Evidence for Multiple Forms of DNA Polymerase in Hodgkin's Disease

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SUMMARY

The DNA-dependent DNA polymerase activities from nodular sclerosis and mixed-cellularity forms of Hodgkin's disease can be distinguished from one another and from control human polymerases by their elution behavior upon diethylaminoethyl cellulose chromatography and Sephadex gel filtration, by the magnesium ion concentration for optimal activity, by the stimulatory or inhibitory effects upon enzymatic activity of monovalent cations, and by sensitivity of enzymatic activity to heparin inhibition. These studies at the molecular level support the mounting evidence of heterogeneity in Hodgkin's disease.

INTRODUCTION

Hodgkin's disease, originally described as a single disease, is currently recognized in at least 4 histologically differentiated forms (18): (a) NS, (b) MC, (c) lymphocyte predominance, and (d) lymphocyte depletion. Within the past few years, there has been mounting epidemiological, clinicopathological, and immunohistological evidence that Hodgkin's disease consists of multiple diseases. For example, MacMahon (19) has built a strong case for two or more diseases in populations of Hodgkin's disease patients, on the basis of differences in age, sex, and geographical distributions (including the absence of an early adult peak in Hodgkin's disease patients in Japan). We felt it would be appropriate to examine tissues with Hodgkin's disease involvement in order to determine whether differences exist at the molecular level which might be detected and correlated with the histological diagnosis.

Interest has recently begun to focus on molecular differences in enzyme activities in malignant compared with normal cells. Various data suggest that human tumor cells have different DNA-dependent DNA polymerase activities (14, 26), RNA-dependent DNA polymerase activities (7, 23), and thymidine kinase activities (28), compared with the normal tissue controls. This report provides preliminary evidence that the DNA-dependent DNA polymerases isolated from NS and MC forms of Hodgkin's disease differ from one another and that both are different from control human polymerases.

MATERIALS AND METHODS

Materials.

dTTP-3H and unlabeled deoxyribonucleotide triphosphates were obtained from Schwarz/Mann, Orangeburg, N. Y. Calf thymus DNA and DNase I were purchased from Worthington Biochemical Corp., Freehold, N. J. Filter paper disks 1 inch in diameter and DE23-cellulose and PI1-cellulose phosphate were obtained from H. Reeve Angel & Co., Inc., Clifton, N. J. Heparin was obtained from LaMotte Chemical Products Co., Chestertown, Md.

Tissues were obtained by laparotomy and were provided by Dr. L. Dabich and Dr. C. Zarafonetis, Department of Hematology, University of Michigan, Ann Arbor, Mich., with the aid of Dr. P. A. Levine, National Cancer Institute, Bethesda, Md.; Dr. W. Y. Inouye, Dr. P. F. Engstrom, and Dr. R. R. Chilow, American Oncologic Hospital, Philadelphia, Pa.; Dr. P. Nowell, Dr. S. A. Hinzstraus, and Dr. E. Edynak, University of Pennsylvania, Philadelphia, Pa.; Dr. F. Gardner and Dr. K. J. Kim, Presbyterian Hospital, Philadelphia, Pa. and Dr. E. Schwartz, Children's Hospital, Philadelphia, Pa.

Tissues were minced with scissors and homogenized with a mortar and pestle in 0.05 M Tris-HCl buffer, pH 8.0, containing 20% glycerol and 20 mM redistilled mercaptoethanol. Buffer for the experiments with Sephadex and the ion effects contained 10 mM mercaptoethanol; glycerol was absent. The homogenates were centrifuged at 75,000 to 100,000 X g for 1 hr. Protein concentrations were determined by the method of Lowry et al. (17).

Enzyme assays were performed at 37° in the following reaction mixture (volume, 30 μl): 75 mM Tris-HCl, pH 8.0; 4 mM KCl; 6 mM redistilled mercaptoethanol; 0.1 mM each dATP, dCTP, and dGTP; 0.01 mM dTTP-3H; 200 μg "activated" DNA per ml (1); and various concentrations of MgCl2, depending upon activity. We terminated reactions by pipetting a 20-μl sample onto a Whatman No. 1 paper disk, followed by repeated washings with 5% trichloroacetic acid (3). Radioactivity was determined by liquid scintillation in a toluene-PPO-POPOP system. Product versus time plots were linear for at least 15 min, and the initial rates were linear with respect to extract concentration. Initial rates were used to calculate specific activity, which is expressed in nmoles/hr/mg protein. Statistical analyses of enzymatic activity determinations yielded a range of 2.7 to 5.8% S.D.

