase in patient serum.

In 1965, Kreutzer [10] reported an anomalous electrophoretic pattern for lactate dehydrogenase (LD) isoenzymes. In 1967, Ganrot [5] reported macromolecular LD in serum from a patient with lupoid cirrhosis and an anomalous electrophoretic isoenzymic pattern, and identified IgA as the binding protein in the complex. The factors leading to discovery of macroamylase and macro LD were high serum activity and/or unusual patterns on electrophoretic isoenzymic analysis.

In the 1970s, macro alkaline phosphatase [EC 3.1.3.1] was reported by Nagamine et al. [11], macro aspartate aminotransferase [EC 2.6.1.1; AST] was reported by Kontinen

et al. [1], alanine aminotransferase [EC 2.6.1.2; ALT] was reported by Kajita et al. [12], and macro creatine kinase [EC 2.7.3.2; CK] was reported by Urdal et al. [7]. Moreover, in the 1980s, γ -glutamyltransferase [EC 2.3.2.2] was reported by Sudo et al. [13], acid phosphatase [EC 3.1.3.2] was reported by Virji [14], leucine aminopeptidase [EC 3.4.11.2] was reported by Maekawa et al. [15], and lipase [EC 3.1.1.3] was reported by Stein et al. [16]. In fact, Maekawa et al. [15] demonstrated various macroenzymes, including macro leucine aminopeptidase, in serum from a patient with rheumatoid arthritis. The author also reported unique and very interesting cases with mitochondrial AST-IgG complex [17] and with salivary amylase-IgG complex [18]. In 1989, Tozawa [19] reported the incidence of macroamylase and macro LD in patients and in blood donors; macroamylase was present in 0.18% and 0.04%, respectively, and macro LD was detected in 0.32% and 0.15%, respectively.

In 1990, we reported the incidence of macro AST in patients with high serum aspartate to alanine aminotransferase ratio (>2.0) using clinical laboratory data [20]; the incidence was 13.1%. It should be noted that macro enzymes are detected in infants and/or children [21-23], and we recently reported transient macro AST in infancy

caused by maternal IgG [24]. To date, there have also been several excellent reviews [21, 25-31].

2. Macro aspartate aminotransferase (AST)

2.1. Molecular forms of AST and Macro AST

AST has two isoenzymes, supernatant or cytosolic AST (sAST or cAST) and mitochondrial AST (mAST), which migrate to the α_2 -globulin region and to the slow γ -globulin region on electrophoresis, respectively [17, 32]. The molecular mass of each isoenzyme is approximately 100,000. Furthermore, these isoenzymes are immunologically independent, and immunochemical cross-reactivity has been reported between them isoenzymes in mammals [32-34]. Utilizing these properties, in 1978, Teranishi et al. [35] established an immunological method for determination of mAST activity using an anti-pig-sAST antibody. In 1983, Sakakibara et al. [36] developed a convenient staining method for electrophoresis of serum AST isoenzymes. AST isoenzyme analysis is important in the diagnosis of liver disorders and myocardial infarction, and determination of mAST is particularly useful in evaluating the severity

of liver and myocardial damage [37].

In 1978, Konttinen et al. [1] reported, for the first time, macro AST in sera from two healthy women with an unexplained persistent elevation of AST activity. Electrophoresis of serum AST isoenzymes displayed an abnormally moving fraction, which migrated between the sAST and mAST positions. One of these two cases was confirmed to be an IgG complexed-AST. Similar cases were also reported by Nakajima et al. [38] in 1980 and Weidner et al. [39] in 1983. Nakajima et al. [38] confirmed that macro AST consists of IgG and sAST isoenzyme. In 1983, Nagamine and Okochi [40] reported that a macro AST of IgG, IgA- κ , λ was present in serum from a patient suffering from lung cancer with metastasis to the liver. They demonstrated that the IgG in the complex binds to sAST, whereas IgA binds to both aAST and mAST isoenzyme [40]. In 1986, the author et al. [17] reported a macro AST of mAST linked to IgG in serum from a patient with benign hypertension. We demonstrated that macro AST is a specific antigen-antibody complex using papain digestion of the IgG in the complex. There have since been a number of papers published describing macro AST [2, 20, 22-24, 31, 41-70].

2.2. Characterization of our cases of macro ASTs

Table 1 shows a summary of the classes and types of immunoglobulins in macro ASTs identified to date in our laboratory, together with those in other macroenzymes. Fifty-four cases with macro AST have been studied to date. The bound immunoglobulins were confirmed as IgA in 32 cases (59.2%), IgG in 14 cases (25.9%), IgG-IgA in 7 cases (13.0%), and not determined in one case. IgA-type macro AST was found most frequently, but the difference was not significant. The average age at discovery was 44.1 years, ranging from 3 days [24] to 84 years (standard deviation, 26.5 years). Thirty cases were male and 24 were female. Total serum AST activity in each of these cases was above the upper value of the normal range (30 U/l), ranging from 51 to 1,408 U/l, except in one mother-to-child case [24] (27 U/l). The mean and standard deviation were 377 and 327 U/l, respectively. The mean and standard deviation of the ratio of AST to ALT were 9.1 and 14.7, respectively, ranging from 1.3 to 70.7. In reconstitution studies (n=10), one case (IgG- κ , λ) was reconstituted with mAST [17], one case (IgG- κ) was reconstituted with sAST and mAST [24], and 8 cases (2 cases of IgA- λ and IgG, IgA- κ , λ , 3 cases of IgG- κ , and 1 case of IgG, IgA- λ) were reconstituted

1 with sAST.

