CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Mar. 1995, p. 205–208 Vol. 2, No. 2
1071-412X/95/$04.00+0

Flow Cytometric Analyses of the Lymphocyte Subsets in Peripheral Blood of Children with Untreated Active Juvenile Dermatomyositis

MAURICE R. G. O'GORMAN,* VIRGINIA CORROCHANO, JEFFREY ROLECK, MARK DONOVAN, AND LAUREN M. PACHMAN

Department of Pediatrics, Northwestern University Medical School, Chicago, Illinois 60614

Received 28 June 1994/Returned for modification 23 August 1994/Accepted 20 October 1994

Juvenile dermatomyositis (JDMS) is a vasculopathy affecting primarily skin and muscle. Although the etiology is unknown, immunopathogenetic mechanisms appear to play a role in both the susceptibility to the disease and its progression. We measured the percentage and absolute numbers of B cells and T-cell subsets in the peripheral blood of untreated JDMS patients with active early disease and compared the results with those obtained from a study of peripheral blood obtained from a healthy age-related control group. The absolute number of total lymphocytes in the peripheral blood of the JDMS patients was significantly lower ($P < 0.002$) than that observed in the healthy control population, with an associated decrease in the absolute number of all T-cell subsets. No concomitant decrease in the absolute number of B lymphocytes was observed in the JDMS patients. In contrast, the percentage of B lymphocytes and the T-helper/T-suppressor cell ratio were significantly higher in the JDMS group than in the control group ($P < 0.001$ and $P < 0.002$, respectively). Retrospective analysis of JDMS patients' serum samples obtained within 1 month of the flow cytometric evaluation indicated that 79% of the sera contained an antinuclear antibody and 46% had immunoglobulin G values above age-adjusted reference ranges. The increased percentage of B cells, the increased T-helper/T-suppressor cell ratio, the positive antinuclear antibody results, and the increased concentration of serum immunoglobulin suggest that humoral immune dysregulation may contribute to the pathogenesis of JDMS.

Juvenile dermatomyositis (JDMS) is a chronic multisystem inflammatory disease that primarily affects the small vasculature of skin and muscle. The diagnostic criteria for definite JDMS established by Bohan and Peter (2) relies on the child having three of the four criteria listed below for muscle involvement in addition to the characteristic cutaneous findings. The rash is the hallmark of the disease and includes violaceous periorbital erythema, with or without edema; Gottron's papules (erythema which may be accompanied by induration over the knuckles of hands); marked erythema of the "shawl" area; and erythema over the flexor surfaces of the knees, elbows, and ankles. The other criteria are (i) symmetrical proximal muscle weakness, (ii) elevated levels of muscle-derived enzymes in serum, (iii) an electromyogram consistent with an inflammatory myopathy, and (iv) muscle biopsy findings typical of JDMS, which include perifascicular atrophy, round-cell infiltration, and capillary occlusion. In addition, affected children have been noted to have decreased gastrointestinal absorption, gastrointestinal ulcerations, cardiac conduction abnormalities, retinal vessel thrombosis, and soft tissue calcification (18).

Epidemiologic data indicate that myositis (including JDMS and polymyositis) is a rare disease affecting 3.2 whites and 7.7 blacks per 10^6. In the United States, myositis affects two females for every male, and there is a bimodal age of onset, with peaks occurring at 5 to 14 and 54 to 64 years (15). Dermatomyositis is approximately 10 to 20 times more frequent than polymyositis in children (8). The etiology and the sequence of events in the pathogenesis of JDMS are undefined. One speculation is that genetic factors control the immune response to an environmental and/or viral stimulus, resulting in expression of disease. Evidence of a viral "trigger" includes the observation of viruslike particles by electron microscopic examination of muscle biopsy specimens (1), increased antibody titers to coxsackie enterovirus strains B2 and B4 (although no increase in antibody titers was detected against 16 other viral antigens [5]), isolation of coxsackie B virus RNA from muscle biopsy specimens of JDMS patients (3), and a seasonal clustering of disease onset (18). In the United States, other groups in addition to our own (20) have examined pediatric and adult human muscle from patients with either polymyositis or dermatomyositis for evidence of persistent viral genomic material and failed to identify positive specimens (14).

