[CANCER RESEARCH 36, 1568–1572, May 1976]

Detection of Liver-bound Metabolites of Azocarcinogens by the Use of Anti-Hapten Antibodies[1]

Christopher Carruthers, Alverna Baumler, Ann Neilson, and David Pressman

Orchard Park Laboratories [C. C., A. B., A. N.] and Department of Immunology Research [D. P.], Roswell Park Memorial Institute,[2] Buffalo, New York 14263

SUMMARY

The presence of the azocompounds, *p*-dimethylaminoazobenzene and 3′-methyl-*p*-dimethylaminoazobenzene, and *p*-amino-*N*-acetyl-*N*-methylaniline (or their metabolites) bound to components of the liver cells of rats fed a single large dose of each compound has been detected using rabbit antibodies raised against the *p*-azo-*N*-acetyl-*N*-methylaniline hapten in the indirect fluorescent antibody technique. Binding of these antibodies was seen on liver sections from rats fed any one of these compounds. When the anti-*p*-azo-*N*-acetyl-*N*-methylaniline antiserum was absorbed with either liver sediments or cytosol fractions from rats fed *p*-amino-*N*-acetyl-*N*-methylaniline, the antibodies reacting with the liver-bound compounds were removed from the antiserum. Also, absorption of the antiserum with liver sediments or cytosol fractions of rats fed either one of the azocompounds selectively removed all of the antibodies reacting with the livers of rats fed that compound but did not remove other antibodies that were still capable of reacting with liver cells of rats fed the other azocompound or *p*-amino-*N*-acetyl-*N*-methylaniline.

Thus this antiserum appears to contain several different anti-*p*-azo-*N*-acetyl-*N*-methylaniline antibodies with different structural requirements for reaction. Some can react with the azocompounds or certain of their metabolites, while others require more of the *p*-azo-*N*-acetyl-*N*-methylaniline structure for reaction. Some of the antibodies appear to react with liver-bound *p*-dimethylaminoazobenzene but not with liver-bound 3′-methyl-*p*-dimethylaminoazobenzene, while still others react with 3′-methyl-*p*-dimethylaminoazobenzene but not with *p*-dimethylaminoazobenzene.

INTRODUCTION

Evidence has been obtained by fluorescent antibody techniques that the metabolites of certain carcinogens, such as 3′-Me-DAB,[3] are bound to liver cells (2). The original carcinogen itself may or may not be bound. Compounds capable of reacting with rabbit antibodies raised against p-azo-AB and antibodies raised against p′-azo-p-DAB were found in the liver cells of rats fed 3′-Me-DAB. Compounds capable of reacting with 1 of these antisera were located in parts of the cells different from the parts carrying compounds capable of binding the other antiserum, although some overlap of localization patterns was observed. These results suggested that at least 2 different compounds, either the azocarcinogen and a metabolite or 2 metabolites, were bound to different cell components with a different distribution.

Reports in the literature suggest possible structures of metabolites of 3′-Me-DAB that may be present in these liver cells. *N*-Hydroxy-*N*-acetyl-*p*-aminoazobenzene has been shown to be a urinary metabolite of DAB, *N*-methyl-*p*-aminoazobenzene, and *p*-aminoazobenzene (5). Some of the simpler metabolites of *N*-methyl-*p*-aminoazobenzene and DAB that have been identified are aniline, *N*-methyl-*p*-phenylenediamine, *p*-phenylenediamine, *p*-aminophenol, *N,N*-dimethyl-*p*-phenylenediamine, and *o*-aminophenol (4).

In the study reported here we have examined the possibility that a metabolite with a structure similar to that of *N*-acetyl-*N*-methyl-*p*-aniline may be present. This metabolite might be produced from 3′-Me-DAB as well as from DAB by a series of reactions, namely, metabolic cleavage of the azo-linkage, demethylation, hydroxylation, and acetylation (5). Rabbit antiserum was raised against the hapten p-azo-AMA, and its reaction with liver cells of rats fed DAB, 3′-Me-DAB, or p-amino-AMA was determined. The results reported here suggest that such a metabolite could be present.

