Differentiation of immunochemically related enzymes in different primate species by monoclonal antibodies

Caprice E. Rogers and Harry Harris

University of Pennsylvania, School of Medicine, Department of Human Genetics, 195 Med Labs/G3 37th and Hamilton Walk, Philadelpia, PA 19104, USA

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1. INTRODUCTION

An unusual heat-stable form of alkaline phosphatase (ALP) that closely resembles human placental ALP in its immunologic characteristics has been found to occur in substantial amounts in lung from certain Old World monkeys [1]. In Ouchterlony double diffusion plates using rabbit antiserum raised against purified human placental ALP, the Old World monkey lung ALPs give precipitin lines continuous with that given by human placental ALP and with no detectable spurring. However, these ALPs can be sharply distinguished from human placental ALP and indeed from other human and non-human primate ALPs by their relative sensitivities to certain amino acid and tripeptide inhibitors [1]. The purpose of this work was to probe the immunochemical characteristics of these ALPs further, by using a series of monoclonal antibodies raised against human placental ALP and known to be directed at a series of different antigenic sites on the surface of the ALP molecule [2,3]. Two different methods were used. The first involves electrophoretic separation of enzyme/antibody complexes from uncomplexed enzyme. The second is based on assays of enzyme activity in immobilized enzyme/antibody complexes. A distinctive immunochemical profile differentiating these ALPs from human placental ALP was obtained.

2. METHODS

Enzyme extraction and assay of human placental ALP and heat-stable (65°C, 1 h) lung ALP from Rhesus monkey, baboon, pig-tailed macaque and Java monkey were carried out as described in [1]. Production and characterization of the 18 monoclonal antibodies used has been described in [2,3]. Polyacrylamide gel electrophoresis and ALP staining of the ALP before and after reaction with monoclonal antibody was carried out as in [4].

ALP assay of immobilized enzyme/antibody complexes was performed as follows: 100 μl 1/2000 rabbit anti-mouse Ig (Cappel) diluted in phosphate-buffered saline (PBS, pH 7.4) was placed in each well of a 96-well, V-bottomed polyvinyl microtiter plate and allowed to stand for 2 h. The wells were then emptied and refilled to the top with 0.5% bovine serum albumin (BSA) in 0.01 M diethanolamine, 0.9% NaCl, to block residual protein-binding sites on the plastic. After standing for 30 min, the plate was washed twice with BSA in PBS. Hybridoma culture fluid (40 μl) was then added to each well and incubated overnight at 4°C to allow immobilization of the monoclonal antibody. The plate was washed twice with BSA in PBS. ALP (100 μl) diluted to 0.5 IU/ml in 0.5% BSA/PBS was then added to each well. After standing overnight at 4°C to allow binding of enzyme to immobilized monoclonal antibody, the plate was washed 6 times in Tris–HCl (pH 7.4) containing 0.9% NaCl. ALP substrate solution (100 μl; 5 mM *p*-nitrophenylphosphate, 1.0 M diethanolamine (pH 9.8), 0.28 M NaCl, 0.5 mM $MgCl_2$) was added to each well and incubated at 30°C for 30 min. The reaction was stopped by addition of 100 μl 0.5 N NaOH. Reaction mixture

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(100 μl) from each well was then transferred to a flat-bottomed E.I.A. microtiter plate (Flow Labs.) and the A_{405} read in a Titertek Multiscan apparatus (Flow Labs.). On each microtiter plate, 4 antibodies were tested against 3 lung ALPs and the control human placental ALP (type 1), each run in triplicate. Individual blanks were obtained by replacing substrate solution by buffer, and the whole plate was zeroed against substrate solution stopped at zero time. The reactivity of a particular lung ALP relative to the reactivity of human placental lung ALP with a particular monoclonal antibody, was determined by dividing the mean absorbance for the test sample by the mean absorbance for the human placental sample with the same antibody on the same plate. This procedure for estimating relative reactivity (*RR*) was adopted so as to minimize plate-to-plate variation.

