

Studies of Lymph Nodes From Patients With Classical Hemophilia

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Within the last 18 months, we have noted the development of unexplained lymph node enlargement in otherwise asymptomatic patients with hemophilia. Because such changes are poorly understood and, in some patient groups, may be related to the acquired immunodeficiency syndrome (AIDS), we studied the enlarged lymph nodes in four patients with severe factor VIII deficiency and abnormally low peripheral blood helper-inducer/suppressor cell (OKT4/OKT8) ratios. Surgically excised lymph nodes were studied for histopathologic, electron microscopic, and chromosomal changes. Cell suspensions from these and normal nodes were also studied using monoclonal antibodies. Excised lymph nodes showed follicular hyperplasia.

RECENTLY, homosexual men and patients with hemophilia have been noted to have chronic lymph node enlargement, abnormal immunologic studies, and occasionally splenomegaly.¹⁻³ Because patients with hemophilia may be at risk for acquired immunodeficiency syndrome (AIDS), and because similar findings have been noted in homosexuals with AIDS, an understanding of these clinical features is important.^{4,5} We have previously noted that hemophiliacs with enlarged lymph nodes have greater abnormalities in lymphocyte function than those without such abnormalities and have joined others in questioning whether this may put them at increased risk for AIDS.^{3,14-18}

With this background, we studied the lymph nodes from four consecutive patients with factor VIII (FVIII) deficiency who were found to develop lymph node enlargement. We compared cell phenotypes, histopathologic and electron microscopic features, and chromosomal evaluations with those of lymph nodes taken from normal subjects.

MATERIALS AND METHODS

Patient Population

All four patients with hemophilia were in good general health and had no past or current illness to explain the development of clinical

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Electron microscopy revealed no viral particles or vesicular rosettes. Chromosomal aberrations included an acrocentric marker chromosome in one patient and monosomy 21 in another. T lymphocyte ratios (OKT4/OKT8) in lymph node suspensions were lower than those in nodes from normal controls (1.2 v 6.1) and reflected the lymphocyte ratio in peripheral blood. Mature B cell percentages were increased in the lymph nodes from patients with hemophilia (38% v 27% in controls). Patients treated with factor VIII concentrates and male homosexuals have similarities in persistent lymph node enlargement, histologic features of follicular hyperplasia, and changes in lymph node and circulating lymphocyte subpopulations.

lymph node changes. Each had been followed in clinic for one to two years before noting their lymph node enlargement (Table 1). These patients fit the description of those previously described with the benign reactive lymphadenopathy syndrome.¹ Patients 1, 3, and 4 brought the enlarged nodes to our attention. The nodes in patient 2 were found during a routine visit. Patient 4 has had palpable splenomegaly, but not lymphadenopathy, since 1981 (Table 1). The other three patients did not have splenomegaly, and no patient had hepatomegaly. The patients were not homosexual and denied the use of recreational or other drugs. Each patient had less than 1% FVIII coagulant activity and used commercial coagulation factor VIII concentrate regularly.¹⁹ None had received other blood components in the two years prior to biopsy. Of a group of approximately 150 patients with factor VIII deficiency seen during 1982 to 1983, we had the most concern for possible malignancy or infection in these four patients because of their immune abnormalities and the sizes of the lymph nodes. All four had typical hemophilic arthropathy, varying from stage I to stage IV.²⁰ Patient 3 was black and had a factor VIII inhibitor of 1 Bethesda unit. The other patients were white with no inhibitor. Patient 3 had had clinical hepatitis 15 years previously, while the others had not experienced jaundice or similar symptoms and none had detectable hepatitis B surface antigen (HBsAG) at the time of study. Each had antibodies to hepatitis A and B. Serology for syphilis was negative in all four patients. Preparations for biopsy and for one to two days postoperatively utilized factor VIII concentrate with a total of 2,600 to 20,000 units per patient.