2 In all cases, one or two macromolecular AST peaks were confirmed by gel
3 permeation chromatography. The amount of free-AST-molecule was observed to vary
4 according to those cases. Typical isoenzyme electrophoretic patterns with macro AST
5 are shown in Fig. 1A. All abnormal bands of IgG complexed-AST showed migration
6 between the sAST and mAST band, while that of IgA complexed-AST showed slightly
7 cathodal migration to the sAST position. However, in the majority of the patients with
8 IgA complexed-AST, the abnormal bands were seen faintly or not detected on ordinary
9 AST isoenzyme electrophoresis [20]. We believe that these abnormal electrophoretic
10 patterns indicate the type, amount and isoenzyme specificity of bound immunoglobulin,
11 from the above studies.

12 In Table 2, morbidities of cases with macro AST for diseases classified according to
13 the international classification of disease [71] are shown, together with those for other
14 macroenzymes. In 54 patients with macro AST, neoplasms (35.2%) including primary
15 and metastatic liver cancer, colon cancer, pancreatic cancer and malignant lymphoma
16 were most common. Diseases of the digestive system (22.2%) including liver cirrhosis,

cholelithiasis and chronic hepatitis were second in frequency. The rest of the cases were patients with circulatory diseases (acute myocardial infarction, abdominal aortic aneurysm and hypertension), and with endocrine and metabolic diseases (hypothyroidism, pituitary puerilism and diabetes mellitus). No patients with autoimmune diseases were seen as far as we have examined. Six patients (11.1%) with no symptoms other than hyper-serum AST activity and abnormal isoenzyme electrophoretic pattern were seen. It is also remarkable that 2 cases with liver cancer and hypothyroidism, respectively, showed both macro AST and macro CK type-1, as reported by Etienne et al. [31].

3. Macroamylase

3.1. Molecular forms of amylase and macroamylase

Human serum amylase can be separated into two principal isoenzymes, pancreatic and salivary amylase, and their isoforms [72-74]. The molecular mass of amylase is, unlike other major serum enzymes, smaller than serum albumin, approximately 60,000 [75]. Consequently, amylase is readily excreted in urine with normal renal function. In

1 1969, Levitt et al. [76] reported that the high serum amylase levels found in patients
2 with macroamylasemia are associated with an extremely low ratio of amylase clearance
3 to creatinine clearance (Cam/Ccr ratio), in contrast to the situation in acute pancreatitis.
4 However, In 1973, Berk et al. [77] reported that the Cam/Ccr ratio was markedly
5 decreased in serum from patients with salivary hyperamylasemia. Consequently, there is
6 a limit to the interpretation of Cam/Ccr ratio. In 1968, Levitt and Cooperband [9]
7 directly identified IgA as a binding protein using an immune-absorption technique.
8 They also demonstrated some important characteristics of the complex: (1) the
9 remaining amylase in serum, intestinal fluid, urine and saliva is of normal size; (2) the
10 complex dissociates at acidic pH levels; (3) the IgA isolated by acid dissociation binds
11 with normal serum amylase; and (4) macroamylase results from binding between
12 normal amylase and abnormal IgA in a unique autoimmune reaction. This report was of
13 great significance in the investigation of macroamylase and macroenzymes.

14 In 1974, Fridhandler et al. [78] showed that the binding immunoglobulins
15 displayed more or less affinity for salivary amylase in reconstitution studies of 30 cases.

16 In 1975, Harada et al. reported a unique (non-fractionation) band, namely the broad

band, in the electrophoretic patterns of serum samples with macroamylase. In 1976, Kanno et al. [79] demonstrated that the binding site of amylase-linked IgG was located in the Fab portion of the IgG molecule and that the complexes are specific antigen-antibody complexes. The author also reported the same findings for IgG [18] and IgA [80] molecules in complexes. Moreover, we reported for the first time a specific salivary amylase-IgG complex in serum from a patient with colon cancer and hyperamylasemia. Until that time, it had been considered that immunoglobulins recognized both salivary and pancreatic amylase because of immunological cross-reactivity. However, at present, monoclonal antibodies that distinguish between salivary and pancreatic amylase are available [81, 82].

