Epitopes of human intestinal alkaline phosphatases, defined by monoclonal antibodies

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Antibody additivity assay *Topology*

1. INTRODUCTION

The human alkaline phosphatases (ALPs) are membrane-bound glycoproteins that occur in different forms in different tissues. Their protein moieties are coded for at least 3 gene loci; one for placental ALP (ALP_P), at least one for the intestinal ALPs (adult and fetal) and at least one for liver/bone/kidney ALPs [1]. We have been investigating the immunological differences and similarities of these various ALPs using monoclonal antibodies. Here, we describe studies aimed at defining immunologic epitopes on the surface of the intestinal ALPs using a series of 7 monoclonal antibodies all of which bind to both adult (ALP_{AI}) and fetal (ALP_{FI}) forms of human intestinal ALP. To do this we define an additivity value (ADD): the amount of a labeled antibody bound to the enzyme after prior saturation with a different unlabeled antibody, calculated as a percentage of label bound to the enzyme without prior saturation by the other antibody. Low ADD values imply that the two antibodies tested bind to determinants that are spatially close on the enzyme surface, if not identical. High ADD values imply that the determinants recognized by the two antibodies are well separated.

Our findings suggest that the 7 antibodies are directed to determinants in 3 distinct epitopes on both adult and fetal intestinal ALP; 3 in one epitope, 3 in another, and one in a third epitope. Each of the epitopes are also represented on ALP_P, though one appears to have diverged structurally in evolution to a greater degree than the others.

2. MATERIALS AND METHODS

2.1. *ALP extracts*

Aqueous solutions containing ALP_{AI}, ALP_{FI} and ALP_P were prepared and assayed as in [2].

2.2. *Monoclonal antibodies*

The 7 monoclonal antibodies used were: ALP_{FI}/Sp2/38, ALP_{FI}/Sp2/37, ALP_{FI}/Sp2/36, ALP_{FI}/Sp2/41, ALP_{FI}/Sp2/39, ALP_P/Sp2/5 and ALP-D98/Sp2/22. Their production, characterization, and the preparation of antibody-rich mouse ascites fluids has been described [3–6]. For simplicity we will refer to the different antibodies in the rest of this paper simply by their numerical listing, e.g., ab38, ab36, etc. The antibodies were purified from ascites fluids using a *Staphylococcus aureus* protein-A conjugated Sepharose 4B column. Polyclonal antiserum to intestinal ALP was made in rabbits as described [3].

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2.3. *^{125}I-labeling of monoclonal antibodies*

Radioiodination using ^{125}I and Chloramin T, as in [7].

2.4. *Determination of antibody concentration required to saturate immobilized ALP*

100 μl rabbit anti-human intestinal ALP polyclonal antiserum at a dilution of 1/4000 was applied per well of a PVC Cooke 96-well microtiter plate. After 2 h at room temperature, the plate was emptied and the wells filled with blocking buffer, 0.67% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), pH 7.2, and incubated at room temperature for 30 min. After emptying the wells, 50 μl human intestinal ALP (ALP_{AI} or ALP_{FI}) at a concentration of 500 mIU/ml in 0.5% BSA in PBS, pH 7.2, was added per well and incubated overnight at 4°C. The wells were washed twice with 0.5% BSA in PBS to remove any unbound ALP. Serial dilutions of purified ascites fluid antibody were then applied and the amount of antibody bound determined with peroxidase labeled rabbit anti-mouse immunoglobulin serum as in [6]. A concentration of antibody at least ten times the maximum required to produce saturation as indicated by this test, was used for the ADD assay.

