Properties of a Slow-Moving Band of Alkaline Phosphatase in a Patient with Liver Cirrhosis

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ABSTRACT

A slow-moving alkaline phosphatase was found by agar gel electrophoresis in serum of a patient with liver cirrhosis. This slow band was detected on clinical remission of hepatitis and disappeared, half a year later, on re-exacerbation of hepatitis. The slow band was confirmed to be the same one as that in ulcerative colitis; Both showed the same electrophoretic mobility or pattern, were made up of an immunoglobulin G - alkaline phosphatase complex in which alkaline phosphatase was derived from liver and bone, and were associated with abnormal immunoglobulin G in patient's serum. The immunoglobulin G - alkaline phosphatase complex was 280,000 in molecular weight and was considered to be composed of one molecule of immunoglobulin G and one molecule of alkaline phosphatase. The cause of forming immunoglobulin G - alkaline phosphatase complex seemed to be the presence of abnormal immunoglobulin G which can bind normal liver or bone alkaline phosphatase in serum.

Key words: alkaline phosphatase; immunoglobulin; liver cirrhosis

INTRODUCTION

The electrophoretically slow-moving, uncommon band of serum alkaline phosphatase (ALP), which was designated ALP_6 after its mobility on agar gel, has been reported occasionally[1)-3)]. This ALP isozyme was once considered to occur exclusively in active stages of ulcerative colitis[2)-4)].

However, it was recently detected in a variety of diseases and accordingly its diagnostic significance or disease specificity was questioned[1)5)6)]. Recently the slow-band ALP was suggested to be a complex of ALP and immunoglobulin G (IgG)[7)]. The occurrence of the slow-band ALP must be evaluated in terms of the ALP-IgG complex, even though the mechanism of the formation of the enzyme-immunoglobulin complex has not been elucidated and the origin of the slow-band ALP is disputed[2)-4),8),9)].

This is the first report of transient appearence of the slow-band ALP in a disease other than ulcerative colitis. The properties and origin of the slow-band ALP in this case were studied and compared with those of ulcerative colitis. Furthermore, the cause of formation and the molecular characteristics of IgG-ALP complex were also investigated.

CASE REPORT

The patient, an 82 year-old woman was admitted to Tokuyama Ishikai Hospital in December, 1976 for the evaluation of increased serum transaminase and alkaline phosphatase activities. Her affliction was diagnosed as liver cirrhosis. She recovered well and was in remission until the re-exacerbation in June, 1978. The slow-band ALP had not been present in July, 1977 and was discovered for the first time in January, 1978 during the remission of the hepatitis. It lasted for half a year and disappeared in June, 1978 simultaneous with the re-exacerbation of hepatitis. Serial laboratory data are shown in Table I. Serum protein electrophoresis revealed a prominent, broad γ-globulin band which comprised 23.5% of total protein. The results of single radial immunodiffusion were as follows; IgG 1,500 mg/dl (normal range, 1,000-1,500), IgA 140 (145-420), IgM 560 (28-132) and IgD 5 (2.8-9.6). Blood urea nitrogen, creatinine and electrolytes were all within normal limits. Serological examinations for C reactive protein, antistreptolysin 0, rheumatoid factor, anti-nuclear factor and syphilis were negative. Tests for hepatitis B surface antigen and antibody, and α-fetoprotein were also negative. Since the liver biopsy was not performed, the diagnosis was made based upon the physical examinations, clinical course and labolatory data.

MATERIALS AND METHODS

The investigation upon the ALP-IgG complex was performed on the patient's serum obtained in January, 1978. Sera which contain ALPs predominantly derived from liver, bone, placenta, or intestine were used