In contrast, there have been some reports describing a macroamylase complex with a specific substance, hydroxyethyl starch [83-86]. Various species of hydroxyethyl starch have been developed for use in the management of hypovolemia, and intravenous infusion results iatrogenic macroamylasemia. However, we confirmed that anomalous bands were present in patient sera only immediately after or two hours after termination of infusion by isoenzyme electrophoretic analysis [87]. Therefore, this macroamylase is

1 rarely found in the clinical laboratories. Accordingly, the descriptive term
2 “macroamylase” is now mostly applied to amylase-immunoglobulin complexes. In 1989,
3 Tozawa reported that the incidence of macroamylase in patients and in blood donors
4 was 0.18% and 0.04%, respectively. A number of papers describing macroamylase have
5 also been published since the 1990s [4, 21, 88-99].

7 **3.2. Characterization of our cases of macroamylases**

8 Forty-seven cases with macroamylase have been studied to date (Table 1 and
9 2). The bound immunoglobulins were identified for all cases; IgA in 39 cases (82.9%,
10 $p < 0.05$), IgG in 7 cases (14.9%) and IgG-IgA in one case. The average age at discovery
11 was 54.3 years (standard deviation, 19.3 years), ranging from 9 years to 84 years.
12 Twenty-eight cases (64.8%) were male and 19 cases (35.2%) were female. The mean
13 and standard deviation of total amylase activity were 516.6 U/l and 43.9 U/l,
14 respectively, ranging from 95 U/l to 2,225 U/l, and almost all (89.4%) cases were above
15 the upper value of the normal range (175 U/l). In reconstitution studies (n=14), one case
16 was only reconstituted with salivary amylase [18] and pancreatic amylase, but the

1 remaining cases were reconstituted with both amylase isoenzymes. A clear
2 macromolecular peak was confirmed in all but two cases, whose amylase activities were
3 detected from a normal-sized amylase molecule peak. During dextran and/or agarose
4 gel permeation chromatography, it is generally observed that the elution of amylase
5 molecules was considerably slower, because these have a chemical structure similar to
6 the substrate of amylase [75, 100]. However, the amylase-immunoglobulin complex is
7 eluted at a similar molecular weight position on gel permeation chromatography.
8 Typical isoenzyme electrophoretic patterns for macroamylase are shown in Fig. 1B.
9 These cases were commonly indicated by a characteristic broad pattern that could not be
10 separated into pancreatic (P) and salivary (S) amylase bands. The broad bands starting
11 from the various positions of P1, P2, S1 and S2 bands showed various widths. We also
12 believe that these abnormal electrophoretic patterns indicate type, amount and
13 isoenzyme specificity of the bound immunoglobulin.

14 In 47 cases with macroamylase, diseases of digestive system (25.5%) were
15 most common. Among the subjects, 4 of 12 cases showed clear evidence of pancreatitis,
16 and one patient with an autoimmune disease, ulcerative colitis, was seen. Other

conditions included gastric ulcer, chronic gastritis and cholelithiasis. There were 9 patients (19.1%) with neoplasms, including pancreatic cancer, colon cancer, gastric cancer, lung cancer, liver cancer and malignant lymphoma. Clinical diagnoses of the patients showed a range of diseases and symptoms, but there were seven patients (14.9%) with no symptoms other than hyperamylasemia.

4. Macro lactate dehydrogenase (LD)

4.1. Molecular forms of LD and macro LD

LD is a tetrameric molecule composed of two subunits designated M (muscle) or A and H (heart) or B. The five isoenzymes are numbered as follows: LD1 (H₄), LD2 (H₃M), LD3 (H₂M₂), LD4 (HM₃) and LD5 (M₄). The relative molecular mass of each subunit is 34,000, and that of each isoenzyme is 134,000 [101, 102]. The determination of LD isoenzyme electrophoresis was the first routine technique in enzymology to be widely adopted by clinical laboratories. In 1963, genetic variants of LD isoenzymes were simultaneously reported by Boyer et al. [103] and Nance et al. [104]. Furthermore, a case of complete deficiency of the H subunit was reported by Kitamura et al. [105] in

1971, and complete M subunit deficiency was reported by Kanno et al. [106] in 1980. These electrophoretic patterns, which reveal variants and/or subunit deficiency, thus showed the expected abnormal pattern.

In contrast, macro LDs are indicated by various anomalous electrophoretic patterns characterized by additional bands, broadening bands, and smearing of LD activity between the typical bands [28]. In 1965, macro LD was confirmed simultaneously by Ganrot [5] and Lundh [107] in patients with lupoid cirrhosis and leukemia, respectively. All reported cases [5, 107-109] of macro LDs have been in patients with underlying basal diseases. However, in 1972, Nagamine [110] reported two cases of macro LD with no abnormalities other than LD activity. In a series of energetic studies by Biewenga et al. [109, 111-117], interesting characteristics of the complex were confirmed: (1) most LD-IgA complexes were restricted to κ type; (2) IgAs were autoantibodies against human LD; and (3) LD-IgG complexes were restricted to IgG3 of IgG subclass. Moreover, Weijers et al. [118-120] demonstrated that IgAs in macro LDs were autoantibodies against LD3. There was an obvious difference in isoenzyme specificity between IgA and IgG in macro LDs in several reconstitution