DEAE-cellulose Chromatography. A small sample (2 to 4 ml) of tissue homogenate was applied to a 9- x 1.7-cm DEAE-cellulose column and was equilibrated with 0.05 M Tris-HCl buffer, pH 8.0, containing 20% glycerol and 20 mM

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3 The abbreviations used are: NS, nodular sclerosis; MC, mixed cellularity; EBV, Epstein-Barr virus.

redistilled mercaptoethanol. The column was washed with 50 ml of the same buffer. A linear gradient (100 ml) of 0–0.8 M NaCl in the same buffer was used to elute the enzymes. Two-ml samples were collected and 5-μl aliquots were assayed for enzymatic activity. Salt concentrations were determined with a Barnstead conductivity meter.

**Sephadex G-200 Chromatography.** Ammonium sulfate fractions (20 to 40%) of the DNA polymerase from the diseased spleens were dialyzed against 0.05 M Tris-HCl, pH 8.0, containing 10 mM redistilled mercaptoethanol, and were applied to a previously equilibrated Sephadex G-200 column (void volume, approximately 56 ml); 1.1-ml fractions were collected.

**Ion Effects.** Enzymes from diseased tissues were partially purified (10- to 15-fold) by chromatography on DEAE-cellulose and phosphocellulose, applied in 0.05 M Tris-HCl buffers, pH 8.0, containing 10 mM redistilled mercaptoethanol, and were eluted with a NaCl gradient and dialyzed as described above.

**RESULTS**

**DNA Polymerase Activity**

DNA-dependent DNA polymerase activity is slightly increased in spleens of patients with Hodgkin’s disease involvement, compared with that in control spleens. DNA polymerase activities from 6 different histologically normal spleens from Hodgkin’s disease patients showed a specific activity (nmoles/hr/mg protein) of 1.34 ± 0.37. The specific activity of 3 different MC-involved Hodgkin’s disease spleens was 2.21 ± 0.20, and that of a NS-involved spleen was 3.36. The specific activities were determined from initial rate measurements, as described in “Materials and Methods.” Most of the samples, which were stored frozen, were assayed within 1 week of surgical removal, and several were assayed within 1 to 3 hr of removal. All of the samples were assayed within 3 to 4 weeks. These time differences did not contribute to the specific activity variations. The most important question is whether the enzymes obtained from pathological tissues are unique. This question is addressed in a later section.

We examined the supernatant fractions for DNA polymerase activity in order to increase the probability of detecting enzymes not bound to cellular DNA.

Some of the homogenates of tissues obtained from patients with Hodgkin’s disease were assayed for RNA-dependent DNA polymerase activity by methods described by Gallo et al. (7); pig kidney and yeast RNA were used as templates, and no significant activity was detected.

**Comparison of DNA Polymerases Obtained from Control and Diseased Tissues**

The DNA polymerase activities from patients with Hodgkin’s disease involvement differ depending on whether the tissue is pathologically involved, as determined by histological examination of the tissues. The enzyme activities show different elution profiles from DEAE-cellulose columns (Table 1); the enzyme from normal tissues elutes later (0.18 M NaCl) than the enzyme from tissue involved with the NS form of Hodgkin’s disease (0.10 M NaCl) and elutes earlier than the enzyme from tissue involved with the MC form of Hodgkin’s disease (0.24 M). Data on polymerases from the spleen of a patient with hypersplenism and a mediastinal mass from a patient with seminoma (obtained at surgery) and from spleens of victims of stroke and myocardial infarct (obtained at autopsy), are included and, upon DEAE-cellulose chromatography, show behavior that is indistinguishable from the polymerases from histologically normal spleens from patients with Hodgkin’s disease. The NS and histologically normal spleen DNA polymerases not only showed the same behavior upon rechromatography on DEAE-cellulose, but mixtures of the different extracts were reproducibly resolved with the same columns (Chart 1).

<table>
<thead>
<tr>
<th>Extract</th>
<th>Tissue</th>
<th>No. of patients</th>
<th>Elution volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls Normal</td>
<td>Spleen</td>
<td>3</td>
<td>32.5 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Lymph node</td>
<td>1</td>
<td>32.6 ± 0.1</td>
</tr>
<tr>
<td>Control material obtained at autopsy</td>
<td>Spleen</td>
<td>2</td>
<td>31.0 ± 0.1</td>
</tr>
<tr>
<td>Hypersplenism</td>
<td>Spleen</td>
<td>1</td>
<td>31.1</td>
</tr>
<tr>
<td>Seminoma</td>
<td>Mediastinal mass</td>
<td>1</td>
<td>32.2</td>
</tr>
<tr>
<td>Hodgkin’s disease tissues</td>
<td>Spleen</td>
<td>1</td>
<td>26.7, 28.9a</td>
</tr>
<tr>
<td></td>
<td>Tumor mass</td>
<td>1</td>
<td>26.5</td>
</tr>
<tr>
<td></td>
<td>Lymph node</td>
<td>1</td>
<td>29.5</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>3</td>
<td>37.5 ± 0.6</td>
</tr>
</tbody>
</table>

*a* The 2nd value was obtained 1 month later than the 1st value.