Table 1 Serial laboratory data

	July '77	January '78	June '78	normal range
serum protein (g/dl)	7.2	7.6	7.3	6.4–8.0
albumin (g/dl)	3.3	3.3	2.2	3.4–5.1
total bilirubin (mg/dl)	0.5	0.7	15.8	0.2–0.8
choline esterase (△pH)	0.47	0.54	0.25	0.73–1.14
ALP (K.A.)	14.8	16.6	23.8	3.8–11
GOT (mI.U.)	20	20	238	4–17
GPT (mI.U.)	9	12	56	1–17
γ-GTP (mI.U.)	40	16	101	0–35
zink sulfate turbidity test (Kunkel)	12	12	22	4–12
C_3 (mg/dl)	–	39.2	27.0	37–71
C_4 (mg/dl)	–	16.2	15.9	15–52
slow-band ALP	(−)	(+)	(−)	

as controls. Serum from a patient in active stage of ulcerative colitis was also used for comparison with the patient's serum. Total ALP activity was determined by a modified method of kind and King[10]. ALP isozymes were fractionated by agar and polyacrylamide gel (PAG) disc electrophoreses by the method of Wieme[11] and Smith et al[12]., respectively. Agarose gel (WAKAO gel) electrophorsis was used in the serial observation of ALP zymograms. After electrophoresis, the ALP bands were visualized by the enzymatic hydrolysis of α-naphthylphosphate and the simultaneous coupling of the product with Fast Blue BB Salts. Serum protein was stained by Amido Black 10 B. Immunoelectrophoresis was performed according to the method of Shirai[13] and was followed by ALP staining. Inhibition by L-phenylalanine, L-homoarginine and urea was carried out by the procedure of Iino[14], and heat inactivation was performed according to the method of Posen et al[15]. Neuraminidase treatment was performed as described by Usategui-Gometz[16]. Molecular weight was determined on a Sephadex G-200 column (100 × 1.5cm) which was equilibrated with 0.1 M carbonate-bicarbonate buffer, pH 10.1. An aliquot (0.5 ml) of sample was applied, and the column was eluted with the equilibration buffer under 20-cm H_2O of hydrostatic pressure. Every 0.5 ml of eluate was collected, and was diluted to 2.0 ml with the same buffer. Protein concentrations were determined by measuring the absorbance at 280 nm of each fraction. Alkaline phosphatase activities of the fractions were assayed by measuring the absorbance at 500 nm after adding β-naphthylphosphate and Fast Blue BB Salts and then being incubated for 15 min at 37°C. Chymotrypsinogen A, hen's egg albumin,

bovine serum albumin, catalase and ferritin were used for calibration. The same column was used throughout the molecular weight study.

RESULTS

Transient appearence of the slow-band ALP

The slow-band ALP had not been present in July, 1977 when only the liver ALP band was detected. The slow-band ALP was first discovered in January, 1978 with concomitant disappearance of the liver ALP band, and persisted for half a year, during which poriod the ALP activity of the slow band gradually decreased. It disappeared almost completely in June, 1978 and the liver ALP band reappeared instead with a minimal activity of intestinal ALP (Fig. 1).

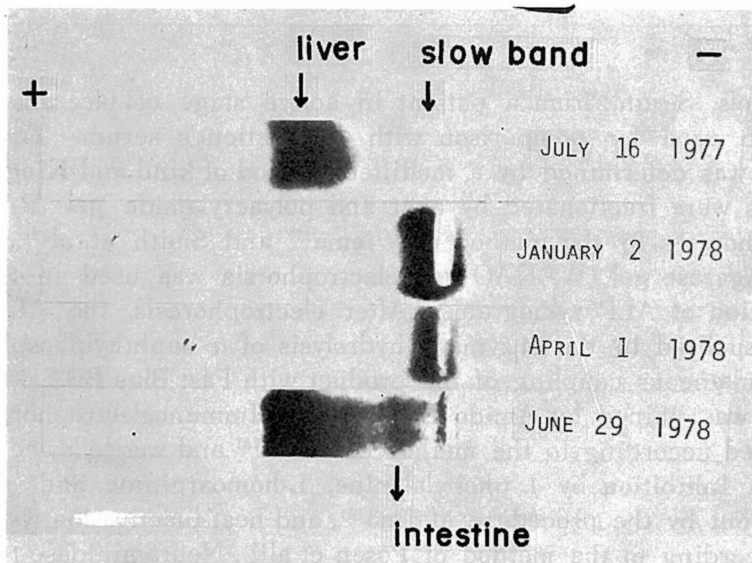

Fig. 1 ALP-zymograms after agarose gel electrophoresis The slow-band ALP remained at the origin.