1 studies [119-123], while the equilibrium constants for LD-IgG complexes were reported
2 by Sudo et al. [124] in 1985. In contrast, it was found that the LD-IgG complex was
3 dissociated by addition of nicotinamide adenine dinucleotide, a coenzyme of LD
4 [125-127]. Conceivably, IgG and coenzyme compete for the same binding site. Fujita
5 [128, 129] demonstrated that LD combines with BJP (or IgG) at the coenzyme binding
6 site, producing a three-dimensional structure similar to the coenzyme. These reports
7 suggest that immunoglobulins have various binding patterns against the LD molecule.

8 On the other hand, there is also another category of macro LDs whose LD
9 activities are partially or fully inhibited by bound immunoglobulins [130-134]. In the
10 reports, the immunoglobulins bind and inhibit the LD subunits. This phenomenon is
11 thought to be as acquired hemophilia A [135, 136]. Tozawa [19] reported that the
12 incidence of macro LD in patients and in blood donors were 0.32% and 0.15%,
13 respectively. From the standpoint of population screening in a local town, we confirmed
14 6 cases (0.36%) with macro LD among 1,666 subjects (not published data), and we
15 include these data in this paper. A number of papers describing macro LDs have also
16 been published since the 1990s [6, 130, 137-141].

4.2. Characterization of our cases of macro LDs

Thirty-seven cases with macro LD have been studied to date (Table 1 and 2). Bound immunoglobulins were confirmed as IgG in 19 cases (51.3%), IgA in 16 cases (43.2%), and IgG-IgA in 2 cases (5.5%). The average age at discovery was 58.4 years, ranging from 19 years to 81 years (standard deviation, 16.4 years). Twenty-three cases (62.1%) were male and 14 cases (37.9%) were female. Total serum activity in each of these cases ranged from 125 U/l to 1,792 U/l, and most (70.3%) of the cases were above the upper value of the normal range (220 U/l). The average and standard deviation were 378.6 U/l and 1,792 U/l, respectively. In reconstitution studies (n=17), IgGs (n=6) were reconstituted with all LD isoenzymes in 5 cases, and with LD2, 3, 4 and 5 isoenzymes in one case. IgA (n=11) was reconstituted with LD2, 3 isoenzymes in 4 cases, LD3 isoenzyme in 3 cases, LD2, 3 and 4 isoenzymes in 2 cases, LD1, 2 and 3 isoenzymes, and LD4 and 5 isoenzymes in one case. There was also an obvious difference in isoenzyme specificity between IgA and IgG in macro LDs in several reconstitution studies [119-123]. Typical isoenzyme electrophoretic patterns with macro LDs are shown in Fig. 1C. These abnormal electrophoretic patterns indicate type and isoenzyme

specificity of the bound immunoglobulin.

In 37 cases with macro LD, diseases of the circulatory system (32.4%), including angina pectoris, aortic stenosis, apoplexy, arteriosclerosis, hypertension and arrhythmia, were most frequent. Digestive system (21.6%) including chronic hepatitis, cholelithiasis and liver cirrhosis were second in frequency. Subsequently, neoplasms (16.2%), including renal cancer, liver cancer, lung cancer and rectal cancer, were seen. No patients with autoimmune diseases were seen. There were 8 patients (21.6%) with no symptoms other than hyper serum LD activity or abnormal isoenzyme electrophoretic pattern. It should be noted that 6 patients (75%) were determined to have IgA-type macro LD.

5. Macro creatine kinase (CK)

5.1. Molecular forms of CK and macro CK

CK is a dimeric molecule composed of two subunits; muscle (M) and brain (B). The three isoenzymes are numbered CK1 (BB), CK2 (MB) and CK3 (MM). The molecular mass of each isoenzyme is approximately 80,000 [142-144]. In 1975, Velletri

1 et al. [145] reported an abnormal CK band that migrated to the cathodic side, to the
2 CK3 position, in serum from a patient with myalgia and depression. Later, similar cases
3 with an unusual CK isoenzyme band were reported [146-149]. In those reports, Yuu et
4 al. [149] demonstrated that the macromolecular CK was present in serum from a patient
5 with breast cancer. In 1979, Urdal and Landaas [7] reported, for the first time, the
6 CK1-IgG complex in serum from a patient with persistently abnormal CK activity. In
7 the same year, several papers describing macro CK were published [150-154]. They
8 demonstrated several characteristics of macro CKs: (1) the unusual band generally
9 migrated between CK2 and CK3 band and/or migrated cathodically to the CK3 position
10 on isoenzyme electrophoresis; (2) total CK activities in patient sera was usually
11 elevated; and (3) macro CKs apparently showed CK1 nature, but there was no reason to
12 suspect any abnormal release of CK1 based on CK activity measurement using
13 antibodies against CK-M or CK-B subunit. In 1981, Stein et al. [155] found that
14 CK1-IgG complex was a specific antigen-antibody, and Urdal and Kierulf [156]
15 established a sensitive and specific radioimmunoassay for CK1 isoenzyme in serum,
16 with the use of an autoantibody from patient serum with macro CK1. Yuu et al. [157]

1 reported that CK activity was not inhibited by anti CK-M antibody in patient serum
2 with CK-IgA complexes.

