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Purification of Hodgkin's Disease Tumor-Associated Antigens

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

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ABSTRACT **Two antigens that exist in high frequency in tumor tissues of patients with Hodgkin's disease have been obtained in relatively concentrated form. Extracts of Hodgkin's spleen tumor tissue, when subjected to chromatography on Sephadex G-200, separate into three major protein peaks of which only the first (peak I) possesses the predominant antigenic activities associated with the disease. Antigenic analysis performed with hyperimmune rabbit antisera obtained after repeated immunizations with peak I proteins demonstrated that this fraction contained both F and S antigens associated with Hodgkin's disease and small contaminant amounts of an antigen associated with normal lymphocytes. The tissue distribution patterns of the Hodgkin's disease tumor-associated antigens suggest that they both originate in lymphoid tissues and that the F antigen may represent a product of reactive lymphocytes while the S antigen may be a dedifferentiation antigen expressed in very immature lymphocytes.**

The development of Hodgkin's disease is associated with a high incidence of abnormalities of the immune system manifested by depression or loss of cell-mediated immunity (delayed hypersensitivity) and impaired resistance to fungal and viral infections (1–3). Recently, a common predominant antigenic specificity, termed F antigen, was identified in Hodgkin's tumor infiltrates by several immunologic techniques with heterologous (rabbit) antisera prepared against Hodgkin's tumor extracts (4–8). This F antigen was subsequently demonstrated in high concentration also in certain fetal tissues (liver and spleen) (7) and in spleens from patients with other clinical disorders associated to varying degrees with known or presumed immunologic abnormalities (6). Moreover, it was recognized from the outset that the F antigen originally observed in Hodgkin's tumor extracts is present in detectable, though considerably lower, quantities in normal spleen extracts (5–8).

In addition to the predominant F antigen, a second antigen occurs less frequently in association with some, but not all, Hodgkin's tumor infiltrates (5–8). The latter specificity, termed S antigen, is distinguishable from F antigen on the basis of slower electrophoretic mobility in immunoelectrophoresis. Moreover, as summarized in Table 1, the two Hodgkin's-associated antigens exhibit clearly different patterns of tissue distribution and frequency (6–8).

The present studies were undertaken in an attempt to purify and characterize the predominant F antigen from Hodgkin's tumor extracts in order to ascertain more precisely its origin, nature, and significance. We have isolated a fraction with high concentration of the F antigen, and by using more potent heterologous antisera prepared against this fraction, we have demonstrated that F antigen is closely associated with S antigen and with another antigen extractable from normal lymphocytes.

Abbreviation: PBS, phosphate-buffered saline.

MATERIALS AND METHODS

Origin of Normal and Tumor Tissue Specimens. Normal splenic tissue was obtained from adult patients undergoing splenectomy for various clinical reasons other than neoplastic disorders. Fetal liver was obtained from a stillbirth at three months gestation. Surgical specimens of spleen and lymph node were obtained from patients with active Hodgkin's disease from the operating suite after staging laparotomy and splenectomy. Tissue diagnoses were confirmed by histologic analysis in all cases.

First Stage Extraction Procedure. For spleens from patients with Hodgkin's disease, grossly visible tumor nodules were carefully dissected apart from uninvolved adjacent splenic tissue, and the resulting specimens were thereafter treated separately. These and normal or fetal tissues were cut into small pieces, immersed in Tyrode's solution (1 g wet weight to 5.0 ml), teased with forceps, and then homogenized at room temperature. The resulting tissue homogenates were centrifuged at 3000 rpm (1500 × *g*) for 30 min at 4°, and the sediments were discarded. Supernatants were stored at −20° until used in further purification procedures.

Purification of Hodgkin's Tumor-Associated Antigens. The presence or absence of Hodgkin's tumor-associated antigens in the various tissue extracts was determined by immunoelectrophoretic analysis with rabbit antiserum to crude Hodgkin's tissue extracts absorbed with normal human spleen as described (6). Protein concentrations of the various tissue supernatant fractions were determined by micro-Kjeldahl analysis.

