Note

Production of Monoclonal Antibodies against Placenta-type Alkaline Phosphatase

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Assay of placenta type alkaline phosphatase (P1-ALP) is important clinically for monitoring malignancies, since P1-ALP can be detected in the blood of a proportion of cancer patients.^{1,2)} Previous assays for detecting P1-ALP in blood have been based on prior heat inactivation of all non-placental ALP isozyme activity and/or the use of polyclonal anti-human ALP sera suitably cross-adsorbed to render them specific for the P1-ALP isozyme.^{3~6)} There remains concern, however, that estimation of low levels of P1-ALP could occur in P1-ALP negative samples with high total ALP levels as a result of either incomplete heat-inactivation or trace amounts of antibody reactive with non-placental ALP isozymes.

To establish a highly sensitive and specific assay for P1-ALP we produced monoclonal antibodies to P1-ALP. Crude P1-ALP (Miles Lab.) was purified on DEAE-Sephrose as described.⁷⁾ The protein thus purified showed a single main band on polyacrylamide gel electrophoresis by protein and also enzyme activity staining with β -

FIG. 1. Immunohistochemical Staining of Human Placenta Section.

1a shows the immunohistochemical staining pattern of human placenta with an monoclonal antibody raised to α -amylase inhibitor in wheat (K. Maeda *et al.*, unpublished data) as a control. Counter staining, (Hematoxylin). $\times 200$.

1b shows the immunohistochemical staining pattern of human placenta with APAP-2. Syncytiotrophoblasts were stained mainly. Moreover, the staining was also observed diffusely in the connective tissue around the cells or around the vessels. Counter staining, (Hematoxylin). $\times 200$.

naphthyl acid phosphate.⁷⁾ Female BALB/c mice were immunized by subcutaneous injection of 30 μ g of protein with Freund's complete adjuvant. A similar injection of 30 μ g of protein was given three weeks later. Another two weeks later 30 μ g of protein was injected intravenously. Three days after the last injection, spleen cell suspensions were prepared for cell fusion. P3-NS-1/1-Ag4-1 mouse myeloma cells were grown in RPMI1640 medium supplemented with 10% fetal calf serum at 37°C in humidified 5% CO₂/95% air. The myeloma cells (2×10^7) were fused with spleen cells (2×10^8) from a hyperimmunized mouse in polyethylene glycol 4000 (Merck, Darmstadt, West Germany) as described⁸⁾ in the above medium. The cells were suspended at 2×10^6 cells/ml in hypoxanthine-aminopterin-thymidine medium⁹⁾ in 2-ml wells of 24-well tissue culture plates. After hybrid clones had appeared (10~14 days after fusion), culture medium were screened for antibodies by cellular radioimmunoassay.^{10,11)} Cultures that secreted antibodies were diluted with medium containing hypoxanthine (1×10^{-4} M) and thymidine (1.6×10^{-5} M) in microtiter plates. After growth and re-assay, the cells were diluted in 2-ml wells in medium without hypoxanthine or thymidine. Cells from these wells were cloned by limiting dilution with thymocytes (4×10^6 /ml) as the feeder layer. Culture medium supernatants of the clones were tested and positive cells in positive wells were cloned and recloned at a density of 1 cell per well until stable. Clones were judged stable when all wells containing a single clone were positive. Two clones producing monoclonal antibodies, APAP-1 and APAP-2, were thus obtained and frozen in liquid nitrogen. The monoclonal antibodies, APAP-1 and APAP-2, are specific for P1-ALP and did not show any affinity towards several proteins examined such as α -amylase inhibitor from wheat¹²⁾ and a protein secreted from *Leucocytozoon caulleryi* (T. Nagasaki *et al.*, unpublished data) in the cellular radio immunoassay. The specific affinity of APAP-1 and APAP-2 to P1-ALP was also confirmed by immunohistochemical staining¹³⁾ with methanol-fixed human placenta sections. The placenta sections were incubated with APAP-1 and APAP-2 and then with secondary antibody, affinity purified F(ab')₂ fragment sheep anti-mouse IgG conjugate with horse radish peroxidase (Cappel Lab. Inc., U.S.A.). After washing the bound antibody, peroxidase was revealed by reacting with 3,3'-diaminobenzidine (Fig. 1a). No such a staining was observed with the control monoclonal antibodies (Fig. 1b). Figure 2 shows the result of competitive inhibition assay¹¹⁾ with APAP-2. A significant competition of free P1-ALP and the enzyme coupled to sheep red blood cells is evident. A similar result was obtained with APAP-1. These facts confirm the specific binding of P1-ALP to the monoclonal antibodies obtained. These antibodies, APAP-1 and APAP-2, did not show any cross-reactivity with the other organ-specific ALP isozymes such as the liver, bone, and intestinal forms of ALP.

The class and subclass of the monoclonal antibodies was

FIG. 2. Competitive Binding Inhibition Assay of APAP-2.

For the competitive inhibition assay, 25 μ g of antibody solution (culture supernatant of hybridoma was diluted 1:1 with phosphate buffered saline containing 0.1% bovine serum albumin), was absorbed with 25 μ l of competitor protein and the mixture was incubated overnight at 4°C. The absorbed antibody was assayed against sheep red blood cells coupled with P1-ALP or an α -amylase inhibitor.¹²⁾ The cellular radioimmunoassay used was described in the references.^{10,11)} The inhibition caused by the original concentration of the competitor protein was set to 100% while the inhibition caused by the competitor with 27 times dilution was taken as 0%, since no significant inhibition was observed with more diluted competitor. The axis labelled 'dilution' shows the dilution of the competitor protein (mg/ml in the original concentration).

determined by immunodiffusion of hybridoma culture supernatants against rabbit antisera to mouse immunoglobulins (Miles Lab., Indiana, U.S.A.). Both APAP-1 and APAP-2 were found to be IgM. Furthermore APAP-2 was shown to cross-react with anti-mouse κ chain serum (Nordic Immunological Lab., Netherland) while APAP-1 did not. An application of these monoclonal antibodies to the assay of P1-ALP in blood and urine is under way.

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