Jpn. J. Cancer Res. 82, 862–870, July 1991

Detection of Bone-type Alkaline Phosphatase by Monoclonal Antibodies Reacting with Human Osteosarcoma-associated Antigen

Satoshi Nagoya,[1] Toshimitsu Uede,[1,2] Takuro Wada,[3] Seiichi Ishii,[3] Shinya Yamawaki[4] and Kokichi Kikuchi[1]

[1]Department of Pathology and [3]Department of Orthopedic Surgery, Sapporo Medical College, S-1, W-17, Chuo-ku, Sapporo 060, [2]Section of Immunopathogenesis, Institute of Immunological Science, Hokkaido University, N-15, W-7, Kita-ku, Sapporo 060 and [4]Department of Orthopedic Surgery, Sapporo National Hospital, Kikusui 4-2, Shiroishi-ku, Sapporo 060

The antigen detected by monoclonal antibodies reacting with human osteosarcoma-associated antigen was shown to be a phosphatidyl-inositol (PI)-glycan-anchored protein, which can be released from the cell surface by PI-specific phospholipase C-treatment. The antigen detected by 2D3 and 2H10 antibodies exhibited alkaline phosphatase activity. Both antibodies strongly reacted with bone-type alkaline phosphatase. However, importantly, immunohistochemical analysis demonstrated that 2D3 and 2H10 did not react with alkaline phosphatase present in kidney or liver. In addition, neither placental nor intestinal alkaline phosphatase was recognized by 2D3 and 2H10 antibodies. These results indicated that two monoclonal antibodies, 2D3 and 2H10, are highly specific for bone-type alkaline phosphatase and can distinguish bone alkaline phosphatase from liver alkaline phosphatase in spite of the fact that liver and bone alkaline phosphatase are encoded by the same gene.

Key words: Bone-type alkaline phosphatase — Monoclonal antibody — Osteosarcoma

Bone alkaline phosphatase (ALP) is an osteoblastic marker and the appearance of this enzyme coincides with bone formation.[1] Furthermore, detection of ALP in tumor tissues might indicate the presence of bone tissue-derived malignancies. Several monoclonal antibodies highly specific for human osteosarcoma have been reported.[2–6] In fact, OST antibodies previously described as monoclonal antibodies reacting with human osteosarcoma have been shown to recognize a serum ALP.[3,4] We also previously reported the generation of monoclonal antibodies, 2D3 and 2H10, highly specific for human osteosarcoma.[5] The physicochemical nature of the antigen detected by these monoclonal antibodies was very similar to that of human ALP. We report here that further immunochemical analysis clearly demonstrated the identity of the antigen detected by 2D3 and 2H10 antibodies to ALP. In man, the products of at least three ALP gene loci, namely, placental, intestinal, and liver/bone/kidney (L/B/K) are distinguishable.[7] Harris *et al.* described the isolation and nucleotide sequence analysis of a complementary DNA (cDNA) corresponding to a human L/B/K ALP[8] and made it possible to address the question of whether the ALPs expressed in liver and bone are encoded by the same gene. The polypeptide encoded by this cDNA is identical to human liver ALP at the 87 amino acid positions that they have determined by sequencing the purified liver ALP. These data indicated that liver and bone ALPs contain the same protein moieties and are likely to be encoded by the same gene. Therefore, it is possible that antibodies raised against human bone ALP may crossreact with liver as well as kidney ALP. Some monoclonal antibodies reacting with human bone ALP also reacted with liver ALP.[9] However, minor molecular heterogeneities among L/B/K ALPs from various tissues are detected on the basis of differences in electrophoretic mobility and thermostability.[10,11] On the other hand, it is also possible to raise monoclonal antibodies against unique epitopes of the same antigen molecule. In fact, some monoclonal antibodies raised against swine kidney ALP crossreacted with human bone as well as kidney ALPs, but not with human liver ALP.[12] We report here that 2D3 and 2H10 monoclonal antibodies reacted with bone ALP, but not with kidney or liver ALP. Therefore, the present report demonstrates that 2D3 and 2H10 antibodies recognized a unique antigenic determinant present on L/K/B type human ALP and confirms the potential usefulness of these monoclonal antibodies for the immunodiagnosis of bone-derived malignancies.

MATERIALS AND METHODS

Normal and tumor tissues Fresh tumor specimens were obtained from patients undergoing surgery. Placental tissues were obtained from normal delivery materials, and normal tissues were obtained at autopsy. Small pieces of viable tissues were snap-frozen in liquid nitrogen and kept at −70°C until cryostat sectioning. Several osteosarcoma, chondrosarcoma, liposarcoma, malignant fibrous histiocytoma and rhabdomyosarcoma tissues ob-

tained from surgical specimens were implanted in athymic BALB/c mice subcutaneously and serially passaged as subcutaneous tumor lines in nude mice. Serum was taken from the tumor-bearing nude mice at various times. At the time of serum sampling, tumor size was measured.

Cell lines and reagents The human osteosarcoma cell line, KIKU established at the Department of Orthopedic Surgery, Sapporo Medical College, and a human osteosarcoma cell line, MG63, were maintained in Dulbecco's minimal essential medium containing 10% fetal calf serum.[5,13] 2D3, 2H10, OST-6, OST-7, and OST-15 monoclonal antibodies reacting with human osteosarcoma cells were used in these experiments.[3,5] My7 (CD13), that reacts with human myeloid cells,[14] was purchased from Coulter Immunology, Hialeah, FL.

Cytofluorography KIKU cells were stained by indirect membrane immunofluorescence by using monoclonal antibodies against human osteosarcoma cell and fluorescein isothiocyanate-conjugated (FITC)-goat anti-mouse immunoglobulin (Ig). Twenty thousand cells were counted by a fluorescence-activated cell sorter and the data were expressed as a histogram in which the fluorescence intensity (log) was plotted on the abscissa against the relative number of cells on the ordinate.

