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Immune and Serologic Profiles of HIV-Infected and Noninfected Hemophilic Children and Adolescents

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Objectives: To assess relationships among the effects of HIV on hemophilic children and adolescents' immunologic parameters and vaccine-related serology.

Methods: We analyzed data from extensive baseline immunologic evaluations of 207 HIV antibody-positive (HIV+) and 126 HIV antibody-negative (HIV−) hemophilic children and adolescents.

Results: HIV+ and HIV− participants differed significantly in T-lymphocyte subpopulation numbers, immunoglobulin levels, and seroprevalence rates for diphtheria toxoid, measles, and mumps antigens. IgG levels, IgM levels, and serologic titers to vaccine antigens showed little correlation with T-cell parameters. Proportionately more HIV+ participants were nonreactive to each and all of a panel of 7 skin test antigens (71% vs 28% anergic, RR 2.6). The odds of anergy increased 1.6 times for every decline of 200 CD4+ cells/μl.

Conclusions: HIV had significant, largely independent T- and B-lymphocyte effects on this pediatric cohort.

Key words: HIV, T cells, hemophilia, anergy, immunodeficiency

INTRODUCTION

The Hemophilia Growth and Development Study (HGDS) is a collaborative multicenter cohort study of human immunodeficiency virus (HIV) infection in hemophilic children and adolescents [1]. Initiated in 1989, this longitudinal study assesses the interactive effects of HIV on these children's physical growth and development and immunologic, neurologic, and neuropsychologic functions. The HGDS is unique in that its participants are preadolescents and adolescents. While there is information on HIV and the acquired immunodeficiency syndrome (AIDS) in infants and adults, few reports examine the effects of HIV during the developmental stage of puberty [2–5].

This article presents the immunologic and vaccine-related serologic findings of the HGDS cohort participants at entry into the study. Although the present data are restricted to those from the baseline evaluation, these analyses are important in three respects. First, they provide insight into the effects of the virus on the immune system of the older child and adolescent. Second, they provide new information on the effects of HIV on humoral immunity to vaccine-related recall antigens. Third, they include an assessment of the relationships among the various cellular and humoral effects of HIV.

PARTICIPANTS AND METHODS

Participants

The HGDS includes 333 hemophilic children and adolescents, 207 of whom were HIV antibody-positive

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(HIV+) (median age 13.2 years, range 7–19 years) and 126 of whom were HIV antibody-negative (HIV−) (median age 10.2 years, range 7–19 years) at the time of enrollment; no seroconversions have occurred subsequently. Fourteen collaborating hemophilia treatment centers across the United States enrolled eligible participants between March 1, 1989 and May 31, 1990. Nonhemophilic male siblings of patients at collaborating centers are also being enrolled; data for these participants will be available at a later time. Details of the study are provided elsewhere [1]. The HGDS was reviewed by the human subjects review committees of all collaborating institutions; informed consent was obtained from the parents of all participants.

Methods

HIV antibody status was determined at a central laboratory [Associated Regional and University Pathologists, Inc. (ARUP)] by an enzyme immunoassay (EIA), with confirmation by a Western blot analysis. Serologic reactions with at least two of the 24-kd, 41-kd, and 120/160-kd HIV proteins/glycoproteins were scored as positive. Absence of any serologic reactivity was scored as negative. Five participants had positive EIA and indeterminate Western blot results; these were considered positive on the basis of clinical symptoms and a history of previous positive antibody assays. Twenty participants had negative EIA and indeterminate Western blot results; these were classified as HIV negative. Of these 20, on subsequent testing, 15 had negative EIA and Western blot results and 5 had negative EIA and indeterminate Western blot results.

