Cold-Reactive Immunoglobulin M Antilymphocyte Antibodies Directed Against B Cells in Thai Children with Dengue Hemorrhagic Fever

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Antilymphocyte antibodies were found in 51 of 83 serum specimens from Thai children with dengue hemorrhagic fever (DHF). The lymphocytotoxic activity was complement dependent, and cytotoxicity was detected in the 19S immunoglobulin M-associated serum fractions at a temperature optimum of 15°C. Sera with lymphocytotoxic activity were cytotoxic to autologous as well as allogeneic lymphocytes from patients and healthy adult donors and were directed primarily against B cells, with some T cell cross-reactivity. This study suggests that infection with DHF induces predominately cold-reactive antilymphocyte antibodies in DHF patients that could potentially interact with peripheral blood cells of patients and modulate the humoral immune responses of patients during infection.

The presence of antilymphocyte antibodies (ALA) in the sera of patients with autoimmune (14), parasitic (16), or viral diseases (5, 12) is well documented. These lymphocytotoxic antibodies differ from conventional cytotoxins in that they are autocytotoxic and are complement and temperature (15°C) dependent (10, 11), and their presence is independent of allogeneic immunization. Although the role of ALA in the immunopathogenesis or manifestation of the diseases in which they are found is unclear, they may interact with cells or other serum factors to modulate the immune responses of the patients.

Recently, Wells et al. (17) have reported that major alterations occur within several component cell subpopulations in the immune system of Thai children with dengue hemorrhagic fever (DHF). These alterations include a significant increase in atypical lymphocytes and non-T, non-B, non-Fc receptor-bearing Null cells and a significant decrease in T cells in the peripheral blood of DHF patients. In addition, Boonpucknavig et al. (2) have used indirect immunofluorescence to show that ALA are present in the sera of patients with DHF; however, they did not determine the incidence, nature, or characteristics of these ALA. We now report that 61.4% of sera from Thai children with DHF contain cold-reactive lymphocytotoxic immunoglobulin M (IgM) antibodies which are cytotoxic to autologous as well as allogeneic lymphocytes in the presence of complement and which are directed primarily against B cells.

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MATERIALS AND METHODS

Collection and processing of specimens. Heparinized and nonheparinized peripheral blood samples were obtained from Thai children examined at the Bangkok Children's Hospital after they were clinically diagnosed as having DHF and after informed consent was obtained from the parents or guardians of the patients. All patients in this study were classified as having secondary dengue infections according to the serological criteria of Winter et al. (20). Nonheparinized blood was clotted at room temperature to obtain serum, and serological confirmation of DHF was proven by using the hemagglutination inhibition test. Control sera were obtained from healthy Thai school children as well as healthy Thai adults. The control sera were also examined by hemagglutination inhibition (see below and Fig. 2).

Isolation of peripheral blood lymphocytes and lymphocyte subpopulations. Peripheral blood lymphocytes were prepared from heparinized venous blood that was centrifuged through Ficoll-Hypaque (3), washed three times with Hanks balanced salt solution, and suspended in RPMI 1640 supplemented with 2 mM glutamine, 50 U of penicillin, 50 µg of streptomycin per ml, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer (HEPES), and 10% heat-inactivated fetal calf serum. In certain experiments, the cells were further separated into subpopulations as previously described (8). Briefly, the isolated cells were passed over a Sephadex G-10 column to remove adherent cells. The nonadherent cells were then sepa-

rated into T, B, and Null cells in a two-stage process. First, the cells were poured over a Sephadex G-200 anti-human F(ab')$_2$ immunoabsorbent column. Then, the nonadherent T plus Null cells were further fractionated by using overnight incubation with sheep erythrocytes, followed by Ficoll-Hypaque density centrifugation. The adherent cells (B cells) were eluted from the column with medium containing 1% human immunoglobulin, incubated overnight with sheep erythrocytes, and centrifuged over Ficoll-Hypaque to remove any contaminating T cells (8). Surface characteristic determinations of the enriched cell populations were performed as previously described (8). All populations were highly enriched, and viability of the final preparation was greater than 95%.