MATERIALS AND METHODS

Male Fischer rats (The Charles River Breeding Laboratories, North Wilmington, Mass.), 6 to 8 months of age and weighing about 300 g, were used.

Carcinogens and Related Compounds. p-Amino-AB, DAB, 3′-Me-DAB, and *N,N*-dimethylformamide were purchased from Eastman Organic Chemicals, Rochester, N. Y., and p-amino-AMA was purchased from Aldrich Chemical Co., Milwaukee, Wis. Ovalbumin was purchased from Mann Research Laboratories, New York, N. Y., and keyhole limpet hemocyanin was obtained from O. A. Roholt of the Roswell Park Memorial Institute.

Protein-Hapten Conjugate. Hemocyanin and ovalbumin conjugates of p-amino-AMA were prepared in the same manner previously described (2) for similar conjugates of p-amino-AB and p′-amino-p-DAB.

[1] This investigation was supported by Grant CA13664 from the National Cancer Institute and General Research Support Grant from the NIH, Department of Health, Education and Welfare.

[2] A unit of the New York State Department of Health.

[3] The abbreviations used are: 3′-Me-DAB, 3′-methyl-*p*-dimethylaminoazobenzene; p-azo-AB, *p*-azoazobenzene; p′-azo-p-DAB, *p*′-azo-*p*-dimethylaminoazobenzene; DAB, *p*-dimethylaminoazobenzene; p-azo-AMA, *p*-azo-*N*-acetyl-*N*-methylaniline; p-amino-AMA, *p*-amino-*N*-acetyl-*N*-methylaniline; p-amino-AB, *p*-aminoazobenzene; p′-amino-p-DAB, *p*′-amino-*p*-dimethylaminoazobenzene.

Received December 2, 1975; accepted January 20, 1976.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/36/5/1568/2397224/cr0360051568.pdf by guest on 03 August 2024

Antisera. Antisera against the hemocyanin conjugate of p-amino-AMA were raised in New Zealand White rabbits, 4 to 7 months old. The sera were assayed for antibody content by the ring test (1), using the ovalbumin conjugate.

The anti-p-azo-AB and anti-p'-azo-p-DAB rabbit antisera have been described previously (2).

Absorption of Antisera. Before use, all antisera, including pooled normal rabbit serum, were absorbed with a pool of normal rat serum and then twice with sediments from normal guinea pig liver in the usual way (2).

Further absorptions of these antisera were carried out either with sediments or the cytosol fractions prepared from normal rat liver or from the livers of rats given DAB, 3'-Me-DAB, or p-amino-AMA by stomach tube. Each absorption was carried out twice. An amount of tissue sediments derived from 2 g of wet tissue was used in each absorption. Anti-p-azo-AMA serum was absorbed twice with 5 to 6 mg or more of cytosol protein per ml of antiserum, while the anti-p-azo-AB and anti-p'-azo-p-DAB sera were absorbed twice with about 4 mg of the cytosol fractions per ml of serum. The cytosol fractions were obtained by differential centrifugation of liver homogenates in 0.25 M sucrose (7). Absorbed antisera were diluted 10-fold with 0.15 M borate-buffered 0.14 M NaCl solution (pH 8) for use in examining liver sections by the fluorescent antibody procedure described previously (2).

Immunodiffusion. Hapten inhibition studies of the reaction of anti-p-azo-AMA sera with the ovalbumin conjugate of p-azo-AMA were carried out by double diffusion analyses in 1.5% agar (buffered with barbital, pH 7.5, $\tau/2 = 0.15$) on microscope slides. Increasing amounts of p-amino-AMA dissolved in *N,N*-dimethylformamide were delivered to the central antisera well. When the compound had diffused out of the center well, 20 μl of the anti-p-azo-AMA serum were added. The slides were then processed as described previously (2).