Separation of the components of the tissue supernatants was initially performed by chromatography on Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) at either 4° or ambient temperature. 2 ml of sample containing 20 mg of protein was applied to a 40 × 2.5 cm column (Pharmacia type K 25/45) filled with Sephadex G-200 equilibrated for 24 hr with phosphate-buffered saline (PBS), pH 7.5. Chromatography was performed under controlled pressure at a constant flow rate of 14 ml per hr and constant spectrophotometric monitoring (280 nm). Eluate fractions were collected in 5-ml volumes; appropriate protein peak

fractions were pooled and then concentrated to around 2.0 ml by ultrafiltration through Diaflo ultrafiltration membranes UM-10 or PM-10 (Amicon Corp., Lexington, Mass.). Peak concentrates were then tested for presence of antigenic activity by immunoelectrophoretic analysis as above.

In addition to Sephadex G-200 chromatography, supernatants of one Hodgkin's spleen were subjected to preparative Pevikon block electrophoresis in barbital buffer, pH 8.6, $r/2 = 0.05$, for 19 hr at 4° and 500 V/cm.

Polyacrylamide Disc Gel Electrophoresis. Portions of the initial tissue supernatants (usually 200 μg) and of the various protein peaks were subjected to alkaline disc gel electrophoresis on 7% gels (pH 9.5). The gels were stained for protein. In some instances, gels were sliced into 1.5-mm segments and eluted with PBS overnight at 4° in order to test for antigenic activity.

Preparation of Rabbit Antisera. Rabbit antisera to crude tissue supernatants were prepared and absorbed with normal human spleen as described (4, 6). Antisera to the isolated Hodgkin's antigen fraction were prepared by immunization of rabbits with 200 μg of antigen emulsified in complete Freund's adjuvant intradermally in the thigh. Rabbits were bled and boosted intraperitoneally with 1 mg of antigen in saline in 4 weeks. Resulting antisera were tested for activity as described below, and if not active, a second booster immunization was administered 1 week later. Antibody activity was again evaluated as described in *Results*.

Radioactive Labeling. Isolated fractions from Sephadex G-200 chromatography of Hodgkin's spleen extract PEN (peak I) were radioactively labeled with ^{125}I by either a chloramine T method (9) or by the lactoperoxidase method (10).

Separation of Peripheral Blood Lymphocytes. Peripheral blood (8.0 ml) from patients and normal donors was collected in a 0.01 M EDTA solution. An appropriate volume of Technicon lymphocyte separator solution was added to the blood, the mixture was agitated for 15 min at ambient

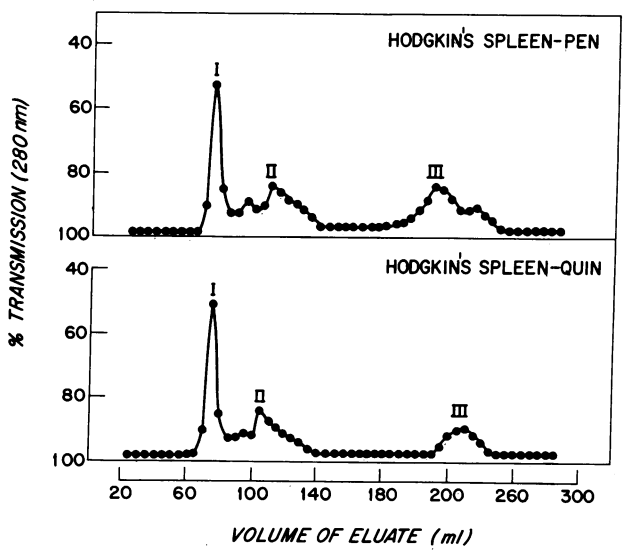

FIG. 1. Elution patterns of extracts from two Hodgkin's disease spleen tumors (PEN and QUIN) subjected to chromatography on Sephadex G-200.