Immunohistochemical analysis Cryostat sections of the various normal and tumor tissues fixed with cold acetone for 10 min at 4°C were incubated with 2H10 and 2D3 monoclonal antibodies (MoAb), OST MoAbs, My7 MoAb or control normal murine IgM for 1 h at room temperature. The sections were washed 3 times with phosphate-buffered saline, pH 7.4 (PBS) for 30 min, and were again reacted with biotinylated horse anti-mouse Ig serum (Vector Laboratories, Inc.) for 1 h. After being washed with PBS, the sections were stained by an avidin-biotin-peroxidase method by using avidin-biotin-peroxidase complexes (Vector Laboratories, Inc). For the localization of ALP activity in tissue sections, cryostat sections of various tissues were incubated with 0.1 *M* Tris HCl buffer, pH 8.5 containing 0.173 m*M* naphthol AS-MX phosphate (Sigma) and 1.32 m*M* 4-benzoylamino-2,5-dimethoxybenzene-diazonium chloride hemi-[zinc chloride] salt at 37°C for 20 min. The sections were washed with distilled water, and nuclei were counterstained with hematoxylin. After being washed, sections were mounted with 1% gelatin and 2% formalin solution.

Immunochemical characterization KIKU cells were externally labeled with ^{125}I by the lactoperoxidase technique as described previously[15] and the cell membrane was disrupted by lysis buffer (0.05 *M* Tris-HCl buffer, pH 7.4 containing 0.14 *M* NaCl, 0.5% Nonidet P-40, 1 m*M* phenylmethylsulfonyl fluoride, 1 μg/ml of pepstatin, 0.05% sodium azide, 0.2 TIU/ml of approtinin, 8 m*M* iodoacetamide, and 5 m*M* ethylenediaminetetraacetic acid) for 90 min at 4°C. Aliquots of the labeled cell membrane were incubated with 2H10, 2D3, or OST MoAbs coupled to Affi Gel (Bio-Rad Japan, Tokyo). Affinity-purified antigens were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and nonreducing conditions. Sequential immunoprecipitation experiments were also carried out. Aliquots of labeled antigen, absorbed with 2H10 or OST MoAb coupled to Affi Gel, were again incubated with 2H10 or OST coupled to Affi Gel. Affinity-purified antigens were analyzed by SDS-PAGE.

Antibody competition study To determine if 2H10, 2D3, and OST recognize different antigenic determinants, a binding inhibition assay was conducted. The affinity-purified 2H10 and 2D3 were biotinylated. Two hundred thousand KIKU cells were incubated with a saturating amount of cold 2H10, 2D3, or OST at 4°C for 30 min. After washing of the cells with PBS, they were reacted with biotinylated 2H10 or 2D3, washed, stained with avidin-FITC, and analyzed by using a fluorescence-activated cell sorter.

Enzyme treatment Phosphatidylinositol-specific phospholipase C (PI-PLC) purified from *Bacillus thuringiensis* was purchased from Wako Pharmaceutical Co. Ltd., Tokyo. KIKU cells or frozen sections were treated with 1 U/ml of PI-PLC for 30 min at 37°C. After being washed with PBS, cells and sections were stained with various antibodies. The presence of ALP activity in tissue sections was also studied after PI-PLC treatment.

Assay of ALP with purified monoclonal antibodies First, 96-well microtiter plates were coated with purified monoclonal antibody, 2H10, 2D3, or OST (24, 12, or 6 μg/ml in 50 m*M* Na_2Co_3, pH 9.5) overnight at 4°C. The plates were washed with sodium phosphate buffer (10 m*M*, pH 7.4) containing 0.14 *M* NaCl, 500 mg of Tween-20, and 200 mg of NaN_3 per liter. The antibody-coated wells were then incubated with 50 μl of sample for 4 h at room temperature. Samples were obtained from KIKU cells, MG63 cells, liver tissues from an obstructive jaundice patient or normal kidney tissues. One ml of PBS buffer containing 0.5% NP-40 was added to cell pellets (1×10^7 cells) or tissues (10 mg) and incubated for 90 min. The lysate was obtained by centrifugation. In some experiments, serum samples were obtained from tumor-bearing nude mice and 50 μl of undiluted serum was added to the plates. The plates were again washed with the same buffer and 100 μl of ALP substrate cocktail (0.1075% disodium phenyl phosphate:0.045% 4-amino-antipyrine in 50 m*M* carbonate buffer, pH 10.1) was added and incubated for up to 20 min at 37°C.[16] The reaction was then stopped with 100 μl of 2 *N* NaOH and A_{405} was determined with an MR600 Microplate Reader (Dynatech Laboratories, Inc., Alexandria, VA).

RESULTS

2D3- and 2H10-defined antigens as PI-glycan-anchored proteins Cells of the human osteosarcoma cell line KIKU were treated with medium or 1 U/ml of PI-PLC at 37°C for 30 min, then washed and stained with OST-15, 2D3, 2H10 or My7 MoAbs. As shown in Fig. 1, the antigen detected by OST-15, 2D3, and 2H10 decreased significantly from 76% to 53%, 68% to 43%, and 71% to 50%, respectively after PI-PLC treatment, whereas the antigen detected by My7 was not affected by PI-PLC treatment (Fig. 1; d). Next we examined whether OST-15-, 2D3-, and 2H10-defined antigens on freshly isolated human osteosarcoma tissues are also PI-glycan-anchored protein. Thus, five human osteosarcoma tissues were treated with medium or 1 U/ml of PI-PLC. The detection of ALP activity on osteosarcoma tissue sections was also carried out before and after PI-PLC treatment by immunohistochemical methods. ALP activity as well as OST-15, 2D3, and 2H10 reactivity was significantly diminished in all cases tested. However, My7 reactivity was not altered. The results of one representative case are shown in Fig. 2. Finally, five normal bone tissues were also treated with 1 U/ml PI-PLC. The PI-glycan-anchored nature of the OST-15-, 2D3-, or 2H10-defined antigen, as well as the ALP activity, was not restricted to KIKU cells and human osteosarcoma tissues. As shown in Fig. 3, ALP activity as well as OST-15, 2D3, 2H10 reactivity were significantly diminished after PI-PLC treatment. My7 weakly stained normal bone tissues and the My7 reactivity was not altered after PI-PLC treatment (Fig. 3; d).

