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Age-Dependent Humoral Responses of Children to Mycobacterial Antigens

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In the United States, disseminated infection with environmental mycobacteria, including the *Mycobacterium avium* complex, is the most common opportunistic bacterial infection seen in AIDS patients. However, the source and relative degree of exposure to environmental mycobacteria during childhood are unknown. To examine the age-related exposure to mycobacteria, we obtained serum samples from 150 children ranging in age from 6 months to 18 years. Each sample was tested against both *M. avium* (serovar 1) sonic extracts and mycobacterial lipoarabinomannan, using an enzyme-linked immunosorbent assay (ELISA). All serum samples were also subjected to immunoblot analysis with the sonic extract antigen. These studies established that elevated ELISA values ($P < 0.0001$) and increased immunoblot reactivity ($P < 0.0001$) against mycobacterial antigens were both associated with increasing age. The seroreactivity differences were most striking when comparing the age groups of children below the age of 6 with the older age groups. Our results suggest that the development of humoral immune responses to mycobacterial antigens in children correlates with increasing age and that there may be an environmental factor predisposing to mycobacterial exposure which is related to advancing age.

Environmental mycobacteria are ubiquitous in nature and have been frequently isolated from soil, water, foods, and a variety of animal sources (13). Over the past decade, disseminated infection with environmental mycobacteria, in particular disseminated infection with organisms of the *Mycobacterium avium* complex (DMAC), has been increasingly recognized as the most common opportunistic bacterial infection seen in both pediatric (18, 25, 36) and adult (3, 10, 16) human immunodeficiency virus (HIV)-infected patients in the developed world. At highest risk are patients with CD4 counts below 50/mm^3 (19). However, DMAC does not seem to occur with the same degree of frequency in African HIV-infected patients who are at the same stage of this viral infection (6, 30), in spite of the ubiquitous presence of MAC serovars in African soil and water (42). To date, it remains unclear whether host factors or the route of colonization is important in establishing the systemic infection. Patients who have been exposed and have immunologically responded to mycobacterial antigens prior to becoming immunocompromised might be at a lesser risk of developing DMAC. Such a possibility is particularly important for the pediatric HIV infection, since most children acquire the viral infection perinatally and thus have a limited window of time during which their immune response to pathogens might be relatively intact.

Previous studies have investigated the development of antibody responses to MAC antigens in both healthy and immunocompromised hosts as a measure of both exposure and response to the organism. Low levels of antibody to a variety of both general mycobacterial (2, 7) and specific MAC antigens have been detected in sera from healthy adults (4, 24, 38). Elevated antibody levels to MAC antigens have been found in sera from nonimmunocompromised patients with MAC pulmonary infections as well as patients with hairy cell leukemia who are also infected with DMAC (29, 43, 45). In contrast, AIDS patients infected with DMAC have universally low levels of antibody against MAC antigens in spite of their infection (4, 29, 44, 45).

An improved understanding of the development of antibody responses to mycobacterial antigens in children has potentially important ramifications. Pilkington et al. have demonstrated that noninfected, non-BCG vaccinated children in the United Kingdom develop an immunoglobulin G (IgG) response to mycobacteria with increasing age (32). However, whether the presence of antibody to mycobacterial antigens varies as a function of age in the United States has not been conclusively established. In one small study, children less than 6 to 10 years of age showed significantly lower humoral responses to MAC antigens than did healthy adults (38), suggesting that there might be an age-related component to the development of antibody against MAC in an American cohort. To further extend these observations using a larger sample size, we investigated whether there is an age-related difference in the appearance of antibody against mycobacterial antigens in children. In this report, we demonstrate an elevated humoral response to mycobacterial antigens with increasing age and discuss some of the possible implications of this age-related immune response.

MATERIALS AND METHODS

Serum samples. Serum samples from 150 subjects ages 6 months to 18 years were obtained from the clinical chemistry lab at Walter Reed Army Medical Center, Washington, D.C. Samples had been sent to the lab for a variety of reasons, including elective surgical screening, dehydration, and evaluation of fever. All patients had no known history of mycobacterial disease, were negative to purified protein derivative, were not suffering from inflammatory bowel disease, and were not known to be immunocompromised. In addition, none of the patients were from the same family or household. Sex, race, birthdate, and medical history were recorded for all subjects.

