Polymorphonuclear Functions in Hodgkin’s Diseases Patients at Diagnosis, in Remission, and in Relapse

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ABSTRACT

Five tests investigating different aspects of the nonspecific defense mechanisms including capillary tube random migration, particle ingestion activity, quantitative and histochemical nitroblue tetrozolium dye reduction by polymorphonuclear neutrophils, and serum lysozyme concentrations were performed in 46 patients with Hodgkin’s disease. The anomalies observed in the active stage of the disease consisted of a decreased random migration, a higher level of serum lysozyme, and an increased nitroblue tetrozolium reduction by resting phagocytes associated with a decrease in nitroblue tetrozolium reduction by stimulated phagocytes. The particle ingestion activity was normal. The serum lysozyme assay was the only test observed to normalize in the group of patients in remission. Its determination, therefore, offers an additional means of evaluating disease activity.

INTRODUCTION

Immune function has been investigated extensively in HD (43). However, only a few studies have been devoted to the nonspecific host defense mechanisms in this disease, and phagocytic activity was usually considered normal (33). The fact is that only a few functions have been studied and only in a small series of patients; moreover, some reports have presented conflicting results.

The present investigation, using several tests in a large series of HD patients, was designed to answer the question of whether phagocytosis was abnormal and related to the clinical state of HD patients.

MATERIALS AND METHODS

Patient Population

Forty-six patients with HD admitted to the Department of Hematology (Purpan Hospital, Toulouse, France) were investigated for PMN function. They gave informed consent to these studies in accordance with the Helsinki Declaration. The population was composed of 21 women and 25 men, ranging in age from 17 to 76 years (average age, 39 years). Table 1 summarizes the disease activity and therapeutic regimen at the time of the tests and also the clinical stage and histological grading, which had been determined at diagnosis. The lymph node histology and the clinical staging were established in each case according to the criteria of Lukes and Butler (26) and the report of the committee on HD-staging classification (9). Despite repeated examination, histological subclasses could not be ascertained in 7 cases. Splenectomy had been performed in 8 patients for pathology staging; the presplenectomy staging was Stage I in one case and Stage II in the other cases. The staging was changed later in 3 subjects in favor of a Stage III classification. Among the 17 patients in remission, 6 patients had been without treatment for several years, the 11 other patients were still under treatment, but in all cases chemotherapy had been suspended for at least 1 month prior to the PMN investigation. In these 17 patients, remission was considered as complete after normal results were obtained for the following parameters: clinical examination; chest X-rays; lymphography; bone marrow biopsy; and blood tests including erythrocyte sedimentation rate, fibrin, serum iron, and iron-binding capacity. Seventeen patients with untreated disease were studied shortly after the diagnosis had been established.

The chemotherapy involved the cyclic administration of nitrogen mustard or cyclophosphamide in association with vincristine, procarbazine, and prednisone (15, 27). The radiotherapy consisted in high-energy irradiation using the “mantle” and/or “inverted Y” fields technique. For the entire population studied, the time interval between diagnosis of HD and the date of PMN testing ranged from 1 week to 18 years.

Controls

The controls were healthy adults, 20 to 42 years old, members of the laboratory personnel and voluntary blood donors, presenting no active disease and taking no drugs with the exception of p.o. contraceptives. Informed consent was obtained from each control. A control subject was studied in parallel with each HD patient without sex or age matching.

Polymorphonuclear Function Tests

Capillary Tube PMN Random Migration. Random migration was tested in whole blood according to the capillary tube method of Ketchel and Favour (23). Two ml of venous blood were collected with 20 μl of calcium heparinate (Calciparine; Laboratory Roche, Neuilly sur Seine, France). Ten microhematocrit tubes (Clay Adams, Parsippany, N. J.) were filled to two-thirds capacity and centrifuged at 4000 rpm (MSE, Crawley, England) for 4 min. The tubes were then placed vertically at 37° for 4 hr. The distance between the advancing front of leukocytes and the erythrocyte level was measured microscopically on each tube. The result was expressed as the arithmetic mean of the distances measured in 10 tubes.