Lymphocytotoxic antibody assays. The methodology for the ALA assays has been described previously (16). Briefly, at either 4, 15, or 37°C, 0.1 ml of each serum or a fraction of the sera obtained by sucrose gradient fractionation was added in duplicate to appropriate wells for 30 min, followed by the addition of 0.1 ml of fresh rabbit serum (as a source of complement). Then, after an additional 3 h of incubation, the percentage of dead cells was determined by eosin dye inclusion. Sera with high lymphocytotoxicity from malarious patients or patients with systemic lupus erythematosus served as positive controls. Some sera with lymphocytotoxic activity were subsequently tested for the presence of ALA against a panel of lymphocytes from 10 normal adults of various human lymphocyte antigen phenotypes.

The microcytotoxicity assays were modified when sera were tested for lymphocytotoxicity against enriched T cell, B cell, Null cell, or macrophage targets. Sera were distributed on Terasaki microtiter trays which had been covered with mineral oil; 1 μl of serum was added to each well, and the trays were stored at −70°C until needed. The assays were performed by putting 1 μl of target cell suspension (2 × 10⁶ lymphocytes per ml) into each well containing test sera. The target cell-serum mixture was incubated for 1 h; 5 μl of rabbit complement (Pel Freeze) was added to each well, and the mixture was further incubated for 3 h. Aqueous eosin (3 μl of a 5% solution) was then added to each well; 2 min later, 10 μl of Formalin was added to the wells, and the tray was covered with a microscope slide (50 by 75 mm). Target cell death was determined by using a phase-contrast microscope.

Sucrose density fractionation of serum. Sucrose density centrifugation of serum was performed by layering the serum over a 10 to 40% linear sucrose gradient from which 12 equal fractions were subsequently collected after centrifugation (7). All fractions were concentrated to their original volume for use in lymphocytotoxicity assays. Quantitative estimation of human IgG, IgM, and IgA in the fractions was performed by using commercially available regular and low-level radial immunodiffusion plates (Hyland Diagnostics, Deerfield, Ill.).

Statistics. Either the two-tailed or the one-tailed Student's *t* test was used to determine statistical significance of difference.

RESULTS

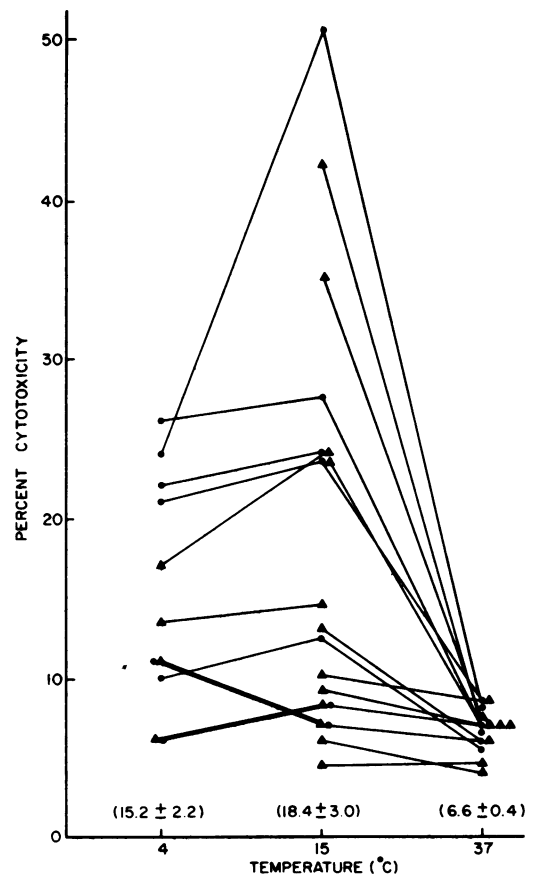Sera from patients with DHF were tested against normal target indicator cells from

