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29 October 1981

A Monoclonal Antibody That Reacts with Nonallelic Enzyme Glycoproteins

Abstract. *One of six monoclonal antibodies raised against purified human placental alkaline phosphatase cross-reacts with the adult and fetal forms of intestinal alkaline phosphatase. The placental and intestinal enzymes are nonallelic. A new electrophoretic titration procedure was used to assess the relative reactivities of the different enzymes with the antibody. The placental enzyme was the most reactive. However, the adult intestinal enzyme showed greater reactivity than the fetal enzyme. The determinants to which the antibody binds on these three forms of alkaline phosphatase presumably differ in their detailed molecular configurations.*

The human alkaline phosphatases (ALP's) are glycoproteins (*1–3*). At least three gene loci code for the protein moieties of the various ALP's that occur in different human tissues: one for the polymorphic placental ALP's, at least one for the intestinal ALP's, and at least one for the liver, bone, and kidney ALP's (*4*). Two forms of intestinal ALP, adult and fetal, have been identified (*2, 5, 6*). These two forms resemble each other closely in thermostability and in their sensitivity to inhibition with a series of different inhibitors, and they are clearly differentiated in these respects from placental and from liver, bone, and kidney ALP's. They differ from one another in that fetal intestinal ALP contains sialic acid residues that do not occur in adult intestinal ALP (*2, 5, 6*). Also it has been found that even after removal of these sialic acid residues with neuraminidase, fetal intestinal ALP still has a slightly greater anodal electrophoretic mobility than the adult form. It is not clear whether this small difference in electrophoretic mobility is due to a difference in the protein moieties of the ALP's which might imply that they are coded at separate loci, or whether they are due to differences in the carbohydrate moieties of the enzyme glycoproteins and reflect posttranslational differences.

Antiserums raised in rabbits against purified human placental ALP do not cross-react with liver, bone, and kidney ALP but cross-react with both adult and fetal intestinal ALP's (*7*). When tested by Ouchterlony double diffusion against placental ALP, the adult and fetal intestinal ALP's give precipitin lines of partial identity. The cross-reacting antibodies can be absorbed with either adult or fetal intestinal ALP leaving an antiserum reacting only with placental ALP. Since such antiserums are polyclonal, the findings indicate that the unabsorbed antiserum to placental ALP contains a mixture of antibodies, some of which cross-react with determinants on the intestinal ALP's and others which do not. We have recently described a series of monoclonal antibodies prepared against purified human placental ALP by the mouse hybridoma technique (*8*). Quantitative binding studies by radioimmunoassay were carried out on six of these antibodies against an extensive panel of placental ALP's of different electrophoretic phenotypes and it was found that each monoclonal antibody gave a distinctive pattern of reactivities with the various phenotypes, suggesting that each is directed at a different antigenic determinant on the surface of the placental ALP molecule. It was of interest to find out whether any of these monoclonal antibodies raised against placental ALP also reacted with either adult or fetal intestinal ALP and if so whether or not differences in the degree of reactivity could be demonstrated. In the present report we describe experiments aimed at answering these questions.

We have recently described how placental ALP–monoclonal antibody complexes can be demonstrated by gel electrophoresis (*9*). In the present study we applied this method to find out whether any of the six monoclonal antibodies whose reactivities with placental ALP's had already been studied in detail also reacted with the different forms of human intestinal ALP. As a source of antibody we used ascites fluid from mice that had been injected intraperitoneally with the hybridoma cells, since these fluids contain much larger quantities of antibody than hybridoma culture fluids. After incubation of ALP containing tissue extracts with ascites fluid, the samples were examined by gel electrophoresis. Typical results are shown in Fig. 1. Only one of the six monoclonal antibodies, Sp2/5, gave evidence of cross-reac-