Particle Ingestion Activity. The particle ingestion activity was expressed by the phagocytic index according to the method of Brandt (7). This index corresponded to the average number of yeasts (Saccharomyces cerevisiae) ingested per PMN. Eight ml of blood were collected in a plastic syringe with 0.2 ml of heparin at 1000 units/ml (Laboratory Vitrum, Vitry sur Seine, France). Two ml of Plasmagel (Laboratory Roger Bellon, Neuilly, France) were added, and the syringe was placed vertically in an incubator at 37°. Twenty min later, the supernatant was collected, and the number of PMNs was calculated from the leukocyte enumeration (Model S Coulter Counter) and the differential. A 0.4-ml amount of a suspension of heat-killed yeasts at a concentration of 40,000/μl was added to a volume of leukocyte suspension to obtain a 7 yeast:PMN ratio of 7:1. After gentle shaking, the
tube was incubated at 37° for 20 min. Then, after adding 0.1 ml of 0.9% disodium EDTA, the tube was centrifuged at 200 x g for 5 min. The supernatant was discarded, and the cells were resuspended. Two smears were stained with panoptic staining (May-Grunwald-Giemsa), and the results were read at x 1000. The number of PMNs which had ingested 0 to 7 yeast particles or more was established. The score obtained for 100 consecutive PMNs divided by 100 provided the phagocytic index.

### Quantitative NBT Dye Reduction

The NBT dye reduction was studied according to the method of Baehner and Nathan (5). Twenty ml of venous blood were collected in a plastic syringe with 2 ml of 5% disodium EDTA. Five ml of Plasmagel were added, and the syringe was placed vertically at 37° for 20 min. The supernatant was discarded, and the cells were resuspended in a plastic tube, which was centrifuged at 200 x g for 10 min after adding a double volume of 0.87% ammonium chloride. The cell pellet was washed twice in Krebs-Ringer buffer, pH 7.4, containing 200 mg/100 ml of glucose. The number of phagocytic cells (mature neutrophil granulocytes plus monocytes) was adjusted to 25,000/µl. Three reaction tubes were prepared in parallel (Tubes A, B, and C). Tubes A and C contained 0.4 ml of Krebs-Ringer buffer plus 0.4 ml of 0.1% NBT solution (BDH Chemicals Ltd., Poole, England) in 0.9% NaCl solution plus 0.1 ml of 0.01 M potassium cyanide. Tube B contained 0.39 ml of Krebs-Ringer buffer plus 0.01 ml of latex particles 0.794 µm in diameter (Serva, Heidelberg, West Germany) plus 0.4 ml of NBT plus 0.1 M potassium cyanide. A 0.1-ml sample of leukocyte suspension was added to each reaction tube after 15 min of preincubation in a waterbath at 37°. The reaction was stopped by adding 10 ml of 0.5 N HCl to each tube, after 10 sec of incubation for Tube C and after 15 min of incubation for Tubes A and B. After 15 min of centrifugation at 1000 x g at 4°, the supernatant was discarded. The reduced NBT was extracted after adding 4 ml of pyridine (RP Prolabo, Paris, France) and boiling for 15 min. After centrifugation the absorbance of Tubes A and B was registered with a Beckman Model 24 spectrophotometer at 515 nm with the Tube C extract as a blank. The absorbance value represented the NBT reduction of resting phagocytes (resting absorbance), that of Tube B represented the NBT reduction in stimulated phagocytes (stimulated absorbance), and the difference between these 2 values (Δ absorbance) represented the potential of metabolic stimulation of the phagocytes.

### Histochemical NBT Reduction

The semiquantitative NBT reduction was studied according to the method of Park (31). One ml of heparinized venous blood (1000 units/ml heparin (Laboratory Vitrum, Vitry sur Seine, France) was added in a siliconized glass tube to 0.50 ml of Krebs-Ringer buffer and 0.50 ml of 0.1% NBT solution (BDH Chemicals Ltd.) in 0.9% NaCl solution and incubated at 37°. After 20 min, smears were stained with nuclear red dye to establish microscopically (x1000) the percentage of PMN with a large black deposit of reduced NBT.

