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# Ferritin, a Hodgkin's Disease Associated Antigen

**(spleen/F- and S-antigens/lymph nodes/fetal liver)**

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ABSTRACT **Antigens which exist in high frequency in tumor tissues of patients with Hodgkin's disease have been obtained in relatively concentrated form by gel chromatography procedures. Further purification and analysis of these antigens performed in the present study have demonstrated that the antigen of fast electrophoretic mobility (F-antigen) is normal tissue ferritin. The identification of F-antigen as ferritin has been made on the basis of comparative physicochemical and immunological analyses of purified F-antigen and normal ferritin. Thus, F-antigen was found to contain iron and to be similar to ferritin in molecular weight, amino-acid composition, electrophoretic mobility, isoelectric distribution, and immunological reactivity. Absorption of a monospecific heterologous anti-F antiserum with normal tissue ferritin completely removed all anti-F antibody activity. Moreover, the absorption of polyspecific heterologous antiserum to crude Hodgkin's extracts, which contains antibodies reacting with F-antigen, the slower migrating S-antigen, and a third specificity present in lysates of normal peripheral blood lymphocytes (PL-antigen), with ferritin, removed only anti-F activity, thus distinguishing the S- and PL-antigens from ferritin. The existence of ferritin in high quantities in serum of Hodgkin's disease patients may provide a tool of potential diagnostic and prognostic importance in the management of this disease.**

Two tumor-associated antigens have been identified in high concentration in Hodgkin's disease (1–3). Using heterologous antisera against tumor tissue extracts which were absorbed with normal tissue, it was demonstrated by immunoelectrophoresis that the antigen of the faster electrophoretic mobility (F-antigen) was found in the spleen of patients with various diseases as well as in thymic and fetal tissues. The slow migrating antigen (S-antigen) was found in infiltrates of Hodgkin's disease as well as in platelets, neonatal thymus, malignant thymoma, and reticulum cell sarcoma. Recently, it was demonstrated that F-antigen is found in least dense lymphocytes derived from spleens of patients with Hodgkin's disease (3), in cultured Burkitt's lymphoma (3), and in purified populations of Hodgkin's disease cells (4). The antigens associated with Hodgkin's disease were partially purified (2) and monospecific antiserum was elicited toward F-antigen (5).

Recently, ferritin has been suggested to be a circulating tumor-associated antigen in Hodgkin's disease (6, 7). Although ferritin occurs intracellularly in a wide variety of tissues (8–10) and its level in the serum of healthy humans is low, it was found in elevated amounts in the serum of patients with Hodgkin's disease as well as in other malignancies and diseases with liver involvement (6, 7, 11, 12). Beta-fetoprotein was found to be ferritin (13) and a characteristic isoferritin profile was obtained for different organs (10) and malignant tissues (14).

In this study we report further purification and characterization of F- and S-antigens and submit evidence that the F-antigen is ferritin.

Abbreviations: F- and S-antigens, electrophoretically fast and slow antigens associated with Hodgkin's disease tumors; PL, peripheral lymphocytes; PBS, phosphate-buffered saline.

## MATERIALS AND METHODS

Tissues and peripheral blood lymphocytes from normal adults and patients with Hodgkin's disease were obtained and processed as described previously (2).

*Gel Chromatography.* Separation of the components in the crude extracts was performed by gel chromatography on Sepharose-6B of the thawed and centrifuged (1500 × *g* for 30 min) supernatants. Five milliliters of sample containing about 50 mg of protein was applied to 100 × 2.5-cm column of Sepharose-6B (Pharmacia) equilibrated and eluted with 0.14 M NaCl–0.01 M phosphate buffer, pH 7.5 (PBS). Chromatography was performed at 4° under controlled pressure at a constant flow rate of 15 ml/hr. Eluate fractions were collected in 5-ml volumes and monitored for absorbance at 280 nm. Appropriate fractions were pooled and then concentrated to 5.0 ml by ultrafiltration through UM-10 Diaflo ultrafiltration membranes (Amicon Corp.).