3 These data show that the presence of these complexes gives false-positive
4 results for CK-B in immune-inhibition test. Subsequently, a case with false-positive
5 CK-MB activity was reported using the immune-inhibition test for CK-MB activity
6 [158]. Of course, the most important diagnosis based on CK isoenzyme analysis is in
7 acute myocardial infarction corresponding to increased CK-MB. Accordingly, such
8 false-positive laboratory diagnoses are becoming a more important problem in routine
9 CK assay in clinical laboratories [159-162]. In 1979, Prabhakaran et al. [154] reported
10 that there is a tendency for IgG and IgA to bind to CK1 and CK3 isoenzyme,
11 respectively. However, Mederios et al. [163] and Venta et al. [164] reported that IgA
12 also bound with CK1 isoenzyme and that this complex migrated to the same position as
13 CK2 on electrophoresis.

14 On the other hand, there is another type of macro CK (type-2), mitochondrial
15 CK, with the same properties as immunoglobulin-bound macro CK (type-1), as reported
16 by James et al. [165], Wevers et al. [166] and Kanemitsu et al. [167]. They

demonstrated the following properties: (1) it migrates cathodically to the CK3 position on isoenzyme electrophoresis; (2) it indicates macro CK; (3) it is not inhibited by CK-M subunit antibody; and (4) it as thermostable as macro CK type-1, as reported by Bohner et al. [168]. It is very difficult to distinguish both types of macro CK by comparative study of their properties. In 1982, Stein et al. [169] reported that both macro CKs could be distinguished by determination of differential activation energies. The activation energy of normal CK and macro CK type-1 are mostly in the range of 40~65 kJ/mol substrate, but that of macro CK type-2 is more than 80 kJ/mol. Kanemitsu et al. [170-174] therefore described research results specifically for macro CK-type 2.

Unless indicated otherwise, macro CK is referred to as type-1 in this paper. In 1983, Whelen and Malkus [175] reported that macro CK confirmed in the serum of apparently healthy individuals whose values for total CK activity were within normal limits. Most macro CKs are associated with basal disease, particularly cardiac diseases or cancer; Kanemitsu [176] reported that the positivity rate for macro CK was significantly higher than the rates in patients with benign diseases or healthy individuals. Subsequently, the prevalence of macro CK in cardiac diseases was reported to be 4.3% by Dalanghe et al.

[177]. Tozawa [19] reported that incidence of macro CKs in patients and in blood donors was 0.61% and 0.23%, respectively. Laureys et al. [162] reported the same incidence in a group of patients in 1991. In 1992, Bayer and Kraus [178] reported an immunological suggestive case of multiple occurrence of macro CK in one family: a mother and two children, each child at an age of approximately 2-month-old. They consider that the maternal IgG involved with macro CK had been transferred the placenta from mother to children. In 2010, Yasuhara et al. [24] the same case was reported in macro AST. A number of papers describing macro CKs have also been published since the 2000s [31, 179-182].

5.2. Characterization of our cases of macro CKs

Thirty-two cases with macro CK-type 1 and four cases with macro CK-type 2 have been studied to date (Table 1 and 2). In macro CK-type 1, bound immunoglobulins were confirmed as IgG in 7 cases (21.9%), IgA in 18 cases (56.3%), IgG-IgA in 5 cases (15.6%) and not determined (not macro CKtype-2) in 2 cases (6.3%). The average age at discovery was 56.4 years. Age ranged from 1 year to 90 years and standard deviation

was 24.6 years. Seventeen cases (53.1%) were male and 15 cases (46.9%) were female. Total serum activity in each of these cases ranged from 55 U/l to 5,505 U/l, and almost (59.4%) all cases were above the upper value of the normal range (male, 216 U/l; female, 165 U/l). Average and standard deviation were 773.4 U/l and 1147.2 U/l, respectively. The average (n=17) for CK-MB (%) determined by the immune-inhibition test using anti-CK-M subunit antibody was 110.6, and ranged from 1.4 to 192.0 (standard deviation, 77.6). Average (n=15) activation energy (kJ/mol) was 50.5, and ranged from 15.6 to 82.4 (standard deviation, 16.5). In contrast, in patients with macro CK type-2 (n=4), average age, total CK activity (U/l), CK-MB (%) and activation energy (kJ/mol) were 66 years (range, 47 to 74 years), 178.5 U/l (range, 44 to 432 U/l), 123.6% (range, 86.8 to 151.1%) and 91.5 kJ/mol (range, 86.7 to 100.0 kJ/mol), respectively.