2.5. *Additivity (ADD) assay*

Enzyme was immobilized in a 96-well microtiter plate as described above for determining antibody saturating concentration. 50 μl of a monoclonal antibody at a saturating concentration in 0.5% BSA in PBS was added per well and incubated at 4°C for 2 h. This was followed by the addition of 50 μl of an ^{125}I-labeled monoclonal antibody in 0.5% BSA in PBS per well (approximately 2×10^5 cpm per well), and the plate was incubated at 4°C for 2 h. The content of the wells was removed by vacuum, and the wells were washed 6 times with cold water and dried. The wells were then cut free of the plate and counted in a gamma counter. Six wells were run with each combination. Twelve wells were used for determining the control binding (i.e., without saturating antibody) and 12 wells for determining background (i.e., without ALP). The entire series was repeated 4 times giving a total of 24 determinations for each combination. The means of the values were calculated and used in the following equation for determining ADD:

ADD = % control binding =
[(cpm in the presence of saturating antibody − background)/(cpm in the absence of saturating antibody − background)] × 100

3. RESULTS

The experimental design was to test each pair of antibodies by first saturating a standardized amount of immobilized enzyme (either ALP_{AI} or ALP_{FI}) with one antibody of a pair, then applying the other antibody labeled with ^{125}I. The amount of ^{125}I bound was then determined. Each pair of antibodies was examined reciprocally. One member of the pair first served as the saturating antibody and then in the reciprocal experiment served as the ^{125}I-labeled antibody. Since the two members of a pair of antibodies are applied separately and not simultaneously to the enzyme, the method is not strictly speaking a competition assay. We therefore refer to the binding of a labeled antibody after prior saturation of the enzyme with the other antibody, as an additivity value (ADD).

Six of the ^{125}I-labeled antibodies were able to bind to immobilized ALP in the absence of saturating cold antibody (i.e., control binding) with counts of between 2000 and 5000 cpm. One antibody (ab37) however gave much lower binding, only about 15% of the cpm obtained with the other labeled antibodies. Since unlabeled ab37 could bind to the enzyme as well as any of the others in saturation studies (not shown) it is likely that iodination of ab37 had affected the antibody binding site, possibly due to the presence of a tyrosine residue in this region. The level of binding obtained with this labeled antibody would not be appropriate for use in further analysis, so ^{125}I-labeled ab37 has been excluded from determinations of ADDs. However, the use of unlabeled ab37 in combination with the other labeled antibodies has been included.

To check the efficacy of saturation, each ^{125}I-labeled antibody was tested for binding after prior saturation of immobilized ALP with the same unlabeled antibody. In all cases the ADD values were less than 1%, indicating that saturation was effectively complete.