*Polyacrylamide Disc Gel Electrophoresis.* Samples containing 20–200 μg of protein were electrophoresed in 7% gels as described previously (2). Polyacrylamide gel electrophoresis in the presence of 0.1% or 1% sodium dodecyl sulfate was performed by the method of Weber and Osborn (15). Aliquots were dialyzed against sample buffer (0.01 M sodium phosphate buffer, pH 7.2) and reduced by heating for 10 min at 70° in the presence of 0.14 M 2-mercaptoethanol, when stated. Electrophoresis in agarose films was performed in the cassette system (Analytic Chemists, Inc.) in ethylenediaminetetraacetate (EDTA)–barbital buffer, pH 8.6, which was used also for immunoelectrophoresis as described before (3). Gels were stained for protein by either Coomassie blue or amido black. Staining for iron was performed with 0.2% potassium ferrocyanide (16).

*Electrofocusing.* Analytical gel electrofocusing was performed at 4° in 4% polyacrylamide gels with 2% ampholytes, pH 5–7, according to the method of Righetti and Drysdale

(17) and Alpert *et al.* (13). The electrofocusing was performed by Dr. E. Alpert from the Gastrointestinal Unit, Massachusetts General Hospital. Protein determinations were made by the Lowry method (18).

*Amino-Acid Analysis.* Four times crystallized ferritins (approximately 2 mg) were hydrolysed in 6 N HCl in the presence of phenol under vacuum at 110° for 18 hr. The hydrolysates were dried and analyzed on a Beckman model 120-C amino-acid analyzer.

*Isolation of Ferritin.* Ferritin was isolated from defrosted human tissues by the method of Drysdale and Munro (16) omitting the carboxymethylcellulose chromatography. Briefly, the tissue was homogenized in water (1 g/4 ml) and the supernatant yielded after centrifugation was heated slowly to 80°. The supernatant obtained after cooling and centrifugation was acidified to pH 4.8 and the ferritin was precipitated from the supernatant by addition of ammonium sulfate to 50% saturation, dialyzed, and chromatographed on Sepharose-6B column equilibrated and eluted with 0.02 M sodium phosphate buffer, pH 7.5. Most of the protein was eluted in one fraction which was concentrated by ultrafiltration. Crystallization of ferritin was performed with 5% $CdSO_4$ according to the method of Granick (19). The ferritin was dialyzed against distilled water before crystallization. The yellow ferritin crystals were dissolved in 2% $(NH_4)_2SO_4$ and recrystallized for three to four times. The yield was about 2 mg of ferritin per 1 g of Hodgkin's splenic tissue and 0.2 mg of ferritin per 1 g of normal spleen.

*Preparation and Absorption of Antisera.* Rabbit antisera against crude Hodgkin's extract and F-antigen were prepared as described previously (2) and were absorbed with soluble proteins as ferritin or $\alpha$-globulins or with peripheral lymphocytes by incubation of the antiserum aliquots with various amounts of the absorbents for 1 hr at 25° and overnight at 4° followed by ultracentrifugation at 105,000 $\times$ *g* for 1.5 hr. Immunoabsorptions were performed also by filtrating the antisera through small columns (1–3 ml) made of either normal human serum or ferritin that was covalently coupled to Sepharose-4B by the cyanogen bromide method (20). After absorption the antisera were brought to their original volumes by pressure-dialysis. Anti-ferritin antisera were prepared in the same way.