The lymph node enlargement was generalized, uniformly nontender, firm, and fairly symmetrical. Axillary nodes were biopsied in patients 1, 2, and 3, and a cervical node was biopsied in patient 4. Computerized tomography of the abdomen in patient 3 revealed retroperitoneal lymph nodes less than 2 cm in diameter. Follow-up for at least 11 months has shown fluctuation, but not resolution, of the nodal enlargement (Table 1). Serologic studies (Table 1) were performed as previously described.³

Lymph Node Studies

The lymph nodes were bisected and one half was processed for histologic examination and bacterial, mycobacterial, and, in two instances (patients 3 and 4), viral cultures. Routine sections were stained with hematoxylin and eosin. For electron microscopy, small fragments (1 mm in diameter) were removed from the formalin fixed lymph nodes and placed in 3% buffered glutaraldehyde, postfixed in osmium tetroxide, dehydrated, and embedded in epoxy resin. Thin sections were examined with Philips 300 and Zeiss 109 electron

Table 1. Clinical Studies in Patients With Hemophilia and Lymph Node Enlargement

	Patient			
	1	2	3	4
Age	20	44	24	11
Years of factor VIII usage	2	2	9	5
Quantity of factor VIII used*	737	820	1,262	3,173
Duration of lymph node enlargement (months)				
Before biopsy	2	4	4	8
After biopsy†	13	12	12	11
Maximal lymph node size (cm)	4.0	2.0	2.0	1.5
SGOT‡	96	42	35	45
Serum IgG	1,510	1,830	3,000	1,190
Cytomegalovirus antibody titer	1:16	1:16	<1:8	1:8
Herpes simplex antibody titer	1:16	1:16	ND	1:8
Home	Rural	Rural	Urban	Urban

*In U/kg/yr for the preceding two years.

†As of December 1983.

‡Normal 5 to 40 U/mL.

microscopes. A single-cell suspension was prepared from the remaining half of each lymph node by teasing in sterile RPMI 1640 medium. The fragments were passed through a stainless steel mesh to separate individual cells. Cell preparations were then passed over Hypaque-Ficoll, and mononuclear cell populations were enumerated as discussed below. Normal lymph nodes were obtained for comparison during elective procedures that required their removal for access to the surgical field during vascular procedures. These nodes were obtained, after informed consent, from three patients, 50 to 62 years old, with normal blood counts, good general health, and no history of malignancy, recent infection, or immunologic disorders.

Chromosomal Analysis

Aliquots of the cell suspension were divided into two parts for chromosomal analysis: one part for a direct harvest and the other for a 24-hour harvest. For the direct harvest, cells were lysed hypotonically at 37 °C for 20 minutes with a solution containing 9 mL of KCl (0.075 mol/L), 1 mL of trypsin-EDTA (GIBCO, Grand Island, NY, Cat. No. 610-5300), and 50 mL of Colcemid (GIBCO, Cat. No. 120-5210, 10 µg/mL). After hypotonic treatment, the cells were fixed in methanol/acetic acid (3:1, vol/vol).

For short-term cultures, cells were incubated in complete culture medium (at a concentration of 10⁶ cells per mL) for 24 hours in a CO₂ incubator at 37 °C. No mitogens were added to any cultures. Thirty minutes before harvest, Colcemid was added to arrest metaphase. Cells were then hypotonically treated with 0.075 mol/L KCl at 37 °C for ten minutes and fixed as described for the direct harvest. Slides were made by an air-dry method and G-banded according to the method of Wang and Federoff.²¹

Isolation of Peripheral Blood Mononuclear Cells

Total white cell counts (by Coulter Counter) and differential cell counts (by Wright's stain) on specimens of whole blood from each participant were performed on the day of lymph node biopsy. Peripheral blood mononuclear cell populations were isolated from heparinized whole blood by centrifugation over Hypaque-Ficoll. Absolute numbers of lymphocytes were calculated by multiplying the total leukocyte count by the lymphocyte percentage of the differential cell count. Viability determinations were performed

before further study and were uniformly greater than 90% by trypan blue dye exclusion.

Mononuclear Cell Enumeration in Lymph Nodes and Peripheral Blood

T lymphocyte populations and subpopulations were enumerated as percentages of the isolated mononuclear cells by direct immunofluorescence on a fluorescence-activated cell sorter (FACS III, Beckton Dickinson Co, Sunnyvale, Calif) as previously described.³ Monoclonal antibodies OKT3 (total T cells), OKT4 (T helper/inducer cells), and OKT8 (T suppressor/cytotoxic cells) (Ortho, Pharmaceutical Co, Raritan, NJ) and Leu-7 (natural killer cells) (Beckton Dickinson) were utilized. B lymphocyte percentages were determined by indirect immunofluorescence using polyclonal F(ab')₂ goat anti-human immunoglobulin (Cappel Laboratories, Cochranville, Pa). Staining of monocytes in isolated mononuclear cell preparations was performed using alpha-naphthyl-butyrate esterase stain (Technicon, Tarryton, NY). Results of peripheral blood studies were compared with ten age-matched, healthy laboratory workers. Lymph node cell phenotypes were compared with the three normal lymph nodes. Statistical analyses were performed using the Student's *t* test, adjusted for several comparisons.