Mycobacterial antigen preparation. The *M. avium* serovar 1 strain used in this study was obtained from the American Type Culture Collection (ATCC 35717).

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The organism was cultured at 37°C in Long's synthetic medium plus 1% glucose (Quality Biologicals Inc., Gaithersburg, Md.). After 17 to 21 days of growth, the cells were harvested by centrifugation, washed in phosphate-buffered saline (PBS), and sonicated with a Vibracell sonicator (Sonics and Materials, Inc., Danbury, Conn.) for 12 min on ice with a microtip. The protein concentration of the sonic extract was determined with the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Richmond, Calif.). Purified *Mycobacterium tuberculosis* lipoarabinomannan (LAM) from H37Rv was provided by Patrick Brennan, Department of Microbiology, Colorado State University, Fort Collins.

Antimycobacterial antibody levels as determined by ELISA. Antimycobacterial antibody levels to the *M. avium* sonic extract and to LAM were determined with standard enzyme-linked immunosorbent assay (ELISA) protocols. The ELISA conditions had been optimized with a checkerboard analysis to determine the appropriate antigen and antibody concentrations. Briefly, *M. avium* antigen was diluted in carbonate buffer and added to 96-well Immulon 2 microtiter plates (Dynatech Laboratories, Chantilly, Va.) at a protein concentration of 1 μg per well. To measure anti-LAM antibody concentrations, 0.2 μg of LAM per well was coated onto Immulon 1 plates. For each serum sample, three wells were coated with antigen and three wells were coated with buffer alone. The plates were incubated for 12 to 16 h at 4°C and then washed five times with PBS containing 0.05% Tween 20 (PBS-T). All wells were then blocked with 0.2 ml of PBS containing 3% bovine serum albumin for 1.5 h at 37°C to reduce nonspecific binding. The plates were washed again five times with PBS-T, and 0.1 ml of a 1:50 dilution of each serum sample was applied in triplicate to wells coated with mycobacterial antigen and to wells coated without antigen. As a consistency control for each plate, six wells (three with antigen and three without) were coated with a 1:100 dilution of a high-titer burro anti-*Mycobacterium intracellulare* polyclonal antibody preparation (33). The plates were reincubated for 1.5 h at 37°C and again washed five times with PBS-T. Goat anti-human IgG alkaline phosphatase conjugate (Sigma Chemical Company, St. Louis, Mo.) was added to wells containing human sera, and the plates were incubated for an additional 1 h at 37°C. The wells containing burro antibody were incubated with rabbit anti-horse IgG alkaline phosphatase conjugate. Next, the plates were rewashed five times with PBS-T, and the substrate (Sigma 104 Phosphatase Substrate dissolved in 1.0 M Tris–0.3 mM $MgCl_2$ [pH 9.8]) was applied. After 25 min at room temperature, the A_{405} was measured, using a model EL3 11 ELISA reader (Bio-Tek Instruments, Burlington, Vt.). To correct for nonspecific background, the readings for each specimen were recorded as the differences in absorbance between the mean values of sera incubated in the presence and in the absence of antigen.

