STUDIES ON HISTOPLASMOSIS IV. ELUTION CHARACTERISTICS OF THE HISTO-INHIBITORY FACTOR (HIF)

by

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(with 5 figs.)

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A heat stable, non-dialyzable growth inhibitor derived from infected tissue has been demonstrated in direct culture of tissue infected with *Histoplasma capsulatum* or with *Blastomyces dermatitidis* and in saline homogenates of such tissue tested with culture grown organisms. (1, 2, 3) This histo-inhibitory factor (HIF) is active against the yeast form of these dimorphic fungi and shows some cross inhibitory activity against the heterologous organism. The mycelial form of *Histoplasma* and *Blastomyces* will adsorb the HIF from homogenates but growth of hyphae is not inhibited. (3) Similarly, bacteria (*Escherichia coli, Bacillus subtilis, Staphylococcus aureus*) and other fungi tested (*Cryptococcus neoformans, Candida albicans*) will adsorb HIF but are not inhibited by it.

The present report describes conditions suitable for elution of the Histoplasma HIF and further characterizes the active fractions.

Experimental

Materials

Sephadex G—150 (particle size: 40—120; water regain: 15 ± 1.5 g/g; bed volume per g dry gel: 20—30 ml); DEAE-Sephadex A—50 anion exchange (particle size 40—120 μ; capacity 3.5 ± 0.5 meq/g: Blue Dextran 2000 (MW 2,000,000) were all obtained from pharmacia Fine Chemicals, Inc., Piscataway, New Jersey.

DEAE-Cellulose (MN-Cellulose powder 300 DEAE, average particle size 10 μ; capacity 0.7 m water/g) was obtained from Macherey, Nagel and Company, Düren, Germany.

Proteins of known molecular weight: gamma globulin (human fraction II); serum albumin (bovine, fraction V); ovalbumin ($3\times$

cryst.); and hemoglobin (bovine, 2×cryst.) were all obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

Preparation of Columns

(a) Sephadex G-150 – The 1.5×30 cm column was packed to a bed volume of 20 cm according to the procedure of WHITAKER.(4)

(b) DEAE-Sephadex A—50 – The procedure of FLODIN (5) was utilized for the preparation of the 1.5×30 cm column.

(c) DEAE-Cellulose – The 1.5×30 cm column was prepared as described by JAMES & STANWORTH (6).

Analysis of Eluates

All spectrophotometric measurements were made in silica cells with a 1 cm light path in a Beckman DU-Spectrophotometer and are expressed as optical density (O.D.). Hemoglobin was estimated at 410 mμ; gamma globulin, serum albumin and ovalbumin were all estimated at 280 mμ.

Molecular Weight Determination by Gel Filtration

Molecular weight of macromolecular fractions was estimated by gel filtration of a Sephadex G—150 column in accordance with the procedures of WHITAKER.(4) Compounds used as reference included human gamma globulin, serum albumin (bovine), hemoglobin (bovine) and ovalbumin, Void volume (Vo) was measured utilizing Blue Dextran (MW 2,000,000).

Preparation and Detection of HIF in Hamster Tissue

One hundred male golden Syrian hamsters (*Mesacricetus auratus*) weighing 60—80 g were infected by subcutaneous inoculation of 0.05 ml of a saline suspension of 5×10^5 yeast cells from a 3-day culture of *H. capsulatum* (strain 6650, DR. C. W. EMMONS) grown on brain heart infusion agar containing 5 % sheep blood (BHIB agar).

Six weeks after infection, the animals' tissues were tested for growth of *H. capsulatum* and for the presence of the HIF by the following procedures:

Culture on BHIB at 37° C

1. Direct culture of tissue (portions of each spleen, kidney and liver).
2. Culture of minced saline homogenate (duplicate portions of sediment from 1—2 g of liver minced with scissors, well mixed with 5 ml saline and allowed to stand approximately 24 hours at 4° C before plating).
3. Culture of minced glycine buffer homogenate (pH 10.2).
4. Test of liver and kidney, homogenized, (Virtis mixer)* in glycine buffer pH 10.2, with in vitro grown *Histoplasma* yeasts (1×10^4 ml) from 3 to 4 day BHIB cultures.

*) Virtis 45, Laboratory Mill/homogenizer, Gardiner, New York.

These cultures were incubated at 37° C, examined daily and held for at least one week if negative.

A small sample of spleen, liver and kidney from each animal was fixed in Zenker's formol solution for preparation of tissue sections.