3. RESULTS

In the electrophoretic system, binding of a particular antibody to a particular ALP is demonstrated by the disappearance of the zone of activity corresponding to that seen in the absence of antibody and the appearance of slower migrating zones corresponding to the enzyme/antibody complexes [5]. With the control human placental ALP type 1, this effect was observed with all 18 antibodies. With the same procedure, all 4 Old World monkey lung ALPs were found to bind to 10 of the antibodies, and with 3 other antibodies the ALP from at least 2 of the lung ALPs from the 4 species showed binding. The remaining 5 antibodies appeared to be unreactive with the Old World monkey ALPs by this test. In those cases where no enzyme binding was detected, activity of the ALP

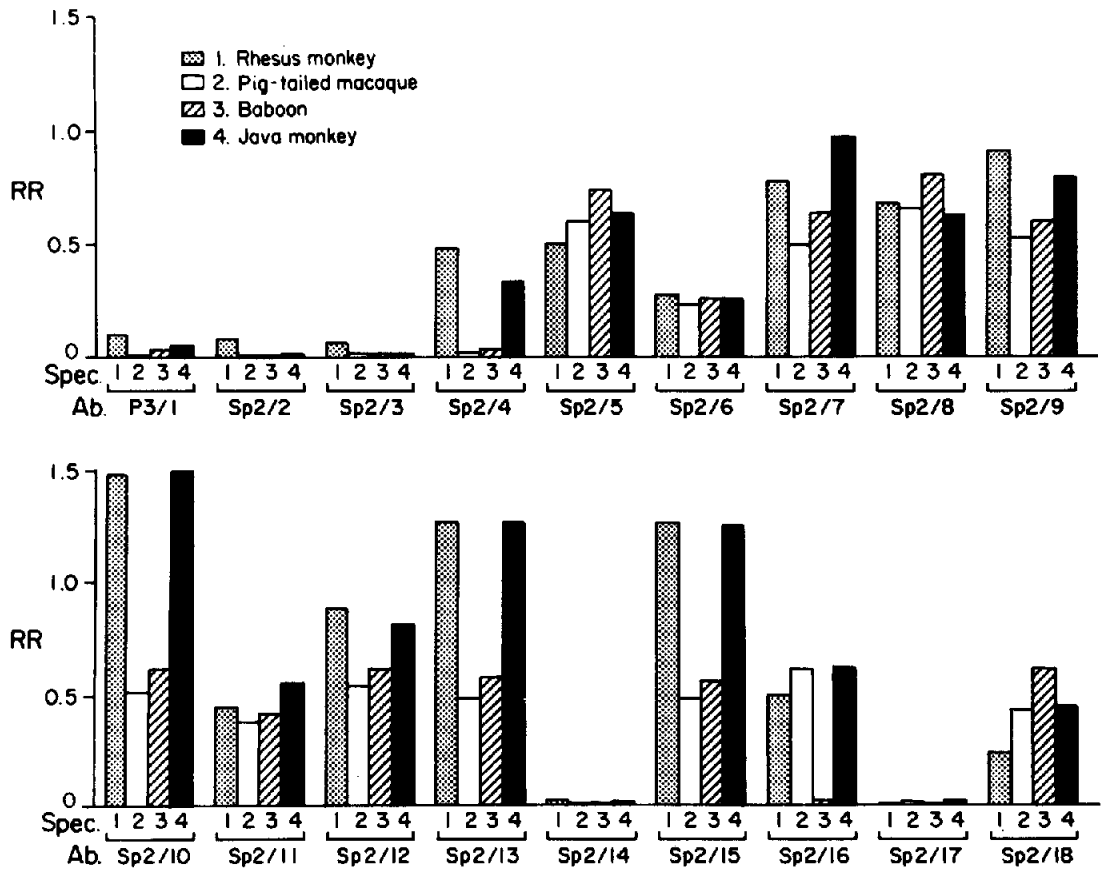

Fig.1. Reactivities of heat-stable lung ALPs from 4 Old World monkeys relative to human placental ALP (*RR*-values) with 18 monoclonal antibodies raised against human placental ALP.