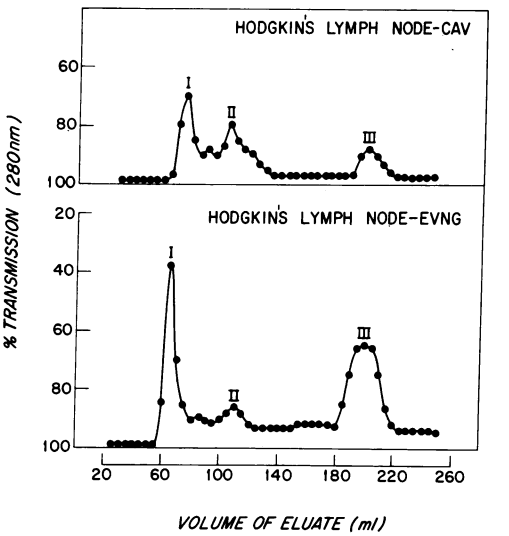

FIG. 2. Sephadex G-200 chromatographic elution patterns of extracts from two Hodgkin's disease lymph node tumors (CAV and EVNG).

temperature, and then stored overnight at 4°. After the blood was equilibrated to room temperature (1 hr), the peripheral lymphocytes were isolated by the lymphocyte separator (Technicon), and either used immediately for absorption of antisera or prepared for soluble antigen extraction. The latter was performed by freezing the lymphocytes (7×10^6 cells/ml) at −20° overnight followed by lysis at room temperature. The lysates were tested by immunoelectrophoresis.

RESULTS

Chromatographic Patterns of Hodgkin's Tumor Extracts. The chromatographic heterogeneity of Hodgkin's splenic tumor extracts on Sephadex G-200 is illustrated in Fig. 1. In all of the splenic tumors examined, a similar elution pattern was obtained. Antigenic activity was found in the eluate fractions collected after about 60 ml of eluate had passed through the column bed. This protein peak (peak I) consisted of usually 20–25 ml of eluate and manifested relative homogeneity by antigenic analysis and by disc gel electrophoresis (see below). No antigenic activity was detected in either peak II or peak III. Repeated chromatography of peak I on Sephadex G-200 yielded a homogeneous elution pattern (not shown). Similarly, preparative Pevikon block electrophoresis yielded a homogeneous peak of antigenically active protein at the anodal end of the block.

Similar elution patterns were obtained with Hodgkin's lymph node extracts subjected to Sephadex G-200 chromatography (Fig. 2). Antigenic activity was again detected in the first protein peak by Ouchterlony gel diffusion and immunoelectrophoretic analysis.

Chromatographic Patterns of Fetal Liver and Normal Spleen. Fetal liver has been demonstrated previously to possess considerable F antigen activity with the antisera prepared against Hodgkin's tumor extracts, whereas normal spleen extracts react poorly with such antisera under conditions of the assay (7). When these tissue extracts were subjected to chromatography on Sephadex G-200, the elution patterns shown in Fig. 3 were obtained. The elution pattern of fetal liver was virtually identical to that obtained with Hodgkin's

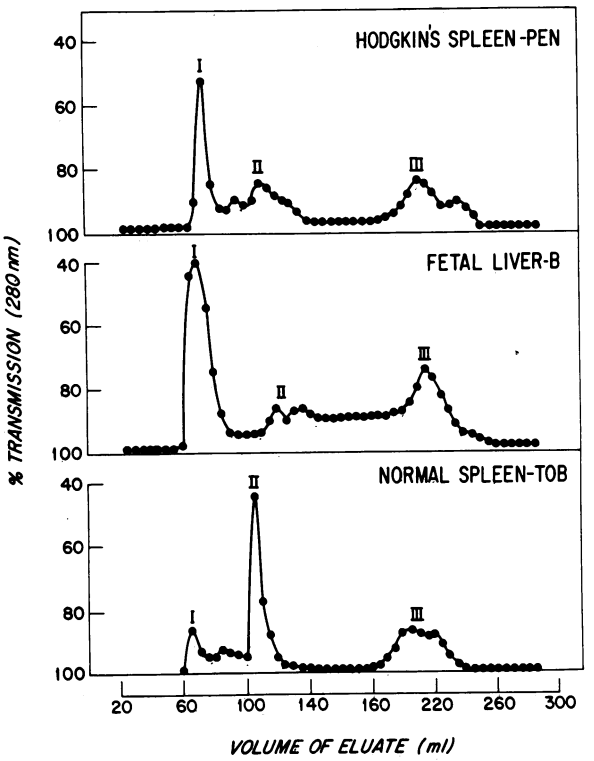

FIG. 3. Chromatographic elution patterns (Sephadex G-200) of extracts of two tissue sources of large quantities of F antigen, i.e., Hodgkin's spleen PEN and fetal liver, and of normal spleen that contains relatively minute amounts of F antigen.