At evaluation, blood was drawn for complete blood cell counts with differentials, done locally, and lymphocyte subpopulation assessments. For participants from 12 of the 14 treatment centers (90% of participants), specimens were collected in heparinized tubes and shipped to the Centers for Disease Control (CDC) for lymphocyte subpopulation quantitation [6–8]. The remaining 10% of participants, from two treatment centers, are involved in multiple studies and have assessments done locally, at Hershey Medical Center (7%) [4] and Mt. Sinai Medical Center in New York City (3%) [9]. For both CDC- and locally performed studies, lymphocyte subpopulations are quantitated using commercial monoclonal antibodies and direct two-color immunofluorescence flow cytometry following whole blood lysis. (For CDC and Hershey Medical Center, FACSCAN flow cytometer and SIMULTEST software by Becton Dickinson Immunocytometry Systems, San Jose, CA; for Mt. Sinai Medical Center, EPICS C. Coulter Instruments Laboratory, Hialeah, FL)[1] [4,6–9].) For CDC-performed studies, monoclonal antibodies used were to CD4 (present on T-helper/inducer cells), CD8 (present on T-suppressor/cytotoxic cells, CD16 [present on natural killer (NK) cells], CD56 (present on NK cells), CD3 (present on mature T cells and absent on NK cells), and CD19 (present on B cells) [7,8]. A small number of participants (n = 8) had CD4 results from both CDC- and locally performed testing of the same blood sample. The median relative difference between the paired results was 11.4%.

The baseline evaluation included delayed hypersensitivity skin testing, using shared lots of the Multitest CMI (cell-mediated immunity) panel (Merieux Institute, Miami, FL) [10–12], distributed centrally from the CDC. One collaborator at each treatment center was instructed in administering the panel and explaining to parents how to interpret skin-test responses. The baseline evaluation also included quantitation of serum immunoglobulin G, A, and M (IgG, IgA, IgM), done at the CDC using a DuPont discrete clinical analyzer [7]; and assessment of serologic responses to a number of vaccine antigens, done at ARUP. Serologic responses assayed included those to diphtheria, using a hemagglutination assay; tetanus, using a passive hemagglutination assay; measles, using Measelisa II test kits (Whittaker Bioproducts, Walkersville, MD); mumps, using Mumpstat test kits (Whittaker Bioproducts, Walkersville, MD); rubella, using SIA Rubella test kits (Sigma Diagnostics, St. Louis, MO); and *Hemophilus influenzae* type B (Hib), using an enzyme-linked immunosorbent assay (ELISA). Levels indicating prior antigen exposure were based on ARUP or test kit recommendations, as follows: diphtheria, reaction at serum dilutions of $\geq$ 1:40, tetanus, reaction at $\geq$ 1:256; measles, an optical density (OD) of >0.13; mumps, an OD of >0.79; rubella, an "arbitrary unit" (AU) value of >14; Hib, detectable antibody as measured in μg/ml.

Definitions. Anergy was defined as a lack of response to all 7 antigens in the CMI panel, as read by the parents at 48 hr and recorded on a specially provided card. A response was considered positive if there were any palpable induration and any visible erythema at a test site. Patients who had received intravenous gammaglobulin (IVIg) in the previous 28 days were not included in serologic analyses or immunoglobulin G assessments (n = 8). Three participants receiving IVIg in the previous 29 to 120 days were included in these analyses; their exclusion would not alter results in any meaningful fashion.

Statistical techniques. Rates of anergy and titers indicative of prior exposure to vaccine antigens from HIV+ and HIV− persons, stratified by age, were compared using the Mantel-Haenszel chi-square test [13]. Age-adjusted relative risks were also estimated using the Mantel-Haenszel method. Lymphocyte counts and immunoglobulin levels were square-root transformed and

[1]The use of any and all trade names and commercial sources is for identification only and does not imply endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

TABLE I. Age-Standardized Medians for Non-Antigen-Specific Immunologic Tests at Study Entry, by HIV Antibody Status, Hemophilia Growth and Development Study†

Parameter	HIV Antibody Status +	HIV Antibody Status −	*P* Value*
CD4+ cells/μl (n = 332)	423	895	<.0001
CD8+ cells/μl (n = 332)	841	678	.0010
NK cells/μl (n = 294)[a]	109	210	<.0001
CD19+ cells/μl (n = 319)	229	387	<.0001
CD4+-to-CD8+ ratio (n = 332)	0.41	1.28	<.0001
IgG (mg/dL)[b] (n = 325)	1788	1232	<.0001
IgA (mg/dL) (n = 333)	219	156	<.0001
IgM (mg/dL) (n = 333)	141	114	.0199

†Standardized according to the age distribution of the total sample (38%, 6–9.9 y.o.; 28%, 10–13.99 y.o.; 34%, ≥14 y.o.)