Treatment of Rats with Compounds. DAB (20 mg/ml), 3'-Me-DAB (20 mg/ml), or p-amino-AMA (20 mg/ml) was dissolved in cottonseed oil with the aid of a hot water bath. The rats were fed these solutions by stomach tube (20 mg/100 g weight of rat) under ether anesthesia. The animals were sacrificed at various time intervals following the administration of the compounds. They were allowed to fast overnight and then were killed by perfusion of cold borate buffer, pH 8, through the abdominal aorta while under Nembutal anesthesia.

RESULTS

Characterization of Anti-p-azo-AMA Antisera. High titer anti-p-azo-AMA rabbit antiserum was obtained from each of 4 rabbits immunized with the p-azo-AMA conjugate of hemocyanin. The specificity of these sera was determined by inhibition of the precipitin reaction using double diffusion analyses in agar. Twenty μl of these sera gave well-defined precipitin bands with 10- and 20-μg amounts of p-azo-AMA ovalbumin conjugate. For the 4 antisera, between 90 and 160 μg of p-amino-AMA added to the antibody well (dissolved in *N,N*-dimethylformamide) were required to prevent this precipitation.

The anti-p-azo-AMA antibodies were only of the IgG class for Rabbit 30 (the only 1 tested), as shown for antibody isolated by the use of an immunoadsorbent (6) when examined by immunoelectrophoresis (3) using goat anti-rabbit globulin antisera. Only an IgG precipitin line was found.

Reaction of Anti-p-azo-AMA Antibodies with Liver of Rats Fed Azocompounds. When liver sections from rats fed p-amino-AMA, DAB, or 3'-Me-DAB were treated with p-azo-AMA antisera by the indirect fluorescence antibody technique, all livers fluoresced strongly (Fig. 1 and Table 1). This strong fluorescence was observed in the livers of rats 17 hr after feeding p-amino-AMA (20 mg/100 g of rat) since we found that there was little fluorescence 42 hr after feeding. The livers of rats fed DAB or 3'-Me-DAB were examined 42 or 67 hr after feeding these azocompounds, since the intensity of the fluorescence did not vary greatly over a long period of time (up to about 500 hr) after feeding (2).

Absorption of the anti-p-azo-AMA antisera twice with liver sediments or with liver cytosol fractions prepared from rats fed p-amino-AMA completely removed the antibodies responsible for staining the liver sections from rats treated with any 1 of the compounds, p-amino-AMA, DAB, or 3'-Me-DAB.

Table 1

Reaction of anti-p-azo-AMA antibodies with liver sections of rats fed p-amino-AMA, 3'-Me-DAB, and DAB

The indirect fluorescent antibody procedure using suitably absorbed antiserum was used.

	Fluorescence of liver sections from rats fed[b]		
Absorption of antiserum[a]	p-amino-AMA[c]	3'-Me-DAB[d]	DAB[d]
Liver of normal rats	+	+	+
Liver of rats fed p-amino-AMA[c]	−	−	−
Liver of rats fed 3'-Me-DAB[d]	+	−	+[e]
Liver of rats fed DAB[d]	+	+[e]	−

[a] See text for details. Both liver sediments and liver cytosol fractions were used and the results were the same for either.

[b] Presence of fluorescence indicated by +, absence by −.

[c] Livers taken from rats 17 hr after feeding.

[d] Livers taken from rats 42 hr after feeding.

[e] Intensity of fluorescence was reduced by these absorptions below that observed in absorptions by normal liver material but was not abolished.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/36/5/1568/2397224/cr0360051568.pdf by guest on 03 August 2024

When the anti-p-azo-AMA antisera were absorbed with liver sediments or liver cytosol fractions from rats fed 3′-Me-DAB, liver sections from rats fed 3′-Me-DAB did not fluoresce; liver sections from DAB- and p-amino-AMA-fed rats, however, still exhibited fluorescence. Similarly, when the sera were absorbed with sediments or cytosol fractions from rats fed DAB, liver sections from 3′-Me-DAB- (Fig. 2) and p-amino-AMA-fed rats did still fluoresce (Table 1). Even when the anti-p-azo-AMA sera were absorbed with larger amounts of cytosol (antiserum, 24 mg/ml), the fluorescence apparently was not diminished (Fig. 3).