Test for Elution of HIF from Histoplasma

Histoplasma yeast cells (approximately 1×10^4/ml) were mixed with HIF in kidney homogenate, allowed to stand at 4° C for two hours, then centrifuged and resuspended in buffer: veronal-acetate buffer pH 4.5—9.0 or glycine – NaOH buffer pH 8.5 – 11.0

The sedimented yeast cells were well mixed with buffer and allowed to stand at 4° C for 12—24 hours, centrifuged again and resuspended in saline before plating on BHIB agar.

The negative control, *Histoplasma* yeast cells in saline, was processed in the buffers as above. The positive control, *Histoplasma* yeast cells in the HIF homogenate, was resuspended in saline after the first and second centrifugation.

Each buffer supernatant was tested for HIF by addition of *Histoplasma* yeast cells to initial concentration, (approximately 1×10^4/ml) at 37° C. All tests were run in duplicate.

Test for Effect of HIF on Heterologous Organisms

Three bacteria (*Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis* at approximately 1×10^5/ml) and three yeast-like organisms (*Candida albicans*, *Cryptococcus neoformans* and *Blastomyces dermatitidis* at 1×10^4/ml) were mixed with HIF in a saline-homogenate of liver or kidney or in a 280 mμ HIF fraction which had been lyophilized and resuspended in saline. A control test with *H. capsulatum* was run in parallel.

The suspensions were allowed to stand at 4° C for two hours, then centrifuged and the supernatants separated from the sediments. The supernatants were boiled (for *B. subtilis* supernatant autoclaved at 115° C) for ten minutes then cooled and *H. capsulatum* yeast cells were added to a final concentration 1×10^4/ml. Organisms in the sediments were resuspended in saline to the original volume. All suspensions were plated in duplicate on BHIB agar. Cultures were incubated at 37° C and checked daily for six days for growth.

Results

Fractionation of Kidney and Liver Homogenates

Kidneys and livers from histoplasma-infected hamsters were separately homogenized with glycine-sodium hydroxide buffer (0.1 M, pH 10.2) in a homogenizer and fractionated initially over a Sephadex G—150 column (prepared as described above). The possible acidic nature of the HIF protein suggested further fractionation over DEAE-cellulose and DEAE-Sephadex anion exchanger. Fig. 1 depicts the elution curves of the histoplasma inhibitory factor

(HIF) from kidney as well as a number of protein standards on a Sephadex G—150 column (1.5×30 cm). HIF, gamma globulin, serum albumin and ovalbumin were estimated at 280 mμ and hemoglobin at 410 mμ.

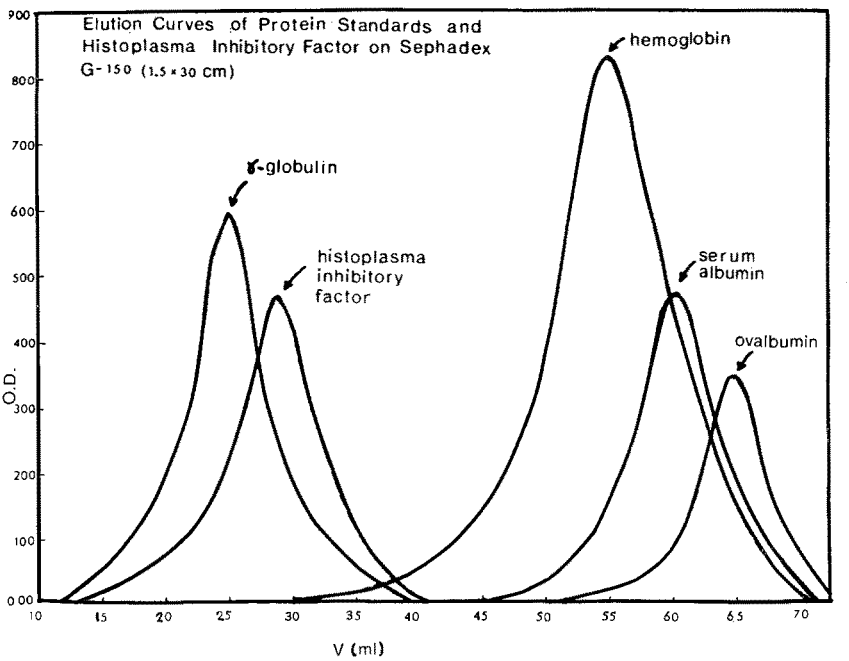

Fig. 1. Elution curves of protein standards and histo-inhibitory factor, (HIF), on Sephadex G—150 (1.5×30 cm) O. D. – optical density

Fig. 2 illustrates the elution curve of HIF (from liver) after an initial fractionation over Sephadex G—150 followed by a passage over DEAE-cellulose. The latter column was developed with 0.02 M phosphate; 0.02 M phosphate – 2 M sodium chloride (1 : 1); 0.02 M phosphate – 4 M sodium chloride (1 : 1) and finally 4 M sodium chloride. The HIF was found in the 0.02 M phosphate – 4 M sodium chloride eluate (fractions 11—17).

