Vol. 112, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 29, 1983 Pages 431-436

MONOCLONAL ANTIBODY WITH HIGH AFFINITY FOR 1,25-DIHYDROXYCHOLECALCIFEROL

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Recieved March 7, 1983

SUMMARY: We have developed a monoclonal antibody capable of detecting 1 pg/ml of 1,25-dihydroxycholecalciferol. At a dilution of 1:80,000 of ascitic fluid this antibody has an apparent K_D of $3.3 \times 10^{-11} ML^{-1}$. The immunogen used was a vitamin D analogue, calcitroic acid [1α, 3 β-dihydroxy-9, 10 seco-24-nor 5,7,10 (19) cholatriene-23-oic acid], conjugated to bovine serum albumin. Although this antibody is extremely sensitive, it also recognizes other important vitamin D_3 metabolites.

The standard assay for 1,25-dihydroxycholecalciferol [$1,25(OH)_2D_3$] in serum employs an ardously obtained, unstable rachitic chick intestinal binding protein. In addition, due to a relative lack of specificity of this cytosolic receptor, each sample requires extensive chromatographic extraction and purification before it can be assayed (1-3). Because of these difficulties, this assay is performed in relatively few clinical laboratories. In an effort to procure more stable and specific reagents, polyclonal antibodies to vitamin D metabolites have been produced and used as high affinity binding agents in radioimmunoassays for these compounds (4). Monoclonal antibodies share the stability of polyclonal antibodies, but may be readily screened for a particular specificity that will discriminate among a large number of closely related compounds (5). Since such clones could be theoretically maintained forever, their development could circumvent the present difficulties of the standard $1,25(OH)_2D_3$ serum assay. In the preliminary evaluation of mono-

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0006-291X/83 $1.50

clonal antibodies to compounds closely related to vitamin D_3, we have isolated an antibody which, although not specific for 1,25(OH)D_3, displays high affinity toward the hormonal metabolite, and can reliably detect 1 pg/ml of 1,25$(OH)_2D_3$.

MATERIALS AND METHODS

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise noted. Calcitroic acid [1α, 3 β-dihydroxy-9, 10 seco-24-nor-5,7,10 (19) cholatriene-23-oic acid] and unlabeled vitamin D metabolites were the gift of Dr. Milan Uskokovic (Hoffmann-LaRoche, Nutley, New Jersey).

Sterol conjugation - 5 μmoles of isobutycholoroformate were added to calcitroic acid (5 μmoles), dissolved in 2 ml of cold, nitrogen-gassed dioxane (Baker, Phillipsburg, New Jersey). After 20 minutes at 5°, this solution was stirred into 3.0 ml of water adjusted to pH 9.0 with .1N NaOH, containing 12 mg of bovine serum albumin (BSA) (Miles Laboratory, Fort Wayne, Indiana). The pH was maintained at 9.0 for 30 minutes. After magnetic stirring for six hours at 5°, the dioxane was evaporated under N_2 and the residual layer was extracted with equal volumes of diethyl ether (Mallinckrodt, St. Louis, MO) until two successive extracts' residues exhibited no absorbance at 264 nm when N_2 dried, and solubilized in 1 ml of ethanol. The aqueous layer was lyophilized, redissolved in 1 ml phosphate buffered saline, pH 7.4, and applied to a 1 x 17 cm column of G-25 Sephadex (Pharmacia, Piscataway, New Jersey), equilibrated in the same buffer. The exclusion volume (Vo) was saved for spectral studies and protein assay (6). Ultraviolet spectroscopic studies of this preparation compared to those of BSA or calcitroic acid alone revealed an increase in absorbance at 264 nm consistent with the covalent conjugation of 5 moles of sterol per mole of protein (7). A similar synthesis was carried out using egg albumin.