Other evidence suggests that genetically susceptible individuals produce autoantibodies which may be associated with the destruction of the tissue. The increased prevalence of antinuclear antibodies (ANAs) in JDMS patients supports this hypothesis (17, 19). In addition, the JDMS population has increased levels of human leukocyte antigens (HLA) B8 and DR3, which have also been associated with autoimmune diseases such as type I diabetes mellitus, Graves' disease, and myasthenia gravis (7, 21). An association between major histocompatibility class II alleles and JDMS has recently been reported (22) and is further evidence that the immune system is involved in the pathogenesis of the disease.

A combination of the foregoing hypotheses may be proffered: a virus initiates a pathological response, and in the genetically susceptible individual autoimmune sequelae result in the ongoing pathogenesis of JDMS.

Increased immunoglobulin G (IgG) levels, increased B cells, and increased CD4/CD8 ratios have been observed in systemic lupus erythematosus (SLE), diabetes mellitus, and Sjögren's syndrome (9, 10, 13). Abnormal immune regulation has been postulated as a mechanism of disease pathogenesis in each of

* Corresponding author. Mailing address: The Children's Memorial Hospital, 2300 Children's Plaza, Chicago, IL 60614. Phone: (312) 880-3070. Fax: (312) 880-3739. Electronic mail address: mogorman@anima.nums.nwu.edu.

these diseases. The purpose of this investigation was to characterize the peripheral blood lymphocyte subsets in untreated active JDMS patients and to compare the results with those obtained in an age-related healthy control group.

MATERIALS AND METHODS

JDMS patient group. Children who fulfilled the criteria of Bohan and Peter (2), had active disease (described below), and had not received immunosuppressive or immunomodulatory therapy for at lease 1 year were included in this study. Active disease was defined as elevated levels of muscle-derived enzymes in serum, documented symmetrical proximal muscle weakness, and various degrees of cutaneous involvement. The project was approved by the IRB committee at the hospital, and informed consent was obtained from both the patients and the control subjects.

Healthy pediatric control group. Peripheral blood was obtained from children (<18 years of age) attending local clinics for "well visit" follow-up with the assistance of the Pediatric Practice Research Group. The latter group is an affiliate of the Children's Memorial Hospital and has established a relationship between Children's Memorial Hospital and many local pediatric private practices for the purpose of conducting research.

MAbs. Samples were analyzed on either a Coulter Profile flow cytometer with monoclonal antibodies (MAbs) obtained from Coulter Cytometry (Hialeah, Fla.) or a Becton Dickinson FACScan flow cytometer, using Becton Dickinson (Mountain View, Calif.) reagents. The two- and three-color combinations of antibodies included CD8-FITC/CD4-PE/CD3-ECD, CD5-FITC/CD19-PE/CD3-ECD, CD3-FITC/CD4-PE, CD3-FITC/CD8-PE, CD3-FITC/CD19-PE, the lymphocyte gating reagent CD45-FITC/CD14-PE, and the isotype control tubes IgG1-FITC/IgG2-PE/IgG1-ECD and IgG2-FITC/IgG1-PE, where FITC is fluorescein isothiocyanate, PE is phycoerythrin, and ECD is energy-coupled dye. When a sample had a particular subset measured more than once, the mean value was reported. Comparisons of samples processed and analyzed by either method did not indicate any significant differences.

Sample preparation. All samples were prepared with standard whole-blood lysing methodology as specified by the manufacturers. Briefly, 100 μl of EDTA-anticoagulated whole blood was added to tubes containing the appropriate combination of MAbs. After a 15-min incubation period, the erythrocytes were lysed and the samples were washed and fixed in a 1% paraformaldehyde solution.

Flow cytometric analysis. Both the Coulter Profile and the Becton Dickinson FACScan flow cytometers contain 15-mW air-cooled argon ion lasers with emission maxima of approximately 488 nm. The light filters and dichroic mirrors were set up in the Profile to detect FITC at 525 nm, PE at 575 nm, and ECD at 615 nm, and the FACScan contains dichroic mirrors and filter arrangements which are factory installed to detect each of these wavelengths. The overlap in fluorescence emission between fluorochromes was corrected electronically prior to analysis.