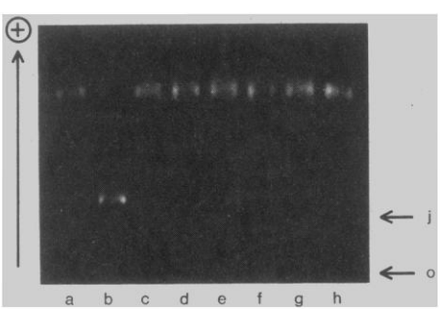

Fig. 1. Electrophoresis of human adult intestinal ALP complexed with six different monoclonal antibodies raised against purified human placental ALP (*8*). Vertical acrylamide slab gel electrophoresis was performed in 9.5 percent acrylamide layered with 5 percent acrylamide prepared as described (*9*) but with the addition of 0.01 percent Zwittergent (TM 314, Calbiochem). The ALP samples were prepared as described (*12*). Portions (50 μl) of the sample, diluted to 0.2 IU/ml, were incubated for 6 hours at 5°C with 10 μl of ascites fluid containing the particular monoclonal antibody (heated at 56°C for 1 hour to destroy any mouse ALP present). Electrophoresis was for 16 hours with a constant current (25 mA). Gels were stained with 4-methylumbelliferyl phosphate as described (*9*). Channels a and h contain uncomplexed adult intestinal ALP; channels b to g contain adult intestinal ALP after incubation with Sp2/5, Sp2/2, Sp2/11, Sp2/3, Sp2/4, and P3/1 ascites fluids, respectively. The letters o and j indicate, respectively, the origin and the junction between 9.5 and 5 percent acrylamide layers.

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tion with the intestinal ALP's, the intestinal ALP–monoclonal antibody complex showing marked retardation compared with the corresponding uncomplexed ALP. The mobilities of either intestinal ALP were not altered after incubation with any of the other antibodies tested, in contrast to the results obtained when placental ALP is tested by the same procedures with these monoclonal antibodies (*9*).

The findings indicate that both forms of intestinal ALP have antigenic determinants reactive with Sp2/5 antibody that are presumably structurally similar to the determinant on placental ALP to which Sp2/5 is directed. In order to compare the reactivities of the intestinal ALP's with placental ALP and with one another, we carried out a series of electrophoretic titrations. Serial one-in-two dilutions of Sp2/5 ascites fluid were incubated with the ALP extracts and the samples were subjected to electrophoresis and stained under standardized conditions (Fig. 2). The end point of the titration was taken as the highest dilution of antibody at which no uncomplexed ALP isozyme could be seen (*10*). The dilution of the Sp2/5 antibody at the end point of the titration for placental ALP was 1/1024; for adult intestinal ALP, 1/128; and for fetal intestinal ALP, 1/1. The variations from experiment to experiment were not greater than one step in the dilution series (that is, one in two).

Thus the titration end point is at a much higher dilution of antibody for adult intestinal ALP than for fetal intestinal ALP. Adult intestinal ALP differs from fetal intestinal ALP by the absence of sialic acid residues. To find out whether the presence of these sialic acid residues in the fetal form accounts for the marked difference in reactivity with Sp2/5 antibody, we treated both forms with neuraminidase and then carried out standard titrations. As expected, the titration end point for adult intestinal ALP, which is unaffected by neuraminidase, was unchanged (1/128). With desialated fetal intestinal ALP the end point of the titration shifted by only one dilution step from that obtained with sialated fetal intestinal ALP (1/2 compared with 1/1). Thus most of the difference in reactivity between the adult and fetal intestinal ALP's cannot be accounted for by differences in sialic acid residues.

Since in these experiments equal activities of the various enzymes were used for the titrations, the observed differences could in principle be attributable to differences in specific activities. If this were the case, the specific activity (that is, activity per milligram of purified protein) for the intestinal ALP's would need to be much less than the specific activity of purified placental ALP in order to account for the observations. This is because greater amounts of the enzyme protein would have been used in the experiments so as to give equal ALP activities and this in turn would have increased the quantity of antibody required to reach the titration end points. But, the experimental evidence indicates that the specific activity of both adult and fetal intestinal ALP is at least two to four times greater than that of placental ALP (*3*, *11*). Thus the titration differences between placental ALP and the intestinal ALP's apparently reflect, for the most part, true differences in reactivity with the Sp2/5 antibody rather than differences in specific activities.