Fig. 3 illustrates the elution curve of HIF (from liver) after an initial fractionation over Sephadex G—150 followed by a passage over DEAE-Sephadex A—50 anion exchanger. The latter column was developed in an analogous manner to that described above for DEAE-cellulose. The HIF active material was eluted with 0.02 M phosphate – 4 M sodium chloride (1 : 1) buffer (fractions 14—17). As shown in Figs. 2 and 3 chromatography of liver homogenates on both DEAE-cellulose as well as DEAE-Sephadex A—50 (following initial passage over Sephadex G—150) indicates essentially similar

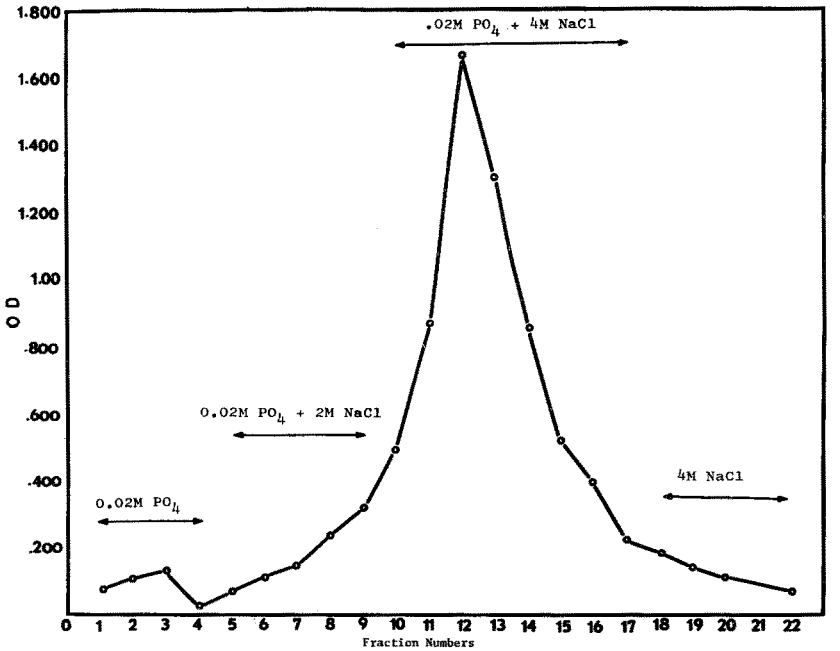

Fig. 2. Elution curve of histo-inhibitory factor, (HIF), (liver) from Sephadex G—150, refractionated over DEAE-cellulose. O,D, – optical density

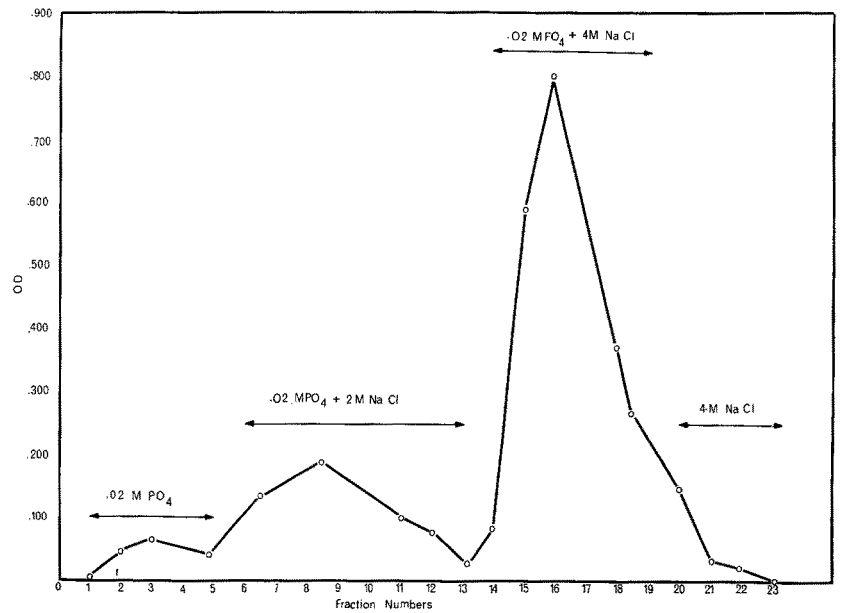

Fig. 3. Elution curve of histo-inhibitory factor, (HIF), (liver) from Sephadex G—150, refractionated over DEAE-Sephadex A-50. O.D. – optical density