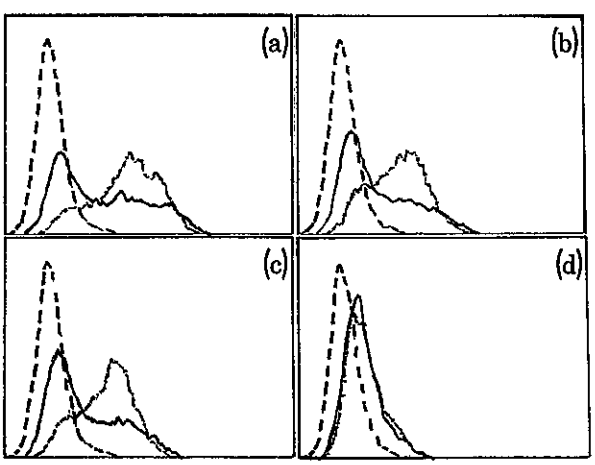

Fig. 1. The effect of PI-PLC treatment on the expression of 2D3- or 2H10-defined antigen. Cells of the human osteosarcoma cell line KIKU were stained with OST-15 (a), 2D3 (b), 2H10 (c), or My7 (d). The dotted, solid, and dashed lines indicate data before and after PI-PLC treatment and the control, respectively.

Immunochemical characterization of antigens defined by 2D3, 2H10 and OST In order to determine whether antigens defied by 2D3 (or 2H10) and OST are the same, sequential immunoprecipitation studies were carried out. As shown in Fig. 4, 2D3 as well as OST-15 antibody immunoprecipitated antigens with an approximate molecular weight of 75,000. When radiolabeled KIKU cell lysates were first absorbed with 2D3 antibody coupled to Affi Gel, 2D3 as well as OST-15 failed to immunoprecipitate the Mr 75,000 component. Furthermore, when the radiolabeled KIKU cell lysates were first absorbed with OST-15 antibody coupled to Affi Gel, 2D3 as well as OST-15 failed to immunoprecipitate the Mr. 75,000 component. Therefore, 2D3 and OST-15 recognize the same antigen molecule. Since previous data reported by Tanaka *et al.* showed that OST recognizes ALP,[4] it is likely that 2D3 and 2H10 also recognize ALP. In order to confirm that 2D3 recognize ALPs, cell lysates obtained from KIKU cells (ALP activity-positive), and MG63 cells (ALP activity-negative) were applied to 2D3 MoAb-coated, 96-well plates. The plates were washed, and ALP substrate was added to the wells.

As shown in Fig. 5, ALP activity was detected in antigen derived from KIKU cells bound to 2D3-coated as well as OST-coated plates. In contrast, ALP activity was not detected in antigen derived from MG63 cells or antigen derived from KIKU cells bound to control antibody-coated plates. ALP activity was detected in antigen derived from obstructive jaundice liver tissues bound to OST-15 antibody-coated plates. However, ALP activity was not detected in antigen derived from kidney tissues bound to OST-15 as well as 2D3 (or 2H10) antibody-coated plates.

Antibody competition study Next we determined whether 2D3 and 2H10, and OST MoAbs recognize different antigenic determinants on the same antigen. When KIKU cells were stained with biotinylated 2D3 antibody followed by avidin-FITC, approximately 58% of cells were positively stained (Fig. 6; a and b). Pretreatment of KIKU cells with saturating a mounts of 2D3 antibody significantly reduced the percentage of cells positively stained with biotinylated 2D3 antibody from 58% to 34% (Fig. 6; a). In contrast pretreatment of KIKU cells with OST-15 antibody did not significantly influence the subsequent staining of KIKU cells with biotinylated 2D3 antibody (Fig. 6; b). In addition, pretreatment of KIKU cells with 2H10 antibody significantly reduced the percentage of cells positively stained with biotinylated 2H10 antibody from 62% to 25% (Fig. 6; c). However, pretreatment of KIKU cells with OST-15 antibody did not influence the subsequent staining of KIKU cells with 2H10 antibody (Fig. 6; d). Thus, 2D3 as well as 2H10