The first tube in each panel contained cells stained with the MAbs CD45 and CD14 (lymphocyte gating reagent) and was used to set an electronic analysis gate on the lymphocyte cluster. The lymphocyte gate was optimized by using both scatter and fluorescence parameters to obtain maximum purity and recovery of the cells expressing $CD45^{bright}$ $CD14^{neg}$. The lymphocytes in all subsequent tubes were analyzed within the same electronic gate on the basis of light scatter (lymphocyte gate), and the percent positive fluorescence of each subsequent lymphocyte subset was measured and corrected to 100% (on the basis of the purity of lymphocytes in the first tube). Discrimination between positive and negative fluorescence was based on the level of fluorescence generated by a non-lymphocyte-specific MAb (isotype control). Integration cursors were set such that more than 95% of the events in the isotype control tube were negative. In subsequent tubes events which fell above and/or to the right of the cursors were considered positive for the marker analyzed. Spectral overlap between fluorochromes (compensation) was checked daily, using the CD4/CD8/CD3 tube. Results were reported as percentages of lymphocytes positive for the particular surface marker(s) of interest.

Absolute numbers of lymphocyte subsets. An automated complete blood count and differential were obtained on a Max M (Coulter) hematology instrument in strict accordance with the manufacturer's protocol. The absolute lymphocyte count was measured as the product of the leukocyte count and the percentage of lymphocytes in the differential. Absolute lymphocyte subsets were calculated as the product of the absolute lymphocyte count and the percentage of the particular lymphocyte subset of interest (e.g., %CD19 × absolute lymphocyte count = absolute number of B cells per mm^3).

Fluorescent ANA assay. The fluorescent ANA assay was performed with the Kallestad Quata Fluor Test Kit (Sanofi Pasteur Diagnostics Inc., Chaska, Minn.) according to the manufacturer's recommendations. This kit employs a HEp-2 substrate. Reactivity in patients' sera is measured as both a nuclear staining pattern and a titer of fluorescence. The control group from which the flow cytometry results were obtained did not have a fluorescent ANA assay performed on their sera. Patient results were considered positive if the endpoint titer was greater than or equal to 1:80.

Quantitative serum immunoglobulins. Serum immunoglobulin results were obtained only from those patients who had a previous result or a frozen serum sample corresponding to the time at which the flow cytometric analysis was performed (±1 month). IgG, IgA, and IgM levels were measured by rate nephelometry on a Beckman Array 360 (Brea, Calif.), using Beckman reagents, and the procedure was performed in strict compliance with the manufacturer's recommendations.

Statistics. Comparisons of absolute lymphocyte counts and percentages were made by subtracting the group means of the control subjects from the JDMS group mean values. Unpaired *t* tests were used, using pooled- and separate-variance estimates as needed, determined by a significant F-test for the ratio of group variances at the 5% level of significance. Satterthwaite approximation was used to compute the degrees of freedom for the separate-variance model. All comparisons were made to detect differences in both directions (two-tailed). Statistical analyses on the immunophenotyping data of the lymphocyte subsets were performed on the mean differences between groups, i.e., JDMS group value − control group value. The Fisher exact test was used to assess relationships between groups and categorical variables.

RESULTS

Patients and controls. From September 1992 until January 1994, a total of 15 new untreated JDMS patients with active disease were seen in our clinic and had peripheral blood drawn and analyzed by flow cytometry. There were 12 females and 3 males, and the average age was 89.1 months, with a range of 31 to 168 months. During this 2-year period, we obtained peripheral blood samples from 6 female and 14 male healthy pediatric volunteers whose average age was 107 months, with a range of 55 to 171 months. No significant age difference between the groups was observed. Although the difference between the groups with respect to sex was significant ($P < 0.002$), a recent study reported no significant difference between male and female individuals with respect to the lymphocyte subsets measured (6). Both sexes were therefore combined within each group for lymphocyte subset analyses.

Flow cytometric analysis of lymphocyte subsets. Results of flow cytometric analyses are summarized in Fig. 1. All results are expressed as means ± standard errors of the means.

(i) Absolute numbers in the JDMS group compared with those in the healthy control group. The total absolute lymphocyte count obtained from the untreated JDMS group was significantly lower than the absolute number obtained from the healthy control population (1,915 ± 219 versus 2,835 ± 187 lymphocytes per mm^3, respectively; $P < 0.002$). Despite the lower total absolute lymphocyte count in the JDMS population, no corresponding decrease in the absolute number of B lymphocytes ($CD19^+$) was observed ($P = 0.30$). The absolute number of all other peripheral blood lymphocyte subsets measured was significantly lower in the JDMS group than the number observed in the healthy control group: i.e., $CD3^+$, $P < 0.001$; $CD3^+$ $CD4^+$, $P < 0.05$; $CD3^+$ $CD8^+$, $P < 0.001$.