## RESULTS

*Molecular Identity of F-antigen and Ferritin.* The crude homogenate obtained from Hodgkin's spleen was separated into four major fractions after gel chromatography on Sepharose-6B (Fig. 1). The second fraction (Peak II) included all the antigenic activity of F-antigen (2). This fraction was eluted at position consistent to molecular weight of 4 to 5 $\times$ $10^5$, appeared yellow, and sedimented after ultracentrifugation at 100,000 $\times$ *g* for 2 hr. Ferritin that was prepared in well established methods from normal or Hodgkin's spleen displayed the same chromatographic pattern as Peak II. The molecular identity between Peak II and ferritin was further confirmed by electrophoresis on polyacrylamide gel (not shown) or agarose gels (Fig. 2) that yielded a single major protein band which was stained also for iron. (The slower electrophoretic mobility of liver ferritin is due to the fact that it was dissolved in PBS while the other ferritins and Peak II were dissolved in 0.02 M phosphate buffer, pH 7.5.)

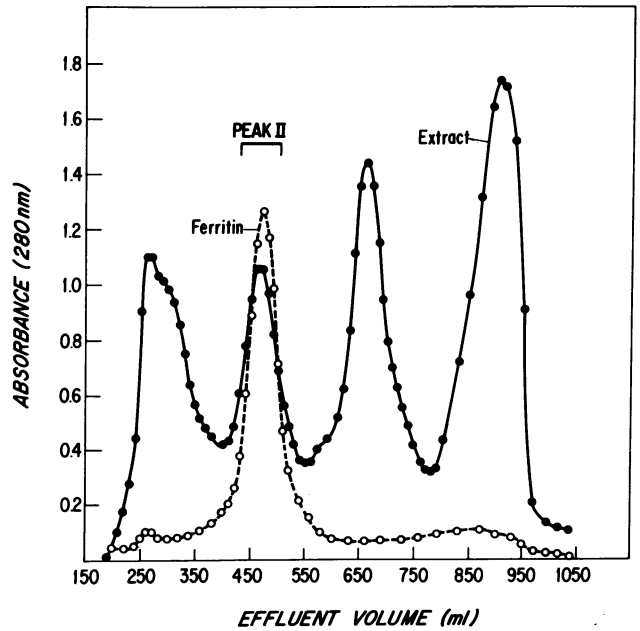


FIG. 1. Gel chromatography on Sepharose-6B of extract (●) and of ferritin (O) prepared from Hodgkin's disease splenic tissue. The fraction eluted between 425 and 500 ml is depicted as peak II.

Since the existence of isoferritins characteristic to different organs has been suggested (14, 21), the isoelectric distribution of normal spleen and liver ferritins was compared to that of ferritin prepared from Hodgkin's splenic tissue (Fig. 3). No significant difference was observed and all of the three ferritins displayed the same major component.

The comparison of amino-acid analysis of crystallized normal human liver and spleen and Hodgkin's splenic ferritin (Table 1) supports the molecular similarity between the different ferritins. The amino-acid composition is consistent, in general, with other findings about human ferritins (10, 21),

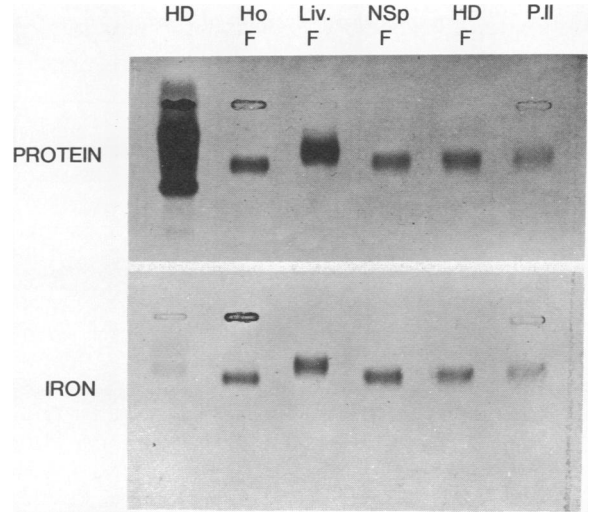


FIG. 2. Electrophoresis in agar slab of Hodgkin's spleen crude extract (HD), ferritins (F) purified from horse spleen (Ho.), normal human liver (Liv.) and spleen (NSp.), and Hodgkin's splenic ferritin or peak II (P.II). The slabs were stained for protein and for iron. The liver ferritin was dissolved in PBS, while the other preparations were in 0.02 M phosphate buffer, pH 7.5, accounting for the somewhat slower mobility.

