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A POST-SYNTHETIC MODIFICATION OF HUMAN ALPHA-FETOPROTEIN REVEALED BY ISOELECTRIC FOCUSING CONTROLS ITS IMMUNOSUPPRESSIVE POTENCY

MASTER

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Short title: Post-Synthetic Modification of HAFP

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ABSTRACT

We have demonstrated 3 variants of human alpha-fetoprotein (HAFP) by crossed immunoelectrophoresis, and have correlated the capacity of HAFP isolates to suppress human lymphocyte transformation in vitro with the relative proportion of the electronegative variant, HAFP-3, present in each isolate. We have now isolated HAFP from the serum, ascitic fluid, and saline extract of tumor from a single hepatoma patient, and from an homogenate of fetal livers. When tested for their capacity to inhibit human lymphocyte transformation in vitro, tumor and fetal liver HAFP were found to be extremely potent; serum HAFP had intermediate potency, and ascitic fluid HAFP was the least potent.

Analysis of these HAFP isolates by crossed immunoelectrophoresis confirmed the correlation between the proportion of HAFP-3 and the immunosuppressive potency of each isolate. In addition, analysis of these HAFP isolates by isoelectric focusing in polyacrylamide gels containing 8 M urea revealed further evidence of microheterogeneity; at least 6 molecular variants were apparent. The proportion of one of these variants, termed HAFP-3a, in each isolate was correlated with the immunosuppressive

potency of the isolate. The sialic acid content of the various HAFP isolates did not vary significantly.

Our data suggest that a post-synthetic modification of HAFP occurs, which modulates its immunosuppressive potency.

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Human alpha fetoprotein (HAFP) is capable of suppressing the response of human lymphocytes in vitro to a variety of mitogenic stimuli (4). HAFP isolates from various fetal and adult human sources vary over 3 orders of magnitude in their immunosuppressive potency independent of their sialic acid content (6). We have previously characterized HAFP microheterogeneity by crossed immunoelectrophoresis in agarose gels, and have demonstrated that HAFP consists of 3 electrophoretically distinct species which we have designated HAFP-1 the most electropositive, HAFP-2, and HAFP-3, the most electronegative. We have shown that total desialylation of HAFP changes, but does not abolish HAFP microheterogeneity, demonstrating that there is a variation of a charged moiety other than sialic acid on the molecule. In addition, total removal of HAFP sialic acid does not interfere with its immunosuppressive potency. We have also shown that the relative proportion of the most electronegative form of HAFP, HAFP-3, in an individual isolate correlates with its immunosuppressive potency (2).

We now report on the microheterogeneity and immunosuppressive potency of HAFP isolated from the

serum, ascitic fluid, and tumor of a single patient (Od.) dying of malignant hepatoma. Analysis of the variation in the HAFP isolated from these three sources suggests that a post-synthetic biochemical modification of the protein occurs which decreases its immunosuppressive potency. The micro-heterogeneity of HAFP has been further characterized by isoelectric focusing in polyacrylamide gel in the presence of 8 M urea (3). By this technique we have found that the proportion of a particular isoelectric variant, termed HAFP-3a, correlates with the immuno-suppressive potency of a given preparation of HAFP from patient Od., and from fetal liver.

HAFP was isolated from serum, ascitic fluid, or saline tissue homogenates by passage over an anti-HAFP affinity column. Impurities were removed by subsequent column chromatography over an anti-human serum protein affinity column and Sephadex G150 (2,4,5,6). All HAFP preparations were pure as judged by immunoelectrophoresis, and polyacrylamide gel electrophoresis. For analytic isoelectric focusing 5% polyacrylamide gels slabs containing 8 M urea and ampholytes with a pH range of 5-7 were employed. Gels were stained with Coomassie Blue and scanned at

540 nm with a linear transport chromatogram spectrophotometer.

The immunosuppressive potencies of HAFP isolated from the ascitic fluid, serum, and tumor homogenate of patient Od. are shown in Table I. Fetal liver and tumor homogenate HAFP were extremely potent, whereas high doses of ascitic fluid HAFP were required for inhibition of lymphocyte transformation; serum HAFP was of intermediate potency. The sialic acid content of all four HAFP preparations were similar.

Crossed immunoelectrophoresis of isolated ascitic fluid HAFP revealed 3 molecular species (Figure 1) as previously described. Serum HAFP contained relatively more HAFP-3, and less HAFP-1 than did ascitic fluid HAFP. Tumor and fetal liver HAFP were nearly identical in that virtually no HAFP-1 was present, and HAFP-3 predominated. The relative content of HAFP-3 in each preparation may be correlated with immunosuppressive potency (Table I).

