INFECTION AND IMMUNITY, May 1980, p. 428-433
0019-9567/80/05-0428/06$02.00/0

Vol. 28, No. 2

Kinetics of Peripheral Blood Leukocyte Alterations in Thai Children with Dengue Hemorrhagic Fever

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Peripheral leukocytes from 16 Thai children with dengue hemorrhagic fever were examined to determine the leukocyte composition on the day of presentation and on convalescent days 15 and 30. Mononuclear cells were isolated each time, and the concentrations of T, B, Fc receptor-bearing, and "null" cells were determined. On the day of hospitalization, in comparison to convalescent values, there was a significant increase in total lymphocytes, primarily due to concentrations of atypical lymphocytes. There was a significant loss of T cells with an increase in non-T, non-B, non-Fc receptor-bearing null cells. There were no changes in the concentrations of monocytes, B cells, or Fc receptor-bearing cells when acute and convalescent values were compared. During the convalescent period, a progressive increase in eosinophils was noted. Also, on day 15 but not on day 30 of the convalescent period, an increase was observed in the total leukocyte number due to an increase in granulocytes. These results indicate that in Thai children with dengue hemorrhagic fever, there are major shifts within several component cell subpopulations of the immune system.

Dengue viruses are a major cause of morbidity and mortality among children residing in tropical and subtropical areas of the world (12). The four serotypes of dengue virus, 1, 2, 3, and 4, form an antigenic subgroup of the flaviviruses (group B arboviruses). Transmission to humans of any of these serotypes initiates a spectrum of host responses, from inapparent to severe and sometimes lethal infections. The most severe host response is dengue hemorrhagic fever (DHF), in which patients present fever, hemorrhagic diathesis, hypotension, thrombocytopenia, and increased vascular permeability. Some of these patients develop vascular collapse and dengue shock syndrome, which may be fatal if not adequately treated (14). The occurrence of the severe aspects of dengue infection correlates in most cases with the patient's immunological status. Although, dengue shock syndrome may occur in primary infections (27), the majority of cases occur in patients with preexisting serum antibody to dengue viruses (15).

Preexisting antibody appears to have multiple roles in the pathogenesis of severe dengue infections. It has been demonstrated that infection of peripheral blood leukocytes by dengue viruses in vitro is markedly enhanced by the presence of non-neutralizing dengue antibody (18) and that dengue virus may be isolated from the peripheral blood leukocytes of naturally infected patients (28). Also, antigen-antibody complexes may lead to massive activation of complement, leading to a release of anaphylotoxins which mediate hypersensitivity in themselves or through interaction with mast cells (5, 36). It is possible that these two phenomena interrelate synergistically to augment adverse reactions to the dengue virus. Since the immune status of the host may determine the severity of the dengue infection in humans, it is worthwhile to consider the makeup of the cellular immune system. Recent in vitro and in vivo studies have demonstrated that the cells of the immune system are important in viral replication and are thereby potentially involved in determining the severity of the illness. It is clear from in vitro studies that T lymphocytes are not capable of being infected with dengue virus and that macrophages can be infected, but whether B lymphocytes can be infected is controversial (19, 31). In addition, dengue virus has been isolated from the peripheral blood leukocytes of dengue patients (Scott et al., manuscript in preparation). Further work is needed to evaluate the cellular immune response to dengue virus in vivo, particularly in naturally infected residents of tropical areas with severe dengue disease. Because few data are available in this area, the present study was undertaken to evaluate changes in leukocyte populations and lymphocyte subpopulations in

children with the severe form of dengue viral infection: DHF and dengue shock syndrome.

MATERIALS AND METHODS

Patients. Patients with histories and physical examinations compatible with DHF were admitted to the Bangkok Children's Hospital. Study patients were examined at least once daily. The day of hospitalization was considered day 1 of disease. Blood was obtained on the day of hospital admission, and convalescent samples were taken approximately 15 and 30 days later. Patient hospital records were reviewed retrospectively, and the cases were grouped into those with and without dengue shock syndrome (23).