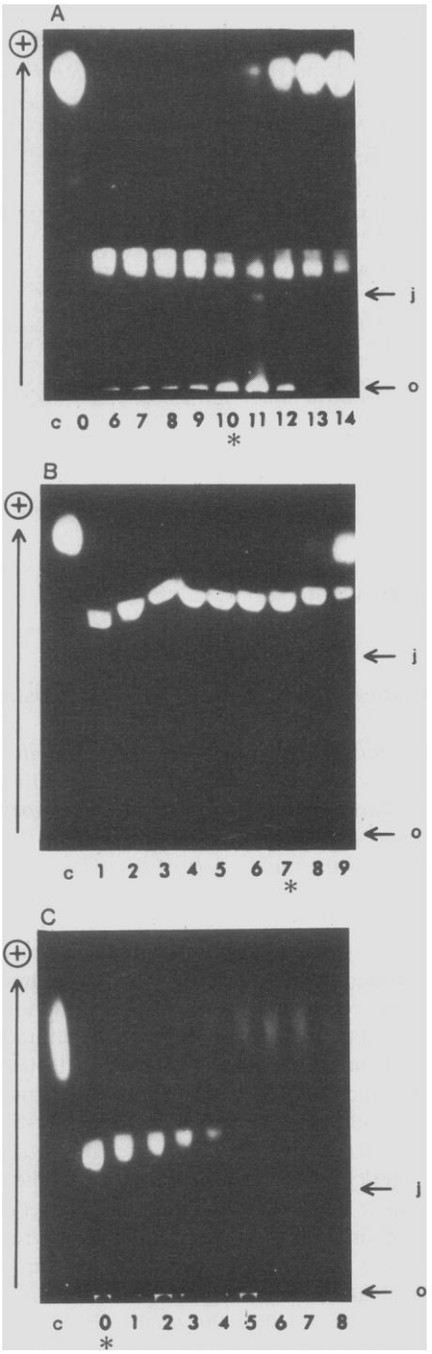

Fig. 2. Acrylamide gel electrophoresis of human (A) placental, (B) adult intestinal, and (C) fetal intestinal ALP after incubation with serial dilutions of Sp2/5 antibody containing ascites fluid. Placental ALP (homozygous type 1) and adult intestinal ALP were prepared as described (*12*); fetal intestinal ALP was extracted from meconium as described (*5*). All extracts were assayed and diluted to 0.2 IU/ml, and 50 μl of each extract was incubated with 10 μl of serial one-in-two dilutions of Sp2/5 ascites fluid for 6 hours at 5°C. Dilutions were made in tris-HCl buffer, *p*H 7.4, containing 0.15*M* NaCl, 0.02 percent sodium azide, 0.05 percent NP40 (BDH Chemicals), and 0.25 percent gelatin. Electrophoresis conditions were as described in Fig. 1. Channel in *c* contains uncomplexed ALP; numbers 0 to 14 indicate the dilution factor of Sp2/5 ascites fluid in the series 2^x where $x = 0$ to 14. Asterisks indicate end points of titration.

The various ALP's are glycoproteins and we have not established whether the antigenic determinant to which the Sp2/5 antibody is directed is in the protein or carbohydrate moieties of the enzyme molecule, or whether indeed the determinant involves both protein and carbohydrate structures. The gene loci that code for the protein moieties of placental and intestinal ALP's are thought to have arisen in evolution from a common ancestral gene by gene duplication. The structural differences in the proteins observed today are presumably the consequence of point mutations which occurred in the course of evolution subsequent to the gene duplication. If so, one would expect that the amino acid sequences of some regions of the enzyme surface would have been more conserved than others so that some antibodies raised against placental ALP would cross-react with intestinal ALP and others would not. It is of interest that antibody Sp2/5, when tested by quantitative binding studies against an extensive panel of placental ALP's that included phenotypes (defined electrophoretically) representing many different pairwise combinations of products of at least 11 alleles at this locus, exhibited no significant differences in reactivity among any of the phenotypes (*8*). In contrast, each of the other five monoclonal antibodies studied here, which did not show cross-reaction with intestinal ALP, revealed significant differences between one or another of the allelic variants represented in the tested panel of placental ALP's. This suggests that if the determinant to which Sp2/5 is directed is in the protein moiety of the molecule, then the region in which it occurs is relatively highly conserved within the species and by inference might have been relatively highly

conserved in evolution. The amino acid structure of such a determinant would not necessarily be identical in placental and intestinal ALP, and such differences as exist could well account for the apparently reduced reactivities of the intestinal ALP's compared with placental ALP.

The structures of the carbohydrate moieties of the ALP's have not been determined, although some analytical data indicating differences in composition are available (*2*). If the Sp2/5 antibody is directed toward an antigenic determinant on the carbohydrate moiety of the ALP molecule, the configuration of sugars in the determinant grouping is presumably similar in the various ALP's but differs in detail, since the various ALP's show differences in reactivity with the antibody.

Our results point the way to detailed studies of the nature of the antigenic determinant at which Sp2/5 antibody is directed and in particular to the question of whether the determinant is in the protein or carbohydrate moieties of these enzyme glycoproteins. The results also illustrate the potential usefulness of such electrophoretic studies in the search for cross-reactions of monoclonal antibodies in other sets of nonallelic enzymes.

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13. This work was supported by grants GM27018, GM20138, and GM07511 from the National Institutes of Health.