Isoelectric focusing in polyacrylamide gel containing 8 M urea revealed the microheterogeneity of human HAFP in greater detail (Figure 2). Six major isoelectric variants could be discerned over a pH

range of 6.0-6.2; these are termed HAFP-1a, the most electropositive, HAFP-2a, HAFP-2b, HAFP-3a, HAFP-3b, and HAFP-3c, the most electronegative. The immunosuppressive potency of each HAFP preparation correlates with the relative content of HAFP-3a in each preparation (Figure 3).

In order to verify that each protein band seen in isoelectric focusing was, in fact, an HAFP variant, we analyzed replicate acrylamide gel slices following isoelectric focusing by crossed immunoelectrophoresis into anti-HAFP containing agarose gel (Figure 4). Reproducible patterns of HAFP composition were thus obtained which closely resemble the spectrophotometric scans of the Coomassie Blue stained isoelectric focusing gels for each HAFP preparation.

Total desialylation of all four HAFP preparations alters the isoelectric focusing bands but results in little or no reduction in their number (Figure 5). Thus it appears from our isoelectric focusing results with native and desialylated HAFP (dsHAFP) that, apart from sialic acid content, there may be multiple biochemical bases for HAFP microheterogeneity. Based on our knowledge of the relative proportions of HAFP isoelectric variants in each

isolate, it appears that dsHAFP-1b is the desialylated derivative of native HAFP-3a, and as such represents a potent immunosuppressive form of desialylated HAFP preparations.

Our data suggest that HAFP is initially synthesized in a relatively electronegative form with a large proportion of HAFP-3 as is seen in both the tumor and fetal liver HAFP isolates. Subsequently, small amounts of HAFP-1 are seen in the serum. As HAFP passes into the extravascular compartment (ascitic fluid) the relative quantity of HAFP-1 increases, and that of HAFP-3 decreases. As these changes in the electrophoretic variants of HAFP occur, there is a simultaneous loss of HAFP-3a, so that ascitic fluid is almost devoid of this isoelectric variant. Two explanations for this progressive shift from electronegative to electropositive species are possible: (a) a post-synthetic, probably post secretory, biochemical modification of the molecule may occur, or (b), the various HAFP species may have differing catabolic rates. Various theoretical and experimental considerations lead us to favor the former hypothesis (1). Thus we believe that HAFP undergoes a biochemical modification following its

synthesis and secretion, which converts the molecule from an electronegative to an electropositive species. We postulate that this conversion modulates the immunosuppressive potency of the HAFP molecule.

Acknowledgments

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TABLE I

Quantitation of Electrophoretic Variants, Isoelectric Variants, Biological Potency, and Sialic Acid Content of Various HAFP Isolates

	IHD_{50}^{*} (µg/ml)	HAFP variants Agarose gel electrophoresis % of total HAFP			HAFP isoelectric variants % of total HAFP						Moles sialic acid/mole HAFP
		HAFP 1	HAFP 2	HAFP 3	HAFP-1a	HAFP-2a	HAFP-2b	HAFP-3a	HAFP-3b	HAFP-3c	
$HAFP_{OD}$ ascites	2000	28	53	19	12	28	22	6	21	13	1.45
$HAFP_{OD}$ serum	822	11	36	53	4	15	23	8	22	27	1.42
$HAFP_{OD}$ tumor	4.7	--	43	57	1	20	15	20	23	22	1.43
$HAFP_{fetal}$ liver	2.7	--	82	72	< 1	10	6	27	26	31	1.56

*Amount of HAFP producing 50% inhibition of lymphocyte mitogenic response to ATS, nonhemagglutinating phytohemagglutinin, or Con-A.

FIG. 1. Crossed immunoelectrophoretic patterns of HAFP isolates. The crossed immunoelectrophoretic pattern of HAFP isolated from Od. ascitic fluid (top) reveals three species, a cathodal HAFP-1, an intermediate HAFP-2, and an anodal HAFP-3. There is more HAFP-3 and less HAFP-1 in the Od. serum isolate. The Od. tumor and fetal liver HAFP lack HAFP-1 and have a high proportion of HAFP-3.