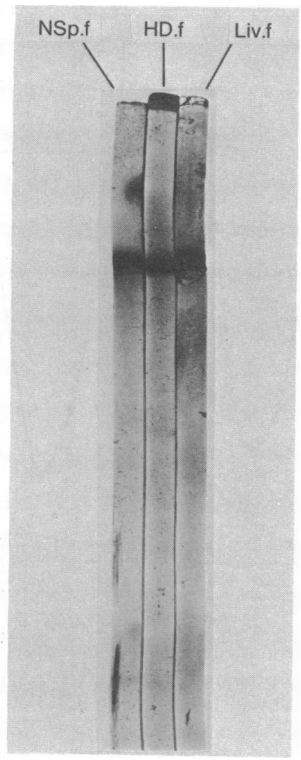


FIG. 3. Analytical isoelectric focusing in 4% polyacrylamide gel using 2% ampholytes, pH range 5–7, of purified human ferritins (f) from (left to right) normal spleen (NSp.), Hodgkin's spleen (HD.), and normal liver (Liv.). The gels were stained for protein by Coomassie blue.

which demonstrated minor but distinct differences between liver and spleen ferritins. There are only small differences in the amino-acid composition between the Hodgkin's spleen ferritin and normal spleen and liver ferritins.

TABLE 1. *Amino-acid composition of crystallized ferritins isolated from normal human spleen, Hodgkin's spleen, and normal human liver*

| Amino acid | Normal spleen | Hodgkin's spleen | Normal liver |
|---|---|---|---|
| Lys | 13.5 | 13.9 | 11.5 |
| His | 7.2 | 8.0 | 6.3 |
| Arg | 6.2 | 8.3 | 8.3 |
| Asx | 20.0 | 19.8 | 19.8 |
| Thr | 6.6 | 6.5 | 6.6 |
| Ser | 9.0 | 7.7 | 7.9 |
| Glx | 22.2 | 24.0 | 23.3 |
| Pro | 4.8 | 4.7 | 3.8 |
| Gly | 12.1 | 10.7 | 11.6 |
| Ala | 15.4 | 14.1 | 14.8 |
| Val | 4.8 | 5.3 | 6.0 |
| Met | 2.3 | 2.5 | 3.1 |
| Ile | 3.0 | 2.6 | 3.1 |
| Leu | 24.2 | 22.6 | 24.5 |
| Tyr | 6.3 | 6.4 | 6.8 |
| Phe | 6.2 | 6.7 | 6.6 |

Results are expressed as residues of each amino acid per ferritin subunit, based on the molecular weight of 18,500 (21). The amounts of cysteine and tryptophan were not determined.