Hybridoma production - BALB/c mice were immunized with 50 μg of calcitroic acid - BSA conjugate emulsified in complete Freund's adjuvant and boosted twice with antigen in saline at three week intervals. Four days after the final boost, spleen cells from these mice were fused with the Sp2/0-Ag 14 cell line (8) using the method of Galfre, et al (9). Cells were cultured in 24-well culture dishes in HAT selection medium (10). Monoclonal antibodies binding calcitroic acid were detected in culture supernatants by a radioimmunoassay in which supernatants were exposed to microtiter plates coated with a calcitroic acid-egg albumin conjugate. Bound monoclonal antibody was detected with a ^{125}I-labeled goat anti-mouse gamma globulin reagent. Cells from positive wells were cloned in soft agar over 3T3 cells (5) and grown in BALB/c mice as ascites tumors.

Preparation of Monoclonal Antibody - Ammonium sulfate was added to ascitic fluid to 40% saturation. The mixture was incubated at 4° for one hour, centrifuged at 1000 x g for 30 minutes, and the supernatant discarded. The pellet was dissolved in .025 M Tris-HCl buffer pH 7.5 with .08 M NaCl (assay buffer) and fractionated on an AcA_{44} column (1.5 x 45 cm) (LKB, Rockville, MD) equilibrated in assay buffer. Antibody eluted in the void volume and Trasylol, 10 mM molybdate and 1 mM EDTA were added. This preparation was diluted 1:10 (initial ascitic fluid vol: final vol) and frozen in 1 cc aliquots at -20°C until use.

Assays for 1,25-Dihydroxyvitamin D_3 - 1α, 25-dihydroxy [26,27-methyl-^3H] cholecalciferol (specific activity 168 Ci/mmole) (Amersham/Searle, Arlington

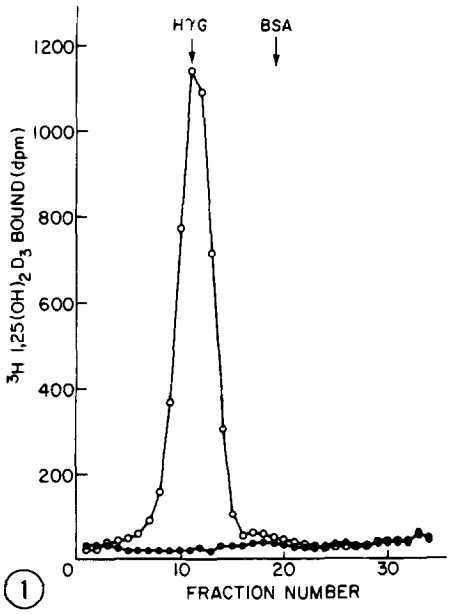
Heights, ILL) was purified on a 1 x 40 cm column of Sephadex LH20 (Sigma) slurried in hexane: chloroform (65:35, v/v) and eluted in the same solvent (3). Antibody at appropriate dilution was incubated at 4°C with reference $1,25(OH)_2D_3$ ranging from 1 pg - 1 ng in 10 λ of ethanol. In assays of binding specificity, $1,25(OH)_2D_3$, 24,25-dihydroxycholecalciferol [$24,25(OH)_2D_3$] and 25-hydroxycholecalciferol ($25OHD_3$) were used. .025 picomoles (10 µl) of radioligand was added; final assay volume was 1 cc. Radioligand was added after 1 hr preincubation in displacement assays; otherwise it was added immediately. After incubation for 18 hours, 10 λ of rabbit anti-mouse Fab' (gift of Dr. Judith Kapp-Pierce, Department of Pathology, Jewish Hospital of St. Louis and Washington University School of Medicine) was added with carrier mouse IgG and tubes were incubated for another 45 minutes at 4°C. Both gamma globulins were partially purified using DEAE - Affigel Blue (Biorad Laboratories, Richmond, CA). After centrifugation at 1000 x g for 20 minutes, the supernatants were aspirated, the pellets suspended in Budget Solve (Research Products International, Mount Prospect, ILL) and their radioactivity determined in a Beckman 7000 Scintillation Counter (Beckman Company, Palo Alto, CA).

For sucrose density gradient analysis, 350 λ of assay mixture or supernatant after immunoprecipitation was layered on a linear 5-20% sucrose gradient and centrifuged at 100,000 x g for 17 hours. Molecular markers, human gamma globulin (16 mg/ml assay buffer) (Calbiochem-Behring, La Jolla, CA) and BSA (12 mg/ml assay buffer) were centrifuged identically. Fractions were collected through a bottom puncture apparatus and their radioactivity assayed.