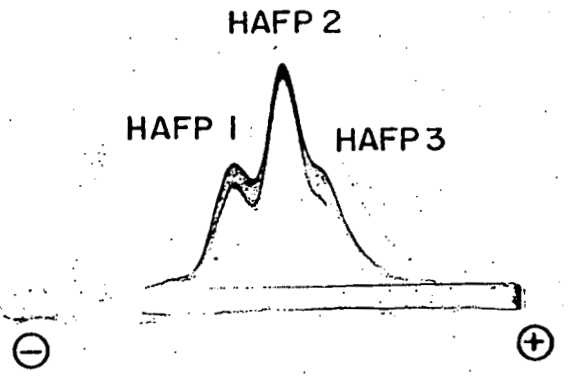
FIG. 2. Isoelectric focusing of HAFP in a polyacrylamide gel in the presence of 8 M urea. The pH range of the ampholytes used was 5-7. The photographic inset (top) reveals the six molecular species of HAFP: HAFP-1a, the most electropositive, HAFP-2a, HAFP-2b, HAFP-3a, HAFP-3b, and HAFP-3c, the most electronegative. Od. tumor and fetal liver HAFP contain proportionately more HAFP-3a than do Od. ascitic fluid or Od. serum HAFP. The pH gradient across the gel, as measured with a surface pH electrode, is displayed on the bottom. A sample of human serum albumin is included in the gel for comparison; no impurities are seen in the HAFP isolates. The anode is to the right.

FIG. 3. Correlation of immunosuppressive potency of various HAFP isolates with their proportion of HAFP-3a. The immunosuppressive potency is plotted on the vertical logarithmic axis as the dose of HAFP required to produce a 50% inhibition of lymphocyte transformation (IHD_{50}). The relative proportion of HAFP-3a (% HAFP-3a) in each isolate is plotted on the linear horizontal axis.

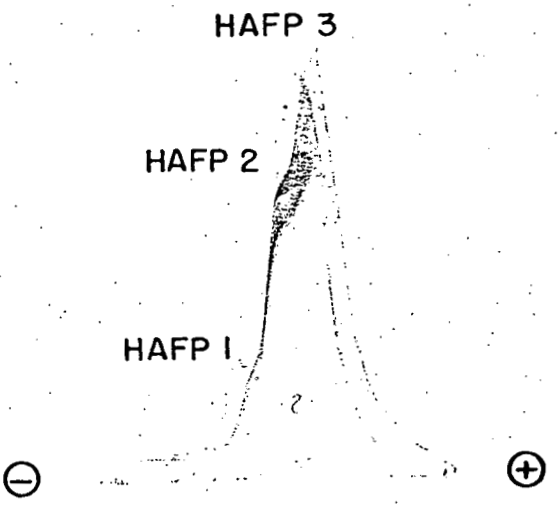
FIG. 4. The molecular species of HAFP as revealed by isoelectric focusing. The solid lines represent the spectrophotometric scans, at 540 nm, of electrofocused samples of HAFP in polyacrylamide gels containing 8 M urea. Beneath each of these is displayed the result of crossed immunoelectrophoresis of the electrofocused HAFP sample, as well as a replicate of the original electrofocused sample reproduced to approximately the same scale. Each band of focused protein is shown to be HAFP and its relative proportion in each isolate may be quantitated as the area under its peak in the spectrophotometric scan.

FIG. 5. A comparison of the isoelectric focusing patterns of native and desialylated (ds) HAFP preparations.