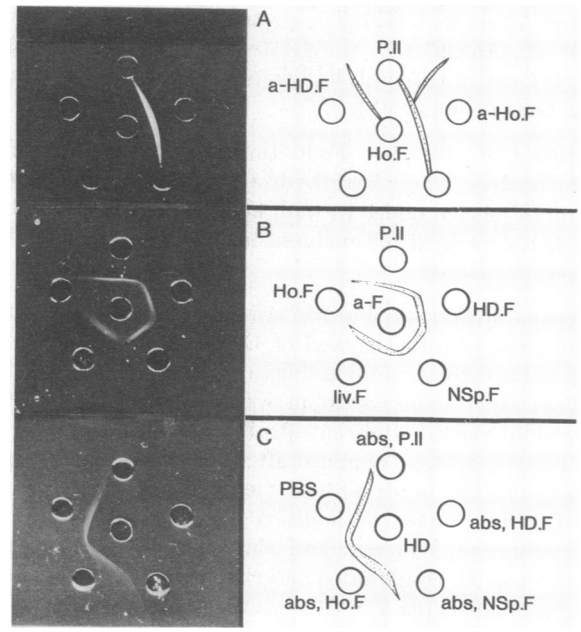


FIG. 4. Immunodiffusion of anti-ferritin and anti-Hodgkin's antisera against different ferritins. Plate A: cross-reactivity between human and horse ferritin; plate B: cross-reactivity between various human ferritins (peripheral wells) and anti-F-antigen (central well); plate C: reaction of Hodgkin's spleen crude extract (central well) with anti-F-antigen absorbed (abs,) with various ferritin preparations (peripheral wells). Antisera: a-F = anti-F-antigen; a-HD.F = anti-Hodgkin's disease spleen ferritin; a-Ho.F = anti-horse spleen ferritin. Antigens: P.II = Peak II; HD.F = Hodgkin's spleen ferritin; NSp.F = normal spleen ferritin; liv.F = normal liver ferritin; Ho.F = horse spleen ferritin; HD = Hodgkin's spleen crude extract.

*Immunological Cross-Reactivity of F-Antigen and Ferritin.* F-antigen shared antigenic determinants with horse ferritin (Fig. 4, plate A) and cross-reacted completely with human ferritins (Fig. 4, plate B). Absorption of antiserum specific for F-antigen with various ferritins obtained from normal or Hodgkin's tissue removed all the anti-F activity when tested against crude Hodgkin's homogenate (Fig. 4, plate C). Identical immunoprecipitating lines were obtained after immunoelectrophoresis of the various ferritins (not shown). Only F-antigen yielded an immunoprecipitating band which was stained also for iron, confirming its identity to ferritin.

Additional proof of the identity between F-antigen and ferritin was obtained when the immunoprecipitate that had been formed by either whole extract or the antigenically active Peak II, after reaction with monospecific anti-F serum, was isolated, washed, dissolved in sodium dodecyl sulfate, and applied to electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate. Fig. 5 demonstrates the electrophoretic pattern obtained after such treatment. No band could be shown with normal rabbit sera, while it is obvious that the high-molecular-weight ferritin is the most predominant component. The lower molecular weight band is immunoglobulin, which dissociates to heavy and light chains after reduction. Ferritin dissociates to subunits of about 18,500 daltons under the same treatment (21).

*Immunological Characterization of S- and PL-Antigens.* Antisera against crude Hodgkin's extract contained, in addition to anti-F antibodies, also antibodies which reacted with slow

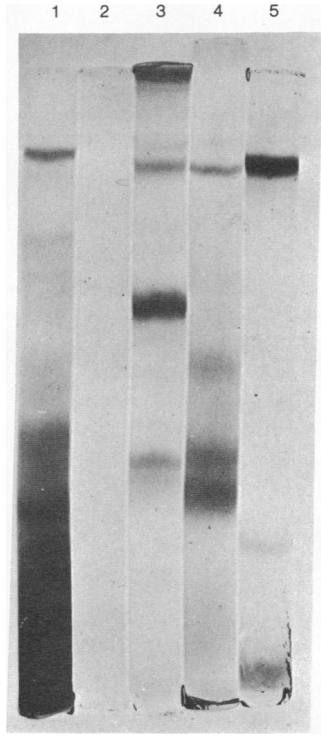


FIG. 5. Electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate. Immunoprecipitates were obtained by co-incubation of 0.1 ml of anti-F-antigen with 0.1 ml of crude extract from Hodgkin's spleen for 18 hr at 4°. The precipitates were washed, dissolved in 0.1% sodium dodecyl sulfate with or without 0.14 M 2-mercaptoethanol, and applied to electrophoresis on 5% polyacrylamide gels containing sodium dodecyl sulfate. Gels were stained with Coomassie blue. From left: (*1*) Hodgkin's spleen crude extract; (*2*) mixture of the extract with normal rabbit serum; (*3*) immunoprecipitate of Hodgkin's spleen extract with anti-F-antigen, unreduced; (*4*) same as (*3*) but reduced; (*5*) unreduced Peak II. Note that the second major band in the third gel, which corresponds to immunoglobulin, was dissociated into heavy and light chains after reduction (gel 4).