In reconstitution studies (n=6), bound IgGs in three cases and IgAs in three cases were reconstituted with CK1 and CK3, respectively, as reported by Prabhakaran et al. [154]. In all cases with macro CK type-1 and type-2, one or two macromolecular CK peaks were confirmed on gel permeation chromatography. In cases with macro CK

type-2, there were two or three macromolecular peaks, at molecular masses of 160,000, 520,000 and 1,000,000. Typical isoenzyme electrophoretic patterns of macro CK type-1 are shown in Fig. 1D-1, lane 2-5, and those of macro CK type-2 are shown in Fig. 1D-2, lane 2-3. Patient serum with macro CK type-1 indicated various patterns characterized by additional bands, changes in intensity of typical CK3 bands, and broadening bands. Additional bands typically appeared at the post-CK3 band or between CK2 and CK1 bands. Anomalous bands in all 7 cases with IgG type complexes were detected between the CK3 and CK2 bands. In IgA type complexes, 13 (72.2%) of 18 cases were detected at the post-CK3 band, 4 cases were detected at the same position as the CK3 band, and one case was detected between CK3 and CK2 bands.

Macro CK type-2 was present in 4 patients; one patient with gastric cancer, one with breast cancer, one with liver cancer, one with senile contracts. In 32 patients with macro CK type-1, neoplasms (43.8%) including liver cancer, gastric cancer, pancreatic cancer, bladder cancer, prostate cancer and lung cancer, were most frequent. Moreover, 7 patients (41.2%) of 17 with malignancies and macro CK type-1 and 2 were terminal. The remaining patients had benign diseases, but this finding of macro CK-associated

cancer was remarkable. In those subjects, no patients with autoimmune diseases were seen. Three patients (9.4%) with no symptoms other than hyper serum CK activity and abnormal isoenzyme electrophoretic patterns were seen. It is remarkable that 2 cases with liver cancer and hypothyroidism, respectively, had both macro CK type-1 and macro AST, as mentioned above.

6. Others

In 1975, Nagamine and Ohkuma [11] reported ALP-IgG complex in skeletal abnormalities due to multiple epiphyseal dysplasias. The electrophoretic pattern indicated a slow migration pattern. The bound IgG had the ability to bind hepatic and osseous isoenzymes. Subsequently, many cases of macro ALP have been reported [183-192]. In these reports, the following characteristics of macro ALPs were demonstrated: (1) most were bound to IgG; (2) isoenzymes in the complexes were all of the liver or bone type, except in one case; (3) most of the complexes showed a broad band at the intestinal ALP on electrophoresis except in one case; (4) macro ALP had a specific antigen-antibody complex; and (5) ALP-IgG complexes were prevalent in

1 serum from patients with ulcerative colitis. In 1983, Nakagawa et al. [193] reported a
2 unique case of macro ALP (ALP linked IgG-IgA complex), in which the IgG had the
3 ability to inhibit ALP in patient serum, as an inhibitory antibody against the LD-subunit
4 described in macro LD. In contrast, Sudo and Kanno [13] demonstrated IgA in all
5 patients (n=23) with macromolecular membrane-associated enzyme complexes (ALP,
6 GGT and LAP) in 1980, whereas Artur et al. [194] demonstrated IgA in 10 patients with
7 hepatobiliary disease. However the antigen-antibody nature of the interaction was not
8 investigated in 1984.

9 In 1978, Kajita et al. [12] reported that macro ALT was present in 16 (3.2%)
10 of 500 patients with chronic liver disease. However, no reports of macro ALT have been
11 published to date. In 1985, Virji [14] reported that prostatic acid phosphatase [EC
12 3.1.3.2]–IgG complex was found in the serum from a patient with Sjogren's syndrome
13 and elevated prostatic acid phosphatase activity. In 1986, Maekawa et al. [15] found that
14 several complexes of enzymes and immunoglobulins were detected at the same time in
15 the serum of a patient with seropositive rheumatoid arthritis. In 1987, Stein et al. [16]
16 reported that lipase-IgG complex was present in the serum of a patient with malignant

1 non-Hodgkin's lymphoma. In 1990, Viallard et al. [195] reported that enolase [EC
2 4.2.1.11]-IgG complex was present in the serum of a patient with squamous cell lung
3 cancer.

4 The question is finally remained why autoantibodies occur against serum
5 enzymes. Unfortunately, that is not fully understood at present. However, immune
6 complexes with numerous serum bioactive substances have been also reported in recent
7 years [135, 196-208]. For example, blood coagulation factor, various hormones
8 (triiodothyroxine, thyroxine, chorionic gonadotropin, pituitary hormones and so on),
9 vitamin B12, myocardial markers and so on complexed with immunoglobulin. The
10 autoantibodies' interference with routine clinical chemical tests is becoming
11 increasingly recognized as a discrepant result. We have to consider in connection with
12 them and we do not miss them. Accurate detection is useful in the day-to-day clinical
13 practice.