(ii) Percentages of lymphocyte subsets in the JDMS group compared with those in the healthy control group. The relative percentage of lymphocytes expressing CD19 averaged approximately 60% higher in the peripheral blood of the JDMS group than in that of the control subjects (28.7% ± 1.6% versus 16.0% ± 0.9%, respectively; $P < 0.001$). The percentage of $CD4^+$ lymphocytes (T-helper cells) was significantly higher ($P < 0.02$) and the percentage of $CD8^+$ T cells (T-cytotoxic or T-suppressor cells) was significantly lower ($P < 0.002$) in the JDMS group than the percentages of each of the corresponding subsets measured in the healthy control group. The increase in T-helper cells and the decrease in T-suppressor or T-cytotoxic cells in the JDMS patients resulted in a T-helper/T-suppressor ratio significantly higher than the ratio observed in the healthy control group ($P < 0.002$).

ANA results and serum IgG, IgM, and IgA levels. The determinations of the ANA pattern and titer, as well as the IgG, IgM, and IgA levels, were made on serum which had been stored from ±1 month of the date of the flow cytometric

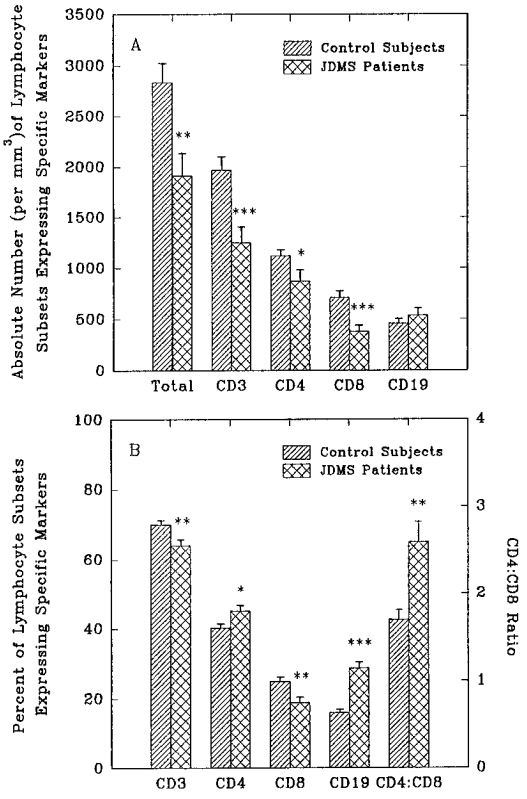

FIG. 1. Results of lymphocyte subset analyses. (A) Mean ± standard error of the mean of each lymphocyte subset represented as the absolute number of the respective subset per cubic millimeter. (B) Analysis as percentage of lymphocytes expressing the particular markers of interest. Level of significance for comparisons between groups are as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

analysis. There was no serum from the control population stored; therefore, a direct comparison of the ANA and serum IgG results from the control group with those from this JDMS group cannot be made. Historically, 4% of healthy control pediatric patients have had a positive ANA in our laboratory (12). Serum samples from 14 JDMS patients in this study were tested for the presence of ANAs. Eleven of 14 samples (79%) were positive at greater than a 1:80 dilution, nine patients (64%) had autoantibodies in their sera which generated a speckled pattern, and two patients' sera (14%) generated homogeneous patterns. Thirteen serum samples were available for the quantization of IgG levels. Six patients (46%) had IgG levels above our age-adjusted 95% confidence limits for normal, one had elevated IgA levels (8%), and none of the patients had elevated IgM levels.

DISCUSSION

We have observed significant abnormalities in both the relative percentages and absolute numbers of lymphocyte subsets in the peripheral blood of untreated JDMS patients. The total absolute lymphocyte count was significantly lower in the JDMS group than that observed in a healthy age-related control population. The lower total count in the JDMS group was reflected in a significantly lower absolute number of T cells and T-cell subsets. The absolute number of B cells, however, was not significantly lower than that observed in the control group. The analysis of lymphocyte subsets can also be expressed as a relative percentage of all lymphocytes. Expressed in this manner, our results clearly indicate that the B-lymphocyte subset in the JDMS group makes up a significantly greater percentage of the total lymphocytes than that observed in the healthy control group. Additionally, there were a higher percentage of CD4-positive lymphocytes and a lower percentage of CD8 lymphocytes, generating a significantly higher T-helper/T-suppressor cell ratio. Miller et al. (16) measured the percentages of various lymphocyte subsets in adult patients with various idiopathic myopathies. Similar to our observation, the group of patients with dermatomyositis had an elevated proportion of B cells ($CD20^+$) in the peripheral blood compared with both the healthy control group and the other myopathy group. In another report, the investigators observed a reduced number of CD8-positive lymphocytes in active JDMS (4), which is consistent with our observation. A recent report from Ishida et al. (11) based on studies of nine active and five inactive JDMS patients concluded that there were no significant differences in the lymphocyte subpopulations regardless of active or inactive disease in JDMS patients compared with healthy children. The differences between the latter study and ours which may contribute to the discrepancies include the fact that all of our patients had active JDMS at the time of the flow cytometric studies and our patients had not been treated for at least 1 year.