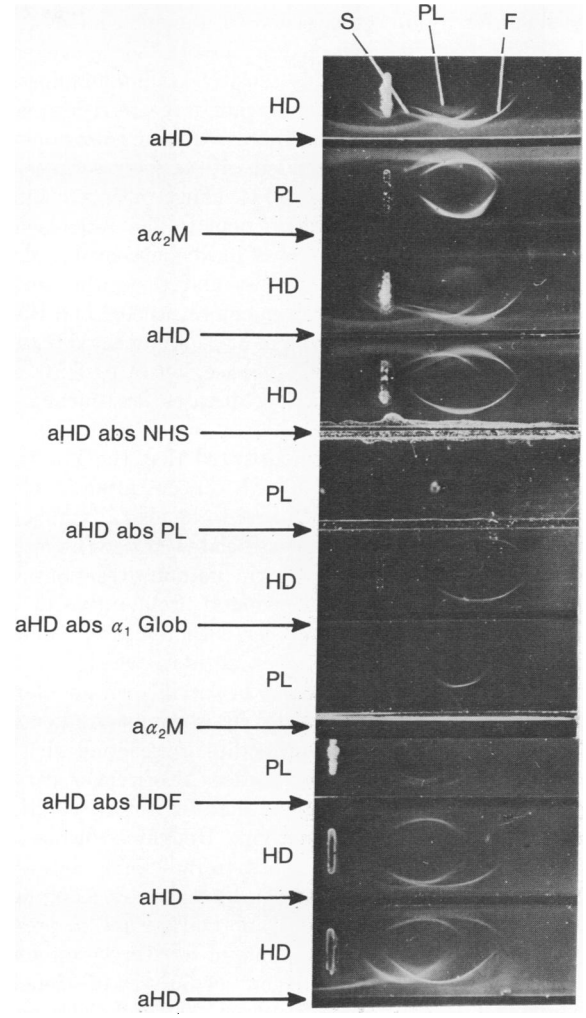


FIG. 6. Immunoelectrophoresis of crude extracts of Hodgkin's spleen and normal peripheral lymphocytes against anti-Hodgkin's extract antiserum that was absorbed by various preparations. The antigens were: HD, crude Hodgkin's spleen extract; PL, crude extract from normal peripheral lymphocytes. The antisera were: aHD, anti-Hodgkin's spleen extract; a$\alpha_2$M, anti-$\alpha_2$ macroglobulin (Microbiological Associates); aHD abs NHS, anti-HD sera that were absorbed through immunoabsorbent made of normal human serum; aHD abs PL, anti-HD sera that were absorbed with normal peripheral lymphocytes; aHD abs $\alpha_1$ Glob, anti-HD sera that were absorbed on $\alpha_1$-globulin (Microbiological Associates); aHD abs HDF, anti-HD sera that were absorbed through immunoabsorbent made of Hodgkin's ferritin.

migrating antigen (S) in Hodgkin's tissue extracts, and antibodies which reacted with an antigen of intermediate mobility (PL-antigen) present in extracts prepared from normal peripheral lymphocytes. As shown in Fig. 6 we could remove either anti-PL or S activity by absorption of the antiserum that was raised against Hodgkin's spleen crude extract with normal human serum. Intact peripheral lymphocytes removed S-activity, while absorption with lysed peripheral lymphocytes removed anti-S and anti-PL antibodies. Absorption with ferritin removed only the F-activity. When serum components were used for absorption, it was found that the $\alpha$-globulin of normal human serum removed S-activity, $\alpha_1$-globulin removed S and PL activity and anti $\alpha_2$-macroglobulin reacted preferentially with PL-antigen which was enriched in extracts made from peripheral lymphocytes. We conclude, therefore, that PL and S antigens react or cross-react with normal serum components which have been absorbed to or constitute one of the lymphocyte membrane components.