Serology. Serum obtained from each patient was tested for antibodies by hemagglutination inhibition with a microtiter method (11, 29). Whole sera were extracted with acetone before testing. Mouse brain antigens were made from prototype strains of dengue virus types 1, 2, 3, and 4, Japanese encephalitis virus, and chikungunya virus (3). Antigens were diluted with a borate saline buffer to contain eight hemagglutinating units at their optimum pH. The serology was used to classify each case as either a primary or a secondary dengue infection (34). Patients with convalescent hemagglutination inhibition titers of 1:640 or greater to two dengue antigens were considered to have secondary infections.

Isolation of virus. Virus isolation was attempted on plasma drawn from each patient, using a direct plaque technique in LLC-MK cells (37). Isolates were identified with a plaque reduction neutralization test with monkey antisera prepared to prototype virus strains (26).

Study of leukocytes. The total leukocyte count was determined in a hemacytometer by standard methods. A blood smear was stained with Wright stain, and a differential count was performed to determine the percentages of normal lymphocytes, atypical lymphocytes, neutrophils, basophils, eosinophils, and monocytes. Each determination (leukocyte count and differential) was done in triplicate on days 1, 15, and 30. The leukocyte count of each individual was multiplied by the percentage of each cell type present to obtain the concentration (number of cells per cubic millimeter) of each cell type. The individual results for each of the patients studied were averaged.

Isolation of mononuclear leukocytes. Peripheral blood mononuclear leukocytes were obtained by the method of Böyum (6). Approximately 5 ml of heparinized blood was drawn from each patient on days 1, 15, and 30 after hospitalization. The blood was diluted 1:2 in Hanks balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.) and layered on Ficoll-Hypaque (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). After centrifugation, the mononuclear leukocytes were removed and adjusted by a hemacytometer count to a concentration of 2×10^6 cells in Hanks balanced salt solution.

Preparation of sheep erythrocytes for rosette-forming assays. Sheep erythrocytes in Alsever solution were filtered with sterile gauze and washed with triethanolamine-buffered salt solution. The sheep erythrocytes were suspended at a concentration of 7×10^9 to 8×10^9 cells per ml in triethanolamine-buffered salt solution containing 0.1% gelatin (BBL Microbiology Systems, Cockeysville, Md.). Unmodified sheep erythrocytes were used to determine the percentage of T receptors; sheep erythrocytes were coated with subagglutinating amounts of 7S (immunoglobulin G [IgG]) anti-sheep erythrocytes (Cordis Laboratories, Miami, Fla.) to form erythrocyte-antibody (EA) rosettes. To detect B cells bearing complement receptors, sheep erythrocytes were coated with subagglutinating amounts of 19S (IgM) anti-sheep erythrocytes (Cordis Laboratories) and then incubated with fresh mouse serum to form erythrocyte-antibody complement (EAC) rosettes.

Procedures for detecting rosette-forming lymphocytes. The methods of Mendes et al. were employed for detecting rosette-forming lymphocytes (22). The percentage of cells forming erythrocyte (E) rosettes was determined after 5 min, 1 h, and 18 h (overnight) at 4°C. The percentage of cells forming EA and EAC rosettes was determined after 30 min at 37°C. In calculating the percentages of lymphocytes forming rosettes with three or more erythrocytes, both sides of a hemacytometer chamber were counted and values of rosette-forming and non-rosette-forming lymphocytes were averaged.

Statistical analyses. For statistical analyses, Student's t test was used, and $P < 0.01$ was considered necessary to obtain significance.

RESULTS

Patient leukocyte characteristics at presentation. Sixteen children with typical signs and symptoms had secondary dengue infections on the basis of the serological tests. Five viruses were isolated, of which two were dengue virus type 2, one was dengue virus type 4, and two could not be typed. Of the 16 patients, 9 exhibited hemorrhagic phenomena without shock, whereas signs of shock developed in the remaining 7.