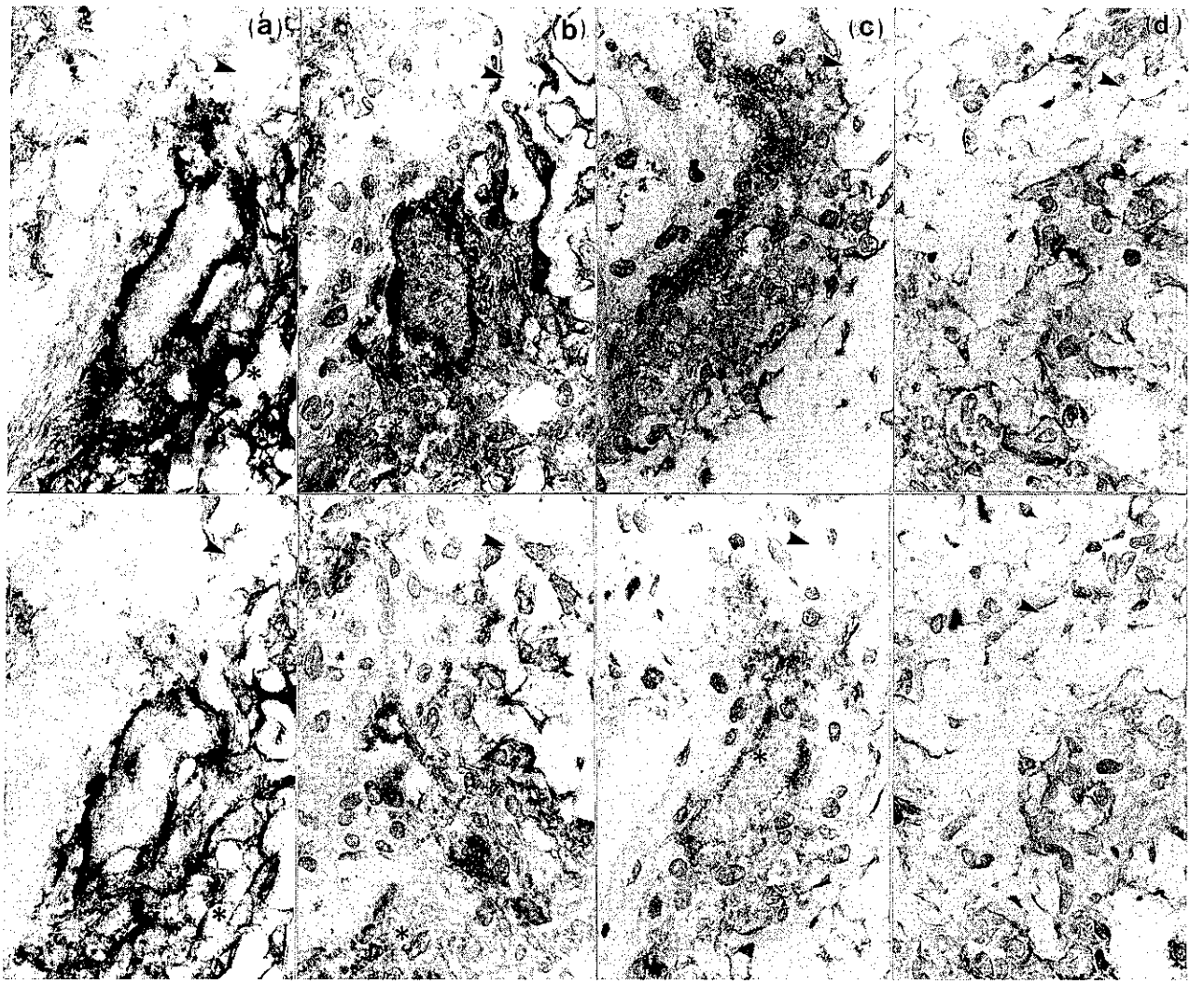

Fig. 2. Immunohistochemical analysis of human osteosarcoma tissues. The ALP activity was detected on tissue sections (a) before (upper row) and after (lower row) PI-PLC treatment and tumor tissues were stained with OST-15 (b), 2D3 (c) and My7 (d) MoAbs before (upper row) and after (lower row) PI-PLC treatment. Note the difference of ALP activity and staining intensity between areas indicated by ∗. Arrows (➞) indicate the presence of osteoid in tumor tissues.

MoAbs recognize an antigenic determinant different from that recognized by OST-15, based on the lack of competition of the two antibodies for binding.

A unique antigenic determinant defined by 2D3 and 2H10 Since OST and 2D3 as well as 2H10 monoclonal antibodies recognized bone-type ALP, we examined whether these antibodies recognize placental as well as kidney and liver-type ALP. For this purpose, each tissue was obtained from five different cases. Strong ALP activity was detected in all samples of human placenta, kidney, and jaundiced liver by immunohistochemical methods. In placenta, ALP activity was clearly detected in Langhans cells of chorionic villi. In kidney, ALP activity was detected in endothelial cells of veins as well as epithelial cells of urinary tubules. In contrast, 2D3, 2H10, and OST antibodies failed to react with any normal kidney or placenta tested. OST antibodies reacted strongly with all liver tissues obtained from five different obstructive jaundice patients with high serum alkaline phosphatase levels. Strong ALP activity was noted in liver tissue derived from the same patient. In contrast, 2D3 as well as 2H10 failed to react with liver tissues obtained from any of the five jaundice patients. 2D3 and 2H10 antibodies did not stain normal liver tissues, as described previously.[5] OST, 2D3, or 2H10 MoAbs failed to react with intestinal tissue that exhibited ALP activity