Immunoblot analyses with MAC sonic extract antigens. Twenty-four micrograms of sonic extract antigen which had been mixed with standard SDS sample buffer and heated at 95°C for 5 min was added into alternate wells of polyacrylamide gradient gels (4 to 20%; Integrated Separation Systems, New York, N.Y.). Prestained molecular weight markers (Bethesda Research Laboratories, Bethesda, Md.) were placed in the remaining wells. Electrophoretic separation was performed under standard Laemmli conditions (22). The antigens were then blotted onto nitrocellulose paper by using the Mini-Protean II transfer system (Bio-Rad Laboratories) as previously described (39). The blots were removed at the completion of transfer and cut into strips containing a prestained marker lane and a MAC sonicate precipitate lane. After blocking the nitrocellulose strips overnight at 5°C with 5% nonfat dry milk in Tris-buffered saline (TBS) (150 mM NaCl, 10 mM Tris [pH 8.0]), they were washed for 5 min with TBS containing 0.05% Tween 20 (TBS-T). Each strip was then incubated for 1.5 h at room temperature with human sera diluted 1:50 in 5% nonfat dry milk dissolved in TBS. After the incubation period, the strips were washed three times with TBS-T for 5 min each and then reincubated for 1 h at room temperature with goat anti-human IgG alkaline phosphatase conjugate (Sigma Chemical Company). The strips were again washed three times (5 min per wash) in TBS-T. Bound antibodies were detected with the Nitro Blue Tetrazolium–5-bromo-4-chloro-3-indolylphosphate toluidinium Immunoselect system (Bethesda Research Laboratories).

Statistical analyses. Results were grouped for analysis into 3-year age groups: 0 to 3, 3 to 6, 6 to 9, 9 to 12, 12 to 15, and 15 to 18 years. Birthdates were known for each subject, and if the sample was obtained on the subject's birthday, the sample was categorized as belonging to the lower age group. For example, if a subject was exactly 3 years old when the sample was obtained, it was assigned to the 0- to 3-year age group. Alternatively, if a sample was obtained when the subject was 3 years and 1 day, the sample was recorded in the 3- to 6-year age group. Summary statistics and regression analyses were performed on all the ELISA values. Single-factor analysis of variance was performed to determine significant differences between age groups. Chi-square analysis was used to determine the significance of categorical factors for the outcome. The Execustat (Strategy Plus, Inc.) computer program was utilized for statistical analyses.

RESULTS

Demographics of the sample population. Subjects were compiled into age groups of 3-year increments for analyses. The numbers of subjects evaluated within each age group and the demographic characteristics related to the age groups are displayed in Table 1. The differences among age groups were not significant for sex or race.

TABLE 1. Demographic characteristics of age groups

Age group (yrs)	No. of subjects	Sex (%)		Race (%)		
		Male	Female	White	Black	Asian
0–3	41	46.5	53.5	61	26.8	12.2
3–6	24	50	50	62.5	33.3	4.2
6–9	16	50	50	56.2	37.5	6.3
9–12	17	41.2	58.8	58.8	35.3	5.9
12–15	24	45.8	54.2	54.2	41.7	4.1
15–18	28	50	50	57.1	39.3	3.6

The association between anti-MAC sonic extract antibody levels and increasing age. The antibody levels against MAC sonic extract antigen, quantified by ELISA, are depicted in Fig. 1 as the mean ELISA value for each age group. The mean value for the entire population is plotted as a dotted line. The ELISA values obtained represent the difference in absorbance between sera incubated in the presence and in the absence of antigen. These data demonstrate that levels of antibody directed against MAC sonic extract antigens, as measured by ELISA, increase with age. As seen in Fig. 1, the mean ELISA value increases with time and remains above the population's mean value by age 6. Thus, children older than age 6 have significantly elevated antibody titers to MAC antigens compared with those for infants. The antibody levels reach a plateau during early adolescence. Scatterplot analysis of the data indicates that this elevation in ELISA values is the result of an increasing number of individuals with above average levels of measurable antibody as the population ages rather than a result of sporadically high levels in a few patients (data not shown). This age-related difference is highly significant when analyzed by regression analysis ($P < 0.0001$). Analysis of differences in the mean ELISA value between age groups was also conducted by using single-factor analysis of variance and Tukey's test. Significant differences ($P < 0.0001$) in mean ELISA values were noted between the 0- to 3-year age group and all other groups except the 3- to 6-year group. Likewise, there were significant differences between the 3- to 6-year age group and all the other, older, age groups ($P < 0.001$). Differences were not significant between any other groups or within age groups. Sex and race were not found to be independent variables in relation to the ELISA values.