## DISCUSSION

Ferritin is one of the main iron storage compounds in the body and is found mainly in the cytoplasm of reticuloendothelial cells, liver cells, and reticulocytes in the bone marrow, as well as in other tissues as the kidney, heart, pancreas, intestine, and placenta (8, 9). Although ferritin is found in normal tissues and serum, hyperferritinemia has been detected in diseases which are characterized by shock, hypertension, edema, and hepatocellular involvement as in hepatitis and cirrhosis. Ferritinemia is also found in some malignant neoplasias such as Hodgkin's and non-Hodgkin's lymphomata and acute myeloblastic leukemia (6, 7, 11). Two mechanisms were suggested for production of the high levels of ferritin detected in tissues and sera of Hodgkin's patients. Aungst (11), who detected ferritin by quantitative immunoprecipitation, stressed its relationship to extensive disease and related it to tissue necrosis which causes release of

intracellular ferritin into the serum. On the other hand, Jones *et al.* (7), in investigations on iron metabolism in leukemia and Hodgkin's disease, used a sensitive radioimmunoassay for the detection of ferritin and related the ferritinemia to reticuloendothelial blockade. Increased concentrations of ferritin in the serum are associated with a decrease in serum iron and transferrin saturation. This change reflects a shift of iron from the plasma transferrin pool to the reticuloendothelial ferritin pool. This shift was most marked in patients with symptoms of systemic disease and therefore may be a useful index of systemic involvement (7). Bieber and Bieber (6) detected ferritin in the serum of 32% of untreated patients with Stages I and II Hodgkin's disease, yet in patients with other inflammatory or necrotizing diseases ferritinemia was not detected.

In this study, we have demonstrated that the F-antigen, which was previously found in high concentration in Hodgkin's disease infiltrates and reported to be also a lymphocyte antigen, is ferritin. This tumor-associated antigen is present also in Burkitt's lymphoma (3), in immune reactions with lymphoid hyperplasia, and in neonatal proliferative thymus gland (1). These findings, together with the recent findings made by other groups using our antisera which (*1*) have localized F-antigen intracellularly in specific cell populations isolated from Hodgkin's infiltrates by isopycnic gradients (4) and (*2*) have shown that the antibodies are reacting with cells obtained from late stages of the disease (M. Eisenger, personal communication), strongly suggest that ferritin distribution is related to the processes underlying Hodgkin's disease. Recently, we have developed a sensitive radioimmunoassay by which we could quantitate ferritin levels as low as 50 ng/ml of serum (Z. Eshhar, S. E. Order, and D. H. Katz, in preparation). Undetectable or low amounts of ferritin were found in healthy human sera, while 54 out of 59 sera of Hodgkin's patients had ferritin levels between 200 and 2500 ng/ml. Thus far, we have not demonstrated distinct differences of ferritin levels in the sera of patients in various stages of the disease. Since it is now possible to detect and quantitate ferritin in extracts prepared from lymphocytes obtained from Hodgkin's disease patients, we are encouraged that, being a circulating and cellular tumor-associated antigen, ferritin may serve as a diagnostic index of activity in Hodgkin's disease.

The existence of organ-specific forms of ferritin and isoferritin molecules in different tissues has been reported by various groups (10, 14, 21, 22). This heterogeneity of ferritin was demonstrated by differences in primary structure (21, 22). The crystallized ferritin obtained by us from Hodgkin's splenic tissue did not show any properties different from those of normal spleen or liver ferritins, as can be judged by its isoelectric point, amino acid composition, and immunological properties. We conclude, therefore, that the Hodgkin's disease associated F-antigen is a normal tissue ferritin.

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