characteristics. For both columns the active HIF fraction was eluted with 0.02 M phosphate – 4 M sodium chloride (1 : 1) buffer following a stepwise increase of the ionic strength of the eluent. The similarity of fractionation characteristics of DEAE-cellulose and DEAE-Sephadex has been previously reported.(7)

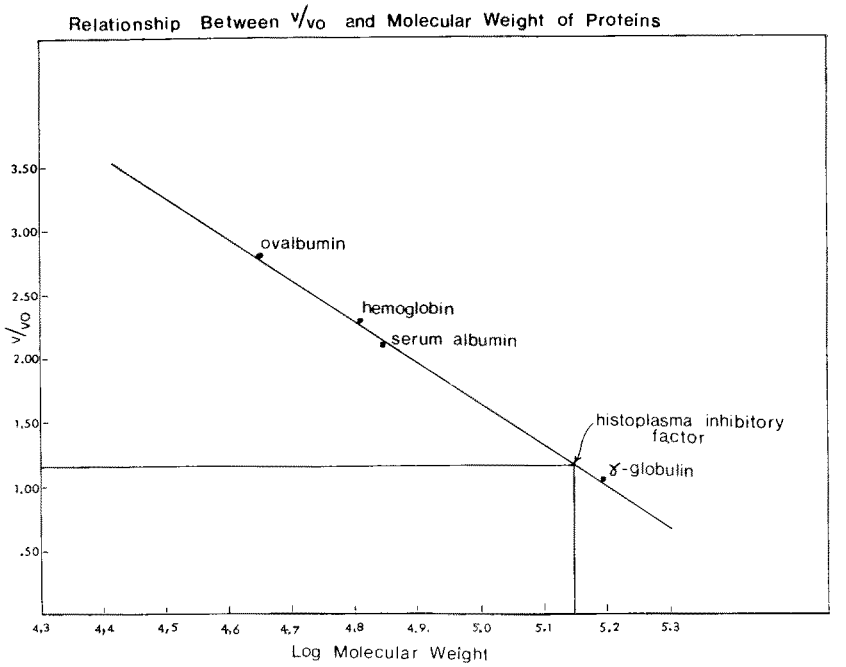

Fig. 4. Relationship between V/Vo and molecular weight of proteins.

TABLE I.
ELUTION OF PROTEINS FROM SEPHADEX G-150
(COLUMN 1.5 x 30 cm)

Material	M.W.	Log M.W.	V	V/Vo
Ovalbumin (3 x cryst)	45,000	4.6532	65	2.78
Hemoglobin (Bovine)	64,500	4.8095	55	2.29
Serum Albumin (Bovine Fraction V)	70,000	4.8451	60	2.09
γ-globulin (Human Fraction II)	160,000	5.1931	25	1.04
Histoplasma Inhibitory Factor (Kidney)	142,000**	5.1500**	28	1.17

*Vo = Void Volume = 24 ml

**Values obtained from standard protein curve (Fig. 4)

Relationship of Elution Volume to Molecular Weight

Fig. 4 depicts the relationship of V/Vo and log molecular weight of the HIF factor (from kidney) as well as a number of standard proteins. Their respective elution volumes molecular and log molecular weights are tabulated in Table I.

WHITAKER (4) has shown that a linear relationship exists between V/Vo and the logarithm of the protein molecular weight, where V is the protein retention volume (initial addition of protein sample to the peak of the eluted protein) and Vo is the column void volume. The column void volume was determined utilizing Blue Dextran (MW 2,000,000) and is the volume of buffer required to elute a substance not retained by inclusion within the column packing (the fractionation range for Sephadex G—150 is 5,000—200,000). The molecular weight of HIF (from kidney) utilizing the above procedure is estimated as 142,000.