Agar gel electrophoresis and PAG disc electrophoresis

The slow-band ALP appeared in the position of "slow-β" in the agar gel electrophoresis (Fig. 2a). It migrated toward the cathode and partially overlapped with the intestinal ALP. On the other hand, in the PAG disc electrophoresis, the slow-band ALP was retarded far behind the intestinal ALP (Fig. 2b). The slow band was relatively broad in band width on both of the electrophoreses. The electrophoretic mobility of the slow-band ALP of the patient was the same as that of a patient

with ulcerative colitis.

Immunoelectrophoresis

ALP activity was evident only in the arcs formed by the reaction between the patient's serum and anti-IgG or anti-λ antiserum, while the arcs formed by the reaction between the patient's serum and anti-IgA, anti-IgM or anti-κ antiserum did not have ALP activity. None of the control sera without slow-band ALP showed ALP activity in the arcs formed by the reaction with these antisera (Fig. 3).

The absorption of the immunoglobulin in the patient's serum by an anti-IgG or anti-λ antiserum removed the slow-band ALP. On the other

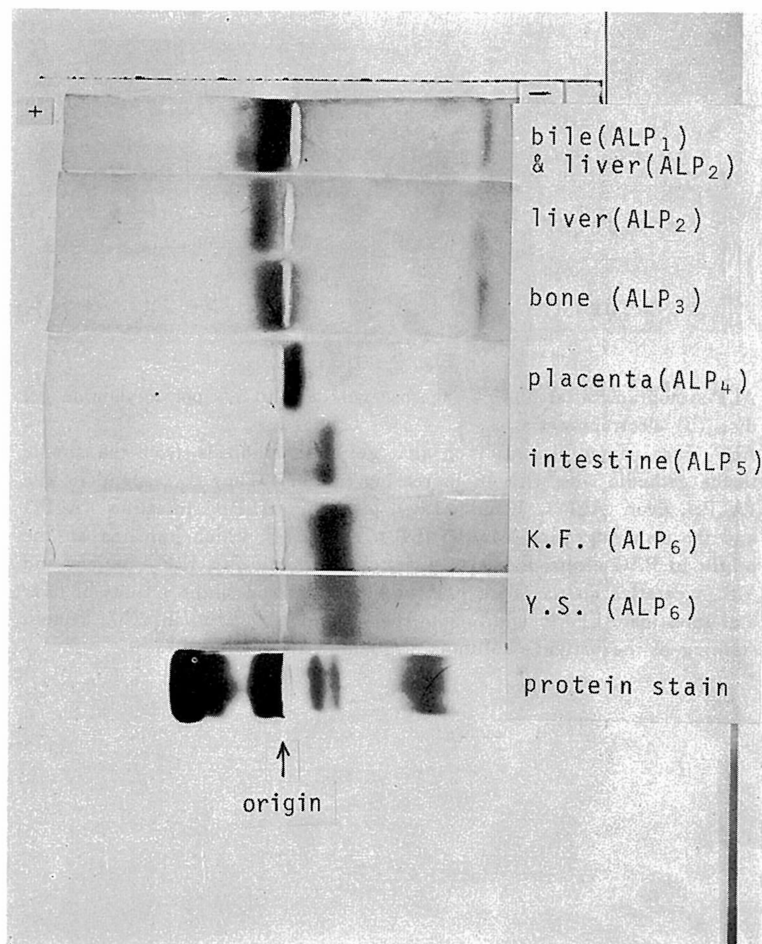

Fig. 2. (a)