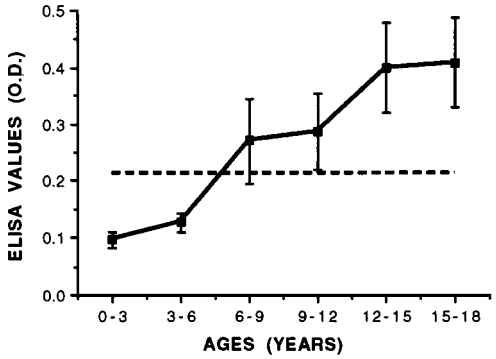

FIG. 1. MAC sonicate ELISA values versus age. Levels of IgG antibody to 1 μg of protein per well of *M. avium* serovar 1 sonic extract were determined as the mean value for each age group. The mean ELISA value for the entire population is represented as a dotted line, and standard error bars are shown. O.D., optical density.

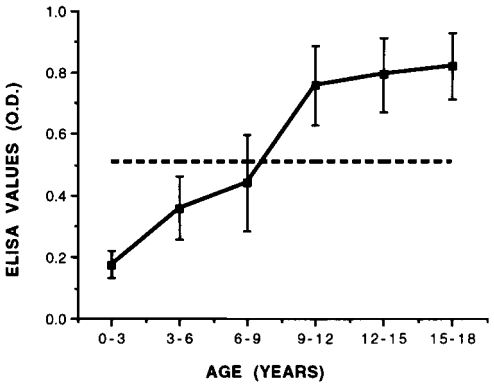

FIG. 2. LAM ELISA values versus age. Levels of IgG antibody to 0.2 μg of protein per well of purified *M. tuberculosis* LAM are shown as the mean value for each age group. The dotted line indicates the average ELISA value for the entire population studied, and standard error bars are shown. O.D., optical density.

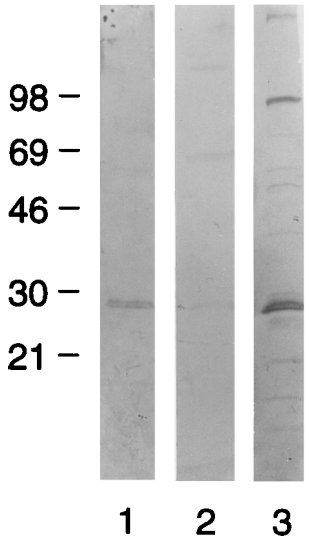

FIG. 3. Immunoblot analysis of sera from representative patients. *M. avium* serovar 1 sonicates were electrophoretically separated by SDS-PAGE, transferred to nitrocellulose, and incubated with a 1/50 dilution of each serum sample. Representative immunoblots are shown. Lane 1 was incubated with sera from a 3-year-old patient, and lanes 2 and 3 were incubated with sera from a 17-year-old patient and a 16-year-old patient, respectively. Molecular mass markers (in kilodaltons) are indicated on the left.

The association between anti-LAM antibody levels and increasing age. The seroreactivity against LAM, a specific mycobacterial antigen, was also measured for each patient. ELISA values were calculated as the difference in absorbance between sera incubated in the presence and in the absence of antigen. Figure 2 demonstrates that the increase in the mean anti-LAM ELISA values when plotted against age groups is similar to the increase shown for anti-sonic extract antibody levels. The anti-LAM ELISA values rise and remain above the average for the overall population (represented on the graph as a dotted line) by age 9. Scatterplot analysis confirms that this result is a consequence of an increasing number of individuals with above average levels of measurable antibody rather than sporadically high levels in the older patients (data not shown). This relationship is also highly significant by regression analysis ($P < 0.0001$). When individual age groups were compared with each other by applying single-factor analysis of variance and testing with Tukey's standard, significant differences ($P < 0.0001$) were identified between the 0- to 3-year age group and all the other age groups older than the 6- to 9-year age group, between the 3- to 6-year age group and all the other age groups older than the 6- to 9-year age group, and between the 6- to 9-year age group and all the older age groups. Sex and race were not significant in relation to the ELISA values.