22 September 1981; revised 13 January 1982

Developmental Changes in the Biliary Excretion of Methylmercury and Glutathione

Abstract. *The long half-time for methylmercury in the neonatal rat is explained by the neonatal liver's inability to secrete the toxin into bile, which in adults is the main route of elimination. The ability to secrete mercury into bile develops between 2 and 4 weeks of age and is correlated with the increasing ability of the developing liver to secrete glutathione into bile.*

The neonatal period is an especially vulnerable time for the accumulation of many heavy metals (*1*). Methylmercury is more toxic to suckling animals and human infants (*2*) than to adults. Metals readily accumulate in young animals due to higher intestinal absorption rates and immaturity of the hepatic and renal excretory functions (*1, 3*). Neonatal mice (*4*) and rats (*5*) treated with methylmercury excrete only a small fraction of the amount administered. At 16 to 18 days of age, however, there is an abrupt increase in mercury excretion. Indirect evidence points to a similar phenomenon in human neonates (*2, 6*). The mechanisms underlying these ontogenetic differences in the excretion of methylmercury are not known. Since the rate of methylmercury elimination is a significant determinant of the body burden, data on differences in the rates of elimination at various developmental stages are important in estimating exposure risks for the fetus and neonate and in therapeutic intervention.

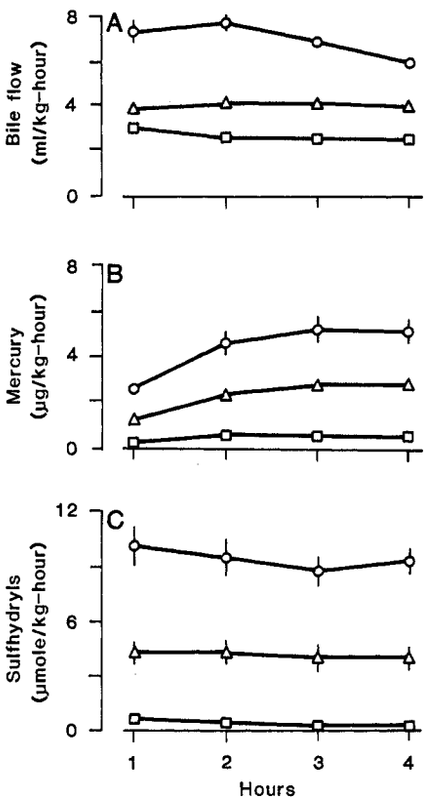

Fig. 1. Developmental changes in bile flow, mercury secretion into bile, and total reduced sulfhydryl secretion into bile. An intravenous injection of mercury (1 mg/kg) as $CH_3{}^{203}HgCl$ was given to 14-day-old (□) (N = 12), 21-day-old (△) (N = 10), and 28-day-old (○) (N = 8) rats, and bile was collected every hour for 4 hours thereafter. Values are means ± standard errors.

Fecal excretion, the main route of mercury elimination, is determined primarily by biliary secretion, and it accounts for over 80 percent of the total excretion in rodents (*7*). An important role in the regulation of the biliary secretion of methylmercury has been ascribed to glutathione (*8–10*). Glutathione, which has a concentration in bile of 1 to 4 mmole/liter, accounts for over 90 percent of the reduced sulfhydryl groups in bile (*8, 11*). Methylmercury has a high affinity for reduced sulfhydryl groups, including those of glutathione, and a methylmercury-glutathione complex has been identified as the main form of methylmercury in bile (*8*). Infusion of glutathione into rats increases the biliary secretion of methylmercury (*12*), while agents that deplete the hepatic content of glutathione inhibit the biliary secretion of methylmercury and simultaneously decrease the reduced glutathione content of bile (*9*).

The biliary pathway of excretion is also important in the design of therapy. A nonabsorbable, thiolated resin, given orally, has been used to increase fecal excretion of methylmercury in animals and humans (*13*). The resin acts by trapping mercury secreted into bile, interrupting the enterohepatic recirculation of the metal (*7*).

In an attempt to explain the rapid increase in methylmercury excretion in young animals, we examined the developmental changes in the biliary pathway for the elimination of methylmercury in relation to the changes in glutathione secretion into bile. The bile ducts of 14-, 21-, and 28-day-old Sprague-Dawley rats were cannulated under sodium pentobarbital anesthesia. Each group had equal numbers of males and females. Labeled methylmercury chloride ($CH_3{}^{203}HgCl$) was injected into a jugular vein cannula in a nontoxic dose (mercury concentration, 1 mg/kg) in a solution of 0.9 percent

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