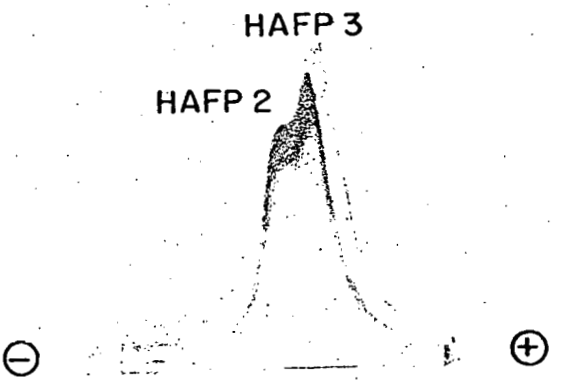
Od. Ascitic fluid



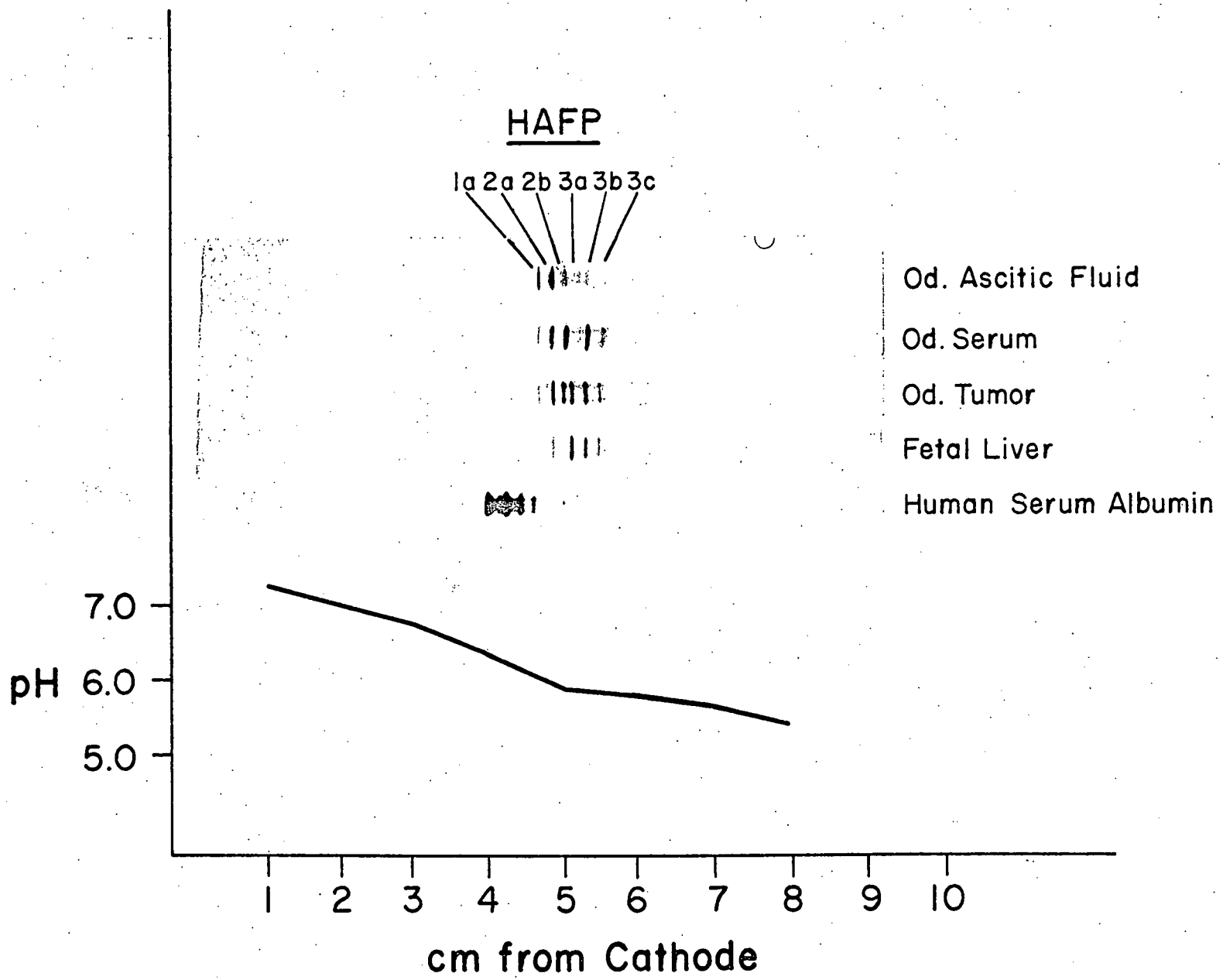
Od. Serum