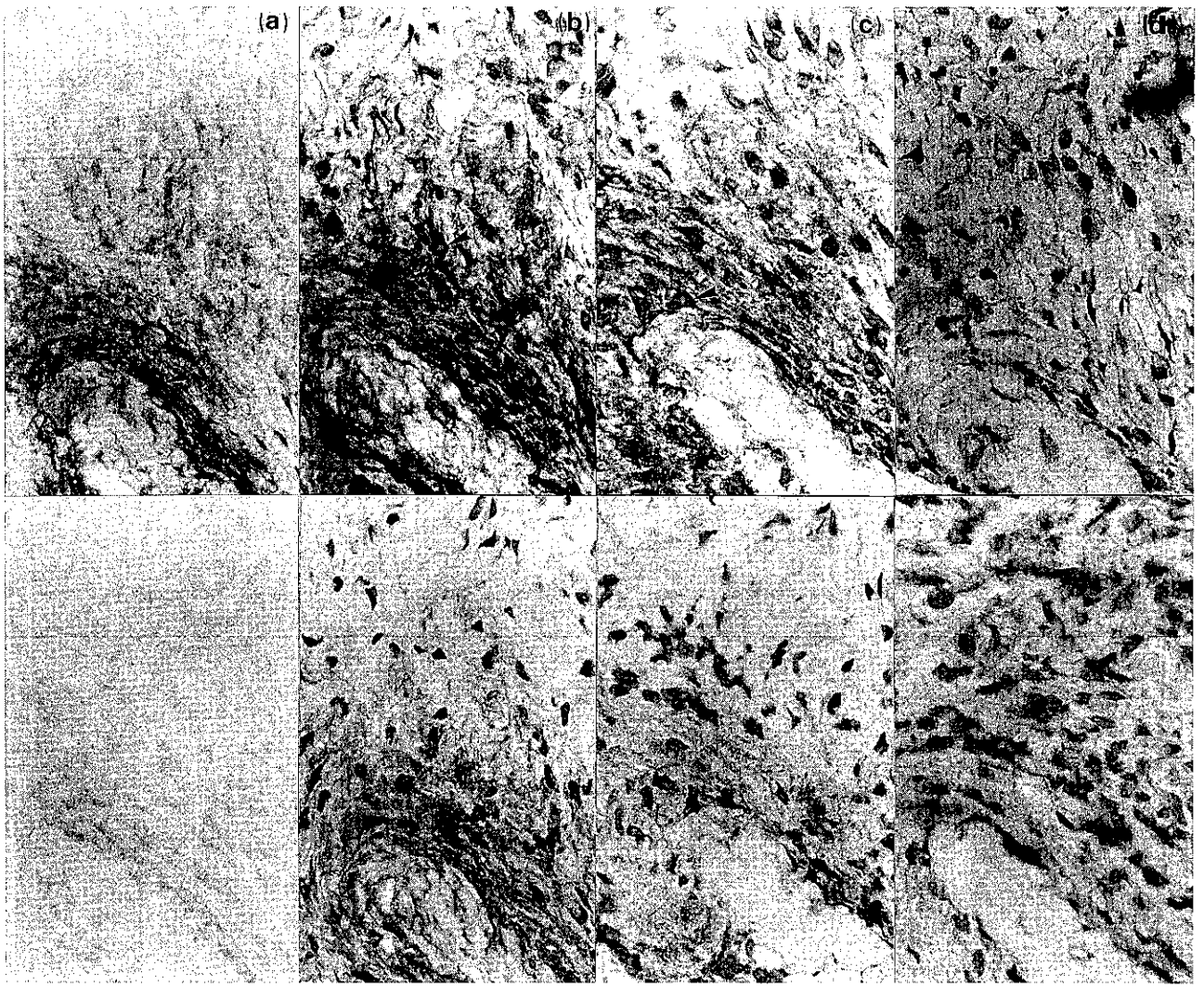

Fig. 3. Immunohistochemical analysis of human normal bone tissues. The ALP activity was detected on tissue sections (a) before (upper row) and after (lower row) PI-PLC treatment and tumor tissues were stained with OST-15 (b), 2D3 (c) and My7 (d) MoAbs before (upper row) and after (lower row) PI-PLC treatment. Note that osteoblasts (→) possess ALP activity and are positively stained with OST-15 and 2D3 antibodies.

(data not shown). Representative results are shown in Fig 7.

The detection of a serum antigen by using 2D3-coated plates Next, we determined whether 2D3 MoAb can be used for the detection of serum ALP. Various human soft tissue and bone derived malignancies were implanted into nude mice. As shown in Fig. 8, the level of antigen detected by using 2D3-coated plates reflected the tumor volume. Furthermore, a high level of ALP was detected in serum of all human osteosarcoma-bearing mice tested, but not in any mice bearing rhabdomyosarcoma, liposarcoma, malignant fibrous histiosarcoma, and chondrosarcoma, or in five normal mice.

DISCUSSION

In this study, we have characterized an antigen defined by 2D3 as well as 2H10. The immunochemical nature of the antigen defined by 2H10 and 2D3 is very similar to that of the antigen recognized by OST.[3-5] In addition, OST MoAbs were found to recognize human bone as well as liver ALP. In order to clarify the relationship among 2D3- or 2H10-defined, and OST-defined antigen, a sequential immunoprecipitation study was carried out. The data clearly demonstrated that antigens defined by 2D3, 2H10, and OST are the same molecule, indicat-

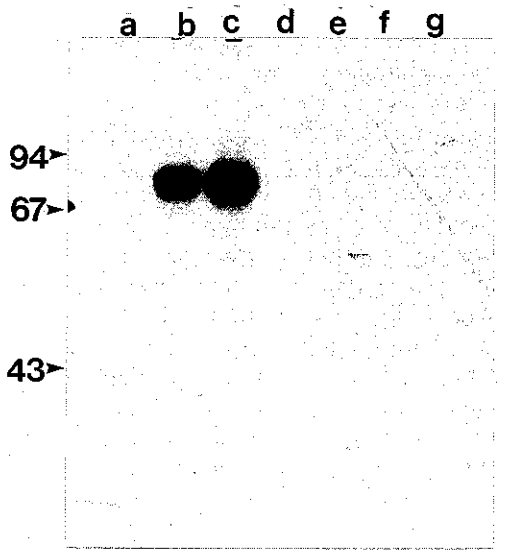

Fig. 4. Relationship between 2D3-defined and OST-15-defined antigen. Sequential immunoprecipitation experiments were performed using 2D3 and OST-15 antibodies. Cell lysates derived from surface radiolabeled KIKU cells were immunopurified by using normal mouse Ig (a), 2D3 (b) or OST-15 antibody (c). A lysate precleared with 2D3 was reimmunoabsorbed with 2D3 (d) or OST-15 (e). A lysate precleared with OST-15 was reimmunoabsorbed with 2D3 (f)or OST-15 (g). Immunopurified antigens were then analyzed by SDS-PAGE. Molecular weight marker proteins: phosphorylase b (Mr 94,000); bovine serum albumin (Mr 67,000); and ovalbumin (Mr 43,000).

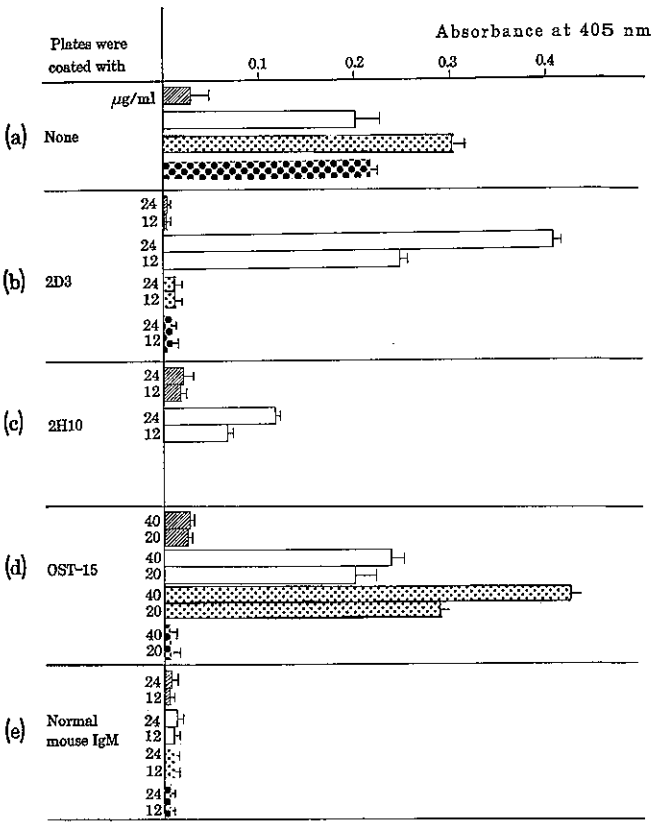

Fig. 5. The detection of ALP activity in 2D3-, 2H10-, and OST-15-defined antigen. One ml of cell lysate was obtained from 1×10^7 MG63 cells (▨) or 1×10^7 KIKU cells (□), 10 mg of liver tissue (⊡), or 10 mg of kidney tissue (⊞). Ten microliters of cell lysate was incubated with 50 μl of ALP substrate (a). Plates were coated with various concentrations of antibodies (b–e). Fifty microliters of cell lysate was added to each well. After washing of the plates, ALP activity was determined as described in "Materials and Methods." Each sample was tested in triplicate and the data are expressed as mean values ± SD.