### Serum Lysozyme

The serum lysozyme was assayed by the microbiological method, using *Micrococcus lysodeikticus* as substrate and hen egg white lysozyme as reference (Seratest Lysozyme, Laboratory Eurobio, Paris, France). Six dilutions, ranging from 50 to 5%, were prepared from a lysozyme stock solution (40 g/ml). The effect of each dilution on a *M. lysodeikticus* suspension was registered on a spectrophotometer (Beckman Model 24) at 580 nm.

From these 6 curves, a reference line was drawn permitting conversion of the absorbance values into lysozyme concentrations. For the determination itself, 0.3 ml of the serum to be tested was added to 0.3 ml of the *M. lysodeikticus* suspension and gently mixed. After 30 sec, the absorbance decrease was recorded for 2 min. The Δ absorbance (difference between the first and last values of the 2-min period) was compared to the reference line, and the serum lysozyme value was expressed in µg of hen egg white lysozyme per ml of the serum.

### Statistical Methods

Comparison of the means was interpreted according to Student's t test. The differences were considered to be significant when the probability (p) was < 0.05.

## RESULTS

The results obtained in the whole population of HD patients are shown in Table 2. The capillary tube PMN random migration was decreased significantly. This decrease was observed in all the subgroups of patients investigated and presented in Table 1. Of particular interest is the fact that this anomaly was present in the group of patients in clinical remission.

The phagocytic index was normal in all the subgroups of HD patients. The group of 13 patients with Stage IV disease tended to have higher values [5.22 ± 0.78 (S.D.)], but this was not a statistically significant finding.

The quantitative NBT reduction was impaired with a moderate increase of the "resting absorbance" value and a decrease of the "stimulated absorbance" value, resulting in a markedly lowered Δ absorbance value. This Δ-absorbance decrease, which represents an impairment of the metabolic reactivity of PMNs under phagocytic stimulation, was also significant in all the subgroups studied.

The histochemical NBT reduction gave mean values identical to the controls, but there were large individual variations. No significant variations were observed in the subgroups.

The value of the serum lysozyme was significantly increased when the entire population of HD patients was considered as a whole. However, there was no statistically significant difference when the group of all patients under treatment (all evolutive phases included) was compared to controls. In addition, the serum lysozyme values of those patients in remission and untreated (for at least 1 month) were not significantly different from those of the controls. However, when compared to the controls, significantly increased values were found in untreated patients (10.4 ± 2.80; p < 0.001) and in those patients with active disease (10.23 ± 3.64; p < 0.001). For the entire HD population, the number of peripheral monocytes was found to be normal (472 ± 281/µl), and no correlation could be established between monocyte numbers and (a) lysozyme values, (b) disease activity, and (c) therapeutic regimen (unreported data). No sequential study was carried out.
Table 2

Results of the 5 tests used to explore the nonspecific defense mechanisms in HD patients (subgroups established according to treatment and disease activity)