Chart 1. DEAE-cellulose elution profile of NS tumor mass DNA polymerase and histologically normal spleen DNA polymerase from the same patient. The enzymes were mixed together and applied to the column. The columns were run as described in “Materials and Methods.”
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The polymerase activities from uninvolved tissues, in comparison with tumor tissues, show the following differences in behavior. The magnesium ion concentration required for optimal activity is substantially higher for the NS-derived enzyme and lower for the MC-derived enzyme, compared to the control polymerase (Table 2), and sensitivity to heparin inhibition is significantly lower for the enzymes obtained from uninvolved tissues (Table 3). The optimum concentration was obtained from magnesium ion concentration-activity profiles over a range of concentrations (0 to 10 mM MgCl₂). For reference, the 2 controls from diseased tissues obtained from patients with hypersplenism and seminoma are included. In addition, control enzymes for sensitivity to inhibition of activity by heparin include a polymerase from a normal spleen obtained at autopsy from a stroke victim.

Comparison of DNA Polymerases Obtained from Tissues with Hodgkin’s Disease Involvement

The enzymes extracted from tissues of patients with Hodgkin’s disease, which have been classified histologically as the NS and MC forms, are distinguishable by the following criteria.

DEAE-cellulose Column Chromatography Characteristics. The elution profiles of the polymerases from NS and MC tissues differ in that the NS enzymes elute at lower NaCl concentrations in a gradient (0.10 M) than do the MC enzymes (0.24 M) (Table 1).

Gel Filtration Characteristics. The MC enzymes are eluted significantly earlier, upon Sephadex G-200 chromatography, compared with the NS-enzyme. The ratio of volume eluted to the void volume for 2 different MC enzyme samples was 1.10 ± 0.02 for 4 determinations, and the ratio for a NS enzyme was 1.24 ± 0.03 for 2 determinations.

Effects upon Activity of Ionic Composition and Concentration. The activities of partially purified polymerases from NS and MC spleens (see “Materials and Methods”) have different magnesium ion concentration optima, i.e., 2 to 4 and 0.6 to 0.8 mM, respectively. The enzymatic activities also show striking differences in their response to monovalent ions. The NS enzymes exhibit 40 to 60% inhibition and the MC enzymes exhibit 50% stimulation with 50 mM NaCl or KCl, and have ammonium ion concentration optima of 5 mM and 50 mM, respectively, when assayed at 3 mM MgCl₂. These ion effects were examined over a wide concentration range, and the data presented were obtained at concentrations showing the most diverse effects.

DISCUSSION

The significance of the different properties of DNA polymerase activities in diseased and normal tissues is at present unclear but may well reflect differences in the primary sequences of the polymerase molecules. The most conclusive demonstration that the different properties reflect different protein molecules requires purification and characterization of the pure proteins, and such studies are presently under way in our laboratory. There are several possibilities regarding the existence of different enzymes in pathological tissues that may well reflect differences in the cellular proliferation, or a difference in the cellular composition of the tissue.

Viral Association with Hodgkin’s Disease. A viral etiology for Hodgkin’s disease has been proposed (19), with supporting evidence provided by observations of elevated antibody levels to EBV in the sera of some patients with Hodgkin’s disease (11, 15) and the report of an “epidemic” within a single school population [Ref. 31 (see also Ref. 30)]. Herpes-like agents have been isolated from tissue cultures of cells obtained from lymph nodes of patients with Hodgkin’s disease, and increased antibody levels to this virus are found in the sera of many patients (6). Some spleens of patients with Hodgkin’s disease reportedly show base sequence homology with the Rauscher murine leukemia virus (9).

Herpes-infected cells contain some enzymes (DNA polymerase, thymidine kinase, and DNase) that are different from
the cellular enzymes and that appear to be viral in origin (13). The viral polymerase has been demonstrated in cells transformed by a murine leukemia virus (24) but, to date, no such experiments have been reported for herpes-transformed cells. If the enzymes detected in the Hodgkin's disease tissues are directly coded for by a virus or if they represent viral-specific altered host enzymes, then the finding of 2 different enzymes would indicate that different viruses are involved (primarily or secondarily) in the NS, as opposed to MC, form of Hodgkin's disease. In further support of these considerations is the characterization of thymidine kinase activities with different Michaelis constants for thymidine in the 2 forms of the disease (unpublished results).

