A Longitudinal Immunologic Evaluation of Hemophiliac Patients

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Over an average span of one year, we performed a prospective clinical and immunologic evaluation of 30 patients with hemophilia. No patient developed life-threatening opportunistic infection or malignancy; however, the immunologic abnormalities and lymphadenopathy initially present in nine patients (lymphadenopathy group) persisted. In addition, five patients, representing 24% of the initial group without lymphadenopathy, developed generalized lymphadenopathy (converter group). One episode of idiopathic thrombocytopenia (ITP) and one episode of staphylococcal sepsis occurred in this converter group; one episode of ITP also occurred in the lymphadenopathy group. Sixteen patients remained asymptomatic. At the time of the follow-up evaluation, those differences in mononuclear cell (MNC) percentages and numbers noted initially among the three hemophilic groups were no longer present. Natural killer cell function alone or in the presence of biologic response modifiers was not different among hemophilic and control groups. Before developing lymphadenopathy, the converter group of patients had significantly better lymphocyte mitogenic function than did the other two groups of patients with hemophilia. However, lymphocyte mitogenic responses of all groups of patients with hemophilia significantly deteriorated over the course of the study. The abnormal mitogenic responses noted in these patients was explained in part by higher levels of spontaneous suppressor cell activity in mononuclear cell preparations from patients with hemophilia. We conclude that long-term immunologic studies of this patient population requires both quantitative and qualitative evaluations. Our data show that patients with hemophilia have progressive dysfunction of cell-mediated immunity.

Twenty-five healthy age- and sex-matched volunteers, with no history of recent infection, drug abuse, or homosexuality, served as concurrent controls.

Immunologic Evaluations

Characterization of proliferation of mononuclear cells. Peripheral blood mononuclear cell (MNC) populations were isolated and enumerated as previously reported. In brief MNCs were isolated from heparinized fresh whole blood over Hypaque-Ficoll gradients. MNC populations and subpopulations were enumerated and reported as the percentage of total MNCs recovered. T lymphocyte populations and subpopulations were identified using OKT11 (E rosette-reactive T lymphocytes), OKT4 (T helper/inducer cells), and OKT8 (T suppressor/cytotoxic) monoclonal antibodies (Ortho Pharmaceutical Co, Raritan, NJ). Cells bearing the Ia-matrix (HLA-DR) and natural killer cell-associated antigens (Leu 7, Leu 11a) were also identified with monoclonal antibodies (Becton Dickinson, Mountain View, Calif). B lymphocytes were detected using the B1 monoclonal antibody (Coulter Immunology, Hialeah, Fla). Cell enumeration with monoclonal antibodies was performed by direct immunofluorescence with analysis on a FACS III fluorescence-activated cell sorter (Becton Dickinson). Each analysis consisted of 25,000 cells using the 488-nm laser line with a gain setting of scatter: 4 × 5, and fluorescence: 2 × 1 at 500 mV. The percentage of positive cells was calculated after correction for background staining, with daily results confirmed by fluorescence microscopy. The absolute numbers of these various MNC populations were

MATERIALS AND METHODS

Study Subjects

Patients were evaluated on at least two occasions at least six months apart while attending the Louisiana Comprehensive Hemophilia Care Center in New Orleans. The group included 30 individuals with classical (factor VIII-deficient) hemophilia, ranging in age from 7 to 70 years. Twenty-five patients had severe (≥1%), 2 had moderate (2% to 5%), and 3 had mild (5% to 18%) factor VIII deficiency. Seventeen of these patients had persistent generalized lymphadenopathy (PGL), manifested by unexplained lymphadenopathy of at least three months duration with nodes ≥1.5 cm in diameter at two or more extralymphatic sites. None of these patients had a recent detectable illness, and no patients had a history of drug abuse or homosexuality.

Lympholized factor concentrates used by patients came from at least four manufacturers and from several lots. Data for calculation of factor usage were available for at least three years. There was a wide variation in the amount of factor VIII used (expressed as units of factor VIII infused/kg/yr). Five patients had also received some cryoprecipitate in the last three years.

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obtained by multiplying the percentage of MNCs comprising that specific population by the total MNC count (calculated from peripheral blood leukocyte count and differential on Wright's-stained smears) and were expressed as cells/μL. Monocytes in the MNC populations were identified by staining with α-naphthyl-butyrate esterase (Technicon, Tarrytown, NY).