ing that 2D3 and 2H10 also recognize human ALP. This result was confirmed by the fact that antigens extracted from KIKU cells, which bound to 2D3 as well as OST antibody-coated plates, exhibited ALP activity. The increase in serum ALP activity may be related to hepatic disease or bone disease. Therefore, ALP isoenzyme analysis is of clinical importance. Although the most common requirement for ALP isoenzyme analysis is to distinguish between bone- and liver-derived ALP, this is a particularly difficult task. Although methods based on subtle differences in electrophoretic mobility or stability to heat or urea denaturation have been developed,[10, 11, 17] these methods do not adequately separate the liver and bone isoenzymes. Two recent developments have improved the separation of bone and liver isoenzymes on electrophoresis.[18, 19] In the first method, bone ALP is precipitated quantitatively by wheat-germ lectin.[18] However, patients with hepatitis often exhibit pathological high pseudo-bone ALP activity.[18] In the second, serum samples are treated with neuraminidase before electrophoresis.[19] This enzyme removes negatively charged sialic acid residues more rapidly from the bone isoenzyme than from the liver isoenzyme, leading to reduced mobility of the bone isoenzyme relative to its liver counterpart. However, the physicochemical nature of isoenzymes circulating in serum may differ from that of isoenzymes extracted from the respective organs, for example, because membrane-localized glycosyltransferase might modify the enzyme molecule as it passes into the intravascular space.[20] The ALP isoenzymes isolated from liver, placenta, and bone are bound by wheat-germ lectin, whereas hepatobiliary and placental ALP in serum are not precipitated.[20, 21] The carbohydrate moieties in ALP may play an important role in the antigenicity of ALP. It should be noted that removal of sialic acid enhances the reactivity of 2D3 with human osteosarcoma tissues.[5]

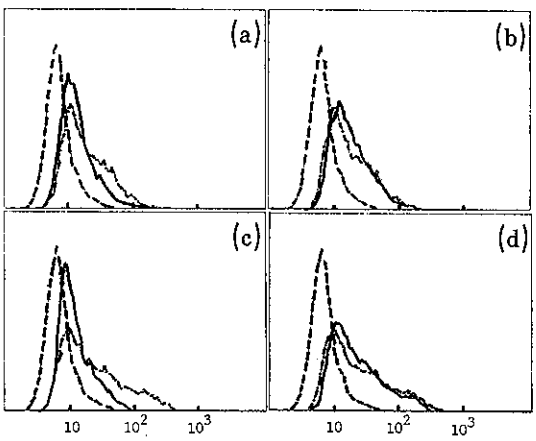

Fig. 6. Antibody competition study. KIKU cells were stained with biotinylated-2D3 (a and b) or 2H10 (c and d) followed by avidin-FITC (dotted lines). Before staining, KIKU cells were pretreated with a saturating amount of 2D3 (a), OST-15 (b and d) or 2H10 (c) MoAb (solid lines). The dashed lines indicate the control.

Several monoclonal antibodies have been raised against universal (L/B/K)-type alkaline phosphatase.[4, 9, 22] Although the bone ALP is the product of L/B/K (universal)-type ALP gene, it is possible to recognize minor immunological differences among liver, bone, and kidney enzymes. In fact, several attempts have been made to raise monoclonal antibodies that can be used for discrimination of bone ALP from liver ALP. Although crossreactivity with human placental and intestinal ALP is rare,[9] 14 antibodies out of 15 hybridoma clones[9] and 24 antibodies out of 31 hybridoma clones[22] did cross-react significantly with liver and bone ALP. However, one antibody raised against human bone ALP, B4-50[9] preferentially reacted with liver isoenzyme of human ALP, but only weakly reacted with bone ALP.[9] Another monoclonal antibody raised against swine kidney ALP crossreacted preferentially with human kidney as well as bone ALP, but not with liver ALP.[12]

These results led us to re-examine the tissue distribution of antigens defined by 2H10, 2D3, and OST MoAbs.

