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ALDEHYDE DEHYDROGENASE IN 2-ACETYLAMINOFLUORENE-INDUCED RAT HEPATOMAS

CHARACTERIZATION OF ANTIGENS RECOGNIZED BY ANTI-HEPATOMA ALDEHYDE DEHYDROGENASE SERA

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Summary

The material in normal rat liver which cross-reacts with anti-hepatoma aldehyde dehydrogenase sera has been partially purified. The normal liver cross-reacting material is a series of proteins electrophoretically identical to the series of hepatoma-specific aldehyde dehydrogenases (aldehyde:$NAD(P)^+$ oxidoreductase, EC 1.2.1.5) induced by 2-acetylaminofluorene. The molecular weights (100000) and the isoelectric points (6.8—7.2) of the cross-reacting material are identical to the molecular weights and pI values of the hepatoma-specific isozymes. Absorption of anti-hepatoma aldehyde dehydrogenase sera with the cross-reacting material removes an antibody population which recognizes a normal liver aldehyde dehydrogenase. This isozyme accounts for 35% of the total normal liver aldehyde dehydrogenase. A second antibody population recognizes an antigen which accounts for 10% of the total normal liver aldehyde dehydrogenase. Approx. 55% of the normal liver aldehyde dehydrogenase activity is not recognized by any antibody population. In hepatomas, 15% of the total aldehyde dehydrogenase activity is immunochemically identical to the normal liver cross-reacting material. The remaining 85% of the tumor aldehyde dehydrogenase is characterizable as hepatoma specific on the basis of these immunochemical studies.

Introduction

We have previously described several physical and functional characteristics of a series of aldehyde dehydrogenase isozymes (aldehyde:$NAD(P)^+$ oxidoreductase, EC 1.2.1.5) found in 2-acetylaminofluorene-induced hepatomas in Sprague-Dawley rats, but not found in normal Sprague-Dawley rat liver [1—4].

Antisera generated in rabbits against this series of purified tumor-specific aldehyde dehydrogenases possess two distinct antibody populations [1]. One antibody population forms enzymatically active immune complexes and is hepatoma specific. The other population forms enzymatically inactive immune complexes with both hepatoma and normal liver preparations [1].

Several lines of indirect evidence indicate the antigen which forms the enzymatically inactive immune complex is an NAD^+-specific isozyme of aldehyde dehydrogenase present in normal rat liver. Firstly, in both agar gel immunoelectrophoresis and gel electrophoresis followed by immunodiffusion in agar, the enzymatically inactive precipitin line forms at a position coincident with the mobility of the normal liver aldehyde dehydrogenase [1]. Secondly, absorption of hepatoma homogenates with anti-hepatoma aldehyde dehydrogenase sera results in the complete removal of aldehyde dehydrogenase activity as judged by gel electrophoresis or gel isoelectric focusing [1]. Absorption of normal liver preparations with the antisera under similar conditions also results in complete loss of the single normal liver aldehyde dehydrogenase isozyme detectable by gel electrophoresis. On the other hand, gel isoelectric focusing resolves normal liver aldehyde dehydrogenase into several isozymes. Of these, two (pI 5.9 and 6.0) are completely lost after absorption with anti-hepatoma aldehyde dehydrogenase sera [1]. Thirdly, the appearance of the enzymatically inactive precipitin line correlates exactly with the appearance of normal liver aldehyde dehydrogenase during its prenatal and early postnatal ontogeny [2].

This paper extends these preliminary immunochemical observations by characterizing the material from normal liver which cross-reacts with anti-hepatoma aldehyde dehydrogenase sera. Then, this partially purified cross-reacting material is employed to further characterize the specificity of anti-hepatoma aldehyde dehydrogenase sera and the various antigens it recognizes. Such information is critical for understanding the relationship between the hepatoma-specific aldehyde dehydrogenases and their normal liver counterparts.