spleen (PEN) shown here for comparison. Antigenic activity was associated with peak I of fetal liver, as was the case with Hodgkin's spleen. In contrast, normal spleen eluted in a quantitatively different pattern. Thus, peak I, the protein peak with which antigenic activity was identifiable in Hodgkin's spleen and lymph node and in fetal liver, was markedly lower in quantity in normal spleen (although eluting at approximately the same place) while peak II was the major protein peak.

Comparative Disc Gel Electrophoresis. A comparison was made of the electrophoretic patterns on polyacrylamide disc gels of the various tissue extract supernatants before chromatography and of the isolated protein peaks eluted from Sephadex G-200.

Fig. 4 illustrates the patterns obtained by disc gel electrophoresis of tissue extracts originating from three sources containing detectable F antigens: Hodgkin's spleen PEN, Hodgkin's lymph node EVNG, and fetal liver. As expected, the unchromatographed extracts exhibited considerable heterogeneity in their migration patterns. In contrast, the first protein peak (peak I) from Sephadex G-200 was relatively homogeneous. This was particularly true in the case of PEN peak I (Hodgkin's spleen), which manifested a single dense band at the top end of the gel. Peak I of fetal liver separated into 3–4 identifiable bands, while 3 bands could also be distinguished in the pattern of ENVG lymph node peak I. Nonetheless, the common feature in all cases is the presence of a very dense band in the top portion of the gel that corresponds to the uppermost band in the unchromatographed extracts. Normal spleen was clearly distinguishable from Hodgkin's spleen before chromatographic separation. As shown in Fig. 5, the obvious difference is the relative absence in normal spleen of the dense band at the top of the gel so readily identified in Hodgkin's tumor. However, when the comparatively small protein peak I of normal spleen eluted from Sephadex G-200 was concentrated sufficiently, a band corresponding in electrophoretic migration to the dense band of PEN peak I could be identified.

To increase the sensitivity of detection of possible contaminants in PEN peak I not recognized by protein staining of the polyacrylamide gels, a small sample that had been radioactively labeled with ^{125}I by the lactoperoxidase method

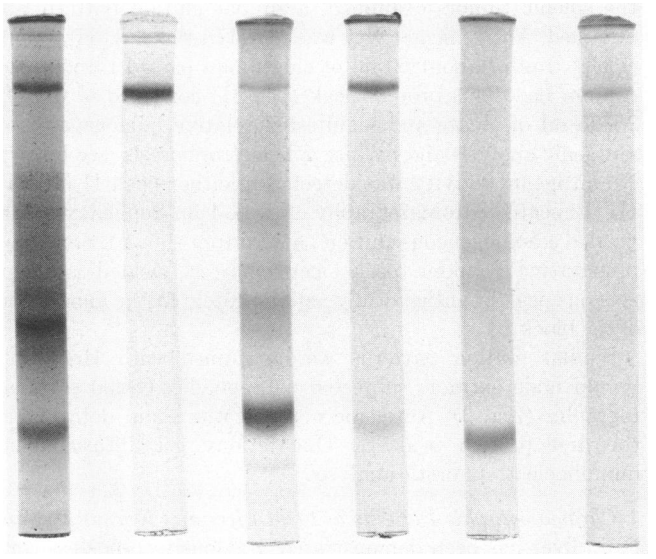

FIG. 4. Comparative electrophoretic patterns on polyacrylamide disc gels of extracts from various sources containing detectable F antigen. The unchromatographed extract and protein peak I from Sephadex G-200 of each tissue are shown as follows: From left: Hodgkin's spleen PEN and PEN peak I; fetal liver extract and fetal liver peak I; Hodgkin's lymph node EVNG and EVNG peak I.