Lymphocyte proliferation induced by the T cell mitogen, phytohemagglutinin (PHA), was evaluated across a dose–response range of 1 to 50 μg/mL of PHA. MNCs (1 x 10^6 per well) were cultured in round-bottom microculture plates (Microtiter Plates, Dynatech, Alexandria, Va) with RPMI 1640 media containing 10% heat-inactivated pooled human serum and antibiotic agents ("enriched media"). Cells were cultured for four days, the time of maximum response in our system. These cultures were harvested using an automated cell harvester 16 hours after the addition of 1 μCi per well of tritiated thymidine (Amersham Corp. Arlington Heights, Ill). Radiolabeled nucleotide was assayed in a liquid scintillation counter and was reported in all mitogenic studies as Δ cpm (Δcpm), where Δ cpm equals cpm from cell cultures stimulated with PHA minus cpm from similar, but unstimulated, cultures.

**Assay for spontaneous suppressor cell activity.** To measure "spontaneous" suppressor cell activity, fresh "responder cells" (RC) for normal subjects (50,000 cells per well) were cocultured with equal numbers of putative "spontaneous suppressor cells" (SC) from unrelated healthy controls or subjects with hemophilia using a published method.4 These putative suppressor cells were pretreated with mitomycin-C (Sigma Chemical Co., St Louis) at a concentration of 100 μg/mL for 30 minutes at 37 °C. Treated cells were then washed and assessed for viability by trypan blue dye exclusion with a mean viability of ≥ 90%. Cocultures of responder cells and putative suppressor cells were stimulated with either a suboptimal (5 μg/mL) or optimal (20 μg/mL) dose of PHA for 96 hours. Sixteen hours before harvest, tritiated thymidine was added as described earlier. Percentage of suppression of response to PHA was calculated by the following formula: Δ = (exp cpm - spontaneous cpm)/spontaneous cpm x 100.

Statistical Methods

Statistical analysis was performed using Student's t test adjusted for several comparisons (paired and unpaired).4 PHA dose–response curves were analyzed by analysis of variance with repeated measures and Tukey's paired comparison procedures.4 Correlation coefficients were calculated using a published method.5

RESULTS

**Clinical Data on Patients in This Study**

Thirty patients with hemophilia were studied on at least two occasions between six and 21 months apart (median 12.8 months). Sixteen patients had no lymphadenopathy when initially evaluated and remained asymptomatic throughout the study (Table 1). Nine patients with generalized lymphadenopathy which persisted were seen. Five patients, initially asymptomatic without lymphadenopathy, developed PGL by the time of their last evaluation 11 to 16 months later. These individuals are listed in Table 1 as "converters."

During the period of observation in this study, several important clinical events occurred. Patient 32 required a tonsilectomy and adenoidecctomy for sleep-apnea associated with marked tonsillar enlargement and lymphadenopathy. Patient 09, on long-term home therapy with factor VIII concentrate, developed persistent staphylococcal sepsis and a

| Table 1. Clinical Data on Patients With Hemophilia in This Study |
|-----------------|-----------------|-----------------|
| Patient No.    | Age (yr)        | Factor VIII Usage (U/kg/yr)* |
| 01              | 22              | 2695            |
| 02              | 9               | 835             |
| 03              | 11              | 4000            |
| 04              | 15              | 581             |
| 05              | 23              | 2900            |
| 06              | 29              | 138             |
| 07              | 27              | 2400            |
| 08              | 59              | 516             |
| 12              | 35              | 698             |
| 13              | 36              | 600             |
| 14              | 33              | 1761            |
| 15              | 43              | 1639            |
| 16              | 70              | 475             |
| 18              | 45              | 10              |
| 22              | 23              | 50              |
| 23              | 29              | 0               |
| 24              | 40              | 363             |
| 33              | 12              | 20              |
| 34              | 41              | 1256            |
| 35              | 42              | 1294            |
| 37              | 25              | 1754            |
| 39              | 36              | 709             |
| Lymphadenopathy |                 |                 |
| 01              | 22              | 2695            |
| 02              | 9               | 835             |
| 03              | 11              | 4000            |
| 04              | 15              | 581             |
| 05              | 23              | 2900            |
| 06              | 29              | 138             |
| 07              | 27              | 2400            |
| 08              | 59              | 516             |
| 12              | 35              | 698             |
| 13              | 36              | 600             |
| 14              | 33              | 1761            |
| 15              | 43              | 1639            |
| 16              | 70              | 475             |
| 18              | 45              | 10              |
| 22              | 23              | 50              |
| 23              | 29              | 0               |
| 24              | 40              | 363             |
| 33              | 12              | 20              |
| 34              | 41              | 1256            |
| 35              | 42              | 1294            |
| 37              | 25              | 1754            |
| 39              | 36              | 709             |
| Converters     |                 |                 |
| 09              | 26              | 1260            |
| 11              | 22              | 1275            |
| 17              | 16              | 42              |
| 20              | 17              | 2979            |
| 21              | 16              | 145             |

*Expressed as units per kilogram of body weight per year calculated from usage data over at least three years.