Immunoblot reactivity and increasing age. Immunoblot analysis was done with the same patient population by incubating nitrocellulose strips containing electrophoretically separated *M. avium* serovar 1 sonic extract with a 1/50 dilution of each serum sample. A summary of the results can be seen in Table 2, and representative immunoblots are shown in Fig. 3. A heterogeneous response was detected by immunoblot analysis, with no distinct banding pattern seen among reactive sera. No common seroreactive bands were identified in all patient sera. However, seroreactive MAC antigens with apparent molecular masses of 22, 27, 43, and 110 kDa were commonly present. The percentage of patients with reactive immunoblots, defined as having at least one seroreactive band, was significantly related to increasing age when comparing the youngest group with the oldest group ($P < 0.0001$). Antibodies to 27- and 43-kDa *M. avium* sonic extract antigens were the most common throughout all age groups. Patients in the oldest group were more likely than patients in the youngest group to react to a 110-kDa antigen ($P < 0.0007$).

TABLE 2. Summary of age-related immunoblot results

Age group (yrs) and no. of patients	No. (%) of patients with positive immunoblots	% of patients with 27-kDa band[a]	No. (%) of patients with multiple bands
0–3 (41)	10 (24)	20	6 (15)
3–6 (24)	13 (54)	25	5 (21)
6–9 (16)	10 (62)	56	6 (37.5)
9–12 (17)	11 (65)	44	10 (59)
12–15 (24)	22 (92.5)	63	14 (58)
15–18 (28)	27 (96)	87	17 (61)

[a] The most frequent band seen was a 27-kDa band.

DISCUSSION

Several reviews of risk factors associated with mycobacterial disease in pediatric AIDS patients have noted a correlation between increasing age and infection (18, 25, 36). Although such an association might be explained by decreasing CD4 counts in these patients, at least one study has shown that the age risk is independent of CD4 counts (25). An alternative explanation could be that the risks of exposure to environmental mycobacteria are higher for older children. A study by Larsson and colleagues which demonstrated that 8- to 9-year-old Swedish children were threefold more likely to react with *M. avium* sensitin in skin test assays than 4- to 5-year-old healthy children supports this hypothesis (23).

The increased-exposure theory also seems to be supported by the data presented in this report. Our results indicate that children below the age of approximately 6 years have significantly lower antibody levels directed against mycobacterial antigens when tested by both ELISA and immunoblot analysis. The same age-related humoral reactivity was observed in assays using both MAC sonic extract antigen and *M. tuberculosis* LAM. Although *M. tuberculosis* is not normally isolated from environmental sources, it is not surprising that sera from children cross-react with purified *M. tuberculosis* LAM. LAM is a

major component of mycobacterial cell walls and is ubiquitously present in the *Mycobacterium* genus. The structural and antigenic similarities between LAMs from environmental mycobacteria, *M. tuberculosis*, and *Mycobacterium leprae* have been previously demonstrated (12, 28). Moreover, we have recently shown by ELISA that sera from HIV-seronegative patients with MAC disease strongly react with *M. tuberculosis* LAM (9a). It is unlikely that the elevated immunoreactivity seen in this study is due to a nonspecific increase, because LAM is a specific mycobacterial antigen and Pilkington et al. have reported that humoral responses of children to common bacterial antigens (heat shock proteins) do not increase with age (32).

Although mycobacteria are ubiquitous in the environment, there has not been conclusive evidence implicating particular routes of exposure with increased mycobacterial disease. While colonization via the gastrointestinal tract or pulmonary tree may predispose individuals to infection, studies investigating the risks and association of colonization with subsequent disease have failed to show a firm association for either route (3, 6, 17, 36). Previously reported results of DNA fingerprinting have suggested that environmental mycobacterial infection was not likely to be transmissible from person to person but rather was acquired from de novo environmental sources (1). Thus, it seems unlikely that the increased exposure to other children in school could account for the observed rise in seroconversion. Although studies by Resnikov and Leggo suggested that soil may serve as an infection source, Wolinsky and Rynearson were doubtful that soil exposure is related to human disease (33, 46). Moreover, no consistent correlation between exposure to pets and reactivity to atypical mycobacterial sensitins has been documented (23, 26).