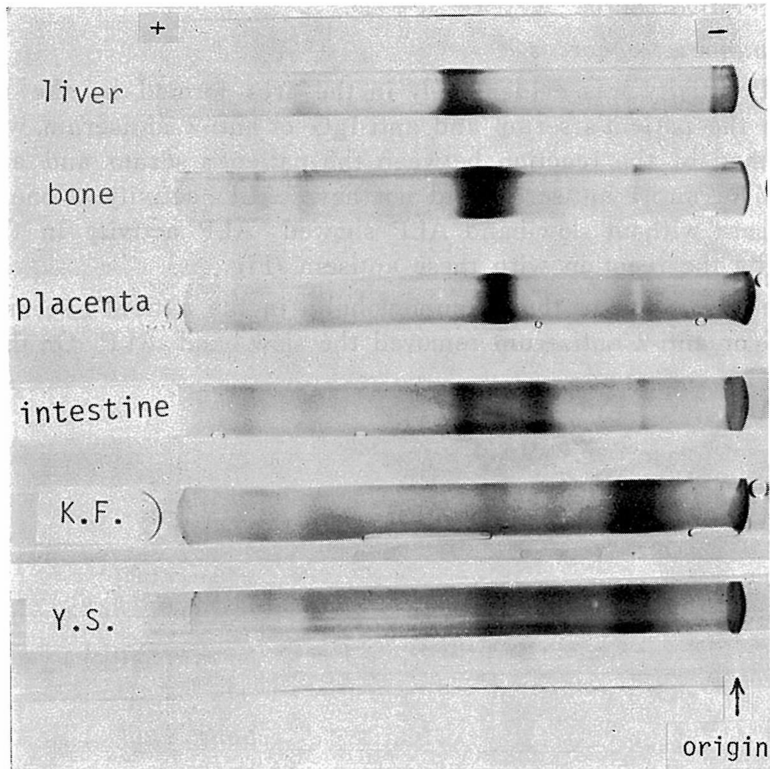

Fig. 2 (b)

ALP-zymograms after 0.75% agarose gel (a) and 5% polyacrylamide gel disc (b) electrophoresis.
ALP_{1-6} are named classically on agar gel electrophoresis from the anodic to the cathodic side with an increasing number. They correspond to bile (ALP_1), liver (ALP_2), bone (ALP_3), placenta (ALP_4). intestine (ALP_5) and the slow migrating band (ALP_6). Bile ALP which remains at the origin in PAG electrophoresis, was not shown in (b). K.F., patient and Y.S., ulcerative colitis Both K.F. and Y.S. showed slight activity of liver and intestine ALPs in addition to the slow moving band in (b). Protein staining of the patient's serum is also shown in (a).

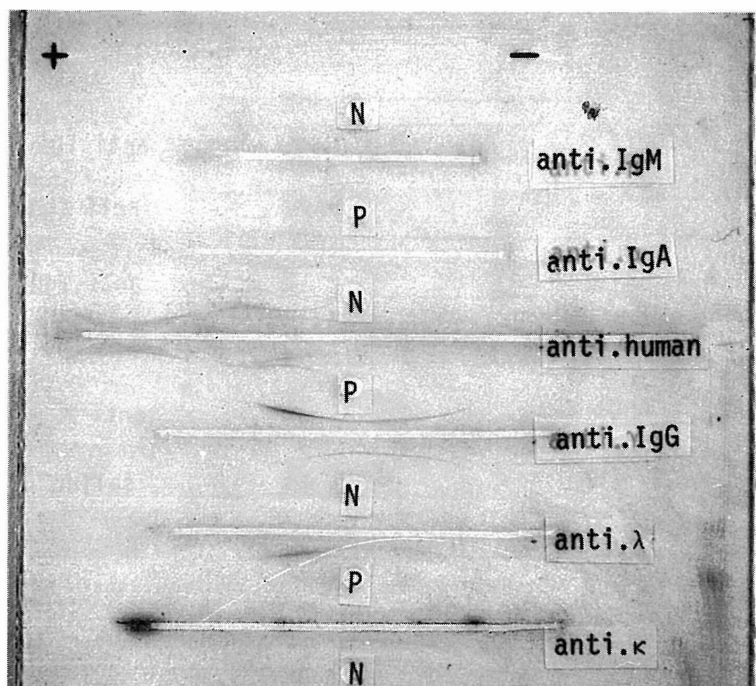

Fig. 3 Immunoelectrophoresis
Electrophoresis was carried out on 1% agarose gel plate for 45 min at 150V in 0.05 M barbital buffer, pH 8.6. After specific antisera were applied to the side groove, the plate was incubated at 4 C° for 48 h and was followed by washing in physiological saline for 24 h. The precipitation arcs formed were opaque and colorless unless any staining were performed. After ALP staining, arcs with ALP activity stained black. Some arcs without ALP activity nonspecifically weakly stained yellow.

hand, the absorption by anti-IgA, anti-IgM or anti-κ antiserum did not exert any effects upon the slow-band ALP (Fig. 4).