Materials and Methods

Chemicals. Propionaldehyde was obtained from Aldrich Chemicals, Inc. and periodically redistilled. NAD^+, 2,2-di-p-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene)ditetrazolium chloride (NBT) and *N*-methylphenazonium methosulfate (PMS) were either from ICN Biochemicals, Inc. or Sigma Chemical Co. Disodium EDTA, β-mercaptoethanol and DEAE-cellulose were from Sigma. Molecular weight determination kits were from Pharmacia Fine Chemicals. All other chemicals were of reagent grade.

Tissue preparation. Normal liver and hepatoma sonicate supernatants were prepared as previously described [2] with a slight buffer modification. In the present studies, 1 mM EDTA and 1 mM β-mercaptoethanol were added to the originally employed 60 mM sodium phosphate buffer (pH 8.5).

Antisera. Pooled anti-(rat hepatoma aldehyde dehydrogenase) sera produced in rabbits were employed [1]. The antisera were stored at −30°C. Aliquots were thawed and centrifuged at 48000 × *g* for 15 min immediately prior to use.

Aldehyde dehydrogenase and cross-reacting material determinations. Alde-

hyde dehydrogenase activity was determined spectrophotometrically as previously described [2] using propionaldehyde as substrate. Normal liver cross-reacting material is defined and qualitatively assayed as that normal liver material which forms the enzymatically inactive precipitin line with anti-hepatoma aldehyde dehydrogenase sera in Ouchterlony double diffusions [1].

Protein was determined by the method of Lowry et al. [5] with bovine serum albumin as standard.

Preparation of normal liver cross-reacting material. The purification scheme for normal liver cross-reacting material was similar to that previously described for the purification of the hepatoma-specific aldehyde dehydrogenases [1] with minor modifications. The buffer employed was 20 mM sodium phosphate/1 mM EDTA/1 mM β-mercaptoethanol (pH 8.5), DEAE-cellulose was employed in place of DEAE-Sephadex. A second column isoelectric focusing was added using as the sample those fractions from the first focusing that possessed cross-reacting material activity. Throughout the purification process, all fractions were tested for cross-reacting material activity and for NAD^+-dependent aldehyde dehydrogenase activity.

Use of cross-reacting material to characterize anti-hepatoma aldehyde dehydrogenase sera. Aliquots of the cross-reacting material obtained from the second isoelectric focusing were used to remove anti-cross-reacting material antibodies from aliquots of anti-hepatoma aldehyde dehydrogenase sera. 150 μl cross-reacting material (1.5 mg/ml) were mixed with equal volumes of varying concentrations of antisera to obtain the following final concentration ratios (v/v) of antisera to cross-reacting material: 0.5 : 1, 1 : 1 and 2 : 1. The immune reactions were carried out for 18 h at 4°C. The mixtures were then centrifuged at 48000 × *g* for 15 min to remove any precipitates formed. 250 μl of resulting supernatants were mixed with equal volumes of either normal liver or hepatoma preparations. The final concentration ratios (v/v) of preabsorbed antisera to liver or hepatoma were: 0.25 : 1, 0.5 : 1 and 1 : 1. At intervals the samples were centrifuged to remove any precipitates and the NAD^+-dependent aldehyde dehydrogenase activity determined. Controls were either non-immune rabbit sera or buffer (60 mM sodium phosphate/1 mM EDTA/1 mM β-mercaptoethanol (pH 8.5) preabsorbed with cross-reacting material and then reacted with normal liver or hepatoma.

Antigenic characterization of normal liver and hepatoma aldehyde dehydrogenase. Aliquots of either normal liver or hepatoma preparations were mixed with equal volumes of varying concentrations of anti-hepatoma aldehyde dehydrogenase sera. The final concentration ratios (v/v) of antisera to liver or hepatoma were 0.5 : 1, 1 : 1, 2 : 1 and 4 : 1. Controls were normal liver or hepatoma treated with either non-immune rabbit sera or buffer. Immune reactions were carried out at 4°C. At intervals, the samples were centrifuged and the NAD^+-dependent aldehyde dehydrogenase activity determined.

