Cross-reactive monoclonal antibodies to alcohol dehydrogenases

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Summary. Three anti-horse liver alcohol dehydrogenase (HLADH) monoclonal antibodies are described. Two are specific for ADH and cross-react with class I and II enzymes from mouse, horse and Chinese hamster. They are specific for the native enzyme but do not inhibit enzyme activity except when combined at high concentration. The third antibody was isolated as a response to rabbit metallothionein. It binds metalloproteins and inhibits ADH activity.
Key words. Monoclonal antibodies; alcohol dehydrogenase.

Alcohol dehydrogenase (ADH). NAD oxidoreductase E. C. 1111 is the major enzyme responsible for ethanol oxidation and the first step in alcohol elimination in mammals. This enzyme exists in many isozyme forms which have been differentiated into three classes on the basis of their electrophoretic mobility, inhibition by pyrazole[1,2] and substrate specificity. Class I ADHs exhibit isoelectric points from 9 to 11 and are inhibited by pyrazole. Class II ADHs, also called π ADH have one isozyme, a pI of 7 to 9 and are insensitive to pyrazole. The isozymes are the principle ADHs found in cell cultures[3]. Class III or χ isozymes have low isoelectric points near pH 5–7 and are more specific for long chain alcohols[4]. They are the principle ADHs found in brain tissue[5]. The genetic relationships of the three mammalian ADH isozymes have not yet been clearly established.

Our earlier work suggested that there is some immunological similarity between class I and class II ADHs of Chinese hamster[6]. In this report we have isolated two monoclonal antibodies to purified horse liver ADH (class I) which also bind to class II ADH. A comparison of species and cell cross-reactivity is described. A third antibody is also described which was derived from an immunization with metallothionein and which reacts with metalloproteins.

Immunization protocol. HLADH was purchased from Boehringer/Mannheim Corp.; it has an isoenzyme composition of 77% EE and 5% SS subunits. Three male BalB/c mice were injected i.p. with 100 µg of purified horse liver ADH (HLADH) once a week for four weeks. The presence of anti-ADH antibodies was detected using an ELISA assay and an anti-mouse peroxidase streptavidin detection system (Amersham Corp.). HLADH was bound to PVC plates (Flow Corp.) at a concentration of 50 ng per well in 0.1 M $NaHCO_3$ (pH 9.0).

Cell fusion protocol. Lymphocyte suspensions were prepared from spleens of immunized mice. In each experiment 10^8 spleen cells were fused with 10^7 mouse myeloma p3 XAg8,653 cells using 37% (v/v) PEG (Baker 1450). Classical techniques[7] were used in the selection of hybridomas. Screening of anti-ADH antibodies was done by ELISA as described above. Control plates of 0.25% gelatin and 1% BSA were used to eliminate false positives.

Immunoglobulin analysis by light chain screening. Monoclonal antibodies were screened for differences in antibody structure by analysis of their immunoglobulin light chain isoelectric focusing patterns[8].