These observations indicate that the rabbit antisera raised by the p-azo-AMA hemocyanin conjugate contain several different kinds of anti-p-azo-AMA antibodies. Some of these react with liver-bound 3′-Me-DAB and DAB or their metabolites, while others do not.

More importantly, they also indicate that some antibodies against p-azo-AMA react with liver-bound DAB or its metabolites but not with liver-bound 3′-Me-DAB, and other of these antibodies react with liver-bound 3′-Me-DAB metabolites but not with liver-bound DAB.

Reactions of Anti-p-azo-AB and Anti-p′-azo-p-DAB Antisera. The specificity of the liver-localizing properties of the 2 antisera (anti-p-azo-AB and anti-p′-azo-p-DAB) described in the previous report (2) has now been determined. These antisera were raised in response to azohaptens similar in structure to the azocarcinogen 3′-Me-DAB. Neither of these 2 sera was found to contain antibodies reacting with liver-bound p-amino-AMA (Table 2).

In agreement with the conclusions reached in the earlier study with these sera, we again found that each apparently contains several different kinds of antibodies reacting with liver-bound azocompounds. Each reacts with liver from rats fed either DAB or 3′-Me-DAB. When either serum was absorbed with sediments or cytosol fractions from the liver of a rat fed DAB or 3′-Me-DAB, the antibodies reacting with the liver of a rat fed the same azocompound were removed, while at least some of the antibodies reacting with the livers of rats fed the other azo compound were not.

DISCUSSION

Rabbit antiserum raised against p-azo-AMA, a hapten with structural features of a possible metabolite of azocarcinogens, appears to be useful in studying the localization of azocarcinogens in liver cells of rats fed these carcinogens. Anti-p-azo-AMA antiserum reacts with liver cells of rats fed DAB or 3′-Me-DAB as well as reacting, as would be expected, with liver cells from rats fed p-amino-AMA, and the antibodies reacting in this manner can be absorbed from the serum with liver sediments or cytosol fractions of rats fed p-amino-AMA.

The absorption experiments reported here indicate that the anti-p-azo-AMA antiserum must contain several antibodies, all of which react with p-amino-AMA or some of its metabolites. Some react preferentially with DAB or its metabolites and others react preferentially with 3′-Me-DAB or its metabolites. Whether the fed azocarcinogens have formed metabolites that react with antibodies against the *N*-methyl-*N*-acetylamino portion is not clear, since the anti-p-azo-AMA antiserum was raised against a structure containing the azo group and it may well be that antibodies reactive with the azo group and adjacent structures are responsible for the reaction.

Although anti-p-azo-AMA antiserum does contain antibodies (of as yet unknown specificity) that react with the azocarcinogens DAB and 3′-Me-DAB or their metabolites, antiserum to the azocarcinogen-like haptens, the anti-p-azo-AB and anti-p′-azo-p-DAB antisera, described in an earlier report (2), do not contain antibodies that react with liver cells of rats fed p-amino-AMA. The reason for this may be simply that the anti-p-azo-AB and anti-p′-azo-p-DAB antisera were raised in response to haptens carrying the azo

Table 2

Reaction of anti-p-azo-AB and anti-p′-azo-p-DAB antibodies with liver sections of rats fed p-amino-AMA, 3′-Me-DAB, and DAB

		Fluorescence of liver sections for rats fed[b]			
Antiserum	Absorption of antiserum[a]	Normal	p-amino-AMA[c]	3′-Me-DAB	DAB
Anti-p-azo-AB	Normal rat liver	−	−	+	+
	Liver of rats fed p-amino-AMA[c]	−	−	+	+
	Liver of rats fed 3′-Me-DAB	−	−	−	+[d]
	Liver of rats fed DAB	−	−	+[d]	−
Anti-p′-azo-p-DAB	Normal rat liver	−	−	+	+
	Liver of rats fed p-amino-AMA[c]	−	−	+	+
	Liver of rats fed 3′-Me-DAB	−	−	−	+[d]
	Liver of rats fed DAB	−	−	+[d]	−