Results

Normal liver cross-reacting material characterization

Using a purification scheme for normal liver aldehyde dehydrogenase-cross-reacting material very similar to that employed in the purification of the hepa-

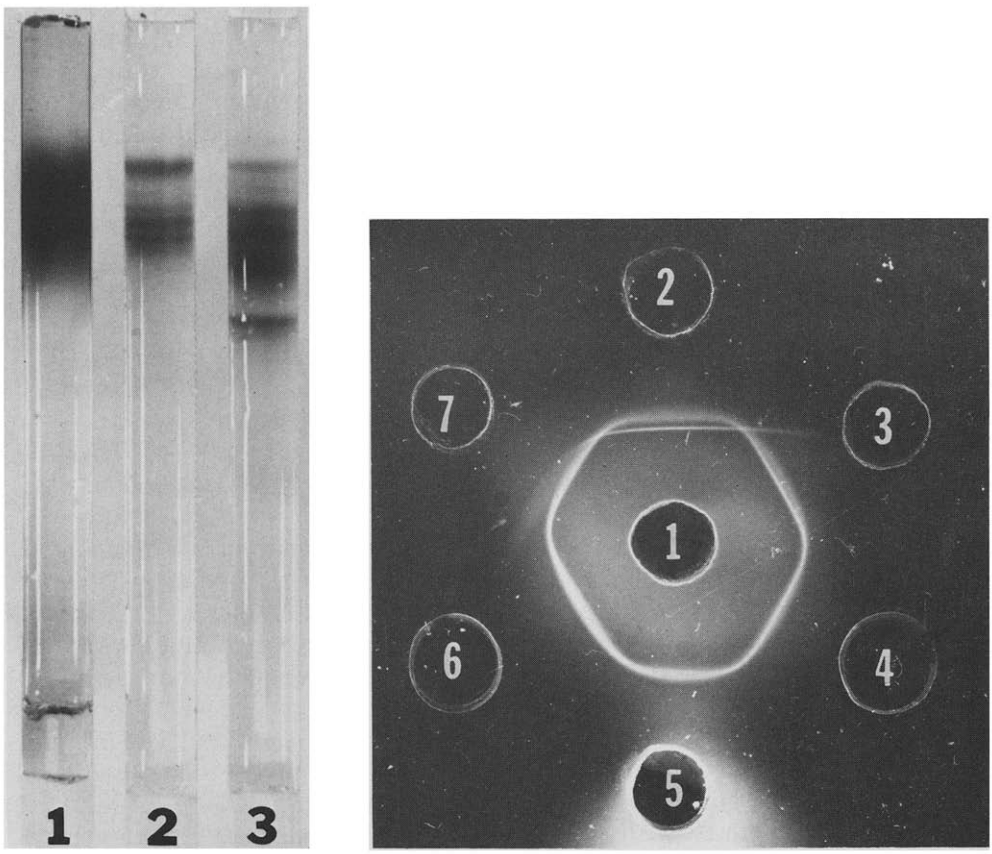

Fig. 1. (a) Gel electrophoresis of normal liver and hepatoma aldehyde dehydrogenase preparations. 1, 50 μg purified hepatoma aldehyde dehydrogenase stained for enzyme activity with propionaldehyde and NAD^+; 2, 50 μg purified hepatoma aldehyde dehydrogenase stained for protein with 0.1% Amido Black. All protein species present in (2) possess aldehyde dehydrogenase activity; 3, 50 μg partially purified normal liver cross-reacting material stained for protein as above. Electrophoresis was performed at 4°C using the Canalco Apparatus (Ames Co) and gels stained as previously described [1]. (b) Ouchterlony double diffusion of various normal liver and hepatoma aldehyde dehydrogenases. 1, anti-hepatoma aldehyde dehydrogenase sera; 2, 20% hepatoma sonicate supernatant; 3, 4, normal liver cross-reacting material preparations I and II; 5, 20% normal liver sonicate supernatant; 6, normal liver cross-reacting material preparation III; 7, purified hepatoma-specific aldehyde dehydrogenase. Gel was stained for protein with 0.1% Ponceau S.