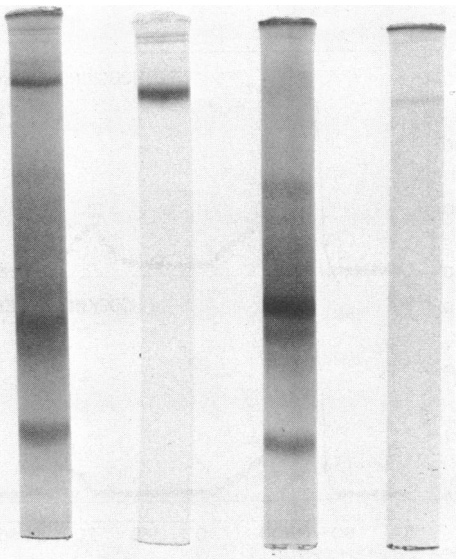

FIG. 5. Comparative disc gel electrophoresis of extracts from Hodgkin's spleen tumor tissue and normal spleen. From left: Hodgkin's spleen PEN and PEN peak I; normal spleen extract and normal spleen peak I.

was subjected to disc gel electrophoresis. The gels were then sliced into 1.5-mm segments, which were then counted for radioactivity in a gamma spectrometer. The pattern obtained is illustrated in Fig. 6. The peak of radioactivity near the top of the gel (slices 4–8) corresponded to the dense protein band observed in stained gels. Moreover, all antigenic activity was associated with this peak of radioactive material after elution of the gel slices in PBS. The smaller peak of radioactivity at the lower end of the gel (slices 25–30) was not apparent by protein staining and did not react with the anti-Hodgkin's antisera either by Ouchterlony gel diffusion or in a sensitive antigen-binding Farr assay.

Antigenic Analysis. Rabbit antisera were prepared against the first protein peak (peak I) of Sephadex G-200 chromatography of Hodgkin's spleen PEN. These antisera were used, with or without absorption, for antigenic analysis of the various tissue extracts and isolated fractions by immunoelectrophoresis and Ouchterlony gel diffusion.

Fig. 7 illustrates the results of Ouchterlony analysis. The center well in all cases contained unabsorbed rabbit antiserum from an early bleeding after immunization with PEN peak I. The unchromatographed tissue extracts from Hodgkin's spleen and lymph node and from fetal liver reacted with single lines of identity, whereas normal spleen reacted with a faint precipitin line (Fig. 7*A*). Concentrated peak I fractions from Sephadex G-200 of fetal liver and Hodgkin's spleen and lymph node also reacted with lines of identity; no reaction was obtained with peak III of lymph node or fetal liver (Fig. 7*B*). Fig. 7*C* illustrates a faint, but identifiable, line of identity between the concentrated peak I of normal spleen and the unfractionated and peak I fraction of Hodgkin's spleen extract, while no precipitation occurred with peak III from either normal or Hodgkin's spleen.

Immunoelectrophoretic analysis demonstrated the predominant antigen in PEN peak I to be F antigen. However, peak I clearly contained S antigen as well, albeit in considerably lower quantities. This became apparent by use of antisera from rabbits that were repeatedly boosted with PEN peak I. Such hyperimmune sera were found, by immunoelectrophoretic analysis, to detect the slower migrating S antigen, in addition to F antigen, in PEN peak I and in other tissues previously found to possess S activity. Furthermore, a weak third specificity present in PEN peak I was also detectable by the use of hyperimmune antisera only. The latter antigen is a lymphocyte-associated antigen, which can be

TABLE 1. *Tissue distribution patterns and frequency of antigenic specificities associated with Hodgkin's disease tumors*

Tissue	F Antigen	S Antigen
Hodgkin's spleen tumors	+++	+
Hodgkin's lymph nodes	+	+++
Normal spleen	+	0
Fetal spleen	+++	0
Fetal liver	+++	0
Neonatal thymus	+++	+++
Malignant thymoma	0	+++
Non-Hodgkin's lymph nodes	0	0

+, Present in small-to-moderate quantities.
+++, Present in relatively large quantities.
0, Not detected.