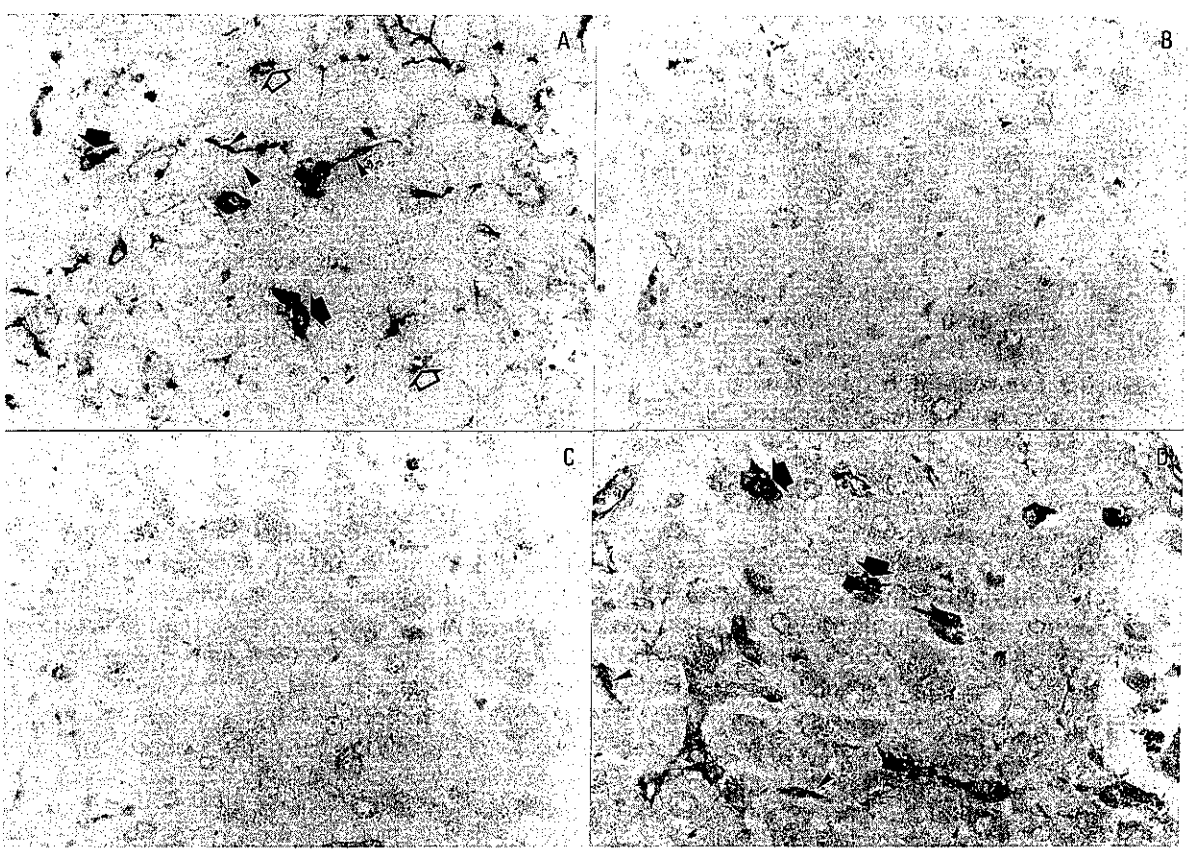

Fig. 7. The detection of ALP activity in liver tissues obtained from an obstructive jaundice patient. (A) ALP activity was detected in endothelial cells (→), Kupffer cells (▶) and epithelial cells of bile canaliculi (▷). Liver tissues were stained with 2D3 (B), 2H10 (C), or OST-15 (D) MoAb. OST-15 antibody stained endothelial cells (→) as well as Kupffer cells (▶).

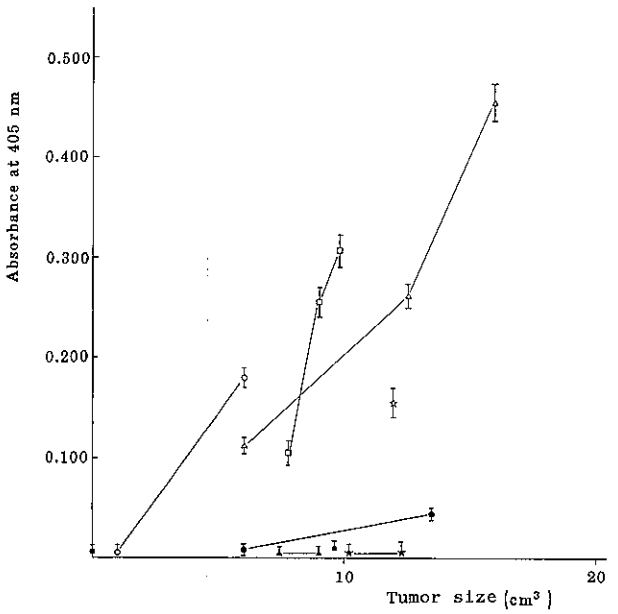

Fig. 8. The detection of ALP activity in 2D3-bound antigen in sera from osteosarcoma-bearing mice. The serum samples were taken from tumor-bearing nude mice and tumor size (X-axis) at the time of serum sampling was plotted. The samples were applied to 2D3-coated plates. After washing of the plates, each well was tested for the presence of ALP activity (Y-axis). Samples were tested in triplicate and the data were expressed as mean values±SD. Serum samples were obtained from normal mice (*) or mice bearing human osteosarcoma tissues (○, △, □, and ☆), chondrosarcoma tissues (●), rhabdomyosarcoma tissues (▲), liposarcoma tissues (■), and malignant fibrous histiocytoma tissues (★).

ALP exists in various tissues such as intestine, placenta, kidney, liver and bone.[8] 2D3 and 2H10 did not react with intestinal ALP, placental ALP, or kidney ALP as determined by immunohistochemical study. OST MoAbs reacted with not only osteosarcoma tissues but also normal as well as jaundiced liver tissues. Antibody competition experiments clearly indicated that 2D3 and 2H10 recognize antigenic determinants distinct from that defined by OST MoAbs. Competition by saturating amounts of unconjugated homologous antibody was not complete (Fig. 6). This result may be due to the low affinity of 2D3 and 2H10, and some of the bound 2D3 or 2H10 antibody may be released from the antigen during washing of the cells. Most importantly, in contrast to OST antibodies, 2D3 and 2H10 did not react with liver tissues, thus confirming the specificity of these antibodies to osteosarcoma tissues.

ALP is a PI-glycan-anchored protein and ALP can be released from the tumor tissues by hydrolysis with PI-PLC as described in this communication (Figs. 2 and 3). In addition, it can be detected on antigenic material in serum recognized by the 2D3 antibody. As shown in Fig. 1, 2D3-, 2H10- and OST-15-defined antigen was still detected on KIKU cells after PI-PLC treatment. ALP activity was also detected by immunohistochemical methods on human osteosarcoma tissue sections after PI-PLC treatment (Fig. 2). Partial resistance to release by PI-PLC has already been reported with various proteins, such as 5′-nucleotidase, Thy-1, Qa, and ALP, in a variety of cell types.[23] It is possible that ALP can exist in two forms, a PI-glycan-anchored form and a transmembrane polypeptide form, since LFA-3 and neural cell adhesion molecule (N-CAM) are also found in two forms.[24] It should be noted that a modification of the inositol ring at 2-OH with palmitate renders acetycholine-esterase insensitive to PI-PLC.[23] At present there is no evidence to suggest that there is a similar molecular modification in ALP. Thus, the basis for PI-PLC resistance of ALP is currently unknown, We have obtained sera from nude mice implanted with various soft tissue- and bone-derived malignant tumors at various intervals. 2D3-defined antigen was detected in serum of osteosarcoma-bearing nude mice, whereas nude mice bearing rhabdomyosarcoma, liposarcoma, malignant fibrous histiocytoma, and chondrosarcoma did not show elevated levels of 2D3-defined antigen in serum. These results suggest that 2D3 and 2H10 may be useful for the serum immunodiagnosis of soft tissue- and bone-derived malignancies.