[a]CD16 and CD56 positive/CD3 negative

[b]Excludes 8 HIV antibody-positive participants treated with intravenous gammaglobulin in the previous 28 days.

*For significance testing, lymphocyte counts and immunoglobulin levels were square-root transformed and modeled to determine differences in mean levels by HIV antibody status, adjusted for age.

analysis of covariance was used to examine differences in mean levels by HIV antibody status, adjusted for age [14]. For anergy, logistic regression was used to examine the slopes of regression lines. The Wilcoxon rank-sum test was used to assess differences in lymphocyte counts by anergy status. Spearman's rank correlation coefficients (r_s) were used to measure associations among lymphocyte and serologic findings. Because of the large number of correlations examined, a correlation was considered significant if the two-tailed *P*-value was <0.01. All other results were considered significant if the two-tailed *P*-value was <0.05. Actual *P*-values are provided for significant results, as are borderline values ($0.05 < P < 0.10$) and insignificant findings, where these are of interest.

RESULTS

Lymphocyte Subpopulations and Immunoglobulin Levels

No HIV− participants had <200 CD4+ lymphocytes/μl, compared with an age-adjusted rate of 25.8% for HIV+ participants. A larger proportion of HIV+, than HIV−, participants had <500 CD4+ lymphocytes/μl: 62% vs. 10%, age-adjusted RR = 5.4, 95% CI 3.6–8.0. HIV+ participants had significantly lower concentrations of circulating CD16+/CD56+/CD3− peripheral blood cells (NK) and CD19+ lymphocytes, compared with HIV− participants (Table I). The HIV+ participants had higher concentrations of circulating CD8+ lymphocytes, IgG, IgA, and IgM (Table I). HIV+ participants also had lower proportions of circulating CD4+ lymphocytes, CD19+ lymphocytes, and NK cells and higher proportions of CD8+ lymphocytes, compared with HIV− participants (data not shown).

Serology Related to Vaccine Antigens

There were no differences in vaccination history by age group or HIV antibody status. A significantly lower proportion of HIV+ participants had serologic titers indicative of prior exposure to diphtheria, measles, and mumps; differences were not significant for tetanus or Hib (Fig. 1). The magnitude of association between HIV status and serologic evidence of exposure to rubella antigen depended on age. For those <14 years of age, a significantly lower proportion of HIV+ participants had levels indicative of prior exposure to rubella; differences by HIV status were not significant for older children (Fig. 2).

Skin Testing

Within the HIV+ and HIV− subgroups, the proportion anergic did not vary significantly by age. HIV+ participants were significantly more likely than HIV− participants to be nonreactive to delayed hypersensitivity skin testing to each of the following antigens: tetanus (79% vs. 40%), diphtheria (89% vs. 56%), *Streptococcus* (96% vs. 87%), old tuberculin (95% vs. 84%), *Proteus* (91% vs. 82%), and *Candida* (94% vs. 81%); the difference for *Trichophyton* was of borderline significance (98% vs. 95%, $P = 0.0939$). Most HIV+ participants (71%) were nonreactive to all antigens, while only 28% of HIV− participants were anergic [n = 289, Relative risk (RR) 2.6, 95% confidence interval (CI) 1.9–3.5, $P < 0.0001$]. Rates of anergy differed significantly among treatment centers: from 43% to 100% for HIV+ participants and from 0 to 89% for HIV− participants. Center variability persisted even after accounting for differences in CD4+ lymphocyte counts.