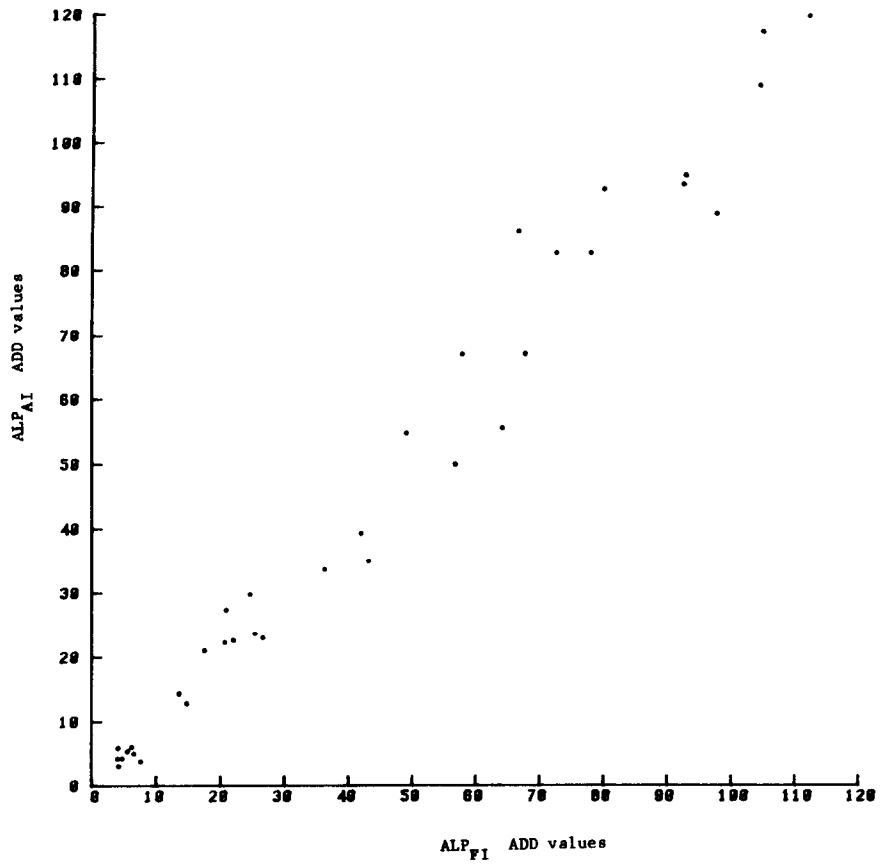

Fig.1. Correlation of ADD values for ALP_{FI} and ALP_{AI} with 6 monoclonal antibodies.

Fig.1 shows ADD values for ALP_{AI} and ALP_{FI} obtained with each pair of antibodies. There is a close correlation (r = 0.99) indicating that all the determinants for these different antibodies are present in both ALP_{AI} and ALP_{FI}. In view of this result, mean ADD values of an antibody for ALP_{AI} and ALP_{FI} have been used in further analysis of the data.

Table 1 shows the reciprocal ADD values for each of the 15 antibody pairs where reciprocal tests were possible. In the case of ab37, only the values when this was used as the unlabeled antibody are shown. In general, there is a good correlation between the ADD values in reciprocal tests on the pairs of antibody (r = 0.87). In a few cases there is a relatively small deviation from equality for the two values in the reciprocal tests. This may be due to appreciable differences in avidity between the two antibodies for the enzyme or to experimental error. The SDs of the individual ADD values for each of the antibody combinations varied between 0.5 and 5.

To examine the topological relationships of the various determinants defined by the different antibodies, we categorized the ADD values shown in table 1 into 4 classes as follows: (A) 0–19; (B) 20–39; (C) 40–59; (D) 60 or greater. Fig.2 illustrates the presumptive topological relationships of the antigenic determinants on the surface of the intestinal ALP molecules recognized by the different antibodies using this classification. Where reciprocal tests on a pair of antibodies were possible the two ADD classes are shown. In the case of ab37, only the ADD values where this was used as a saturating antibody are shown. ADD values falling into the lowest class, AA or A, suggest that the antigenic determinants recognized by a pair of antibodies lie close together on the enzyme surface

Table 1

ADD values for different combinations of 7 antibodies with intestinal ALP (data for ALP_{AI} and ALP_{FI} combined)

^{125}I-labeled antibody	Unlabeled saturating antibody	ADD value	^{125}I-labeled antibody	Unlabeled saturating antibody	ADD value
(a) Reciprocal ADD tests					
ab5	ab22	76.4	ab39	ab36	24.6
ab22	ab5	39.1	ab36	ab39	35.1
ab39	ab22	4.2	ab41	ab36	24.8
ab22	ab39	5.8	ab36	ab41	22.3
ab39	ab5	40.8	ab22	ab38	93.5
ab5	ab39	62.5	ab38	ab22	67.5
ab41	ab22	116.3	ab5	ab38	4.5
ab22	ab41	93.2	ab38	ab5	5.1
ab41	ab5	111.3	ab39	ab38	21.5
ab5	ab41	80.5	ab38	ab39	24.1
ab39	ab41	93.2	ab41	ab38	27.3
ab41	ab39	86.5	ab38	ab41	14.0
ab22	ab36	13.9	ab36	ab38	19.3
ab36	ab22	53.5	ab38	ab36	0.2
ab36	ab5	5.7			
ab5	ab36	3.7			
(b) One way ADD tests					
ab38	ab37	51.9	ab5	ab37	77.9
ab41	ab37	106.8	ab36	ab37	60.0
ab39	ab37	6.2	ab22	ab37	5.8

	ab38	ab36	ab5	ab39	ab37	ab22	ab41
ab38	-						
ab36	AA	-					
ab5	AA	AA	-				
ab39	BB	BB	CD	-			
ab37	C	D	D	A	-		
ab22	DD	AC	DB	AA	A	-	
ab41	BA	BB	DD	DD	D	DD	-