RESULTS

As shown in Figure 1, partially purified B2B4 binds labeled sterol and sediments on sucrose gradient identically with human IgG immunoglobulin (150,000 daltons). When 1 ng/ml of $1,25(OH)_2D_3$ is added to the mixture prior to centrifugation, the radioligand is almost completely displaced (data not shown). Following immunoprecipitation by the addition of rabbit anti-mouse Fab', the antibody peak is lost but all of the radioactivity is recovered in the immunoprecipitate (Figure 1).

Using immunoprecipitation, we next examined the association kinetics of antibody and radioligand. Antibody B2B4 (1:80,000 dilution) and radioligand were incubated for various time intervals, immunoprecipitated and the radioligand in the pellet quantitated. As shown in Figure 2, maximum binding is reached in 4 and 6 hours at 4°C and 22°C, respectively. The magnitude of specific binding is slightly higher at 4°C. Although the reasons for this observation are not readily apparent, this is a reproducible phenomena. Consequently, all further assays were performed at 4°C for 18 hours.



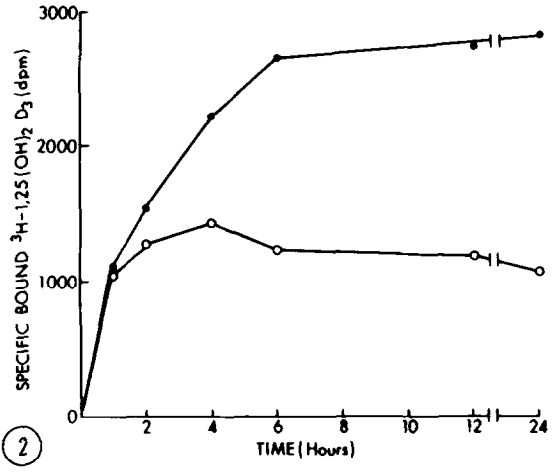


Figure 1: Linear sucrose density ultracentrifugation of antibody B2B4 and ^{3}H-1,25(OH)$_2$D$_3$ before (o——o) and after (●——●) immunoprecipitation with 40 µl of purified rabbit anti-mouse gamma globulin. Human gamma globulin (HγG) and bovine serum albumin (BSA), peaks (determined by absorption at 280 nm) are indicated by arrows.

Figure 2: Association of radioligand and antibody at 4° (o——o) and at 22° (●——●). The specific counts bound equal the mean of three replicates containing antibody and radioligand minus the mean of three replicates containing antibody, radioligand and 1 ng of 1,25(OH)$_2$D$_3$ at the indicated time point.

Figure 3 illustrates a displacement curve for this assay using a 1:80,000 dilution of partially purified B2B4. In the absence of unlabeled 1,25(OH)$_2$D$_3$ approximately 3000 dpm are bound, half of the radioligand is displaced by 26 pg/ml of 1,25(OH)$_2$D$_3$. Higher dilutions (1:120,000) of the B2B4 preparation can be used to reliably detect 1 pg/ml using this same displacement assay. The apparent K_D at 1:80,000 dilution of the B2B4 preparation is $3.3 \times 10^{-11} ML^{-1}$. Thus, this preparation provides a high affinity binding protein for very sensitive serological assays. However, B2B4 discriminates poorly among various vitamin D$_3$ metabolites. At a dilution of 1:80,000 half of the reversibly bound ^{3}H-1,25(OH)$_2$D$_3$ is placed by 48 pg/ml 24,25(OH)$_2$D$_3$ or 68 pg/ml of 25OHD$_3$.