<table>
<thead>
<tr>
<th></th>
<th>Controls (47)</th>
<th>All patients (48)</th>
<th>Untreated (17)</th>
<th>Remission (17)</th>
<th>Relapse (8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Random migration (mm/4 hr)</td>
<td>1.66 ± 0.33° (44)</td>
<td>1.15 ± 0.35° (40)</td>
<td>1.28 ± 0.36° (14)</td>
<td>1.13 ± 0.55° (17)</td>
<td>0.99 ± 0.32° (8)</td>
</tr>
<tr>
<td>Phagocytic index</td>
<td>4.71 ± 0.85 (31)</td>
<td>4.62 ± 1.04 (43)</td>
<td>4.38 ± 1.52 (17)</td>
<td>4.90 ± 1.03 (17)</td>
<td>5.01 ± 0.83 (7)</td>
</tr>
<tr>
<td>Quantitative NBT dye reduction</td>
<td>0.062 ± 0.023 (37)</td>
<td>0.083 ± 0.070 (46)</td>
<td>0.078 ± 0.068 (17)</td>
<td>0.090 ± 0.068 (17)</td>
<td>0.071 ± 0.041 (8)</td>
</tr>
<tr>
<td>Resting absorbance</td>
<td>0.468 ± 0.139 (37)</td>
<td>0.376 ± 0.095 (46)</td>
<td>0.389 ± 0.099 (17)</td>
<td>0.374 ± 0.083 (17)</td>
<td>0.385 ± 0.079 (8)</td>
</tr>
<tr>
<td>Stimulated absorbance</td>
<td>0.405 ± 0.123 (37)</td>
<td>0.293 ± 0.073 (46)</td>
<td>0.311 ± 0.070 (17)</td>
<td>0.284 ± 0.048 (17)</td>
<td>0.314 ± 0.069 (8)</td>
</tr>
<tr>
<td>Δ absorbance</td>
<td>12.1 ± 11.9 (28)</td>
<td>13.3 ± 15.6 (27)</td>
<td>20.1 ± 21.9 (10)</td>
<td>17.3 ± 20.9 (12)</td>
<td>14.6 ± 23.2 (5)</td>
</tr>
<tr>
<td>Histochemical (% of positive PMNs)</td>
<td>7.96 ± 2.04 (47)</td>
<td>9.96 ± 3.42° (42)</td>
<td>10.4 ± 2.80° (17)</td>
<td>8.74 ± 3.40 (17)</td>
<td></td>
</tr>
</tbody>
</table>

* Significant difference; p < 0.01.
* Mean ± S.D.
* n = number of subjects tested.
* Number in parentheses, number of subjects tested.

**DISCUSSION**

The present study was intended to evaluate simultaneously various aspects of the nonspecific host defense mechanisms observed in HD. The techniques used involved both isolated PMNs and whole blood.

In the whole-blood technique used to test random migration, PMNs remain in autologous plasma throughout the test; thus, the influence of plasma constituents is maximal. The absence of leukocytosis assures that the decrease of the migration found in HD patients is not due to a phenomenon of overcrowding, as may be observed in chronic granulocytic leukemia (13). Even in the absence of cross-tests, which permit us to define the origin of the anomaly as being either the PMNs themselves or the plasma, the differences observed between the controls (tested in parallel) and the groups of HD patients are highly significant in all cases. Moreover, our results can be correlated with those of authors who had studied previously PMN motility in HD. Thus, Senn and Jungi (36), using a skin window technique, found a significant impairment of neutrophil migration in a series of 26 HD patients, and Ward and Berenberg (42) described a chemotactic inactivator in HD patients. The lack of difference between the values of treated and untreated patients, as described previously by others (18, 21, 25), would indicate that random migration of PMNs is apparently unaffected by the therapeutic regimen. We cannot, however, explain why our results of random migration remained abnormally low in patients in remission. We are presently observing the clinical outcome of these patients.

The phagocytic index established according to the procedure of Brandt (7), expresses both the opsonic and phagocytic activities, since the yeast particles are added to a suspension of leukocytes in autologous plasma. The more complicated kinetic methods for the determination of opsonophagocytosis were considered useless for the present study, since the observed phagocytic index was normal and in agreement with the results reported by previous workers (11, 17, 19). Although diminished phagocytosis has been observed previously in subjects treated with corticosteroids (16) and after splenectomy (28), we could find no decrease of the phagocytic index either in our 16 patients under nitrogen mustard-vincristine-procarbazine-prednisone or cyclophosphamide-vincristine-procarbazine-prednisone chemotherapy regimen, or in our 8 splenectomized patients. These latter results are identical to those of Hancock et al. (19).