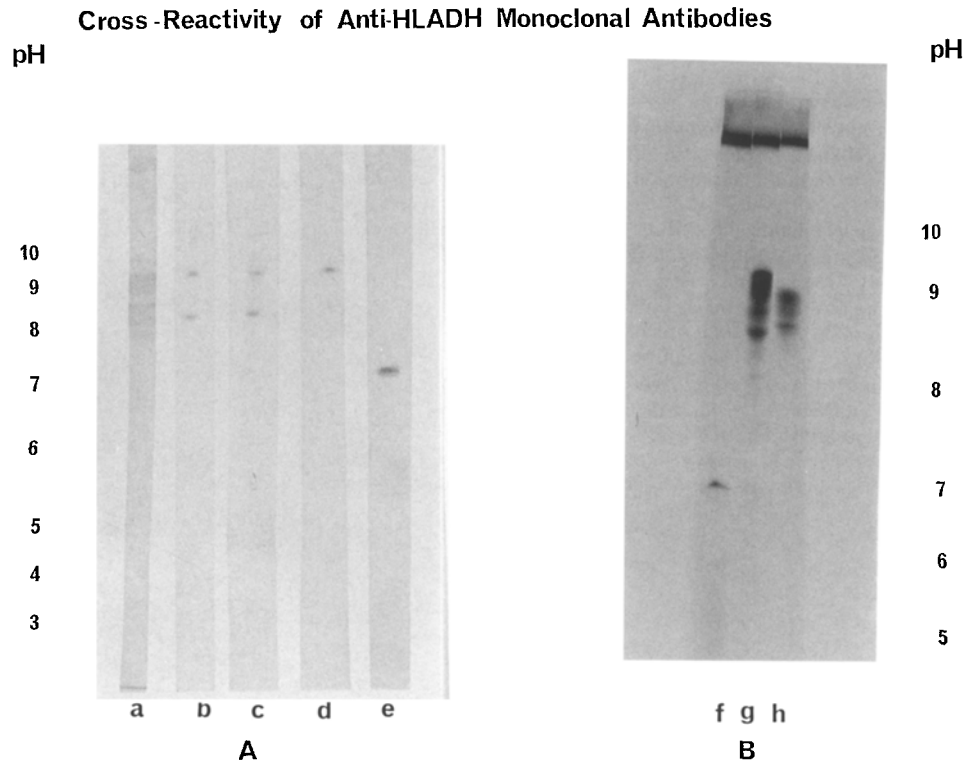

Figure 1. *A* Immunoblot of an isoelectric focusing gel pH 3–10 using TA (or TB). a) HLADH, 5 µg stained by imido black; b) mouse liver extract; c) Chinese hamster liver extract; d) purified horse liver ADH; e) Chinese hamster V 79 cell extract. Coloration was performed using a peroxidase based streptavidin system. *B* Isoelectric focusing gel pH 5–10 stained for alcohol dehydrogenase activity. f) Chinese hamster V 79 cell extract; g) horse liver extract; h) pure HLADH.

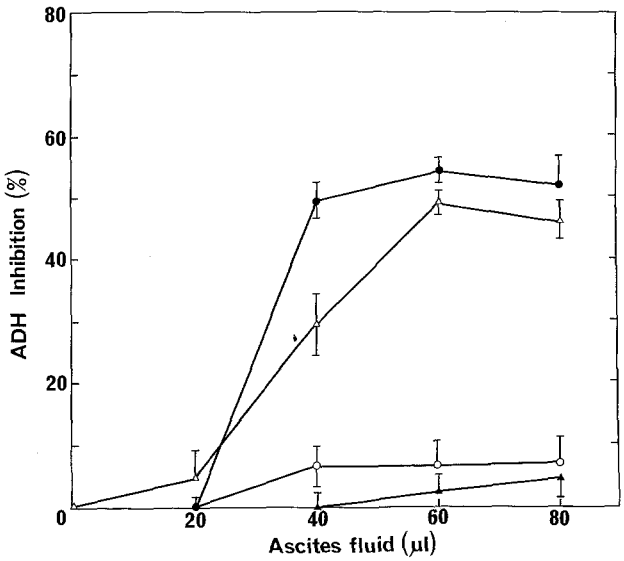

Figure 2. Inhibition of ADH activity. The results are expressed as % inhibition of activity relative to a control ascites fluid. ●—● Equal quantities of TA + TB incubated for 4 h at 4 °C with 200 µl of V_6 extract (4.6 mU, 5.6 mg protein). △—△ Equal quantities of TA + TB incubated for 4 h at 4 °C with 200 µl of commercial HLADH (16 mU, 3 µg protein), ▲—▲ TA alone, ○—○ TB alone. Assays were performed in triplicate and SEM is included. Activity was measured as the change in O.D. 340 nm in 0.1 M pyrophosphate buffer, 0.1 M ethanol, 2.8 mM NAD.

Purification and immunoprecipitation. ADHs from tissue or cell extracts were partially purified on the affinity column AG-AMP Type 2-(P-LBiochem) followed by elution with NADH[6]. This preparation was used for the electrophoretic and electrofocusing analyses. Immunoaffinity columns were made using Moab, purified by FPLC (Pharmacia), attached to Sepharose CL-4B. ADH was eluted at low pH, iodinated with chloramine T or iodogen (Pierce Chem. Co) and revealed by SDS gel electrophoresis.

Isoelectric focusing and native protein blotting. Isoelectric focusing was carried out on 7% polyacrylamide slab gels with 0.2% ampholines (Bio-Rad) and either a 3–10, or a 5–10 pH gradient[6]. The samples were applied directly to the surface of the prefocused gel because ADHs bind to paper applicators. Focusing was for 3 h at a constant power of 20 W and a maximum voltage of 1200 V. The gels were stained for ADH activity using NAD, Nitroblue tetrazolium, phenazine methosulfate and either ethanol or pentanol as substrates as described previously. 50 mM pyrazole was added in some cases to identify class II and III ADHs[6].

Starch gel electrophoresis. Starch gel electrophoresis was performed as described by Bosron et al.[9]. Partially purified ADH preparations were concentrated and desalted on Sephadex G-25 and applied 1 cm from the cathode side of the gel. Electrophoresis was performed for 7 h at 450 V, 8 °C with a 1.5 mm thick gel. ADH activity was revealed as described under isoelectric focusing above.