14 15 **7. Identification Methods for Macroenzymes**

16 Upon identification for macroenzymes, we always perform by a combination of

following identification methods and gel filtration assay. In some cases that was containing little amount of macromolecular enzyme in gel filtration assay, we had to apply two identification methods to identify bound immunoglobulin. However, a few cases were not able to identify the bound immunoglobulin class and type (Table 1). In those cases, a reasonable macromolecular peak was detected in gel filtration assay. Below, we briefly describe in descending order the detection sensitivity for the identification methods of the bound immunoglobulin and described their merits and demerits.

7.1. Immunoprecipitation reaction

This technique was developed for the identification of macroenzymes by Tozawa in 1991 [28]. Patient serum is mixed with each type of antiserum at an appropriate antigen/antibody ratio, incubated at 4°C overnight, and the resultant precipitate is separated and washed several times with phosphate buffered saline (pH 7.4). Isoenzymic reagents are then added directly to the precipitates, and enzyme activity is measured by spectrophotometry after centrifugation. Fig. 2A shows the results using this method for macro AST. This method is the most highly sensitive, but antiserera

large amount of need.

7.2. Counter current immunoelectrophoresis

This classical technique was first applied to identify macro AST by Nagamine and Okochi [40] in 1983. We use a modified version; antisera are applied at 5 mm from the cathode side from the application position of serum sample and after electrophoresis, the supporting membrane is directly subjected to enzymatic staining. We are then able to observe the identification results and isoenzyme electrophoretic patterns. Fig. 2B shows the identification results using this method of macroamylase. This method has a great advantage that the patient's serum electrophoretic pattern and the identification results can be verified at a same time.

7.3. Immunoelectrophoresis

Since 1970, beginning with the study of Biewenga and Thijs [109], immunoelectrophoresis on a 1.0% agarose gel plate has been used for identification of macroenzymes. The same method using cellulose acetate membranes has also been used, particularly for macroamylase [18]. Fig. 2C shows the results using this method for macro LD. This method is time-consuming to detect but the persuasive and attractive

ways.

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Table 1. Classes and types of immunoglobulins in macroenzymes

A, immunoglobulin A; G, immunoglobulin G;

ND, not-determined.

*, $p < 0.005$ (IgA-type vs IgG-type)

Table 2. Morbidities of cases with macroenzymes classified according to international classification of diseases

Figure legends

Fig. 1 Typical isoenzyme electrophoretic patterns of patient sera with macroenzymes [209].

A, Macro AST.

sAST, supernatant aspartate aminotransferase; mAST, mitochondrial aspartate aminotransferase; 1, control serum from acute hepatitis; 2, sAST-IgG, IgA- λ complex; 3, sAST-IgA- λ complex; 4, sAST-IgG- κ complex; 5, sAST-IgG- κ , λ complex; 6, mAST-IgG- κ , λ complex.

B, Macroamylase [18].

P1, pancreatic amylase 1; S1, salivary amylase1; 1, Control serum; 2, IgA- λ complex; 3, IgA- κ complex; 4, IgA- κ , λ complex; 5, IgG- κ complex; 6, IgA- λ complex. The cases in lanes 2, 3, 4 and 6 were reconstituted with both salivary and pancreatic amylase. The case in lane 5 was only reconstituted with salivary amylase. Remain cases were reconstituted with salivary and pancreatic amylase.

C, Macro LD.

1, LD control serum; 2, IgG- κ , λ complex; 3, IgG- κ , λ complex; 4, IgA- κ complex; 5,

IgG- λ complex; 6, IgG- κ , λ complex; 7, IgG- κ type. In reconstitution study, patient IgG in lane 2 was reconstituted with LD1~5 isoenzymes. Patient IgG in lanes 5, 6 and 7 were reconstituted with LD2-5 isoenzymes. IgA in lane 4 was reconstituted with LD2, LD3 and LD4 isoenzyme. Dot marks indicate the position of each LD isoenzyme.

D, Macro CK.

1) Macro CK type-1. 1, CK control serum; 2, IgG, IgA- κ , λ complex; 3, CK₁-IgG- κ , λ complex; 4, CK₁-IgG- κ complex; 5, CK₃-IgA- λ complex.

2) Macro CK type-2. 1, CK control serum; 2, 3, patient serum with macro CK type-2 (mitochondrial CK). The arrow indicates abnormal band.

Dot marks indicate the position of each CK isoenzyme.

Fig. 2 Identification methods for macroenzymes.

2A. Immunoprecipitation reaction for macro AST

1, Patient's serum mixed with anti-IgG antibody; 2, patient's serum mixed with anti-IgA antibody; 3, patient's serum mixed with anti-IgM antibody; 4, patient's serum mixed with anti-L κ antibody; 5, patient's serum mixed with anti-L λ antibody; 6, reagent blank. Tube number 1, 2 and 5 were significantly colored, then bound immunoglobulin was identified as IgG, IgA- λ type.