Fig.2. ADD levels grouped into four classes A, B, C and D as described in the text, for different antibody combinations with intestinal ALP (ALP_{AI} and ALP_{FI} combined).

and may possibly overlap or be identical. Higher ADD values (e.g., BB, BC, CD, etc.) imply that the two determinants are further apart. Fig.2 suggests that the antibodies can be divided into 3 groups. In group 1 ab38, ab36 and ab5 give low ADD values (AA) with each other but higher values with the 4 other antibodies. Group 2 (ab39, ab37 and ab22) give low ADD values (AA or A) with each other but high values with the other antibodies. Group 3, which includes only one antibody (ab41), is distinct from either of the other two groups.

4. DISCUSSION

In general the experimental design, which involves the sequential application of a saturating antibody followed by ^{125}I-labeled antibody to the immobilized enzyme, is similar to that used in other studies of the same sort, and appears to provide a satisfactory and systematic approach to the topological analysis of antigenic determinants on enzymes. If the two members of a pair of antibodies have the same avidity for the enzyme, then one expects the ADD values in the reciprocal tests to be equal, apart from experimental error. Significant deviations from equality would imply avidity differences which might be confounded with the effects of topological separation. With the set of antibodies studied here, such avidity differences that may exist do not appear to have influenced the ADD values sufficiently to invalidate their use as topological markers.

In occasional cases ^{125}I-labeling markedly reduces the binding of the antibody to the enzyme (1 out of the 7 antibodies used here). This is probably due to the insertion of the ^{125}I into tyrosine residues close to the binding site of the antibody. If so, it precludes the use of such labeled antibodies in ADD assays, though the antibody can still be used as a saturating antibody. This difficulty might in principle be dealt with by some other method of antibody labeling, e.g., labeling with peroxidase.

The findings indicate that the antibodies fall into three separate groups which are distinguishing different epitopes on the enzyme surface. Epitope 1 contains determinants for ab38, ab36 and ab5. Epitope 2 contains determinants for ab39, ab37 and ab22. Epitope 3 contains the determinant for ab41. The epitopes appear to be well separated on the enzyme surface, though epitope 3 may be somewhat closer to epitope 1 than epitope 2.

It is of interest to relate these findings to other information about the characteristics of the various antibodies. As noted in section 3, all of the determinants recognized by this set of antibodies occur on both ALP_{AI} and ALP_{FI}. Antibodies ab38, ab36, ab5 (epitope 1), ab22 (epitope 2) and ab41 (epitope 3) have in other studies also been shown to bind to ALP_P [3,4,5]. ALP_P is known to be structurally distinct from the intestinal ALPs and its protein moiety to be encoded by a separate gene locus. It has been suggested that ALP_P and the intestinal ALPs arose in evolution by duplication of a common ancestral gene, and that they subsequently diverged in structure by point mutations [1]. The present results suggest that each of the three epitopes are represented on the placental ALP molecule though presumably with structural modifications. The fact that of the three antibodies recognizing determinants in epitope 2, two do not cross-react with placental ALP (ab37 and ab39), suggests that this epitope may have been less conserved structurally than the other epitopes.

Placental ALP, unlike the intestinal ALPs, is known to be genetically highly polymorphic, with three common alleles found in most human populations. In various studies using monoclonal antibodies generated against placental ALP it has been shown that the products of the three common alleles can in a number of cases be discriminated by monoclonal antibodies [6,8]. Of the series of antibodies discussed here, one (ab41) has been found to give significantly reduced binding with the product of the type 3 allele than with the product of either the type 1 and type 2 alleles [3]. This implies that the antibody is directed to a determinant on the ALP molecule whose structure was altered by the mutation giving rise to the type 3 allele. The present results indicate that this mutation must lie in the DNA region coding for epitope 3.

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