**Multiple DNA Polymerases.** The presence of multiple DNA polymerases in the diseased and control spleens, as noted previously, need not reflect a viral origin. Multiple DNA and RNA polymerases have been reported for most cells including human, that have been examined (27, 32). Two DNA polymerase activities have been isolated from human cells: Polymerases I and II (27) or the "cytoplasmic" and "nuclear" enzymes (32). In our studies on spleens obtained at autopsy (specific activities, approximately 0.2 n mole/hr/mg protein; homogenized with a blender), we find evidence for these 2 enzymes, which appear to be similar to those described by Weissbach et al. (32). Due to the uncertainties associated with the use of autopsy material, we have focused our attention on the histologically normal spleens obtained by laparotomy from Hodgkin's disease patients. We find 1 enzyme in these tissues (homogenized with mortar and pestle) that behaves identically (on DEAE-cellulose chromatography) to the "cytoplasmic" (32) or Polymerase I (27) enzyme activity from the spleen obtained at autopsy. However, upon further examination, we find that the enzymatic activities of material obtained at autopsy are characterized by a higher magnesium ion concentration optimum for enzymatic activity [the same as described in the literature (16)] and by considerably less sensitivity to heparin inhibition, compared with the enzyme derived from histologically normal spleens from patients with Hodgkin's disease (Table 1). Further conclusions regarding differences between material from laparotomy and that obtained at autopsy must await the availability of spleens surgically removed from healthy patients (e.g., patients with splenic rupture).

The possibility of a derepressed host enzyme might be considered attractive, when applied to the NS form of Hodgkin's disease, since that form has been described as being similar to an autoimmune disease (19), perhaps triggered by a virus (21).

The diseased spleens used in this study were several times the normal mass and showed widespread involvement by pathological tissues. To some extent this minimizes, but does not eliminate, the uncertainties caused by the occurrence of multiple cell types in splenic material. In the diseased tissue, detectable levels of what appears to be normal polymerase (by the criteria listed in Tables 1 to 3) also appear to be present. The MC and NS enzymes appear to be more labile than the control enzymes, and these activities are lost, upon tissue storage for several months. Thus, following prolonged storage, the residual activity behaves increasingly like the control enzyme (Table 1, NS spleen, 2 sequential determinations). The tumor mass and large diseased spleens (450 g or more) appear to have the greatest relative amounts of NS or MC enzyme activity, compared with control enzyme. Tissue with less tumor (the lymph node, for example) shows characteristics in terms of DEAE-cellulose chromatography, magnesium ion concentration effects upon enzymatic activity, and sensitivity of enzymatic activity to heparin inhibition that would be predictable if there were a combination of the 2 enzymes, with the NS enzyme in excess.

**Heterogeneity in Hodgkin's Disease.** Regardless of the origin of the different enzyme activities in NS and MC forms of Hodgkin's disease, the differences demonstrated at the molecular level support the mounting evidence for heterogeneity in Hodgkin's disease. In addition to MacMahon's study (19), other evidence has recently accumulated that supports the concept of 2 or more diseases, as follows. (a) Analysis of predicted survival time of patients according to sex, symptoms, and stage of disease shows clear-cut differences that are correlated with histological type (2). (b) The histological consistency of the NS type of Hodgkin's disease (less than 9% show variation in histological picture with time) provides further support for the concept of more than 1 disease, rather than different temporal stages of the same disease (29). (c) In NS patients, the early spread of the disease is to adjacent areas, while the MC form spreads in a random manner (10). (d) The EBV antibody studies of Levine et al. (15) and Johansson et al. (11) [despite an earlier negative study (8)] further support the concept of heterogeneity. Thus, Levine et al. (15) found elevated levels of EBV antibody in patients with the MC and lymphocyte depletion but not with the lymphocyte predominance and NS forms of Hodgkin's disease. (e) There is an increased remission duration when Stage I and II MC patients are treated with chemotherapy following radiation therapy, while NS patients showed no increased beneficial effect from the chemotherapy (22). (f) Sites of clinical involvement at the time of diagnosis differ with histological type: NS patients show a high frequency of mediastinal involvement, while MC patients present with splenic involvement twice as frequently as do NS patients (12).

**Potential Therapeutic Value of Heparin.** In view of the great effort toward the development of selective inhibitors for RNA-dependent DNA polymerase for potential therapeutic use, the reported selectivity of heparin inhibition for enzymes from Hodgkin's disease tissues is of interest. It is possible that heparin or a derivative might be effective in Hodgkin's disease, since heparin is concentrated and remains in lymph tissue (mature lymph nodes, thymus, and spleen) (5) and tumors (4) even after its disappearance from the blood. A nonanticoagulant derivative of heparin has been used to transport a number of alkylating agents selectively to the site of tumors (20) and has been reported to be effective in the treatment of some patients with Hodgkin's disease (25).

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