Good correlation has been shown between gel-filtration (molecular-sieve chromatography) and the molecular weights of many enzymes and other proteins. (4, 8-12)

Elution of HIF from Histoplasma

Fig. 5 presents the results of elution tests at pH 6.0 to 10.5. Control *H. capsulatum* (1×10^4/ml) failed to grow below pH 6.5, but growth was good at pH 10.0 and 10.2. By comparison, at pH 5.5 to 9.0 test *H. capsulatum* (1×10^4/ml kidney homogenate) failed to grow after resuspension in buffer pH 6.5 to pH 9.0 following two hours at 4° C in contact with the HIF in kidney homogenate. Above pH 10.0 growth of *H. capsulatum* freed from HIF was equal to that of the control at the same pH. Buffer supernatants showed HIF activity above pH 10.0.

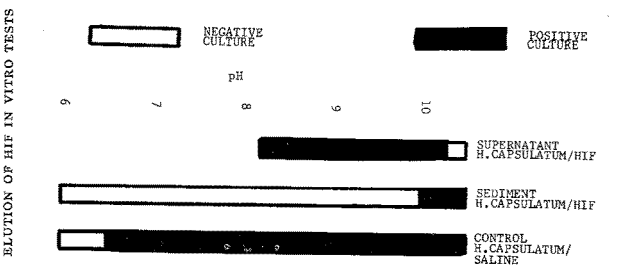

Fig. 5. Effect of pH on elution of histo-inhibitory factor, (HIF), from *Histoplasma capsulatum* yeast cells cultured on brain heart infusion blood agar at 37° C.

Table II shows the close correlation between activity of HIF in 280 mμ fraction and in the liver or kidney homogenate from which the fraction was obtained. Positive homogenates from infected liver and kidney tissue yielded positive fractions which inhibited *H.*

capsulatum completely, *B. dermatitides* slightly and the other organisms tested not al all. Negative homogenates from normal tissues eluted at pH 10.2 (not shown) lacked the peak at 280 mμ and failed to inhibit *H. capsulatum* or other organisms tested.

TABLE II.

Results of BHIB cultures of sediment (sed) or supernatant (super) from test organisms suspended in Histoplasma-infected tissue homogenate (liver or kidney) or 280 mμ fraction thereof. Complete inhibition - 0; no inhibition - +. Sed (10^5/ml) resuspended in saline; super + Histoplasma (10^4/ml).

	CULTURE INHIBITION													
Test Organism:	Histoplasma capsulatum		Blastomyces dermatitides		Candida albicans		Cryptococcus neoformans		Escherichia coli		Staphylococcus aureus		Bacillus subtilis	
TEST MATERIAL	Sed	Super	Sed	Super	Sed	Super	Sed	Super	Sed	Super	Sed	Super	Sed	Super
HISTOPLASMA Tissue Homogenate	0 (1-100)	+	0 (1-10)	+	+	+	+	+	+	+	+	+	+	+
280 mμ Fraction	0	+	0	+	+	+	-	-	+	-	+	-	-	-

Discussion

Chemical characterization of HIF makes possible a more precise study of the production of the inhibitors during the course of an infection, provides the basis for a meaningful study of the mode of action of HIF and establishes a basis for determining the identity or non-identity of HIF and other known inhibitors found in normal-pathological tissues. (1—3, 13—16)

Activity of HIF fractions at 280 mμ would suggest a compound that is at least in part protein. A molecular weight of 142,000 is within range of known antibody molecules (17) and specificity of the HIF inhibitory activity would also suggest this possibility.

Elution of HIF from *Histoplasma* at pH 10.2 has shown that the inhibition is not irreversible the organisms are not killed by the inhibitor, at least not within the two hour test period, at 4° C. This finding increases the probability of isolating *Histoplasma* from infected tissue which has been processed in buffer at approximately pH 10.2, centrifuged and resuspended in saline before culturing.

Summary

The histo-inhibitory factor (HIF) derived from homogenates of liver or kidney from hamsters infected with *Histoplasma capsulatum* has been fractionated by column chromatography. It shows maximum absorption at 280 mμ, has a molecular weight of 142,000 and can be eluted from DEAE-cellulose or DEAE-Sephadex A—50 with 0.02 M phosphate – 4 M sodium chloride (1 : 1)

HIF can be eluted from *Histoplasma* yeast cells at pH 10.0, thus a greater number of positive cultures from chronic histoplasmosis could be expected to result from pretreatment of clinical specimens in glycine buffer pH 10.0 prior to culture.

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