toma-specific aldehyde dehydrogenases allows a direct comparison of the normal liver materials and their hepatoma counterparts. Through salt fractionation and ion-exchange chromatography, the results of the normal liver aldehyde dehydrogenase, cross-reacting material and hepatoma aldehyde dehydrogenase purifications are qualitatively identical. The first differences between the normal liver and hepatoma aldehyde dehydrogenases are found during Sephadex G-200 gel filtration, indicating differences in molecular weight. The hepatoma aldehyde dehydrogenase activity elutes as a single peak at a volume correspind-ind to molecular weight 105000. All the normal liver aldehyde dehydrogenase

activity elutes in the void volume indicating a molecular weight in excess of 300000. No cross-reactivity is detectable between this high molecular weight material and anti-hepatoma aldehyde dehydrogenase sera. The normal liver material which cross-reacts with anti-hepatoma aldehyde dehydrogenase sera elutes at a volume corresponding to molecular weight approx. 100000. However, this cross-reacting material does not possess aldehyde dehydrogenase activity.

Preparative isoelectric focusing as a final purification step yields a normal liver cross-reacting material preparation which gel electrophoresis resolves into a series of proteins, five of which are identical to the several purified hepatoma-specific aldehyde dehydrogenases (Fig. 1a). The pI values of the normal liver cross-reacting material species are 6.8—7.2 (at 25°C), identical to the pI values of the hepatoma-specific aldehyde dehydrogenases. Only those proteins present in both the purified hepatoma and partially purified cross-reacting material preparations can be involved in the formation of the enzymatically inactive precipitin line (Fig. 1b), eliminating the two most anodal cross-reacting material preparation proteins as the normal liver cross-reacting material.

The molecular weights, isoelectric points and the electrophoretic behavior of the normal liver cross-reacting material are all very similar to those same physical characteristics of the hepatoma-specific aldehyde dehydrogenases. However, the lack of aldehyde dehydrogenase activity necessitates the use of immunochemical methods to demonstrate conclusively that this material is related to normal liver aldehyde dehydrogenase.

Use of cross-reacting material to characterize anti-hepatoma aldehyde dehydrogenase sera.

This experiment is designed to determine whether preabsorption of anti-hepatoma aldehyde dehydrogenase sera with normal liver cross-reacting material removes antibodies that would otherwise react with normal liver aldehyde dehydrogenase. The results of such experiments are uniquivocal (Fig. 2a). At all times and at all antisera concentrations tested, normal liver supernatants reacted with antisera previously absorbed with normal liver cross-reacting material retain significantly more aldehyde dehydrogenase activity than their corresponding controls. Thus, although it is enzymatically inactive, the normal liver cross-reacting material is very closely related to a normal liver dehydrogenase.

In addition, those cross-reacting material-preabsorbed samples not containing excess anti-cross-reacting material antibody are consistently 10% lower in aldehyde dehydrogenase activity than their corresponding non-immune sera-treated controls (Fig. 2a). This observation indicates that a second minor antibody population recognizes another normal liver aldehyde dehydrogenase as antigen.