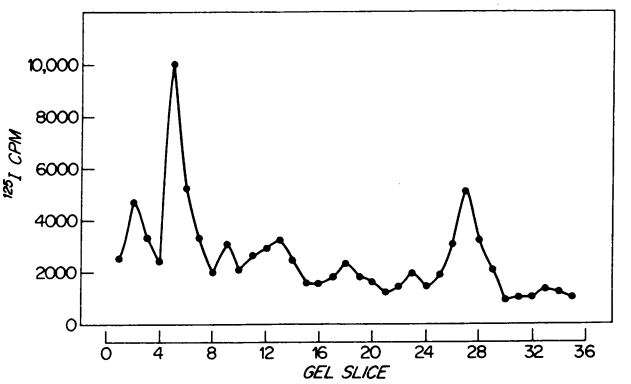

FIG. 6. Polyacrylamide disc gel electrophoresis of ^{125}I-labeled PEN peak I. Gels were sliced into 1.5-mm segments, which were individually counted for radioactivity.

easily identified in soluble extracts of lymphocyte preparations as described below.

Cell lysates were prepared from viable normal peripheral lymphocytes, peripheral lymphocytes from patients with Hodgkin's disease, and lymphocytes from Hodgkin's spleen tumors as described in *Methods*. Equal quantities of lysates from an equivalent number of cells were tested by Ouchterlony gel diffusion and by immunoelectrophoresis with a late rabbit antiserum to PEN peak I. Positive reactions were obtained with all such lymphocyte lysates when tested against unabsorbed antiserum. Moreover, in Ouchterlony gels there were lines of identity between all lymphocyte lysates and a second faint precipitin line formed with crude PEN spleen extract and the PEN peak I isolated by chromatography. The latter two preparations also reacted with antiserum to give very sharp, distinct F antigen precipitin lines of identity with one another that did not crossreact with the antigen commonly present in lymphocyte lysates. Absorption of the antiserum to PEN peak I with viable peripheral lymphocytes (three times with 10^8 cells per ml of antiserum at 4°) specifically removed all reactivity with lysate antigen but did not affect the predominant reaction with F or S antigens in PEN peak I or crude PEN spleen extract.

DISCUSSION

The present study has demonstrated that two antigens, previously reported to exist in high frequency in Hodgkin's

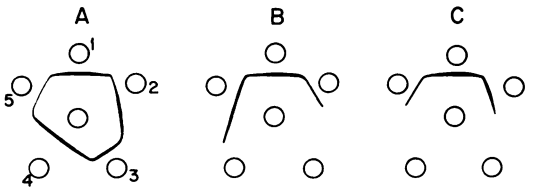

FIG. 7. Ouchterlony gel diffusion patterns of various tissue extracts and isolated fractions. *Center wells* in all cases contain unabsorbed rabbit antiserum from an early bleeding after immunization with PEN peak I. *A:* (1) Hodgkin's spleen PEN (unchromatographed); (2) PEN peak I from Sephadex G-200; (3) EVNG lymph node (unchromatographed); (4) fetal liver (unchromatographed); (5) normal spleen (unchromatographed). *B:* (1) PEN peak I (spleen); (2) EVNG peak I (lymph node); (3) ENVG peak III; (4) fetal liver peak III; (5) fetal liver peak I. *C:* (1) Hodgkin's spleen PEN (unchromatographed); (2) PEN peak I; (3) PEN peak III; (4) normal spleen peak III; (5) normal spleen peak I.

tumor patients (5–8), can be obtained in relatively concentrated form by rather uncomplicated methodology. Extracts of Hodgkin's spleen tumor tissue when subjected to chromatography on Sephadex G-200 separate into three major protein peaks of which only the first (peak I) possesses the predominant antigenic activities associated with the disease. The chromatographic patterns of other tissue extracts, known to give positive antigenic reactivity, have been found to be remarkably similar to that of Hodgkin's spleen, whereas normal spleen extracts elute in a quantitatively different manner. However, antigenic activity has been restricted to peak I proteins in all cases.