Previous studies from this laboratory have evaluated serum immunoglobulin concentrations in definite JDMS (19) and found that this disease differed from other B8/DR3-associated connective tissue diseases such as Sjögren's syndrome and SLE in several important aspects. The first concerns concentrations of immunoglobulin isotype in serum; as opposed to children with SLE and Sjögren's syndrome, children with JDMS did not have evidence of polyclonal B-cell activation when concentrations of serum immunoglobulins were measured. In Sjögren's syndrome and SLE it is common to find elevations of IgG, IgA, and IgM in active disease: children with JDMS had elevation of one or, at most, two immunoglobulin isotypes. We report that 46% of our patients in this study had elevated IgG levels, whereas none had elevated IgM and only one (8%) had elevated IgA levels. The other aspect of the serum analysis in JDMS concerns the specificity of the ANA. Sera obtained from SLE patients contain many well-characterized autoantibodies. In contrast, the autoantibody specificity in ANA-positive sera obtained from 90 JDMS patients could be not determined when examined for specificity against 17 tissue and organ-specific antigens (19). Furthermore, unlike children with SLE whose sera contained detectable antibodies against many of the 16 viral antigens in the panel, the JDMS patients' sera contained neutralizing and complement-fixing antibodies only to coxsackieviruses B2 and B4 (5). These results suggest that the patients with SLE have a generalized polyclonal B-cell activation, whereas in JDMS the response may be antigen driven. We are currently investigating B-cell function with respect to regulation of in vitro IgG synthesis and secretion in JDMS patients.

In summary, the relative percentage of B lymphocytes in the peripheral blood of JDMS patients is significantly higher than that observed in a healthy control group. Additionally, the ratio of regulatory T-cell subsets (T-helper/T-suppressor cells) is significantly higher than that in the control group. The sera obtained from most JDMS patients contain autoantibodies against an unidentified component of the cell nucleus, and approximately 50% of the patients' sera contained elevated levels of IgG. These results suggest that humoral dysregulation may contribute to the pathogenesis of JDMS.

ACKNOWLEDGMENTS

We gratefully acknowledge the collaboration of the Pediatric Practice Research Group for their involvement in obtaining blood samples from healthy pediatric patients.

This work was supported by grants from the Children's Memorial Institute for Education and Research at Children's Memorial Hospital in Chicago and National Institute of Arthritis and Musculoskeletal and Skin Diseases grant P60-AR-30692 from the National Institutes of Health.