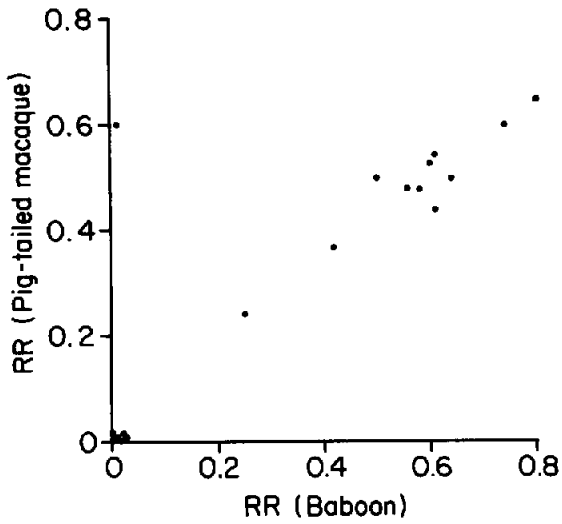

Fig.2. Correlation of *RR*-values for heat-stable lung ALPs from baboon and from pig-tailed macaque with 18 monoclonal antibodies raised against human placental ALP.

was unchanged after incubation with antibody.

The reactivities of the various lung ALPs relative to the reactivity of placental ALP with the 18 different antibodies estimated by the plate assay (*RR* determination) are shown in fig.1. There is a wide range of *RR*-values. Some show reactivities very similar to those obtained with human placental ALP ($RR \sim 1.0$). Others show virtually no cross-reactivity ($RR < 0.1$). The majority show intermediate levels of cross-reactivity. All the lung ALP:monoclonal antibody combinations which showed no apparent binding in the electrophoretic system gave $RR < 0.1$, the majority being close to zero. In general, there is a good correlation between the *RR*-values for the lung ALPs from the 4 Old World monkey species with the 18 monoclonal antibodies. This is illustrated for baboon and pig-tailed macaque in fig.2. However, there are occasional exceptions where with a particular antibody, a marked difference in cross-reactivity is seen with the lung ALP from different species. Thus in fig.2, one antibody gives an *RR* of 0.6 for pig-tailed macaque, but a value close to zero for baboon. Other examples are seen in fig.2.

4. DISCUSSION

The results show how the close immunologic similarity of the thermo-stable Old World monkey lung ALPs with human placental ALP, originally detected with polyclonal antisera, can be dissected using a panel of monoclonal antibodies. Each monoclonal antibody is thought to bind to an antigenic determinant that represents a small region on the enzyme surface made up of just a few not necessarily sequential amino acids. The Old World monkey lung ALPs and human placental ALP may be presumed to have evolved from a common ancestral form, the differences in structure they now exhibit being the consequence of point mutations (amino acid substitutions, etc.) which occurred in the course of their divergent evolutions. Cross-reactivity with a given monoclonal antibody implies that the antigenic determinant in human placental ALP, against which the antibody was raised, is represented in the Old World monkey ALP, though perhaps with modifications derived from structural changes in evolution. The relative degree of cross-reactivity provides an indication of the extent of such structural differences. The wide variation in *RR*-values indicates the degree to which the structures of different determinant sites have been modified. Those antibodies that show strong cross-reactivity with each of the Old World monkey lung ALPs are presumably directed to antigenic determinant sites that have been relatively highly conserved in evolution.

The generally high correlation of the *RR*-values for the different monoclonal antibodies among the ALPs from the 4 Old World monkey species indicates that they share common immunologic and therefore structural characteristics that distinguish them from human placental ALP. However, departures from this common reactivity profile, in that with a given antibody, the ALP from one of the species may cross-react strongly and from another only weakly or not at all, do occur. These exceptions indicate structural changes in the particular determinant region that have occurred in the separate evolutions of the four related species, or are polymorphic within the species. The fact that in those cases where very little or no antibody binding was detected in the quantitative study, the ALP in the electrophoretic study after incubation with the particular antibody showed no change in

activity from its control, indicates that the low *RR*-values in these cases cannot be attributed to inhibition of enzyme activity by the antibody.

In general, the results show the potential value of quantitative studies with a panel of monoclonal antibodies in investigations on enzyme evolution.

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