Electroblotting. Focused gels were rinsed in water. A piece of wet (0.85% acetic acid) nitrocellulose was then carefully placed on the upper surface of the gel. Three layers of wet Whatman 3 MM paper were placed on each side of the gel and the whole was sandwiched between 'Scotchbright' pads in an electroblot apparatus. Electro-transfer was performed in 0.85% acetic acid toward the positive electrode for 2 h at 0.6 A and a maximum of 25 °C. Immunodetection of the transferred proteins was performed using peroxidase anti-mouse streptavidin (Amersham Corp.) and 4-chloro-1-naphthol as chromogem.

Results. After screening of the stable hybridomas for ADH binding a total of 21 independently cloned lines were isolated. An analysis of the antibodies secreted by these cells demonstrated that there were only two different types of immunoglobulin light chains among the 21 antibodies. This suggests that these two antibodies must have constituted a major antibody response to HLADH in this mouse. In a separate experiment, using rabbit metallothionein as antigen in an in vitro immunization, an antibody was obtained which binds to HLADH. This antibody (TN-1) appears to be specific for metal binding proteins and is included here because of its inhibitory effects on HLADH[10]. The two anti-HLADH specific antibodies are named TA and TB, the anti-'metalloprotein' antibody is TN-1. Antibodies TA and TB are of isotype IgG1, antibody TN-1 is IgM, the latter antibody is unstable to freezing and tends to lose activity on purification. TA and TB are stable and can be purified by FPLC chromatography using a Mono Q (Pharmacia Corp.) column.

Specificity. Antibodies TA and TB bind only to the native ADH and thus were not reactive with SDS polyacrylamide nitrocellulose blots of HLADH. Antibody TN-1 reacts with SDS-denatured and blotted HLADH as well as metallothionein and carbonic anhydrase (not shown). Figure 1 shows the immunoreactivity of the TA and TB on non dissociating isoelectric focusing nitrocellulose blots of purified HLADH, Chinese hamster liver extracts, Chinese hamster fibroblast culture (V79) cell extracts and ADH-enriched liver extracts of mouse and Chinese hamster. Both TA and TB gave the same pattern of binding specificity. The V79 extract shows a single band at pH 7.0, the commercial HLADH shows a single band at pH 9. The two liver extracts showed two bands of immunoreactive protein at pHs 8 and 9. A protein stain of the commercial HLADH blot clearly showed that the normal series of isozymes was present and transferred to the nitrocellulose between pHs 8 and 9. Figure 1B shows an isoelectric focusing gel on a pH 5–10 gradient after coloration for ADH activity. The single isozyme of the V79 extract is clearly visible as are the series of isozymes present in the commercial HLADH and the horse liver extract. ADH from hamster or mouse brain extracts did not react with the antibodies after isoelectric focusing. In order to be certain that the ADHs used as target for the antibodies were in fact the isoenzymes class I, II and III a starch gel electrophoresis was carried out using partially purified ADHs from hamster liver, brain, and fibroblast cells (V79) in culture. The three classes of enzyme were clearly defined, class III (brain) being most anodic, class II (V79) less anodic and the major liver isozyme class I being cathodic at that pH. Class III had to be concentrated before use and was not visible in the liver preparations. As expected, the brain and V79 ADHs were not inhibited by pyrazole and the brain ADH was more active with pentanol than ethanol. These results are in agreement with the classification established by Vallee[2].

The more 'nonspecific antibody' TN-1 reacted with a large number of bands after isoelectric focusing as would be expected (not shown).

Immunoprecipitation. In all cases a 40000 Dalton subunit protein was bound by the antibodies and in the crude extracts some undissociated 80000 Dalton ADH was seen on the SDS gels after immunoprecipitation.

Inhibition of ADH activity. Figure 2 shows how TA, TB and TN-1 affected the ADH activities of commercial HLADH and V79 cell extracts. TA and TB combined, inhibit both enzymes to a maximum of 65%, whereas individually the inhibition is very low. There was no effect on the ADH activity of hamster brain extracts. TN-1 inhibits ADH at high concentration as would be expected for an antibody that affects metal binding[10].

Discussion. We have described the production and properties of two ADH specific monoclonal antibodies TA and TB and one metalloprotein binding antibody TN-1 which reacts with ADH. The results clearly confirm the immunological relationship between classes I and II of ADH isozymes which we reported earlier[6] and suggest that this is not necessarily linked to the amino acid sequence since the two antibodies described do not bind to denatured ADH. The only difference between TA and TB is their immunoglobulin light chain isoelectric points. Yet it

appears that they must bind to separate, but related, epitopes in order to cooperatively inhibit ADH activities. These results complement the report of Lad et al.[11], who isolated inhibitory anti-rat liver ADH monoclonal antibodies. Their antibodies, in contrast to ours, were species specific. TA, TB and TN-1 are all cross-reactive between horse, hamster and mouse and thus must be considered as auto-antibodies produced in response to the hyperimmunization of the mice by the original antigens. It is interesting to note that V79 cells and brain tissue express only ADH class II and III respectively, these tissues could be used in conjunction with antibodies specific for the two types of enzyme for the study of ADH gene regulation. It is expected that our antibodies will be useful to purify ADH in its native form (they do not bind to the denatured ADH) and also to help distinguish between the isozymes of the different classes since they are unreactive with class III ADHs.