The above findings indicate that the slow-band ALP exists as the ALP-IgG complex.

Molecular weight

To evaluate the molecular weight of the slow-band ALP the patient's serum was applied on the Sephadex G-200 column (Fig. 5). From the elution profile of the ALP activity, the molecular weight of the ALP-IgG complex was estimated at 280,000, whereas that of the liver and bone ALP was approximately 146,000 (Fig. 5b). Since the molecular weight of IgG is 150,000-160,000, the binding of one molecule of IgG with one molecule of ALP was assumed for the slow-band ALP, or IgG-ALP complex.

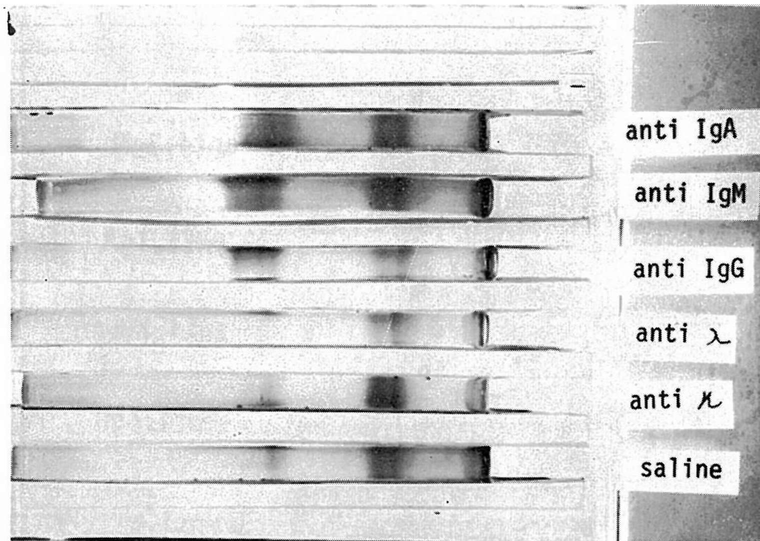

Fig. 4 Polyacrylamide disc electrophoresis of the patient's serum after absorption of immunoglobulin by specific antisera.
Optimal ratio of antigen (patient's serum) and antibody (each specific antiserum) was first determined. The serum-antiserum mixture in optimal ratio was incubated for 24 h in a hematocrit tube and the precipitates formed were removed by centrifugation. The whole fluid phase was subjected to electrophoresis. Equal amount of original patient's serum was used throughout this experiment to enable a comparison between the effect of each specific antiserum to patient's serum. Anodic broad bands in anti-IgA, IgM and IgG gels are rabbit's ALP from which these antisera were derived.

Effects of inhibitors, heat denaturation and neuraminidase

The results of inhibition by L-phenylalanine, L-homoarginine, and urea showed that the slow-band ALP was similar to the liver ALP of the control serum. In the heat denaturation test, the same characteristic was revealed (Table Ⅱ). Electrophoretic mobility of the slow-band ALP was retarded after the neuraminidase treatment as were the bone and liver ALP's in control sera, while only intestinal ALP was unaffected. Since the slow band was affected by neuraminidase, the ALP in slow band was likely derived from liver and/or bone. (Table Ⅱ).

Effect of inactivated patient's serum on control sera

The control sera which contained predominantly liver, bone, placenta or intestine ALP were incubated overnight with heat inactivated (56°C, 30 min) patient's serum, and were then subjected to PAG electrophorsis. The ALP activity of the liver and bone isozyme bands in the control