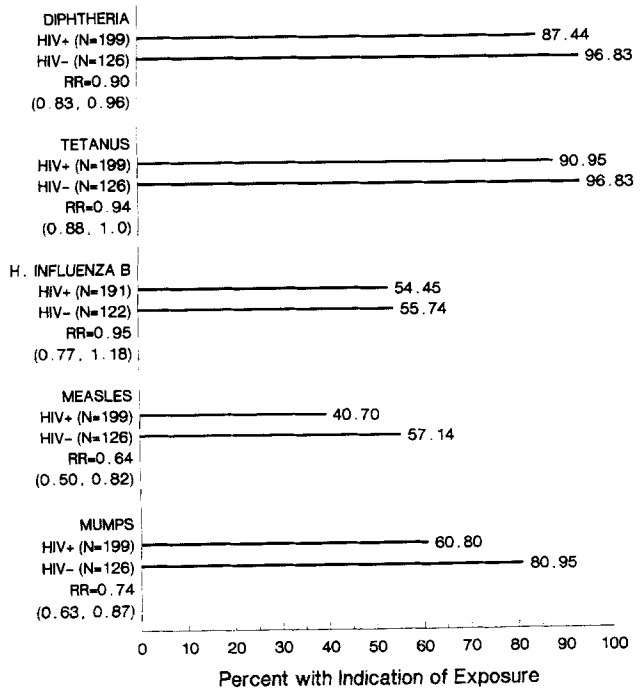

Fig. 1. Participants with titers indicative of prior exposure, by HIV status. Age-adjusted relative risks and 95% confidence intervals.

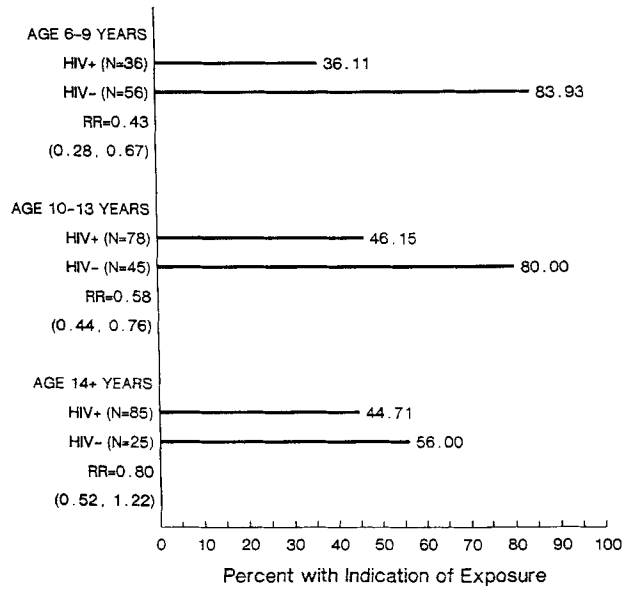

Fig. 2. Age-specific rates of rubella titers indicative of prior exposure, by HIV status. Relative risks and 95% confidence intervals.

Relationships Among Immune Parameters

CD4+ lymphocytes. For both the HIV+ and HIV− participants, significant positive rank correlations were found between CD4+ lymphocyte counts and CD8+ lymphocyte counts, NK cell counts, and CD19+ lymphocyte counts (Table II A, B). For HIV+ participants, a strong negative correlation was found with IgA levels (Table IIA). CD4+ lymphocyte cell concentrations were not significantly correlated with any vaccine antigen-specific antibody titer. The rate of anergy significantly increased with decreasing CD4+ lymphocyte counts ($P < 0.0001$); the odds that an HIV+ participant would be anergic increased 1.6 times for every decline of 200 CD4+ lymphocytes/μl.

Other nonvaccine antigen-specific immune parameters. Concentrations of lymphocyte subpopulations were significantly correlated with one another (Tables IIA, B). For HIV− participants, immunoglobulin levels were not significantly correlated with any lymphocyte subpopulation concentrations (Table IIB). For HIV+ participants, IgA levels were significantly and negatively correlated with concentrations of all cell types, the strongest relation being with CD4+ lymphocyte counts; IgM levels had a weak, negative correlation with NK cell concentrations (Table IIA). For all participants, class-specific immunoglobulin levels were significantly correlated with one another (Table IIA,B).

Vaccine antigen-specific immune parameters. There were no significant correlations (i.e., $P < 0.01$) between vaccine antigen-specific antibody levels and non-antigen-specific immune parameters; the coefficients were all <0.23 in magnitude. For both HIV+ and HIV− participants, antibody titers to Hib were unrelated to antibody titers to other vaccine-related recall antigens. Four significant ($P < 0.0001$) correlations were identified in the HIV+, as well as HIV−, group: diphtheria vs. tetanus (HIV+: R = 0.42, HIV−: R = 0.58); measles vs mumps (HIV+: R = 0.43, HIV−: R = 0.40); measles vs rubella (HIV+: R = 0.33, HIV−: R = 0.40); and mumps vs rubella (HIV+: R = 0.41, HIV−: R = 0.47).