Early rabbit antisera prepared against the peak I protein of Hodgkin's spleen gave a single clear precipitin line of identity when reacted with concentrated peak I fractions of fetal liver, Hodgkin's spleen and lymph node, and also a faint but detectable reaction with normal spleen peak I. This activity corresponds to the F antigen by immunoelectrophoretic analysis.

Antigenic analysis performed with hyperimmune rabbit antisera obtained after repeated immunizations with PEN peak I demonstrated antigenic heterogeneity in the peak I protein fractions not detectable by the chemical methods used. Thus, in addition to F antigen, such antisera reacted as well with S antigen. The latter specificity is known to exist in small quantities in some, but not all, Hodgkin's spleen tumors and in larger amounts in Hodgkin's lymph node tumors (Table 1). The fact that both antigens elute in the same protein peak from Sephadex G-200 suggests that they may be similar in terms of molecular size. In addition to F and S antigenic activity, PEN peak I also contained an antigen associated with normal lymphocytes that could be detected with hyperimmune rabbit antisera against peak I. This unexpected contaminant was present in small amounts and could be clearly distinguished from F and S antigens by absorption of such antisera with viable peripheral blood lymphocytes. Antisera absorbed in this way maintain reactivity with F and S antigens but no longer react with soluble lymphocyte extracts.

The present studies represent an initial step in the attempt to isolate these antigens. Further and more sophisticated preparative procedures must be used before it will be possible to isolate and characterize these substances in molecular terms.

The fact that F and S antigens have tissue distributions clearly related to lymphoid cells raises fundamental questions concerning their origin, nature, and significance. These tumor-associated antigens are clearly not absolutely tumor-specific. F antigen is present in small quantities in normal spleens. Its considerable quantitative increase in Hodgkin's spleen tumors and in other conditions associated with heightened lymphocyte proliferation and/or reactivity makes it tempting to consider that F antigen reflects a relatively normal product of reactive lymphoid cells. S antigen, however, has rarely been demonstrated in normal adult tissues (6–8). Its occurrence in neonatal thymus and malignant thymoma may indicate that it originates from very immature lymphocytes, possibly representing a dedifferentiation antigen.

The clear quantitatively significant association of these antigens in patients with Hodgkin's disease may provide a useful diagnostic and/or prognostic tool. The relatively uncomplicated isolation procedures for obtaining the predominant antigens, described herein, should facilitate development of simple and rapid assay procedures that will distinguish patients with Hodgkin's disease from normal individuals or those with other diseases. We are currently developing a radioimmunoassay to apply to studies in such patients in the hope of determining what relationship higher or lower quantities of these antigens have to the course of disease and the nature of the immunologic abnormalities associated with it.

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1. Aisenberg, A. C. (1966) *Cancer* **19**, 385–394.
2. Gendel, B. R., Ende, M. & Norman, S. L. (1950) *Amer. J. Med.* **9**, 343–355.
3. Casazza, A. R., Duvall, C. P. & Carbone, P. P. (1966) *Cancer Res.* **126**, 1290–1296.
4. Order, S. E., Porter, M. & Hellman, S. (1971) *New Engl. J. Med.* **285**, 471–474.
5. Order, S. E. & Hellman, S. (1972) *Front. Rad. Ther.* **7**, 155–167.
6. Order, S. E., Chism, S. E. & Hellman, S. (1972) *Blood* **40**, 621–633.
7. Chism, S. E., Order, S. E. & Hellman, S. (1972) *Amer. J. Roentgenol.*, in press.
8. Order, S. E., Chism, S. E. & Hellman, S. (1972) *J. Nat. Cancer Inst.*, in press.
9. Greenwood, F. C., Hunter, W. M. & Glover, J. S. (1963) *Biochem. J.* **89**, 114–117.
10. Marchalonis, J. J. (1969) *Biochem. J.* **113**, 299–303.