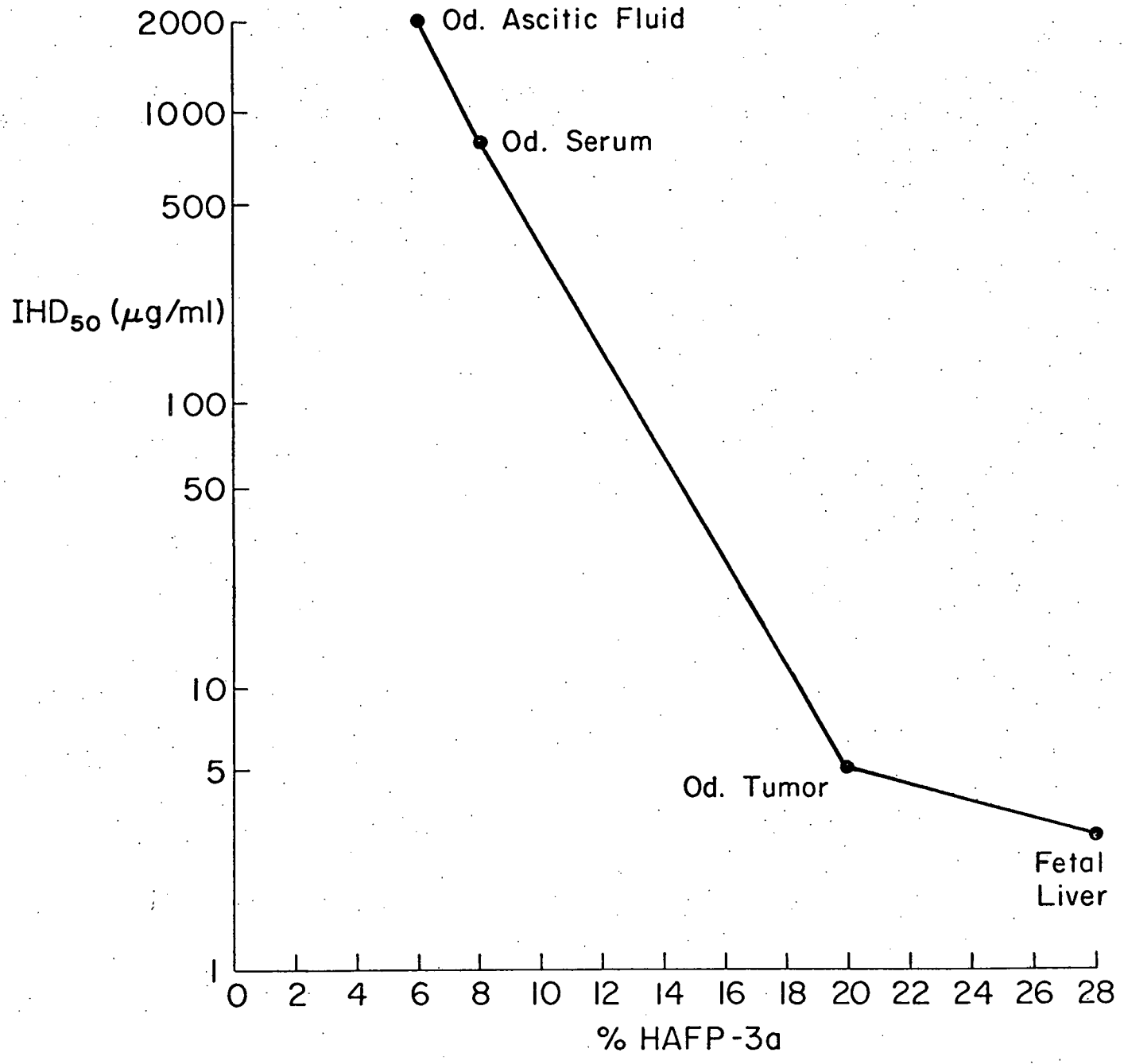


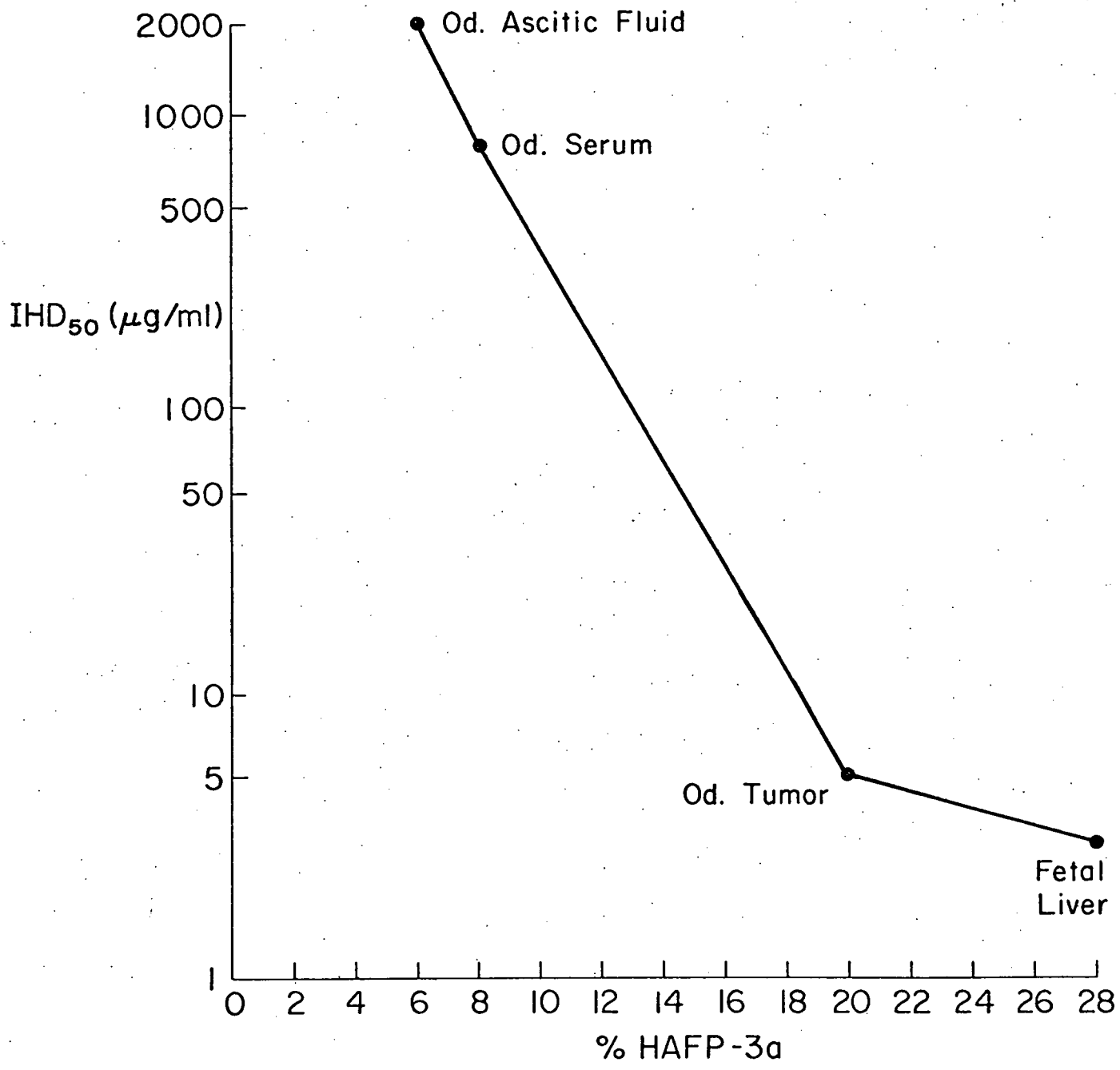
Od. Tumor

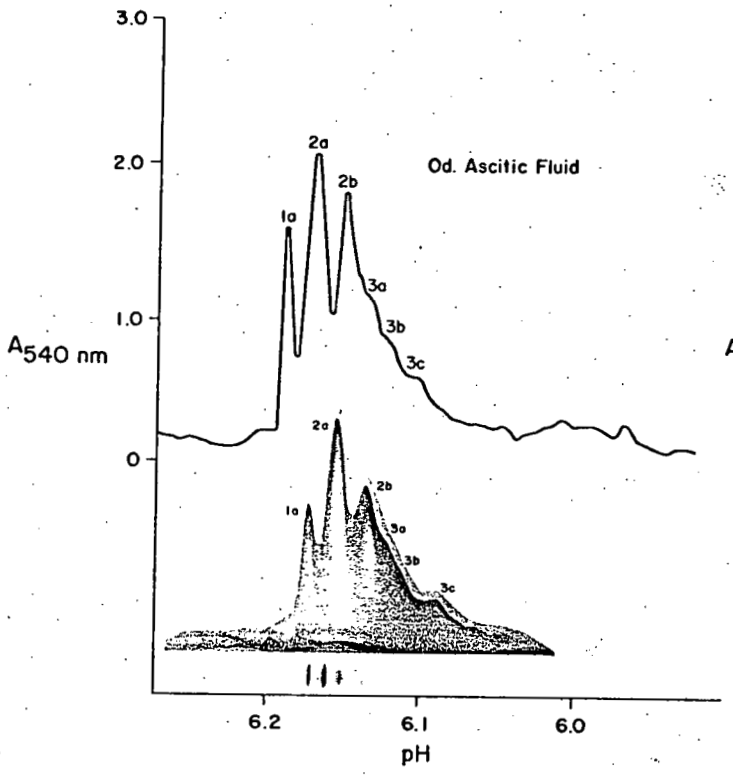