Skin test anergy. The anergy status of HIV+ participants was not significantly associated with concentrations of CD8+, CD19+, or NK cells.

DISCUSSION

A number of studies support age as a cofactor for the clinical course of HIV infection, with the time to development of AIDS varying inversely with age [4,5,15]. The HGDS cohort is unique in representing HIV− infected adolescents and preadolescents. The effects of HIV on infected hemophilic youth in this study are severe, broad, and not necessarily all secondary to infection of CD4+ lymphocytes. We found a lack of correlation between most nonspecific T-lymphocyte- and nonspecific/specific B-lymphocyte-related parameters, consistent with the hypothesis that the immunologic effects of HIV are not due solely to infection of T-helper cells, or

TABLE IIA. Correlations Among Non-Antigen-Specific Immune Parameters for HIV Antibody-Positive Participants† Baseline Findings, Hemophilia Growth and Development Study

	CD4+ (n)	CD8+ (n)	NK (n)	CD19+ (n)	IgG (n)	IgA (n)	IgM (n)
CD4+	1.00* (206)	—	—	—	—	—	—
CD8+	.61* (206)	1.00* (206)	—	—	—	—	—
NK[a]	.26** (181)	.31* (181)	1.00* (181)	—	—	—	—
CD19+	.55* (196)	.52* (196)	.29* (181)	1.00* (181)	—	—	—
IgG[b]	.06 (198)	.12 (198)	−.05 (173)	−.00 (188)	1.00* (199)	—	—
IgA	−.41* (198)	−.28* (198)	−.15 (173)	−.15 (188)	.26** (199)	1.00* (199)	—
IgM	−.03 (198)	−.08 (198)	−.18 (173)	−.02 (188)	.45* (199)	.34* (199)	1.00* (199)

†Number tested given in parentheses.
[a]CD16 and CD56 positive/CD3 negative.
[b]Excludes 8 HIV antibody-positive participants treated with intravenous gammaglobulin in the previous 28 days.
*Spearman's rank correlation coefficients (r_s) are significant at $P < .0001$; **, $.0001 < P < .01$.

TABLE IIB. Correlations Among Non-Antigen-Specific Immune Parameters for HIV Antibody-Negative Participants (n = 126)† Baseline Findings, Hemophilia Growth and Development Study

	CD4+	CD8+	NK	CD19+	IgG	IgA	IgM
CD4+	1.00*	—	—	—	—	—	—
CD8+	.69*	1.00*	—	—	—	—	—
NK	.29**	.53*	1.00*	—	—	—	—
CD19+	.63*	.51*	.26**	1.00*	—	—	—
IgG	−.04	−.08	−.04	.03	1.00*	—	—
IgA	−.15	−.08	.01	−.12	.44*	1.00*	—
IgM	−.02	−.03	−.07	−.03	.28**	.25**	1.00*

†n = 113 for all correlations involving CD16 and CD56 positive/CD3 negative (NK) cells; n = 123 for all correlations involving CD19+ cells.
*Spearman's rank correlation coefficients (r_s) are significant at $P < .0001$; **, $.0001 < P < .01$.

T lymphocytes in general. Rather, our results support additional, independent effects on B-lymphocytes, antigen presenting cells, and/or cytokine production [16,17].

Our findings concerning humoral immunity, including seroprevalence rates to vaccine-related recall antigens, are uniquely extensive but consistent with more limited reports of impaired in vivo and in vitro specific antibody responsiveness [18–23]. We found HIV-infected children to have lower seroprevalence rates for five of six vaccine-related recall antigens. Similar data from other studies of HIV-infected persons are limited to one or a few antigens and are not assessed in relation to other immune findings. One previous report found no significant difference between 17 HIV-infected homosexuals without AIDS and 17 uninfected homosexuals in seroprevalence rates to diphtheria and tetanus toxoids [19]. The results from our much larger study differ in regard to diphtheria but are similar in regard to tetanus serology, consistent with a strongly sustained immune response to the latter antigen.