†Time in months that persistent generalized lymphadenopathy (PGL) has been presented.
subsequent large biceps abscess requiring surgery. No source was found for the staphylococcal sepsis. Patients 03 and 20 developed idiopathic thrombocytopenic purpura (ITP), each demonstrating a precipitous fall in their platelet count after the onset of lymphadenopathy.

Thrombocytopenia was not associated with clinical events such as viral illness or vaccination, and reached life-threatening levels of 10 to 50,000 platelets µL. Corticosteroid therapy resulted in some improvement in platelet count and simultaneous partial resolution of their lymphadenopathy; and lymph nodes enlarged when steroids were discontinued. Immuneologic studies in these patients were carried out several months before and after corticosteroid therapy. The clinical course of these patients will be the subject of a separate report.

Four patients (patient numbers 01, 03, 06, and 19) with PGL underwent lymph node biopsy. All lymph nodes were classified histopathologically as benign reactive hyperplasia by previously published criteria.10

**MNC Populations in Hemophiliac Patients.** MNC populations and subpopulations in patients with hemophilia and controls are shown in Table 2. Statistical comparisons were made (1) between patient values and similar values for controls, (2) among patient groups, (3) and between initial and follow-up determinations on the same patient groups. Although data are expressed as the percentage of total MNC in Table 2 and in subsequent figures, similar comparisons were made with the data expressed as absolute numbers. Thus, the total MNC numbers are given for reference. Because the conclusions were the same, only the percentage data are shown.

When compared with control values, percentages of helper/inducer (T4+) cells were significantly diminished in all patient groups (P ≤ .05) throughout the study, which also resulted in lower helper/suppressor (T4/T8) ratios. These decreases in ratios reached significance (P ≤ .05) in patients with and without lymphadenopathy, but not in the converters. Percentages of E rosette receptor positive T cells (T11+) were usually lower and percentages of suppressor/cytotoxic (T8+) and natural killer (Leu 7+) lymphocytes were higher in patient groups as compared with control values. However, significant differences (P ≤ .05) between patient and control values of these cell populations occurred only in the percentages of T8+ cells in the patients with lymphadenopathy, and then only on the first evaluation.

There were few significant differences in MNC percentages and ratios among the three patient groups. Initial T4–T8 ratios of patients with lymphadenopathy were significantly lower than those of patients without lymphadenopathy. Although these same ratios remained lower in the lymphadenopathy group on the follow-up evaluation, differences among groups were no longer significant.

Individual changes in T4–T8 lymphocyte ratios which occurred between the initial and follow-up evaluations are shown in Fig 1. These ratios reflect changes in the percentages of the T lymphocyte subpopulations that occurred during this interval. Ratios of T4–T8 cells in the lymphadenopathy group were significantly greater on the second evaluation when compared to the first. This improvement reflected a decrease (P ≤ .05) in the percentage of T8+ T cells between the first and second evaluations in that group (Table 2). Except for a decrease in the percentage of monocytes in patients without lymphadenopathy, no other significant changes were noted between initial and follow-up values in any group including the converters.

**Lymphocyte Proliferative Responses.** Lymphocyte proliferative responses to the mitogen PHA among patient groups and controls are shown in Fig 2. On initial evaluation, the dose–response curves of patients with hemophilia with or without lymphadenopathy (excluding the converters) were lower (P ≤ .05) than the responses of control individuals. Patients with hemophilia with lymphadenopathy had the lowest blastogenic responses, although their dose–response curves were not significantly different from subjects with hemophilia who had no lymph node involvement. Those individuals who subsequently developed lymphadenopathy (converters), initially demonstrated lymphocyte proliferative responses that were not different from those of control subjects.

At follow-up evaluation, the mitogenic responses of all hemophilic groups were lower (P ≤ .05) than were control responses. Those individuals with long-standing lymphadenopathy had the lowest responses, whereas those with newly developed lymphadenopathy had the least abnormal proliferation. Patients without lymphadenopathy had intermediate responses. However, there were no significant (P ≤ .05)
differences among the follow-up dose–response curves of patient groups.

When the peak response to PHA was analyzed (Fig 3), the lymphocyte responses of subjects with hemophilia were lower ($P < .005$) at the time of follow-up than at initial evaluation. No significant differences were observed between mean lymphocyte proliferative responses of controls studied on two occasions six months or more apart (data not shown).

Effect of Biologic Response Modifiers on Natural Killer Cell Activity. MNC populations from nine control subjects, 9 patients with hemophilia without lymphadenopathy, and 8 patients with hemophilia with lymphadenopathy were evaluated for natural killer cell activity. Studies were performed immediately after isolation of MNCs or after culture with the following biologic response modifiers (BRM): γIFN, αIFN, or IL-2. Natural killer cell activity among patients and control subjects was not different and was significantly increased by exposure to any of the BRM (Fig 4). Values obtained after incubation with BRM were not different among the study groups or control subjects.