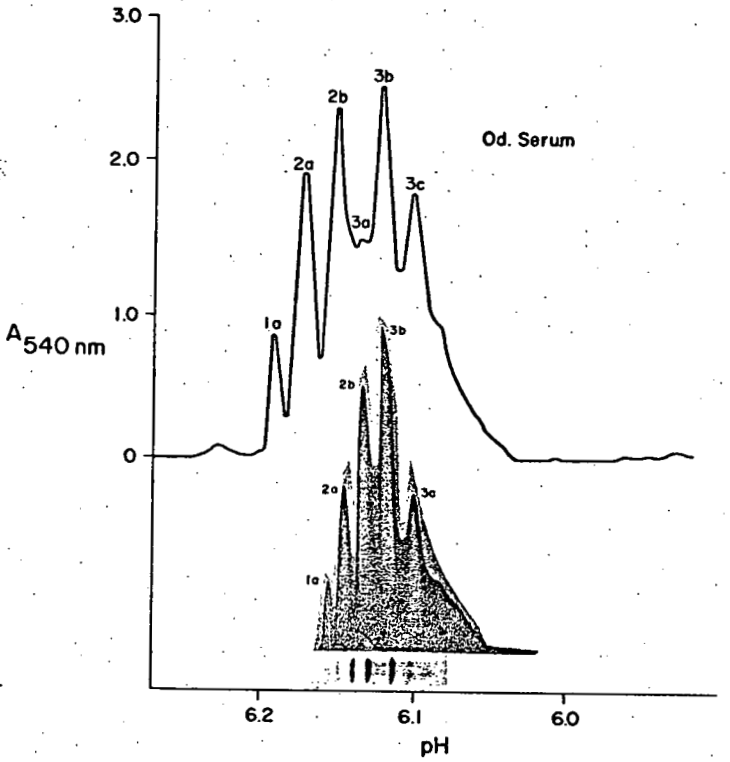
Fetal liver

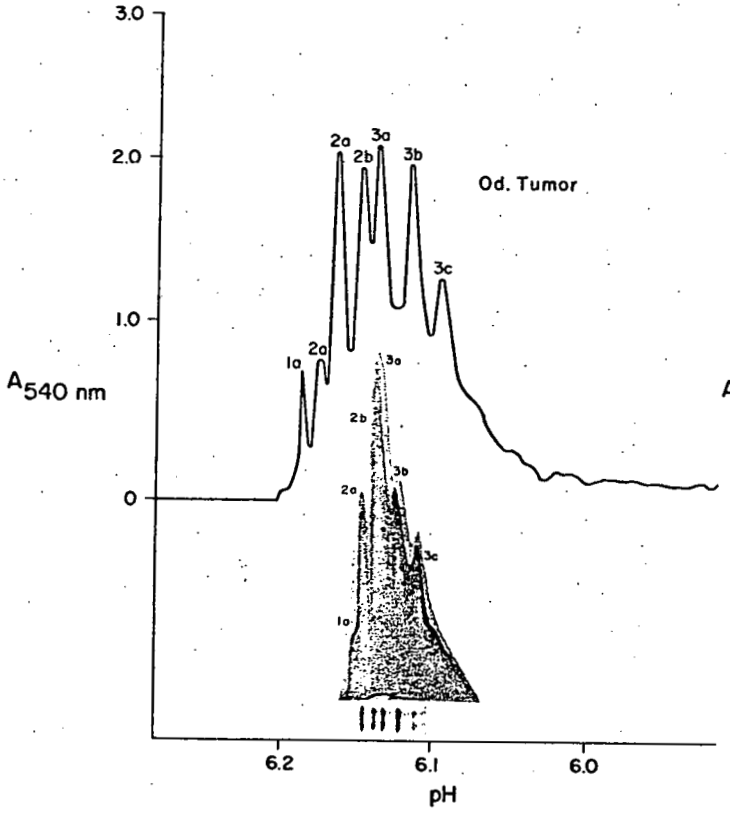

HAFP
1a 2a 2b 3a 3b 3c
Od. Ascitic Fluid
Od. Serum
Od. Tumor
Fetal Liver
Human Serum Albumin
pH
7.0
6.0
5.0
1 2 3 4 5 6 7 8 9 10
cm from Cathode