[a] See text for details. Both liver sediments and liver cytosol fractions were used and the results were the same.
[b] Presence of fluorescence indicated by +, absence by −.
[c] Livers taken from rats 17 hr after feeding; all other livers were taken from rats 42 hr after feeding.
[d] Intensity of fluorescence was reduced by these absorptions below that observed in absorptions by normal liver material but was not abolished.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/36/5/1568/2397224/cr0360051568.pdf by guest on 03 August 2024

group that appears to be a major determinant, whereas neither p-amino-AMA nor its metabolites carry this group. The reactions of the 2 antisera described here do, however, confirm the conclusions of our previous report, namely, that each of these antisera, raised against azo compounds similar to the azocarcinogen 3′-Me-DAB, contains 2 or more antibodies that react with the azo compounds or metabolites in the liver cells of rats fed 3′-Me-DAB. The results here also indicate that these 2 antisera also contain antibodies reacting with liver cells of rats fed another azo compound, DAB.

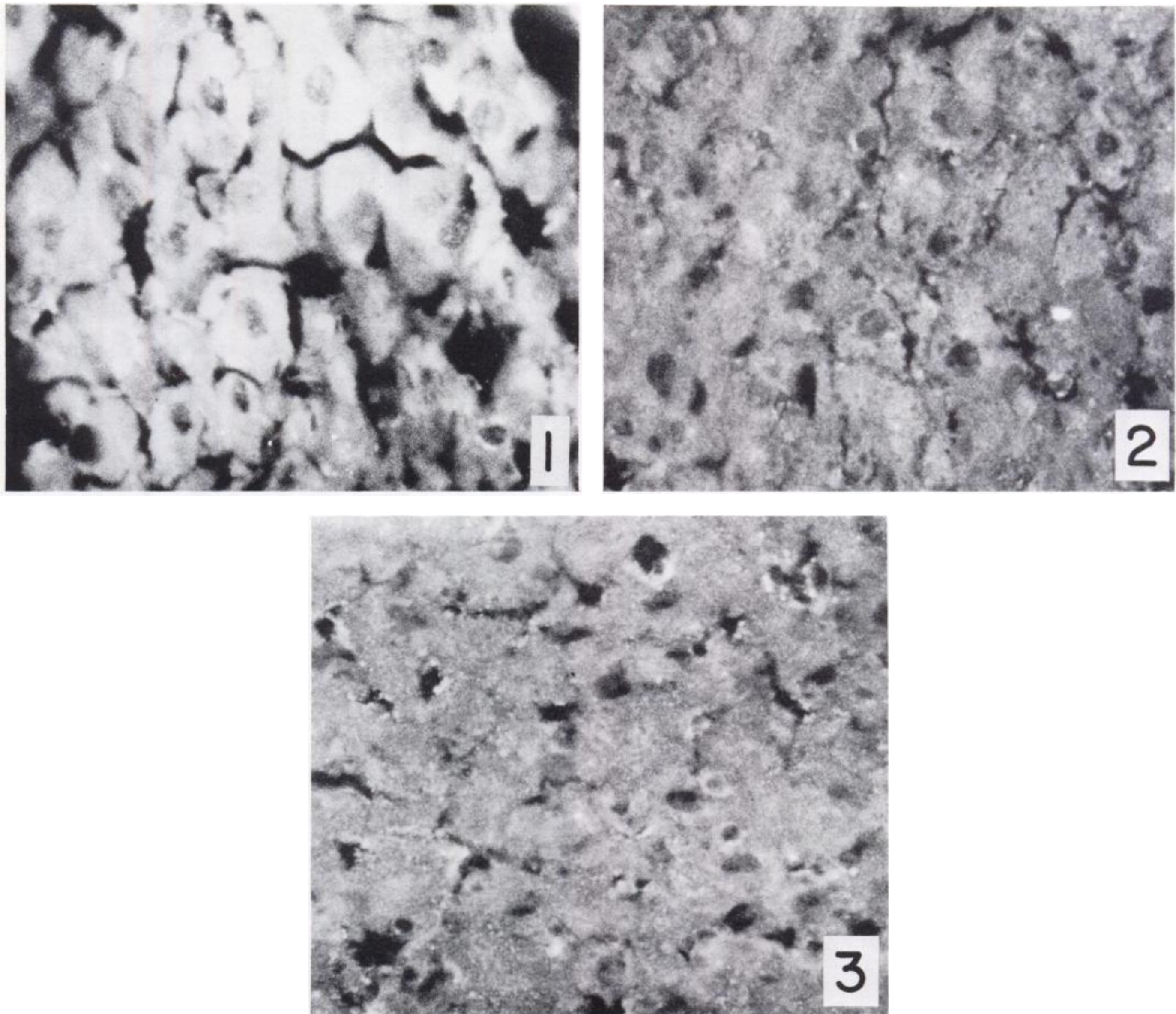
ACKNOWLEDGMENTS

We wish to thank Dr. V. Peter Kreiter for valuable aid in preparing the manuscript.

REFERENCES

1. Campbell, D. H., Garvey, J. S., Cremer, N. E., and Sussdorf, D. H. Methods in Immunology. A Laboratory Text for Instruction and Research, pp. 130–135. New York: W. A. Benjamin, Inc., 1964.
2. Carruthers, C., Baumler, A., Neilson, A., and Pressman, D. Binding of the Azocarcinogen 3′-Methyl-*p*-dimethylaminoazobenzene to Cellular Components of Normal Rat Liver and Azocarcinogen-induced Hepatomas. Cancer Res., *35:* 3798–3806, 1975.
3. Kitagawa, M., Yagi, Y., Planinsek, J., and Pressman, D. *In Vivo* Localization of Anticarcinogen Antibody in Organs of Carcinogen-treated Rats. Cancer Res., *26:* 221–228, 1966.
4. Miller, J. A., and Miller, E. C. The Carcinogenicity of Certain Derivatives of *p*-Dimethylaminoazobenzene in the Rat. Cancer Res., *7:* 139–156, 1947.