A variety of mycobacterial species, including *M. avium*, can be cultured from environmental sources and treated water supplies (8, 11, 15), and it has been speculated that showering may provide an aerosolized exposure to MAC. Since children are less likely to take showers in early childhood, this could account for the difference in antibody response in this group. However, a recent report which concludes that showering was not associated with an increased risk of MAC disease in persons with HIV infection and low CD4 counts (19) suggests that this route of exposure may be of limited importance. Good has speculated that AIDS-related MAC disease is not due to direct infection by drinking water or by inhalation of aerosols but results from an activation of silent MAC foci in the lymphatic tissue (13). Under this circumstance, the MAC bacilli may translocate the epithelial lining of the gastrointestinal tract into the lymphatic tissue after a childhood exposure. The significant number of cervical adenitis cases identified in young children (27) indicates that MAC bacilli are pathogenic in early childhood, although most of these infections are self-limiting and eventually resolve. Since the portal of entry for cervical adenitis is probably oral, gastrointestinal colonization with MAC seems likely to occur during early childhood. One risk factor that has been associated with MAC disease in AIDS patients is the consumption of hard cheese (19). Therefore, it is possible that the changing dietary habits of children as they grow older increase the risk of gastrointestinal colonization by environmental mycobacteria, eventually resulting in elevated immune responses to mycobacterial antigens.

Several observations have suggested that previous exposure to mycobacterial antigens may protect the host against nontuberculous mycobacterial disease. First, the rate of incidence of DMAC in African AIDS patients is lower than the rate in AIDS patients from industrialized nations with similar CD4 levels, in spite of the documented presence of MAC in the African environment (30, 31, 42). The finding that the percentages of individuals that responded to *M. avium* sensitin in developed and developing countries were similar (41) indicates that exposure is not substantially different and is unlikely to account for the difference seen in DMAC disease in the two populations. Although different routes of exposure in the two geographic areas may be important with respect to this difference, it is more likely that frequent exposure to the virulent *M. tuberculosis* in developing countries may provide broad mycobacterial immunity and significant protection against DMAC disease.

Second, BCG vaccination appears to protect against MAC disease. While the incidence of MAC disease in Swedish HIV-infected patients, most of whom received *Mycobacterium bovis* BCG vaccination at infancy, is 10%, the incidences of AIDS-related MAC disease in the United States and The Netherlands, two countries where BCG vaccination has not been given, are 30 and 50%, respectively (14). In addition, the incidence of lymphadenitis in Sweden and in the Czech Republic caused by atypical mycobacteria increased significantly when mass neonatal BCG vaccination was discontinued in those countries (35, 40). Consistent with these and our observations, Katilla has suggested that neonatal BCG vaccination protects children against nontuberculous mycobacterial disease, especially from 1 to 4 years of age (21). It is also intriguing that the incidence of DMAC in AIDS patients located in the southeastern United States is low, in spite of CD4 levels comparable to those found in other regions of the United States (20), and is perhaps due to higher environmental exposure to mycobacteria in the Southeast (5, 9, 10, 11). Thus, previous exposure to mycobacterial antigens may be protective against DMAC. Given these observations, our results suggest that younger children, who in general have less antimycobacterial immunity, would be more vulnerable to DMAC if exposed to MAC colonization than would older children with a primed immune system. A recent study which demonstrated that the average age for serious invasive MAC infection in children without predisposing conditions was 4.6 years seems to support this contention (37).

Because of the resurgence of tuberculosis, the emergence of multiple-drug-resistant tuberculosis, and the prevalence of DMAC in AIDS patients, a primary goal of mycobacterial research should be the development of improved mycobacterial vaccines. As discussed above, a preexisting immune response to mycobacterial antigens may be protective against nontuberculous mycobacterial disease, including DMAC. Therefore, vaccines developed to protect susceptible patients against mycobacterial disease might best be administered in early childhood in order to prime the immune system prior to significant exposure. Our study suggests that children under age 6 have substantially lower rates of natural exposure of the immune system to environmental mycobacteria, and targeting this age group for vaccination would be worthwhile.

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