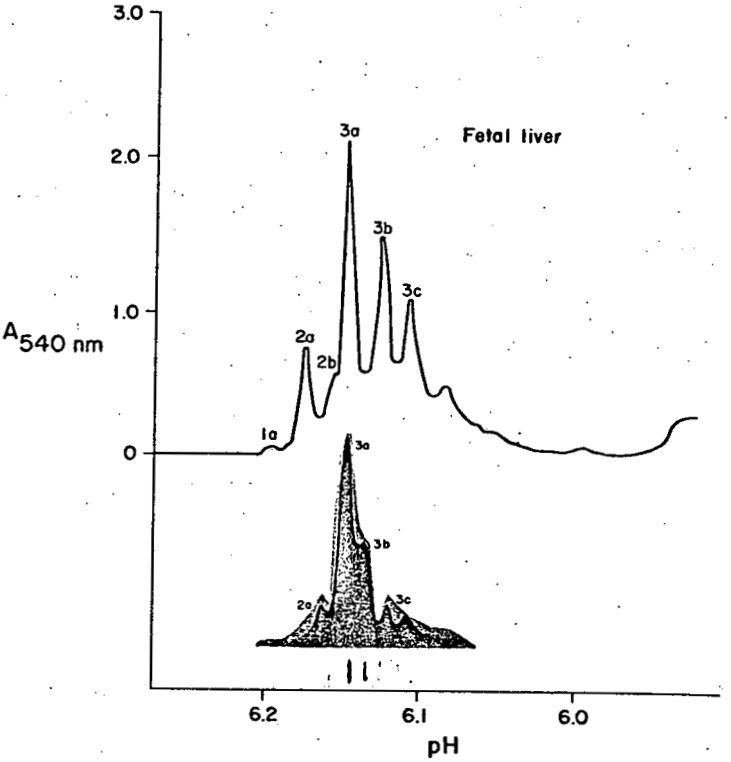

2000
1000
500
IHD50 (μg/ml)
100
50
10
5
1
Od. Ascitic Fluid
Od. Serum
Od. Tumor
Fetal Liver
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
% HAFP-3a

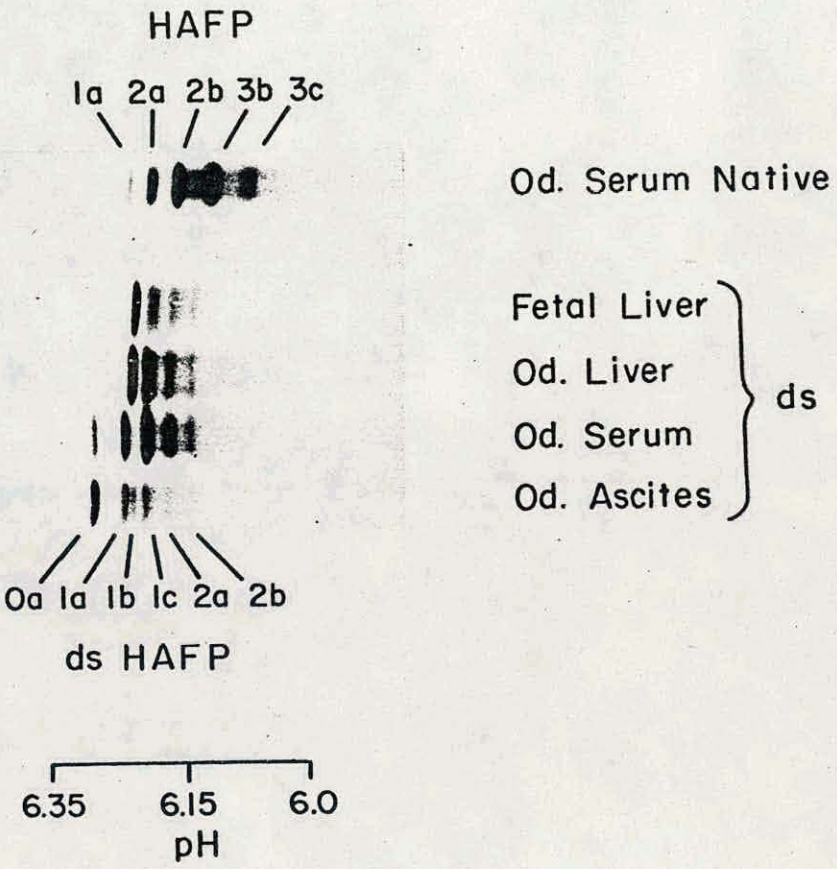

2000
1000
500
IHD50 (μg/ml)
100
50
10
5
1
Od. Ascitic Fluid
Od. Serum
Od. Tumor
Fetal Liver
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
% HAFP-3a


Od. Ascitic Fluid
$A_{540\ nm}$
3.0
2.0
1.0
0
1a
2a
2b
3a
3b
3c
6.2
6.1
6.0
pH


Od. Serum
$A_{540\ nm}$
3.0
2.0
1.0
0
1a
2a
2b
3a
3b
3c
6.2
6.1
6.0
pH


Od. Tumor
$A_{540\ nm}$
3.0
2.0
1.0
0
1a
2a
2b
3a
3b
3c
6.2
6.1
6.0
pH


Fetal liver
$A_{540\ nm}$
3.0
2.0
1.0
0
1a
2a
2b
3a
3b
3c
6.2
6.1
6.0
pH


HAFP
1a 2a 2b 3b 3c
Od. Serum Native
Fetal Liver
Od. Liver
Od. Serum
Od. Ascites
ds
0a 1a 1b 1c 2a 2b
ds HAFP
6.35 6.15 6.0
pH