RESULTS

Histopathology

The basic pattern was characterized by lymphoid hyperplasia, which involved B and T cell domains. Germinal centers were prominent, with stainable macrophages, and were cuffed by uniform mantles of small lymphocytes. The paracortical zones were widened and contained a mixed infiltrate of small and immature lymphocytes, eosinophils, pale histiocytes, and scattered plasma cells. Occasional immunoblastic cells with marginated chromatin and prominent nucleoli were present in the paracortical zones. Vessels in the paracortical zones were somewhat increased in number, with swollen endothelium. Clusters of interdigitating reticulum cells with occasional melanized cells were present in paracortical zones. Medullary sinuses showed reticuloendothelial hyperplasia with lymphoid cells and occasional neutrophils in the spaces between histiocytes. Plasma cells were prominent in the medullary cords. The peripheral sinus was preserved with histiocytes and admixtures of lymphocytes, neutrophils, and eosinophils.

The lymph nodes from patients 1, 2, and 3 showed this basic pattern, but also contained rare foci of clustered histiocytes with enlarged nuclei and stippled chromatin. The lymph node in patient 3 also showed scattered areas of lymphocyte depletion, with a vascularized delicate fibrous matrix containing a sprinkling of lymphoid cells, many of which had elongated cleaved nuclei.

In patients 1 and 2, focal changes in paracortical zones qualified as a mild "dermatopathic" pattern, with hyperplasia of interdigitating reticulum cells. Special stains and cultures for acid-fast organisms and

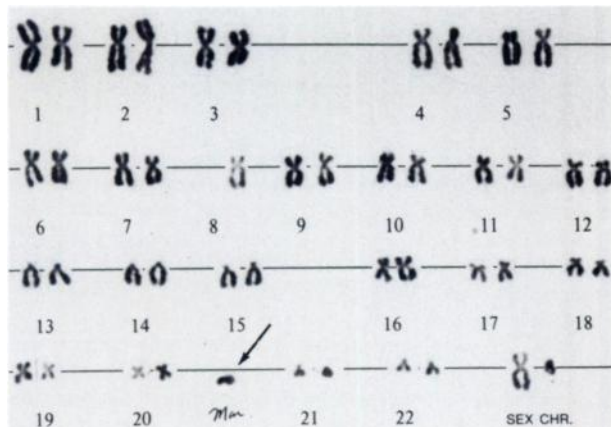


Fig 1. Karyotype prepared from lymph node mononuclear cells shows the existence of a tiny acrocentric chromosome (arrow) in a patient with hemophilia and lymph node enlargement.

bacteria were negative. Viral cultures were negative in two lymph nodes.

In patient 3, the nodal architecture was preserved, but small active germinal centers were uniformly distributed in the subcapsular zone. Sinusoids were preserved and medullary cords contained plasma cells. The paracortical zones were markedly expanded and contained a plexus of tortuous, thick-walled vessels with swollen endothelium. There was a sparse infiltrate of mature and immature lymphocytes, pale histiocytes, and eosinophils. Most of the lymphoid cells had irregular nuclear membranes and slightly open nuclear chromatin patterns. Scattered foci of cells with scanty basophilic cytoplasm, plump nuclei, marginated chromatin, and prominent nucleoli were seen in the paracortical regions. The sinusoids showed reticuloendothelial hyperplasia, with a few neutrophils and immature lymphoid cells.

The lymph node of patient 4 showed a preponderance of follicular lymphoid hyperplasia. The follicles were large, with prominent pale histiocytes and immature lymphoid cells. Small, scattered collections of epithelioid histiocytes with granulomatous qualities were present in the germinal centers. The histiocytes were relatively confined to the enlarged follicles and not in the paracortical zones. The paracortical zones were somewhat compressed between hyperplastic folli-

cles. Such zones showed a pleomorphic infiltrate with numerous immunoblasts. Most of the lymphoid cells had intermediate-sized nuclei with open and stippled chromatin patterns. There were scattered mitotic figures and plasma cells in the paracortical areas. The peripheral sinus was preserved and contained a loose infiltrate of small lymphoid cells. Sinusoids exhibited reticuloendothelial hyperplasia and contained mature and immature lymphoid cells.