(Received February 20, 1991/Accepted April 19, 1991)

REFERENCES

1) Farley, J. R. and Baylink, D. J. Skeletal alkaline phosphatase activity as a bone formation index *in vitro*. *Metabolism*, **35**, 563–571 (1986).
2) Tsang, K. Y., Warren, R. Q., Bishop, L., Pathak, S., Koger, B. and Lavia, M. F. Monoclonal antibodies to human osteosarcoma-associated antigen(s). *J. Natl. Cancer Inst.*, **77**, 1175–1180 (1986).
3) Hosoi, S., Nakamura, T., Yamamuro, T., Toyama, S., Shinomiya, K. and Mikawa, H. Detection of human osteosarcoma-associated antigen(s) by monoclonal antibodies. *Cancer Res.*, **42**, 654–659 (1982).
4) Tanaka, C., Yamamuro, T., Masuda, T., Tanaka, H., Matsumoto, M., Katoura, Y., Tsuji, T., Toguchida, J., Miyama-Inaba, M. and Ueda, M. Recognition of serum alkaline phosphatase by murine monoclonal antibodies against human osteosarcoma cells. *Cancer Res.*, **46**, 4863–

4857 (1986).
5) Wada, T., Uede, T., Ishii, S., Matsuyama, K., Yamawaki, S., and Kikuchi, K. Monoclonal antibodies that detect different antigenic determinants of the same human osteosarcoma-associated antigen. *Cancer Res.*, **48**, 2273–2279 (1988).
6) Tsai, C. C., McGuire, M. H., Mellitt, R. J., Ritter, R. A., III, Xu, J., Litwicki, D. J. and Roodman, S. T. Monoclonal antibody to human osteosarcoma: a novel Mr 26,000 protein recognized by murine hybridoma TMMR.2. *Cancer Res.*, **50**, 152–158 (1990).
7) Seargeant, L. E. and Stinson, R. A. Evidence that three structural genes code for human alkaline phosphatases. *Nature*, **281**, 152–154 (1979).
8) Weiss, M. J., Henthorn, P. S., Lafferty, M. A., Slaughter, C., Raducha, M. and Harris, H. Isolation and characterization of a cDNA encoding a human liver/bone/kidney-type alkaline phosphatase. *Proc. Natl. Acad. Sci. USA*, **83**, 7182–7186 (1986).
9) Lawson, G. M., Katzmann, J. A., Kimlinger, T. K. and O'Brien, F. Isolation and preliminary characterization of a monoclonal antibody that interacts preferentially with the liver isoenzyme of human alkaline phosphatase. *Clin. Chem.*, **31**, 381–385 (1985).
10) Rosalki, S. B. and Ying Foo, A. Two new methods for separation and quantifying bone and liver alkaline phosphatase isoenzymes in plasma. *Clin. Chem.*, **30**, 1182–1186 (1984).
11) Moss, D. W. and Whitby, L. G. A simplified heat-inactivation method for investigating alkaline phosphatase isoenzymes in serum. *Clin. Chim. Acta*, **61**, 63–71 (1975).
12) Goseki, M., Oida, S. and Sasaki, S. Detection of minor immunological differences among human universal-type alkaline phosphatases. *J. Cell. Biochem.*, **38**, 155–163 (1988).
13) Billian, A., Edy, V-G., Heremans, H., Damme, J. V., Desmyter, J., Georgiades, J. A. and Somer, P. D. Human interferon: mass production in a newly established cell line, MG63. *Antimicrob. Agents Chemother.*, **12**, 11–15 (1977).
14) Griffin, J. D., Ritz, J., Naddler, L. M. and Schlossman, S. F. Expression of myeloid differentiation antigens on normal and malignant myeloid cells. *J. Clin. Invest.*, **68**, 932–941 (1981).
15) Uede, T., Kohda, H., Yuasa, H., Osawa, H., Diamantstein, T., Yodoi, J., Ishii, Y. and Kikuchi, K. 5C6-F4, a novel 100KD rat lymphocyte activation antigen defined by monoclonal antibody. *J. Immunol.*, **136**, 3968–3976 (1986).
16) Kind, P. R. N. and King, E. J. Estimation of plasma phosphatase by determination of hydrolysed phenol with amino-antipyrine. *J. Clin. Pathol.*, **7**, 322–326 (1954).
17) Horne, M., Cornish, C. J. and Posen, S. The use of urea denaturation in the identification of human alkaline phosphatases. *J. Lab. Clin. Med.*, **72**, 905–915 (1968).
18) Behr, W. and Barnert, J. Quantification of bone alkaline phosphatase in serum by precipitation with wheat-germ lectin: a simplified method and its clinical plausibility. *Clin. Chem.*, **32**, 1960–1966 (1986).
19) Schreiber, W. E. and Sadro, L. C. Agarose gel patterns of alkaline phosphatase isoenzymes before and after treatment with neuraminidase. *Am. J. Clin. Pathol.*, **89**, 181–186 (1988).
20) Lorentz, K. Katalytische Konsentration, multiple Formen und Lektin affinitat von mikrosomalen Enzymen menschliches Gewebe. *J. Clin. Chem. Clin. Biochem.*, **19**, 1181–1187 (1981).
21) Lehmann, F. G. Differentiation of human alkaline phosphatases by lectin binding affinity. *Klin. Wochenshr.*, **58**, 947–951 (1980).
22) Seabrook, R. N., Bailyes, E. M., Price, C. P., Siddle, K. and Luzio, J. P. The distinction of bone and liver isoenzymes of alkaline phosphatase in serum using a monoclonal antibody. *Clin. Chim. Acta*, **172**, 261–266 (1988).
23) Low, M. G. and Satiel, A. R. Structural and functional roles of glycosylphosphatidylinositol in membranes. *Science*, **239**, 268–275 (1988).
24) Low, M. G. Glycosyl-phosphatidylinositol: a versatile anchor for cell surface proteins. *FASEB J.*, **3**, 1600–1608 (1989).