Acknowledgment. This work was supported by the National Cancer Institute of Canada.

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0014-4754/87/040426-03$1.50 + 0.20/0

Co-localization of glucagon and pancreatic polypeptide in testudine pancreas

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Summary. Immunocytochemistry was carried out on sections of pancreas from the gopher tortoise, *Gopherus polyphemus*. Combined immunofluorescent and peroxidase-anti-peroxidase techniques showed unequivocally that some of the cells were immunoreactive for both glucagon and pancreatic polypeptide (PP). Antibodies directed against avian PP, bovine PP, and human PP all have a positive reaction. Co-localization of glucagon and PP in the pancreas of the gopher tortoise indicates that the occurrence of these hormones in the same cells is widespread in higher vertebrates.
Key words. Antibodies; hormones; pancreatic; peptides; localization; immunocytochemical.

The four major hormones of the vertebrate pancreas, insulin, glucagon, pancreatic polypeptide (PP), and somatostatin, are generally regarded as being found in separate cell-types which may[2], or may not[3,4], have particular spatial relationships with one another. However, cells positive for both glucagon and PP have been found in a lower vertebrate, the frog *Rana pipiens*[5], and more recent studies have found cells reactive for both glucagon and PP in mammalian pancreas, including human islets[4,6,7]. I report here the occurrence of glucagon and PP in the same cells in a reptilian pancreas.

Materials and methods. Gopher tortoise, *Gopherus polyphemus*, pancreas was fixed overnight in Bouin's fluid and processed for embedding in Paraplast-Plus (Lancer, St. Louis, MO). Sections of pancreas were reacted with the peroxidase-antiperoxidase (PAP) immunocytochemical technique of Sternberger et al.[8], with one recycling[9]. Sequential reactions were carried out using indirect immunofluorescence followed by PAP. Immunocytochemical controls consisted of both methodological and antisera controls. Controls for specificity of the method consisted of replacing the primary antisera with normal (non-immunized) rabbit serum and omitting coupling immunoglobulin or PAP, or including an excess of unlabeled second antibody with indirect immunofluorescence. Controls for antibody specificity consisted of absorption of the primary antisera with an excess of the antigen for which the particular antiserum was known or believed to be selective. The antisera absorptions were carried out with the following hormones at a concentration of hormone expressed as mass per microl of undiluted serum: glucagon (bovine-porcine, lot number 258-V016235, Lilly Research Laboratories), 5 µg/µl; pancreatic polypeptide (bovine, lot 615-D63-295, Lilly Research Laboratories, 50 µg/µl; or turkey, HPLC grade from H. Gail Pollock, 10 µg/µl). All of the controls gave the appropriate negative result.

Immunocytochemical reagents were obtained from the following sources. Rabbit anti-bovine pancreatic polypeptide serum (RABPP), used at a concentration of 1:4000–1:20,000, was a gift from Dr Ronald E. Chance, Lilly Research Laboratories, Eli Lilly and Co. (lot 615-R110-146-16). RABPP was routinely absorbed with glucagon and insulin, 10 µg and 2 µg respectively per µl of undiluted serum (see above). Rabbit anti-avian pancreatic polypeptide, prepared in the laboratory of the late Dr Joe R. Kimmel and used at a concentration of 1:1000–1:10,000, was the gift of H. Gail Pollock and the Medical Research Service, Medical Center, Veterans Administration. Rabbit anti-human pancreatic polypeptide (lot No. 0010), used at a concentration of 1:500–1:2000, was purchased from Miles Scientific, Naperville, IL. Rabbit antiserum to glucagon, used at a concentration of 1:5000–1:20,000, was generously provided by Dr Howard Tager (R-1, 11–20)[10]. Ovine anti-rabbit immunoglobulin G (heavy and light chains), soluble horseradish peroxidase-rabbit anti-peroxidase complex, and normal ovine serum were obtained from CooperBiomedical, Inc., Malvern, PA. Donkey anti-rabbit immunoglobulin conjugated to fluorescein was purchased from Amersham Int., Buckinghamshire, England.

Results and discussion. Endocrine cells of the gopher tortoise pancreas which were immunoreactive by the PAP method for PP were widely distributed, including duct epithelium (fig. 1). The PP-positive cells occurred singly or in small groups and were relatively abundant (fig. 1). Glucagon-containing cells generally had a distribution similar to the PP-containing cells, but were less abundant. Many of the PP-positive cells were not reactive for glucagon and glucagon-positive cells were absent from some