Electron Microscopic Findings

Electron microscopy revealed slight variations from normal in the profiles of the endoplasmic reticulum in small and large lymphocytes. No viral particles or vesicular rosettes were found that could be considered identical to those previously reported in the lymphocytes in lymph nodes obtained from patients with AIDS.^{17,22}

Chromosomes

Chromosomes of the lymph node suspensions in patients 3 and 4 revealed certain abnormalities in several of the 25 spreads examined. Monosomy of chromosome 21 was found in 10% of the spreads in patient 3, but the peripheral blood chromosome studies were normal, 46,XY. Also, a tiny acrocentric marker chromosome (Fig 1) was identified in at least 20% of lymph node spreads in patient 4, whereas the remaining 80% of spreads had a normal chromosomal composition. This patient and his parents had normal blood chromosome composition. Chromosome analysis was not performed on the normal nodes.

Analysis of Lymph Node Mononuclear Cell Populations

Percentages of helper-inducer T lymphocytes were greatly decreased in nodes from hemophilic patients, whereas percentages of suppressor-cytotoxic cells were increased ($P < .05$) (Table 2). These changes resulted in significantly lower OKT4/OKT8 ratios of T cells from hemophilic nodes (1.2 ± 0.3) compared with normal nodes (6.1 ± 0.8). All OKT4/OKT8 ratios in hemophilic nodes were less than 2. Interestingly, there was a significant increase in the percentage of B

Table 2. Cell Surface Marker Studies in Lymph Nodes

	Mono	B	T11	T3	T4	T8	T4/T8	Ia	NK
Hemophilic (N = 4)	1.8 ± 0.5	38* ± 5	52 ± 8	51 ± 2	25* ± 4	23* ± 3	1.2* ± 0.3	27 ± 5	2.7 ± 1
Normal (N = 3)	1.2 ± 0.2	27 ± 1	47 ± 6	46 ± 7	40 ± 6	6.6 ± 1	6.1 ± 0.8	20 ± 6	2.6 ± 1

Data expressed as mean percent ± SE of the mononuclear cell population.

* $P < .05$ compared with normal lymph nodes.

lymphocytes in the nodes obtained from hemophilic patients.

Analysis of Peripheral Blood Mononuclear Cell Populations

Percentages of blood mononuclear cell populations are shown in Table 3. Significant decreases in the percentages and absolute numbers of helper/inducer (OKT4+) cells and increases in percentages and absolute numbers of suppressor/cytotoxic (OKT8+) cells, as well as increases in percentages and absolute numbers of cells bearing the natural killer phenotype (Leu-7), were noted in patients compared with matched controls. Changes in the T lymphocyte subpopulations resulted in significantly lower OKT4/OKT8 ratios ($P < .05$) in patients than in controls. All patients had OKT4/OKT8 ratios less than 1.0.

DISCUSSION

We have characterized certain aspects of the lymph nodes from four patients with classical hemophilia who developed chronic lymph node enlargement.^{2,3,12} Concurrent lymph node enlargement and abnormalities in T cell subpopulations have been reported in at least four patients with hemophilia at the time of recognition of AIDS.^{8,11,13} The present investigation, therefore, sought further information to compare the lymph node enlargement in hemophilic patients with such enlargement in other groups at risk for AIDS.

The location and duration of enlarged nodes in homosexual and hemophilic patients have been similar, with no predominance in a single lymph node-bearing area. In contrast to many patients with AIDS or malignant conditions, our four patients with hemophilia and lymph node enlargement did not have constitutional symptoms. Fluctuation in node sizes, but not progression, has been noted, which is similar to that in some homosexuals with lymph node enlargement.¹⁶ Our concern for their risk of AIDS or malignancy has mounted, as the lymph node enlargement in patients with hemophilia may persist for 12 or more months.¹² Similarly, a large group of homosexuals with

lymph node enlargement in San Francisco has now been followed for over two years with little change.¹⁶ However, homosexuals evaluated for lymph node changes in New York or Atlanta have developed AIDS, lymphoma, or Kaposi's sarcoma, even occasionally following disappearance of the nodes.^{14,18} Prospective studies will be necessary to better compare the prognosis associated with such enlargement in patients with various risk factors or in different geographic regions.