Similar preabsorption experiments using hepatomas as antigen source also present unequivocal results (Fig. 2b). Preabsorption of the antisera with cross-reacting material is largely, but not entirely, without effect on the ability of the antisera to remove aldehyde dehydrogenase activity from hepatoma preparations. Absorbed hepatoma preparations retain approx. 15% of the total detectable hepatoma aldehyde dehydrogenase. This observation indicates that

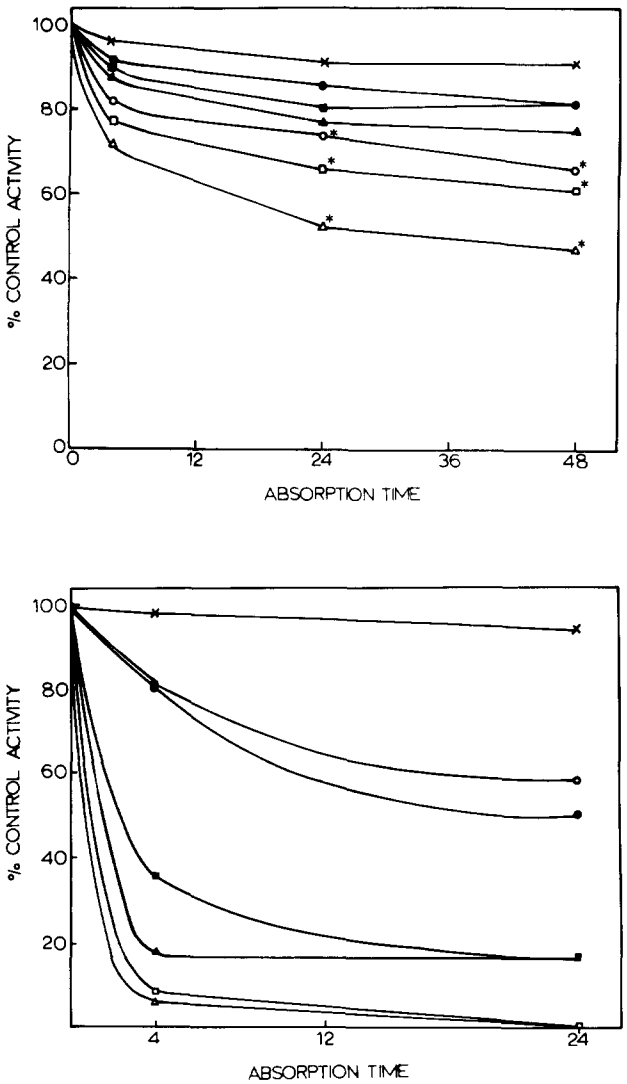

Fig. 2. Effect of prior absorption of anti-hepatoma aldehyde dehydrogenase sera with normal liver cross-reacting material on normal liver and hepatoma aldehyde dehydrogenase activity. Following an 18 h pre-absorption with normal liver cross-reacting material, anti-hepatoma aldehyde dehydrogenase sera was tested for its ability to remove aldehyde dehydrogenase from both normal liver and hepatoma preparations. Percent control activity is the activity of various experimental samples relative to their corresponding same-time buffer-treated controls. Absorption time is the time of absorption of normal liver or hepatoma with various antisera at three different (v/v) antibody:antigen ratios. Normal liver or hepatoma were absorbed with the following: X, cross-reacting material preabsorbed non-immune sera, 1 : 1; ●, cross-reacting material preabsorbed immune sera, 0.25 : 1; ■, cross-reacting material preabsorbed immune sera, 0.5 : 1; ▲, cross-reacting material preabsorbed immune sera, 1 : 1; ○, buffer preabsorbed immune sera, 0.25 : 1; □, buffer preabsorbed immune sera, 0.5 : 1; △, buffer preabsorbed immune sera, 1 : 1. (A) Normal liver aldehyde dehydrogenase activity following absorption with various preabsorbed anti-hepatoma aldehyde dehydrogenase sera. * indicates activity is significantly less than its corresponding same-time buffer or non-immune sera controls at at least the $P < 0.05$ level of significance by a one-sided t-test. (B) Hepatoma aldehyde dehydrogenase activity following absorption with various preabsorbed anti-hepatoma aldehyde dehydrogenase sera. All activities except those of the 4 h 0.25 : 1 samples are significantly less than their corresponding same-time buffer and non-immune sera controls at at least the $P < 0.05$ level of significance by a one-sided t-test.