FIG. 1. Comparison of DHF lymphocytotoxic antibody activity at different incubation temperatures. Percent lymphocytotoxicity was determined by averaging values from duplicate wells and subtracting the control serum cytotoxicity value. Symbols: ●, serum samples tested at three temperatures; ▲, serum samples tested at only two temperatures, owing to the small volume of serum available. The serum of each patient was assayed by using allogeneic MNC as target indicator cells. Values indicate mean lymphocytotoxicity ± SD for the sera assayed at the temperatures indicated. The value at 37°C represented a statistically significant difference from the cytotoxicity at 15°C ($P < 0.001$).

healthy donors in assays performed at three different incubation temperatures (Fig. 1). Control sera from normal Thai children were cytotoxic for 5.8 ± 2.3% (mean ± standard deviation [SD]) of the normal mononuclear cells (MNC). Positive lymphocytotoxicity by patient serum was thus defined as greater than 10.4% (mean of control plus 2 SD). Eight of 11 DHF sera were positive for ALA at 4°C, 11 of 19 were positive at 15°C, and 0 of 14 were positive at 37°C. The ALA and DHF are therefore "cold-reactive" antibodies.

Fifty-one of 83 (61.4%) serum specimens from patients with DHF had definite cytotoxic activity (defined as greater than 10.4% killing) against lymphocytes from random donors when the microcytotoxicity assays were done at 15°C with a 4-h incubation period (Fig. 2). ALA activity in individual sera ranged from a low of 4.5% normal MNC lysed to a high of 58.5%, whereas only 3 of 19 (15.8%) serum specimens from healthy Thai schoolchildren (mean age ± SD = 9.1 ± 0.2 years) and 0 of 48 healthy Thai adult control sera were cytotoxic to the target MNC. Eight of the control sera from healthy children which did not contain ALA were serologically positive when examined for antidengue antibodies. Moreover, the three healthy control sera demonstrating lymphocytotoxic activity were serologically negative for antidengue antibodies. Mean lymphocytotoxicity of the sera of the patients differed significantly from the mean lymphocytotoxicity in both the schoolchildren's sera ($0.05 < P < 0.02$) and the adult control sera ($P < 0.05$). Furthermore, when 13 individual serum specimens containing high (<25%) lymphocytotoxic activity were tested against a panel of lymphocytes from 10 normal donors, 7 of the 13 individual serum specimens showed at least 20% cytotoxicity against 50 to 100% of the donor target MNC at 4°C, whereas little or no activity was present at 37°C.
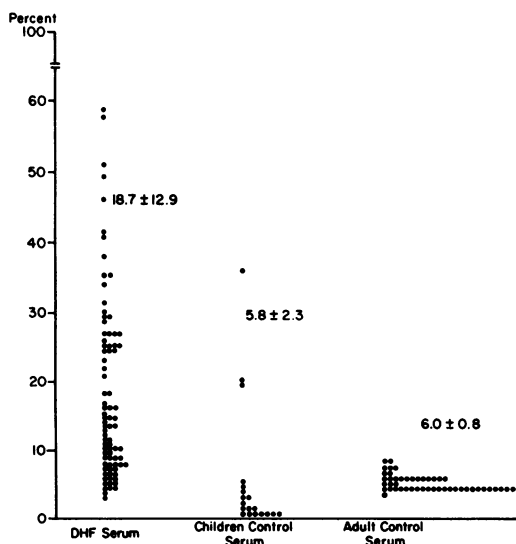

Percent

FIG. 2. Percentage of normal mononuclear cells lysed after incubation with sera from Thai children with DHF. Numerical values indicate mean cytotoxicity ± SD. The DHF serum was obtained on day 1 of hospitalization and was confirmed serologically as DHF.