We had expected to find relationships between CD4+ lymphocyte numbers and antibody titers to vaccine-related antigens and/or seroprevalence rates to vaccine-related antigens. This was intuitively plausible and would have been consistent with studies reporting correlations between CD4+ lymphocyte numbers and postvaccination responses to tetanus [19,22], poliovirus [24], and (conjugated but not nonconjugated) Hib vaccines [23]. However, we found that parameters representing specific B-cell function had minimal relationship to nonspecific T- and B-lymphocyte parameters, including CD4+ lymphocyte numbers.[2] Participants in this study are being revaccinated if their titers are below laboratory-defined protective levels. Thus, we will be able to assess relation-

[2]The lack of correlation between specific antibody titers and total immunoglobulin levels supports the true specificity of the vaccine-related antibodies.

ships between CD4+ lymphocyte numbers and revaccination titers in future phases of this study.

The skin-testing data presented in this article are problematic in that the HIV-negative participants had a higher rate of anergy than would be expected from the literature [10–12]. However, they are otherwise consistent with the literature and are relevant to the emerging problem of tuberculosis in HIV-infected patients [25–28]. Only one other study provides in-depth information on skin testing of HIV-infected persons [29]. Our results illustrate the importance of using multiple antigens for the assessment of skin reactivity and the relative strong antigenicity of tetanus and diphtheria toxoids as skin test reagents. Our finding that anergy rates increased 1.6 times for every decrease of 200 CD4+ lymphocytes/μl may provide a useful guideline for clinicians assessing HIV-infected patients with suspected tuberculosis but nonreactive tuberculin skin tests.

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APPENDIX

The following persons and institutions constitute the Hemophilia Growth and Development Study: National Institute of Child Health and Human Development, Bethesda, Md.—A. Willoughby, MD, MPH; Bureau of Maternal and Child Health and Resources Development—W. Kessel, MD, MPH, S. Barrett, MS; Centers for Disease Control and Prevention—B. Evatt, MD, J. Jason, MD; National Hemophilia Foundation—J. Wasserman, RN, MBA; National Institute of Mental Health—W. Pequegnat, PhD; Children's Hospital of Los Angeles—E. Gomperts, MD, F. Kaufman, MD, M. Nelson, MD, M.E. Schultze, RN, S. Pearson, RN; The New York Hospital–Cornell Medical Center—M. Hilgartner, MD, J. Gertner, MD, J. Sciacca, RN, BS; University of Texas Medical School/Houston—W. K. Hoots, MD, K. Loveland, PhD, M. Cantini, RN, C. Curry; New England Research Institute, Inc.—S. McKinlay, PhD, S. Donfield, PhD, M.A. Maeder, MHS, B. Willis, P. Connell, L. Sleeper, SCD; Baylor College of Medicine—C. Contant, Jr., PhD, University of Iowa Hospitals and Clinics—C.T. Kisker, MD, J. Stehbens, PhD, J. Bale, MD, V. Cool, PhD, Tulane University—W.A. Andes, MD, P. Sirois, PhD,; Childrens Hospital of Oklahoma—C. Sexauer, MD, R. Olson, PhD, M. Bowman, MS, S. Hawk, PA; Mount Sinai Medical Center—L. Aledort, MD, S. Arkin, MD, M. Arroyo; University of Nebraska Medical Center—W. Haire, MD, J. Erickson, RN; University of Texas Health Science Center at San Antonio—R. Parmley, MD, J. Mangos, MD, A. Scott, PhD, L. Honeck, RN; Childrens Hospital of Michigan—J. Lusher, MD, I. Warrier, MD, K. Baird-Cox, RN; Milton S. Hershey Medical Center—M.E. Eyster, MD, E. Pattishall, MD, J. Schaefer, RN, C.S. Neagley, RN, MA; University of Indiana, James Whitcomb Riley Hospital for Children—A. Shapiro, MD, S. Hatcher, RN; University of California-San Diego Medical Center—G. Davignon, MD, P. Mollen, RN; Kansas City School of Medicine, Childrens Mercy Hospital—B. Wicklund, MD, M. Spoor, RN.