In the peripheral blood, our patients had decreased percentages and absolute numbers of T helper/inducer lymphocytes and increased percentages and numbers of suppressor/cytotoxic T lymphocytes, which resulted in lower than normal ratios of these populations. Single-cell preparations of lymph nodes had a similar reversal of these populations, a finding similar to studies in homosexuals.^{23,24} This suggests that changes in the T lymphocyte subpopulations and function may be taking place within the node and may affect immunoregulatory mechanisms for control of B cell proliferation. Such proliferation was reflected by enlargement of B cell-dependent regions of the node and increased percentages of mature B cells in lymph node suspensions. Fungal, viral, and bacterial cultures of the nodes did not establish the presence of a microbial agent, although in the future more extensive laboratory investigations must be applied in these efforts.

Histologic studies revealed that features of follicular and paracortical hyperplasia in our cases are similar to those reported in three hemophiliacs and in some homosexual patients from various institutions.^{2,12,15,18} In these studies and our own, the lymph nodes did not show a specific histologic pattern, but showed instead a nonspecific pattern of reactive lymphoid hyperplasia. The distribution of small granulomas containing epithelioid histiocytes in clusters was unusual and remains unexplained. Also, in the lymph nodes of three of our patients, histiocytes with a mild admixture of neutrophils were focally clustered. Clusters of this type are seen in some infectious diseases, such as cat scratch fever or toxoplasmosis. They were reported by Guarda et al, but were described as sinuses that were focally

Table 3. Cell Surface Marker Studies in Peripheral Blood (% Cells Positive*)

	Mono	Surface Ig	T11	T3	T4	T8	T4/T8	Ia	Leu-7
Hemophiliacs (N = 4)	17 ± 5.9	15 ± 6.0	63 ± 8.4	62 ± 6.0	25 ± 4.3†	39 ± 1.6†	0.65 ± 0.1†	32 ± 4.2	15 ± 4.0†
Controls (N = 10)	15 ± 4.5	12 ± 3.0	70 ± 0.7	57 ± 2.7	38 ± 2.6	19 ± 1.7	2.1 ± 0.2	25 ± 3.0	5 ± 0.9

*Data are expressed as mean ± 1 SD of the mononuclear cell population. Total mononuclear counts (cells/μL) were 2,329 ± 160 for the hemophiliac group and 2,228 ± 153 for the control group.

† $P < .05$ compared with control values.

packed with round histiocytes.¹⁵ We could not relate the clusters to preexisting sinusoids. However, admixtures of histiocytes and immature lymphoid cells were focally prominent in the peripheral sinuses of several cases. No example of nodal effacement and vascular proliferation, as previously reported, was seen in this small series.¹⁸ Our patients have not had repeat biopsy of their lymph nodes, as all have remained well. By electron microscopy, no "vesicular rosettes" were found.^{17,22} Although we suspect that a chronic viral infection may be causing the changes noted in patients with hemophilia, viral hepatitis would not seem to be a likely cause. Various forms of hepatitis have been known to be present in these patients for a number of years, whereas the development of AIDS and lymph node enlargement is more recent.^{2,3,12}

Chromosome aberrations were identified in some of the lymph node cells of both patients 3 and 4. The significance of these cytogenetic alterations in relation to lymphadenopathy is not clear yet. However, the normal peripheral blood chromosome composition of both patients indicates that the chromosomal changes identified in their lymph nodes are very likely "ac-

quired" instead of "inborn." It is of interest that chromosomal abnormalities have also been found by Han et al in prison-acquired lymphoproliferative syndrome.²⁵

To date, lymph node enlargement in hemophiliacs has not been documented to undergo malignant transformation, although the development of Burkitt's lymphoma has been reported recently.²⁶ Since most biopsies have not revealed specific causes, an initially conservative approach to the biopsy of enlarged lymph nodes in patients with hemophilia is warranted.² Histopathologic features, abnormal helper-inducer/suppressor T lymphocyte ratios in the blood, and persistent but fluctuating lymph node enlargement all suggest a close similarity to the changes noted in homosexual patients. Perhaps further studies of the lymph nodes, especially microbiologic investigations, may be more informative than those studies using cells from peripheral blood. Careful follow-up of such cases, with continued search for an etiologic agent(s) or mechanism causing immunodeficiency and lymph node enlargement, would all seem to be important concerns for the near future.

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