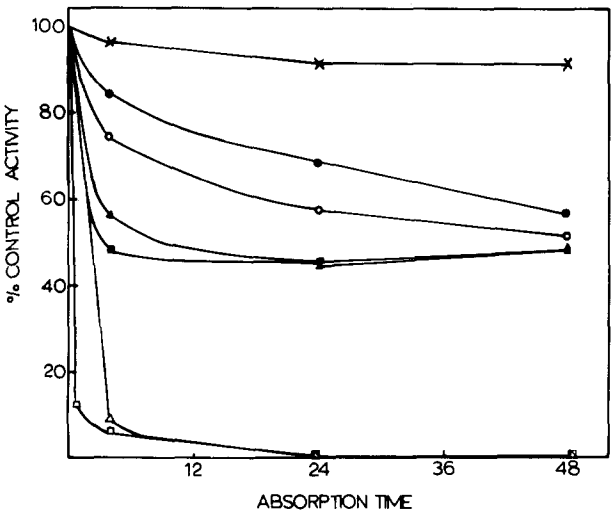

Fig. 3. Effect of anti-hepatoma aldehyde dehydrogenase sera on normal liver and hepatoma aldehyde dehydrogenase activity. Normal liver or hepatoma preparations were treated with various concentrations of anti-hepatoma aldehyde dehydrogenase sera. The amount of normal liver or hepatoma aldehyde dehydrogenase activity removed at various absorption times is expressed as percent control activity relative to the corresponding same-time buffer-treated controls. X, normal liver absorbed with non-immune sera, 2 : 1; ●, normal liver absorbed with immune sera, 0.5 : 1; ○, normal liver absorbed with immune sera, 1 : 1; ■, normal liver absorbed with immune sera, 2 : 1; ▲, normal liver absorbed with immune sera, 4 : 1; △, hepatoma absorbed with immune sera, 0.5 : 1; □, hepatoma absorbed with immune sera, 1 : 1; All activities except the 4 h 0.5 : 1 and 1 : 1 normal liver immune sera samples are significantly less than their corresponding same-time buffer and non-immune sera controls at at least the $P < 0.05$ level of significance by a one-sided t-test.

approx. 15% of tumor aldehyde dehydrogenase is related to normal liver cross-reacting material.

Antigenic characterization of normal liver and hepatoma aldehyde dehydrogenase

Having identified the reactive species in the anti-hepatoma aldehyde dehydrogenase sera, the antisera can now be used to further characterize the antigenic composition of both normal liver and hepatoma aldehyde dehydrogenase. Various antisera concentrations were used to absorb aldehyde dehydrogenase from normal liver preparations (Fig. 3). By 48 h absorption, between 44% (0.5 : 1) and 52% (4 : 1) of the total normal liver aldehyde dehydrogenase activity is removed. The equivalance point is estimated to be approx. 1.5 parts antisera to 1 part normal liver (Fig. 3). At the equivalance point, the antisera removes 45% of the total normal liver aldehyde dehydrogenase when corrected for the non-specific absorption observed with non-immune rabbit sera. Of this 45%, 10% is due to antibodies which do not recognize the normal liver cross-reacting material (Fig. 2a).

In contrast to the incompete inhibition of normal liver aldehyde dehydrogenase by anti-hepatoma aldehyde dehydrogenase sera, the loss of hepatoma aldehyde dehydrogenase activity in the presence of its homologous antisera is rapid (95% by 4 h) and complte by 24 h (Fig. 3).