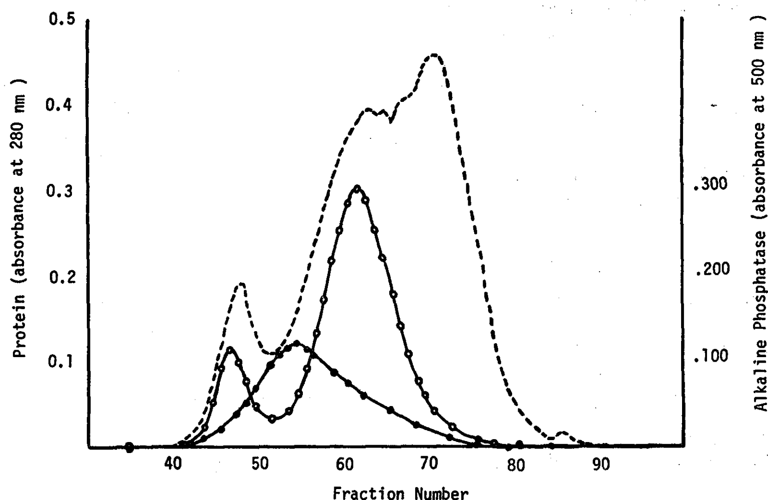

Fig. 5a Gel filtration patterns on the Sephadex G-200 column.
ALP activities of patient's serum (•——•) and of the serum from a case with obstructive jaundice (○——○). The latter serum contains bile ALP and liver ALP. Protein concentrations of the patient's serum were also plotted (·········).

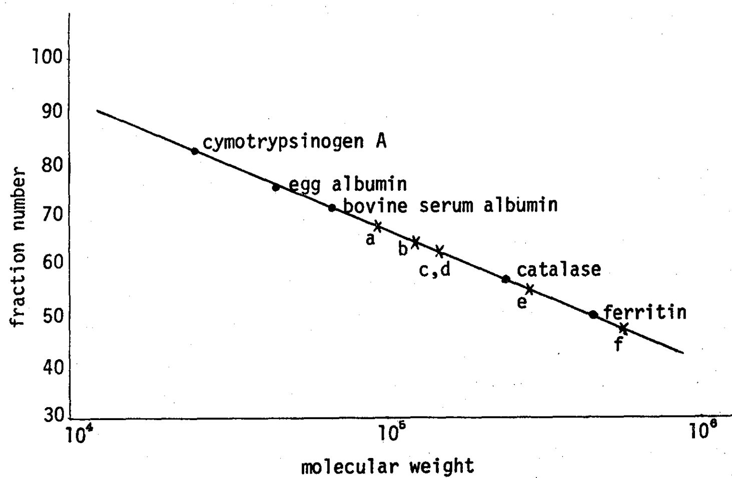

Fig. 5b Molecular weight of ALP isozymes and the IgG-ALP complex a, placenta 94, 000; b, intestine 123, 000; c, liver 146, 000; d, bone 146, 000; e, IgG-ALP complex 280, 000; f, bile 560, 000

Table Ⅱ Effects of amino acids, urea, heat and neuraminidase treatment on patient's serum

treatment	% residual activity of isozyme				
	liver	bone	intestine	K.F.	Y.S.
L-phenylalanine (3 mM)	78	74	39	78	71
L-homoarginine (3 mM)	28	24	71	28	35
Urea (3 M)	18	6	35	16	25
Heat inactivation (56°C, 10 min)	8.9	5	31	10	9
Neuraminidase*	81	76	100	76.55	

* % retardation of electrophoretic migration after neuraminidase treatment

% retardation $= \frac{b}{a} \times 100$, where, a and b were migration distances from the origin before and after neuraminidase treatment, respectively.

K.F., patient and Y.S., ulcerative colitis

sera decreased and a slower band corresponding to the original slow-band ALP of the patient developed (Fig. 6). This phenomenon was prevented by the absorption of IgG from patient's serum by anti-IgG antiserum prior to mixing with the control sera. The bile ALP in control serum was not influenced by the mixing with the inactivated patient's serum in the agar gel electrophoresis (not cited in the figure) as was the placenta ALP. The same results were obtained with the serum from a patient with ulcerative colitis.

DISCUSSION

The slow-band ALP in this patient was confirmed to be an ALP-IgG complex. The results of the inhibition of ALP activity in the complex by amino acids, urea or heat, and retardation of the slow band by neuraminidase treatment, revealed that the ALP in the complex was liver and/or bone ALPs. The cause for forming the complex was the presence of an abnormal IgG in patient's serum, which can combine with human ALP of liver or bone origin. The results on the case of ulcerative colitis also indicated the presence of the abnormal IgG in the serum as well as the two kinds of ALP for the formation of the complex. Thus the ALP-IgG complex in both cases have been proved to be of the same nature.