2B. Counter current immunoelectrophoresis for macroamylase [74]

1, Patient' serum and anti-L, λ antibody; patient' serum and anti-L, κ antibody; 3, patient' serum and anti-IgM antibody; 4, patient' serum and anti-IgA antibody; patient' serum and anti-IgG antibody; 6, original patient' serum; 7, normal serum. O1 is origin of patient's serum and normal serum. O2 is origin of various antibodies. After amylase isoenzyme electrophoresis, the membrane was subjected to blue starch staining technique. Amylase activities were detected on the precipitation lines indicated by the arrows (lane 1 and 4). As bound immunoglobulin, IgA- λ was identified.

2C. Immunoelectrophoresis

N, Normal serum; P, patient's serum. Immunoelectrophoresis was performed 1.0% agarose plate, after incubation and washing, the plate was subjected to LD isoenzyme

1 staining technique (blue color). Finally, after drying the plate, the plate was subjected to
2 protein staining with ponceau 3R (red color). LD activities were detected in the
3 precipitation lines against anti-IgA and anti-L, κ antibody, indicated by the arrows. As
4 bound immunoglobulin, IgA- κ was identified.

Table 1. The classes and types of immunoglobulins of the macroenzymes

Macroenzyme	Class	κ	λ	κ, λ	ND	Total (%)
Macro AST	G	10	1	3	0	14 (25.9)
	A	0	31	1	0	32 (59.2)
	G, A	0	4	3	0	7 (13.0)
	ND	0	1	0	0	1 (1.9)
	Total	10 (18.5)	37 (68.5)	7 (13.0)	0	54 (100)
Macroamylase	G	6	1	0	0	7 (14.9)
	A	14	19	6	0	39 (82.9)*
	G, A	1	0	0	0	1 (2.2)
	Total	21(46.8)	20 (42.5)	6 (12.7)	0	47 (100)
Macro LD	G	8	3	7	1	19 (51.3)
	A	11	3	1	1	16 (43.2)
	G, A	0	0	1	1	2 (5.5)
	Total	19 (40.2)	6 (16.2)	9 (24.3)	3 (19.3)	37 (100)
Macro CK type-1	G	2	2	3	0	7 (21.9)
	A	0	11	5	2	18 (56.3)
	G, A	0	1	4	0	5 (15.6)
	ND	0	0	0	2	2 (6.3)
	Total	2 (6.2)	14 (43.8)	12 (37.5)	4 (12.5)	32(100)

A, immunoglobulin A; G, immunoglobulin G; ND, not-determined.

*, $p < 0.005$ (IgA-type vs IgG-type)

Table 2. Morbidities of cases with macroenzymes for diseases classified according to the international classification of diseases

Classification	Macro AST	Macroamylase	Macro LD	Macro CK Type-1	Macro CK Type-2
I. Certain infectious and parasitic diseases	1 (1.9)	1 (2.1)		1 (3.1)	
II. Neoplasms	19 (35.2)	9 (19.1)	6 (16.2)	14 (43.8)	3 (75.0)
III. Diseases of the blood and blood-forming organs and certain disorders involving the immune mechanism	1 (1.9)		2 (5.4)		
IV. Endocrine, nutritional and metabolic diseases	5 (9.3)	7 (14.9)		6 (18.8)	
V. Mental and behavioural disorders		2 (4.3)	1 (2.7)	2 (6.5)	
VI. Diseases of the nervous system	1 (1.9)	3 (6.4)			
VII. Diseases of the eye and adnexa					1 (25.0)
VIII. Diseases of the ear and mastoid process					
IX. Diseases of the circulatory system	4 (7.4)	1 (2.1)	12 (32.4)	4 (12.5)	
X. Diseases of the respiratory system		1 (2.1)			
XI. Diseases of the digestive system	12 (22.2)	12 (25.5)	8 (21.6)	2 (6.5)	
XII. Diseases of the skin and subcutaneous tissue	1 (1.9)	1 (2.1)			
XIII. Diseases of the musculoskeletal system and connective tissue	2 (3.7)	2 (4.3)			
IV. Diseases of the genitourinary system		1 (2.1)			
XV. Pregnancy, childbirth and the puerperium					
XVI. Certain conditions originating in the perinatal period	2 (3.7)				
XVII. Congenital malformations, deformations and chromosomal abnormalities					
XVIII. Symptoms, signs and abnormal clinical and laboratory findings, not elsewhere classified	6 (11.1)	7 (14.9)	8 (21.6)	3 (9.4)	
XIX. Injury, poisoning and certain other consequences of external causes					
XX. External causes of morbidity and mortality					
XXI. Factors influencing health status and contact with health services					
XXII. Codes for special purposes					
Total	54	47	37	32	4

Fig. 1 A

Fig. 1B

Fig. 1C

Fig. 1D

Fig. 2A

Fig. 2B

Fig. 2C