The NBT dye reduction is regarded as an index of the superoxide anion production (40), a reactive metabolite of oxygen essential to the bactericidal activity of the phagocytes (4). The quantity of reduced NBT, formazan, which accumulates in the cell is correlated to its phagocytic activity (number and size of ingested particles) (35). Several techniques for NBT reduction have been described, including histochemical and quantitative methods. In the latter, total formazan is measured spectrophotometrically in both resting and stimulated phagocytes. The results obtained with resting cells reflect their basic metabolic activity, while those results obtained with stimulated cells provide an index of the metabolic activation. This activation depends on both the ability to ingest particles and the functional state of the glycolytic and oxidative metabolism of the PMNs. The results that we observed in our patients for the quantitative NBT reduction test when compared to the controls (tested in parallel) were unquestionably abnormal and indicate that the histology, clinical stage, disease activity, WBC, and treatment have no correlation with this test, as has been stated previously (2, 10, 14, 19, 32). The NBT dye reduction has been tested extensively in malignant lymphomas with conflicting results (2, 10, 18, 24, 32, 37, 38). Most of these studies were based upon histochemical methods, the microscopic interpretation of which is often difficult (3, 6). In our experience, we have never found any correlation between the quantitative and the histochemical methods, and we therefore rely only on the quantitative method. Pickering et al. (32), while evaluating the hexose monophosphate shunt activity in HD patients, found the same type of disturbance of oxidative metabolism as we have observed when investigating NBT reduction. In both cases, an increased activity of resting PMNs and a decreased activity of stimulated PMNs were observed. This observed decrease of NBT reduction in stimulated cells is not related to a decrease of particle ingestion. Indeed, the phagocytic index was normal, and the number of ingested latex particles observed in the normal and HD PMNs (from the NBT assay) did not differ. However, these metabolic alterations are minor, and the bactericidal activity has usually been found to be normal (18, 39, 41) or only slightly decreased (11, 34).

In the absence of renal insufficiency and peripheral monocytosis, serum lysozyme levels reflect only the turnover rate of...
neutrophilic granulocytes (20). Since none of our investigated HD patients presented with renal insufficiency or peripheral monocytosis, we conclude that the observed elevation in serum lysozyme levels must be the reflection of an increased rate of neutrophilic turnover, a turnover rate which would be accelerated in active phases of HD and return to normal during remission. Our results are in contradiction with earlier published papers in which serum lysozyme levels were reported to be normal in malignant lymphomas (29, 44, 45). Recently, Karle et al. (22) observed a significant increase of serum lysozyme without a change of the intracellular content of the enzyme. Our results showing increased levels of lysozyme in patients during active stages of HD and normal values in patients in remission, lead us to propose the serum lysozyme determination as an additional parameter for determining disease activity.

The present study reveals the extent and the frequency of anomalies of nonspecific defense mechanisms in a series of HD patients. These include alterations of random migration, NBT reduction, and serum lysozyme activity. This combination of anomalies suggests influence of serum factors upon circulating PMNs; however, our whole-blood technique does not permit us to confirm the plasma origin of the observed defect. It should be pointed out that we have found previously the same type of impairment of NBT production by circulating PMNs in rheumatoid arthritis (12). CIC are implied in the pathogenesis of rheumatoid arthritis and may be responsible for the activation of circulating PMNs (30). Recently, Amlot et al. (1) and Brown et al. (8) have reported CIC in more than 50% of the HD patients that they tested. We, therefore, propose a pathophysiological scheme in which CIC, after binding to the cytoplasmic membrane of PMNs, would be phagocytized and thereby induce the observed migratory and metabolic anomalies. These “activated” PMNs would experience a shortened life span with a compensatory acceleration of their turnover rate and the observed increase in serum lysozyme levels. The persistence of anomalies of PMN function in patients considered to be in remission justifies further investigations. The normalization of the lysozyme value in this group may express only the disappearance of the inflammatory process and re-active monocytosis, the PMN dysfunction being the sequela of the chemotherapy. In order to minimize this possibility, the functional tests were performed after a treatment-free interval, which we believe to have been long enough to eliminate this factor. The ultimate solution of this problem will necessitate the study of a selected population of cured patients, which should be performed at least 1 year after the cessation of all treatment.

REFERENCES

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