The observation of transient appearance of the slow-band ALP suggests that the slow band ALP is acquired, although no investigations upon relatives of the patient were performed. The correlation between

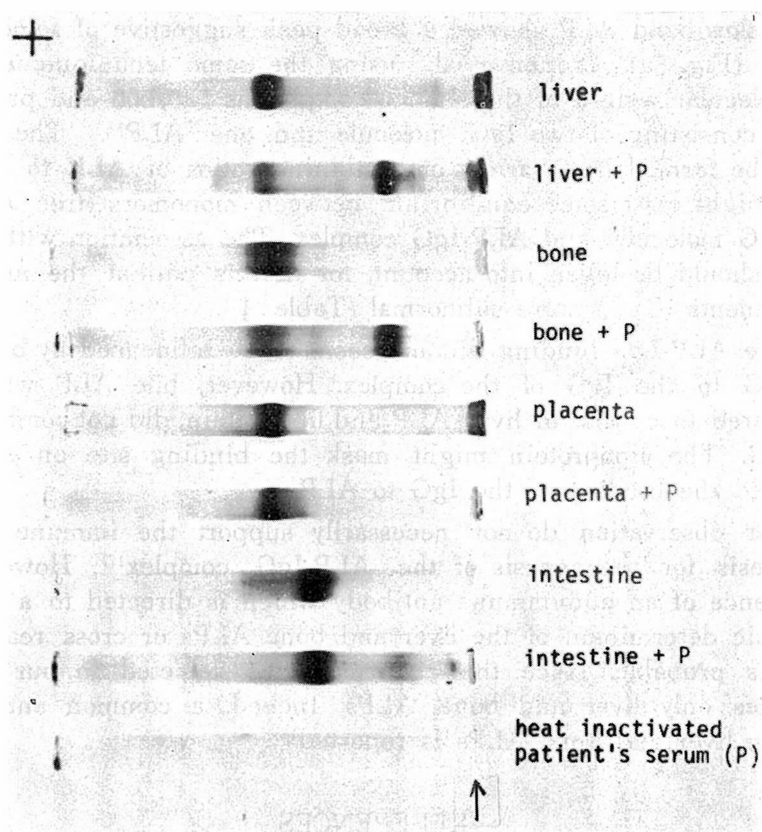

Fig. 6 Effects of inactivated patient's serum on control sera 5 % PAG disc electrophoresis P, heat inactivated patient's serum in which ALP activities were completely lost. By the addition of P to control sera, only the liver and bone ALPs in control sera diminished in their activities and a new slow moving band appeared instead. Arrow indicates the origin. Slight activity of slow-band ALP was also detected in a mixure of control serum of intestine ALP and P. This was due to slight contamination of bone ALP in the intestinal control.

the development of the slow band and activity of the disease is the reverse of that in ulcerative colitis in which the slow band appears in active stage of the disease. Although hepatitis and ulcerative colitis might be different in the etiology of the disease, the pathogenesis of the slow band, or ALP-IgG complex is suspected to be the same, since an abnormal IgG was detected in both cases.

Molecular weight study suggested that the complex was made up of one molecule of ALP and one molecule of IgG. However, the elution profile

of the slow-band ALP showed a broad peak suggestive of some heterogeneity (Fig. 5a). Crofton et al., using the same technique, estimated the molecular weight of the similar complex as 540,000 and proposed a trimer consisting of two IgG molecule and one ALP[6]. The complex might be formed in a variety of combining ratios of ALP to IgG and there might exist some equilibrium between monomers (free ALP and free IgG molecule) and ALP-IgG complex. The association with complements should be taken into account, for in this patient the amount of complements (C_3,C_4) were subnormal (Table I).

The ALP-IgG binding did not seem to be influenced by binding of anti-IgG to the IgG of the complex. However, bile ALP which was considered to consist of liver ALP and lipoprotein, did not combine with the IgG. The lipoprotein might mask the binding site on ALP and inhibited the binding of the IgG to ALP.

Our observation do not necessarily support the immune complex hypothesis for the genesis of the ALP-IgG complex[17]. However, the occurrence of an autoimmune antibody which is directed to a common antigenic determinant of the liver and bone ALPs or cross reacts with them is probable, since the abnormal IgG detected in our patients combines only liver and bone ALPs. Indeed, a common antigenicity between liver and bone ALPs is reported[18].

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