5. Miller, J. A., and Miller, E. C. A Survey of Molecular Aspects of Chemical Carcinogenesis. Lab. Invest., *15:* 217–241, 1966.
6. Onoue, K., Yagi, Y., and Pressman, D. Immunoadsorbents with High Capacity. Immunochemistry, *2:* 181–194, 1965.
7. Schneider, W. C. Intracellular Distribution of Enzymes. III. The Oxidation of Octanoic Acid by Rat Liver Fractions. J. Biol. Chem., *176:* 259–266, 1948.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/36/5/1568/2397224/cr0360051568.pdf by guest on 03 August 2024



Figs. 1 to 3. Liver sections of rats fed 3′-Me-DAB were treated first with anti-p-azo-AMA antiserum and then with fluorescent goat anti-rabbit IgG globulins. The rats received 3′-Me-DAB (20 mg/100 g weight of rat) by gastric intubation and were sacrificed after an overnight fast at the indicated time intervals. Antiserum was first absorbed once with pooled normal rat serum, twice with guinea pig liver sediments, and then with tissue sediments or cytosol proteins as indicated.

Fig. 1. Liver of rat at 67 hr, stained with anti-p-azo-AMA antiserum absorbed with liver sediments obtained from normal rat liver. Note strong cytoplasmic as well as some intranuclear fluorescence. × 200.

Fig. 2. Liver of rat at 41 hr, stained with anti-p-azo-AMA antiserum absorbed with cytosol proteins (12 mg/ml antiserum) obtained from the liver of a DAB-treated rat (20 mg DAB per 100 g weight of rat by gastric intubation) sacrificed 41 hr after treatment, following an overnight fast. Note fluorescence in cytoplasm with only traces of intranuclear fluorescence. × 200.

Fig. 3. Liver of rat at 41 hr, stained with anti-p-azo-AMA antiserum absorbed with cytosol proteins (24 mg/ml antiserum) obtained from the liver of a DAB-treated rat as in Fig. 2. The fluorescence is comparable to that shown in Fig. 2. × 200.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/36/5/1568/2397224/cr0360051568.pdf by guest on 03 August 2024