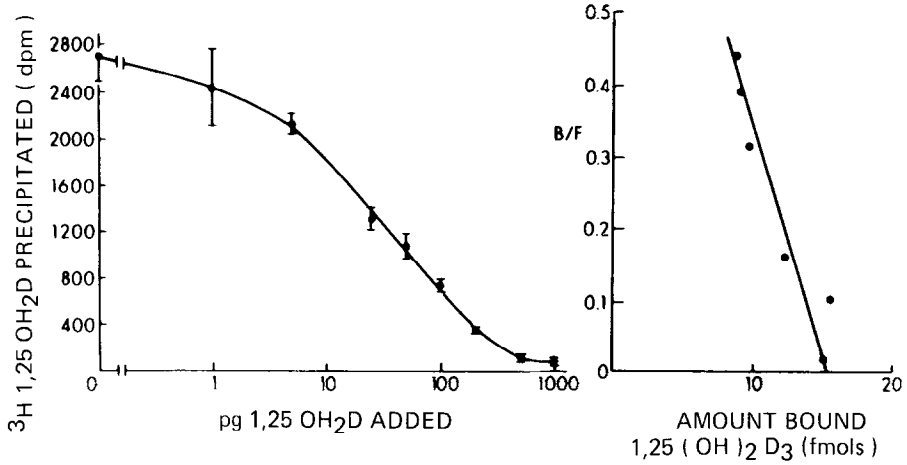


Figure 3: Antibody (1:80,000 dilution) with radioligand and varying amounts of unlabeled 1,25(OH)$_2$D$_3$. Counts bound equal the mean of three replicates containing antibody, radioligand and various amounts of 1,25(OH)$_2$D$_3$ minus the mean of three samples containing only radioligand (no antibody present). The bars show the standard deviation for each point. Inset shows the Scatchard analysis of data presented; apparent K_D is 3.3 x 10-11ML-1.

DISCUSSION

Calcitroic acid was used in an attempt to produce an antibody directed against the A ring and 1α-hydroxyl moiety of 1,25(OH)$_2$D$_3$. Since this conjugated compound lacks side chain carbons (C24-26), antibodies raised against this hapten might not recognize metabolites hydroxylated in those positions. However, since this particular antibody displays only slight preference for the 1-hydroxyl metabolite, we have not as yet achieved this specificity. However, the antibody is a superior assay receptor for 1,25(OH)$_2$D$_3$ as compared to the rachitic chick intestinal binding protein. It has remained stable after repeated freezing and thawing and its binding characteristics are unchanged after twenty-two months storage at -20^{0}C. At an antibody dilution of 1:120,000 the system can detect 1 pg/ml of 1,25(OH)$_2$D$_3$, which is at least an order of magnitude less than normal circulating levels. This study, therefore, demonstrates the possibility of producing high affinity monoclonal antibodies to vitamin D metabolites. Current work is directed toward analyzing other clones which may produce antibodies with more selective

recognition of certain sterols, since such agents could abbreviate the presently available protein binding assays which require extensive chromatographic preparation.

REFERENCES:

1. Dokoh, S., Pike, J.W., Chandler, J.S., Mancini, J.M., and Haussler, M.R. (1981) Anal. Biochem. 116: 211-222.
2. Eisman, J.A., Hamstra, A.J., Kream, B.A., DeLuca, H.F. (1976) Arch. Biochem. Biophys. 176: 235-243.
3. Gray, R.W., Wilz, D.R., Caldas, A.E., Lemann, J.R. (1977) J. Clin. Endo. and Metab. 45: 299-306.
4. Clemens, T.L., Hendy, G.N., Papapoulus, S.E., Fraher, L.H., Care, A.D., O'Riordan, J.L.H. (1979) Clin. Endo. 11: 225-234.
5. Coffino, P., Baumal, R., Laskov, R., Scharff, M.D. (1972) J. Cell Physiol. 79: 429-440.
6. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951) J. Biol. Chem. 193: 265-275.
7. Lieberman, S., Erlanger, B.F., Beiser, S.M., Agale, F.J. (1959) Rec. Prog. Hor. Res. 15: 165-200.
8. Shulman, M., Wilde, C.D., Kohler, G. (1978) Nature 276: 269-270.
9. Galfre, G., Howe, S.C., Milstein, C., Butcher, G.W., Howard, J.C. (1977) Nature 266: 550-552.
10. Littlefield, J.W. (1964) Science 145: 709-710.
11. Scatchard, G. (1949) Ann. NY Acad. Sci. 51: 660-666.