In 6 of 13 cases, the sera of patients with DHF were cytotoxic (>7.6% mean ± SD of adult control sera cytotoxicity) to the patients' own lymphocytes. An equivalent level of lymphocytotoxic antibody activity was found when the serum specimens containing antibody were tested against allogeneic lymphocytes from other patients and healthy donors. Thus, sera from patients with DHF which contained antibodies cytotoxic to allogeneic lymphocytes contained antibodies against the patients' own lymphocytes as well. When patients' positive ALA sera were serially diluted and tested against allogeneic mononuclear target cells from healthy Thai donors, ALA were absent or substantially decreased to between a 1:4 and a 1:16 dilution.

Thirteen sera from patients with acute DHF were tested against T cell-, B cell-, and Null cell-enriched target subpopulations. In a small number of experiments, patient sera with strong ALA against unfractionated MNC were tested against monocyte-macrophage-enriched (>85% esterase positive) target subpopulations. ALA were primarily directed against B cells (38%), and, in a number of instances (31%), against both T cells and B cells. Only occasional sera (8%) reacted with T cells alone. The remaining 23% did not react with any cells. No reactivity was seen against Null cells or monocyte-macrophage-enriched populations at any temperature. Of 16 normal (control) serum samples, none reacted with T or B cells.

When a comparison was made between a limited number of paired antisera collected during the acute period of illness and antisera collected 15 and 30 days later during the convalescent period, the antibodies had higher titers during the acute period of infection than during the convalescence period. The mean (±SD) percent lymphocytotoxicity induced by sera from 25 patients was as follows: 21.9 ± 2.2 at the acute stage; 18.0 ± 2.5 after 15 days; and 15.4 ± 2.3 after 30 days. For sera from healthy controls, it was 6.3 ± 0.2%. Furthermore, none of four paired patient sera which contained less than 10.4% ALA during the acute period of infection developed increased ALA during the convalescent period of illness.

Six individual sera were examined after sucrose density gradient fractionation, and the ALA were consistently found in the 19S (IgM) fraction near the bottom of the gradient (tubes 2, 3, and 4 in a 12-tube gradient) (Table 1). In addition, the lymphocytotoxic activity in the sera was inhibited by rabbit antiserum specific for human IgM (μ-chain specific) as well as after reduction by 2-mercaptoethanol, but it was not affected by rabbit antisera specific for human IgG (γ-chain specific) or human IgA (α-chain specific). In five of six individual sera, lympho-

TABLE 1. Lymphocytotoxic activity mediated by different classes of immunoglobulin as assessed by analysis of sucrose density fractionation of six individual patients' sera

Fraction (tube) no.	Activity at the following temp[a]:	
	15°C	37°C
2	21.9 ± 5.1	23.6 ± 5.4
3	19.9 ± 4.7	22.6 ± 4.1
4	13.9 ± 2.5	19.6 ± 3.6
6	6.8 ± 0.6	17.8 ± 3.6
7	6.2 ± 0.4	12.0 ± 1.6
8	6.2 ± 0.3	10.3 ± 1.1
9	6.2 ± 0.3	9.5 ± 1.5

[a] Percent lymphocytes killed (mean ± SD) by six individually fractionated sera from patients with DHF. Sera from healthy controls exhibited less than 6.0% killing.

cytotoxic activity greater than 10% was found in the IgM-containing fraction when assayed at 37°C. Lymphocytotoxic activity was always less than 8% in the undiluted patient sera at 37°C. Thus, ALA are detectable by the use of unfractionated mononuclear target cells at 37°C if isolated IgM fractions are obtained from the patient sera.

In several experiments (data not shown), we pooled the IgM fractions and IgG/IgA fractions of the patients to test for IgG/IgA-associated blocking activity. We then added to the IgM fraction equal volumes of either media, autologous IgG/IgA, or allogeneic IgG/IgA fractions from a healthy donor. The IgM fractions of the various patient sera were equally cytotoxic, irrespective of whether they were combined with media, autologous IgG/IgA, or IgG/IgA from an uninfected donor, suggesting that patient IgG/IgA fractions are unable to significantly block or enhance IgM ALA.