Discussion

These immunochemical studies indicate that two antibody populations in rabbit antisera generated against a series of rat hepatoma-specific aldehyde dehydrogenase isozymes recognize antigens in normal liver. Together these antigens account for 45% of the total aldehyde dehydrogenase activity. Of the total enzyme activity, 35% is recognized by the same antibody population which generates the enzymatically inactive precipitin line in Ouchterlony double diffusion. This cross-reacting material, although enzymatically inactive, is very similar to the hepatoma-specific aldehyde dehydrogenases in molecular weight, isoelectric point and electrophoretic mobility [1]. Whether the lack of enzymatic activity of the cross-reacting material is due to instability [1] or whether the cross-reacting material represents a normally inactive form of aldehyde dehydrogenase is currently under investigation.

10% of the total normal liver aldehyde dehydrogenase activity is recognized by a second antibody population not previously identified as a component of the antisera. Since only the single cross-reacting material precipitin line is observed in double diffusion, this minor antibody population may consist of enzyme inactivating, non-precipitating antibodies. A second possibility is that the antibody population considered to be hepatoma specific can partially recognize a normal liver aldehyde dehydrogenase. The antibodies may form enzymatically inactive immune complexes but may be unable to form large enough aggregates to function as precipitating antibodies. It is also possible that this normal liver antigen is present in such small quantities that, even though it may be fully recognized by the hepatoma-specific antibody population, the dynamics of immune complex formation (great antibody excess) prevents the formation of precipitating aggregates.

The incomplete inhibition of normal liver aldehyde dehydrogenase by anti-hepatoma aldehyde dehydrogenase sera (Fig. 3) is similar to the phenomenon of antibody-associated enzyme neutralization first described by Cinader [6] for ribonuclease and subsequently by others for several additional enzymes [7–10]. Whether the high (approx. 55%) residual activity seen with normal liver aldehyde dehydrogenase is due to the same type of non-inactivating, non-precipitating antibody is unknown but seems unlikely. The large number of isozymes of normal liver aldehyde dehydrogenase detectable by a variety of techniques (e.g. ref. 10) makes it more likely that the activity remaining after absorption of liver supernatant with anti-hepatoma aldehyde dehydrogenase sera is due to aldehyde dehydrogenases not recognized by any antibody population in the antisera.

In hepatomas, all of the aldehyde dehydrogenase activity is removed by its homologous antisera. Even though all of the hepatoma aldehyde dehydrogenase is physically and functionally identifiable as hepatoma specific [1,2], some 15% is antigenically related to functional normal liver aldehyde dehydrogenase.

Deitrich et al. [11] have recently reported the induction by 2,3,7,8-tetrachlorodibenz-*p*-dioxin of a low molecular weight, soluble aldehyde dehydrogenase isozyme not normally present in rat liver. Their data indicate that activation of a gene normally repressed in liver is responsible for the appearance of this isozyme. We have recently demonstrated that the hepatoma-specific

aldehyde dehydrogenases are very closely related, if not identical, immunochemically to the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-inducible isozyme (Lindahl et al., ref. 12). The antibody population of the anti-hepatoma aldehyde dehydrogenase sera which recognizes both the inducible and hepatoma-specific isozymes is the hepatoma-specific antibody population, not the cross-reacting material antibody population which recognizes antigens in both hepatoma and normal liver aldehyde dehydrogenase. This observation indicates that gene derepression is at least partially responsible for the production of the hepatoma-specific aldehyde dehydrogenase phenotype.

The results reported in this study indicate that events in addition to new gene derepression contribute to the generation of the hepatoma-specific aldehyde dehydrogenases. The series of hepatoma-specific aldehyde dehydrogenases possess antigenic determinants which are detectable in normal liver aldehyde dehydrogenases, in addition to the set(s) of determinants shared by the hepatoma-specific and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-inducible aldehyde dehydrogenases. These observations are consistent with the hypothesis that the hepatoma-specific aldehyde dehydrogenase phenotype is composed, in part, of aldehyde dehydrogenases present in normal, uninduced rat liver [1,2].

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