REFERENCES

1. **Ben-Basset, M., and I. Machtey.** 1972. Picornavirus-like structures in acute dermatomyositis. Am. J. Clin. Pathol. **58:**245–249.
2. **Bohan, A., and J. B. Peter.** 1975. Polymyositis and dermatomyositis. N. Engl. J. Med. **292:**344–347.
3. **Bowles, N. E., V. Dubowitz, and C. A. Sewry.** 1987. Dermatomyositis, polymyositis, and coxsackie B virus infection. Lancet **i:**1004–1007.
4. **Bresnan, M. J., S. L. Hauser, H. L. Weiner, E. Reinhertz, Y. Borel, and A. Bhan.** 1981. Characterization of T-cell subsets in peripheral blood and muscle in childhood dermatomyositis. Ann. Neurol. **10:**283.
5. **Christiansen, M. L., L. M. Pachman, R. Scheiderman, D. C. Patel, and J. M. Friedman.** 1986. Prevalence of coxsackie virus antibodies in patients with juvenile dermatomyositis. Arthritis Rheum. **29:**1365–1370.
6. **Denny, T., R. Yogev, R. Gelman, C. Skuza, J. Oleske, E. Chadwick, S.-C. Cheng, and E. Connor.** 1992. Lymphocyte subsets in healthy children during the first 5 years of life. JAMA **267:**1484–1488.
7. **Friedman, J. M., L. M. Pachman, M. L. Maryjowski, R. M. Radvany, W. E. Crowe, V. Hanson, J. E. Levinson, and C. H. Spencer.** 1983. Immunogenetic studies of juvenile dermatomyositis: HLA-DR antigen frequencies. Arthritis Rheum. **26:**214–216.
8. **Hanson, V.** 1976. Dermatomyositis, scleroderma and polyarteritis nodosa. Clin. Rheum. Dis. **2:**445–465.
9. **Ichibawa, Y., H. Shimizu, M. Yoshida, and S. Arimori.** 1990. Activation of T cell subsets in the peripheral blood of patients with Sjögren's syndrome. Arthritis Rheum. **33:**1674–1681.
10. **Illonen, J., M. Surcel, and M. L. Kaar.** 1991. Abnormalities within CD4 and CD8 T lymphocyte subsets in type 1 diabetes. Clin. Exp. Immunol. **85:**278–281.
11. **Ishida, T., Y. Matsumoto, M. Ohashi, and R. Sasaki.** 1993. Analysis of lymphocyte subpopulations in peripheral blood of adult and juvenile cases of dermatomyositis. J. Dermatol. **20:**30–34.
12. **Jacobitz, J., K. Rymut, J. Hayford, and L. M. Pachman.** 1987. Anti-nuclear and anti-cytoplasmic antibodies in pediatric rheumatic disease. Clin. Res. **35:**916A.
13. **Klinman, D., A. Shirai, Y. Ishigatsubo, J. Conover, and A. D. Steinberg.** 1991. Quantization of IgM and IgG secreting B cells in the peripheral blood of patients with systemic lupus erythematosus. Arthritis Rheum. **34:**1404–1410.
14. **Leff, R. L., F. W. Miller, S. J. Greenberg, E. A. Klein, M. C. Dalakas, and P. H. Plotz.** 1992. Viruses in idiopathic inflammatory myopathies: absence of candidate viral genomes in muscle. Lancet **339:**1192–1195.
15. **Medsger, T. A., W. N. Dawson, and A. T. Masi.** 1970. Epidemiology of polymyositis. Am. J. Med. **48:**715–723.
16. **Miller, F. W., L. A. Love, S. A. Barbieri, J. E. Balow, and P. H. Plotz.** 1990. Lymphocyte activation markers in idiopathic myositis: changes with disease activity and differences among clinical and autoantibody subgroups. Clin. Exp. Immunol. **81:**373–379.
17. **Montecucco, C., A. Ravelli, R. Caporali, S. Viola, F. De Gennaro, S. Albani, and A. Martini.** 1990. Autoantibodies in juvenile dermatomyositis. Clin. Exp. Rheum. **8:**193–196.
18. **Pachman, L. M.** 1990. Juvenile dermatomyositis: a clinical overview. Pediatr. Rev. **12:**117–125.
19. **Pachman, L. M., J. M. Friedman, M. L. Maryjowski-Sweeny, O. Jonnason, R. M. Radvany, G. C. Sharp, M. A. Cobb, N. D. Battles, W. E. Crowe, C. W. Fink, V. Hanson, J. E. Levinson, C. H. Spencer, and D. B. Sullivan.** 1985. Immunogenetic studies of juvenile dermatomyositis. III. Study of antibody to organ-specific and nuclear antigens. Arthritis Rheum. **28:**151–157.
20. **Pachman, L. M., D. L. Litt, A. H. Rowley, J. R. Hayford, J. Caliendo, S. Heller, M. L. Christensen, B. K. Patterson, and S. R. Ytterberg.** 1993. Lack of detection of enteroviral, coxsackievirus B (CVB) mRNA or bacterial DNA in fresh frozen biopsies from juvenile dermatomyositis (JDMS) by polymerase chain reaction (PCR). Pediatr. Res. **33:**156A.
21. **Pachman, L. M., and M. C. Maryjowski.** 1984. Juvenile dermatomyositis and polymyositis. Clin. Rheum. Dis. **10:**95–115.
22. **Reed, A. M., L. M. Pachman, and C. Ober.** 1991. Molecular genetic studies of major histocompatibility complex genes in children with juvenile dermatomyositis: increased risk associated with HLA-DQA1*0501. Hum. Immunol. **32:**235–240.