DISCUSSION

In this study, greater than 60% of sera obtained on day 1 of hospitalization of Thai children with DHF contained ALA, and as patients recovered from DHF, the ALA began to diminish. The ALA were cold-reactive (15°C) IgM antibodies and did not appear to be due to anti-human lymphocyte antigen antibody activity since the DHF serum ALA were not associated with any history of transfusions or allogeneic stimulation. The ALA were found to be present in a titer of 1:4 to 1:16 and to display comparable activity for autologous as well as allogeneic MNC targets. The ALA were primarily directed against B cells and, to a lesser extent, against T cells. It is thus apparent that the cold-reactive ALA in DHF are similar to cold-reactive ALA reported for other diseases (19).

Although the absence of cytotoxicity in whole serum at 37°C raises the possibility that lymphocytotoxic antibodies in DHF sera may not cause in vivo complement-mediated lymphocytolysis (18), it should be noted that the temperature in the peripheral circulation of patients is lower and that the antibodies could attach and lead to cell surface modulation in the periphery. It is possible that optimal cytotoxicity at 15°C represents a compromise between lower and higher temperature for binding and complement fixation, respectively. In vivo and in vitro observations on lymphocytotoxins in other human diseases suggest that at 37°C, lymphocytes may be able to process lymphocytotoxic antibodies effectively. This has been proposed to result from pinocytosis (13) or shedding of the cytotoxic factor and its receptor (P. Wernet, M. Fotino, R. Thoburn, A. Moore, and H. G. Kunkel, Arthritis Rheum. 16:137, 1973). At temperatures less than 37°C, which can be seen in vivo in the peripheral circulation of patients, altered metabolism might impair this process with resultant cell lysis.

On the other hand, unexpected cytotoxicity seen when the 19S gradient fractions were tested at 37°C suggests that lymphocytotoxic IgM antibodies present in the sera of DHF patients have in vivo functions. A likely explanation for the enhanced lymphocytotoxicity at 37°C is that (i) the degree of avidity of the cytotoxic antibodies in the DHF serum varies, and (ii) the IgM eluates are more reactive at 37°C, owing to a higher concentration of specific lymphocytotoxic antibodies in the total 19S immunoglobulin pool. A similar phenomenon was noted by Cicciarelli et al. (4) in their studies on the identification of surface IgM as the target antigen of cold lymphocytotoxins.

ALA in the sera of patients may function as auto-regulatory feedback antibodies. The anti-T cell ALA could function in an auto-regulatory manner by reacting with specific T-suppressor or T-helper cell subpopulations at physiological temperatures, leading to the sequestering of the respective subpopulations at physiological temperatures. On the other hand, since ALA react with B lymphocytes, they could function in modulating antibody production by the B lymphocytes. Hypothetically, autoreactive B cells may develop during infection and have the potential to synthesize antibodies against autologous cell subpopulations. The previously observed decrease in T cells (17) in dengue patients could relate in a causative manner to the present studies if a loss of suppressor T cells resulted in autoantibody production of anti-B cell antibodies.

Because dengue virus invades and replicates

poorly in resting lymphocytes but well in stimulated transformed B lymphoblast cells (6, 9, 15), it has been postulated that in secondary dengue infection, antigenic stimulation by the virus of cells primed to recognize the virus leads to blast transformation, with subsequent enhanced viral invasion and replication in the atypical lymphocytes (21). Thus, since polyclonal antibody formation is known to occur as a result of virus infection (1), it would be important to determine whether polyclonal B cell activation exists in humans as a result of dengue infections. Whether or not lymphocytotoxic activity in DHF serum results from activation of autoreactive B cells during the course of infection could be of practical significance for future vaccine programs if immunosuppressive capabilities are associated with ALA.

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