Spontaneous Suppressor Cell Activity. Coculture of control mitomycin-C–treated MNCs (putative suppressor cells) with homologous control responder cells resulted in decreased mitogenic responses to PHA. At 5 μg of PHA/mL, these proliferative responses were decreased by 35% ± 8% and at 20 μg of PHA/mL by 37% ± 8% (mean ± SEM, Table 3). Levels of similar spontaneous suppression were significantly greater in all cultures in which patient cells were used as putative suppressors than in those in which control cells were used similarly. Levels of suppression between patients with lymphadenopathy and those without were not different. Percentage values reflected a consistent suppression of proliferation and not merely a large degree of suppression generated by cells from a few patients. For instance, at the dose of 5 μg of PHA/mL, 12/17 control subjects, 12/12 subjects with hemophilia without lymphadenopathy, and 9/9 subjects with hemophilia with lymphadenopathy had demonstrable suppression. Ten of the 12 subjects with hemophilia without lymphadenopathy and 7/9 subjects with hemophilia with lymphadenopathy had suppression greater than mean control values of suppression at the same PHA doses.

DISCUSSION

The data presented in this study were obtained while monitoring the clinical course of 30 patients with hemophilia who also have a variety of immunologic abnormalities.
Patients in the lymphadenopathy group have remained stable, whereas 24% of patients without lymphadenopathy subsequently have developed lymph node involvement. Several of these individuals, termed “converters,” have developed symptom complexes seen in homosexual patients with AIDS, including one case of ITP. It is curious that the only immunologic finding which separated this group of patients from other subjects with hemophilia prior to their development of lymphadenopathy was their normal lymphocyte proliferative responses to PHA. This finding is difficult to explain as their MNC preparations contained decreased percentages and numbers of OKT4+ lymphocytes, the primary lymphocyte population responsive to PHA. Percentages and numbers of these cells were similar to those of the other groups of subjects with hemophilia. Perhaps SC activity was lower in the group initially, although this possibility was not addressed in our study.

Abnormalities of MNC populations in patients with hemophilia, as noted in our previous study, have been confirmed in this larger prospective study. No consistent changes in the abnormal distributions of these cell populations were noted during this study, and the abnormalities noted both initially and at follow-up were similar among patient groups whether lymphadenopathy was present or not. Although abnormalities of T lymphocyte subpopulations remained relatively constant, T lymphocyte function did not. Lymphocyte proliferative responses deteriorated in all three patient groups between the times of initial and follow-up evaluations. It is unlikely that this finding occurred on the basis of trivial explanations such as technical differences between initial and follow-up studies, as this possibility was carefully considered and controlled for in the study design. The finding may be explained, at least in part, by high levels of active SC activity present in patient MNC populations, levels twice that seen in cultures of cells from normal subjects evaluated in this and other studies. The cell type(s) responsible for this activity has not been conclusively determined, but preliminary studies in our laboratory suggest that both monocytes and lymphocytes are necessary for its expression.

Natural killer cell activity has been noted to be decreased in patients with AIDS. Because natural killer cell activity has been associated with host defense against neoplasia, the possibility that abnormalities of this cell population are reflected in the high incidence of Kaposi’s sarcoma in homosexual patients with AIDS has been considered. Numbers of cells with the natural killer cell-associated antigen, Leu 7, have consistently been found to be increased in our patients, perhaps reflecting the cross-specificity of this reagent for some OKT8+ T lymphocytes, a population of cells also increased in our patients. Numbers of cells reactive with Leu 11a, a monoclonal antibody not cross-reactive with T lymphocytes, have been normal in our patients. Furthermore, as shown here, unstimulated NK cell function as well as NK cell function boosted by BRM was normal in our patients. These findings may explain why Kaposi’s sarcoma and other malignancies have not been reported in patients with hemophilia and AIDS.

Finally, the data presented here emphasize the necessity for continued close clinical and immunologic monitoring of patients with hemophilia who are receiving factor concentrate therapy. Preliminary studies in our laboratory suggest that factor VIII concentrates may directly suppress the
proliferation of normal lymphocytes. The progressive
decrease in lymphocyte proliferative function noted in our
patients followed for more than one year is of special concern
and indicates that evaluation of T cell phenotype without
functional assessment may not accurately reflect the immu-
nologic status of these individuals. The clinical significance
of these immunologic abnormalities should be clarified by
further prospective studies. Studies in progress include those
assessing the relationship of HTLV III infection, likely to be
detectable in most of these patients, to the various immuno-
logic abnormalities noted. These studies should provide

information useful in making decisions concerning alternate
forms of therapy and the potential use of BRM in patients
with hemophilia and in immunologically compromised patients.

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