The changes in relative percentages and concentrations of peripheral blood leukocytes in the patients during the acute and convalescent phases of DHF are shown in Tables 1 and 2. During the acute illness there was a significant increase in both the percentage and the concentration of total lymphocytes, which was due to a marked increase in both the percentage and the number of atypical lymphocytes, whereas normal lymphocytes were essentially unchanged. There was essentially no change in monocyte percentage and number, but during day 15 of convalescence a transient increase in the total number of leukocytes due to an increase in both the percentage and the absolute number of granulocytes was observed. The absolute number, but not the percentage, of basophils increased during convalescence. Finally, a marked increase during convalescence in both the percentage and the concentration of eosinophils was observed. Thus, the major changes

TABLE 1. *Changes in the relative percentages of peripheral leukocytes in children during acute and convalescent phases of DHF*

Phase of illness	Mean % ± standard error of the mean						
	Total lymphocytes	Normal lymphocytes	Atypical lymphocytes	Neutrophils	Basophils	Eosinophils	Monocytes
Acute day 1 (n = 16)[a]	53.6 ± 5.2	43.1 ± 4.2	10.5 ± 2.9	40.7 ± 5.4	0.2 ± 0.1	0.5 ± 0.3	5.0 ± 0.7
Convalescent day 15 (n = 16)	39.1 ± 3.2 ($P < 0.001$)[b]	38.0 ± 3.2 ($P < 0.01$)	1.1 ± 0.3 ($P < 0.001$)	53.4 ± 3.1 ($P < 0.01$)	0.4 ± 0.2	3.7 ± 1.2 ($P < 0.01$)	3.4 ± 0.6
Convalescent day 30 (n = 15)	45.4 ± 3.0 ($P < 0.01$)	44.8 ± 3.0	0.6 ± 0.2 ($P < 0.001$)	43.4 ± 2.9	0.4 ± 0.2	6.3 ± 0.85 ($P < 0.001$)	4.5 ± 0.7

[a] n, Number of patients studied.

[b] Numbers within parentheses indicate the P value for convalescent value in comparison with acute illness (day 1) value.

noted in the leukocytes were a marked increase in atypical lymphocytes during the acute stage of DHF and the appearance of increasing numbers of eosinophils during the convalescent stages of the illness.

Leukocyte composition over time. We next examined alterations in lymphocyte subpopulations during the acute and convalescent stages. There were significantly fewer T cells as judged by both percentages and absolute numbers of E-rosette-forming cells during the acute stage (Tables 3 and 4). There were no changes in the percentage or number of either EA or EAC rosette-forming cells. By calculation, both the percentage and the concentration of non-T, non-B, non-Fc receptor-bearing cells was significantly increased during acute DHF. Thus, the major changes noted in the lymphocyte subpopulations were a decreased percentage and number of T cells and an increased percentage and number of non-T, non-B, non-Fc receptor-bearing cells during the acute stage of illness.

When the data were analyzed to compare dengue with and without shock, no differences were found in the mean percentages or concentration values for either the leukocyte differentials or the lymphocyte subpopulation determinations.

DISCUSSION

There are few data available on the alterations in peripheral blood leukocytes that occur during dengue infection. Simmons et al. (30) reported a decrease in the leukocyte count during the illness which was due to a decrease in neutrophils. Halstead and co-workers (17, 23) found that early in the course of illness, patients with either primary or secondary dengue infections exhibited a fall in the leukocyte count associated with a rise in the percentage of lymphocytes. Recent advances in the understanding of the immunopathophysiology of dengue shock syndrome prompted us to study changes in the lymphocyte subpopulations and to reexamine alterations in the concentrations of leukocytes.

Our studies demonstrated that naturally infected Thai children with DHF have significant alterations in their leukocyte populations during the acute stage of illness. Most prominent were an increase in the percentage and total number of lymphocytes and an increase in the percentage and number of atypical lymphocytes.

Atypical lymphocytes are known to occur in numerous viral illnesses, including infectious mononucleosis, viral hepatitis, herpes, rubella, influenza, and others. Although the function of atypical lymphocytes is unclear, they incorporate increased amounts of [^{3}H]thymidine into deoxyribonucleic acid and are similar in appearance to lymphocytes which undergo blast transformation after stimulation with mitogens (such as phytohemagglutinin) or specific antigens as reported by Wood and Frenkel (35). Thus, it is possible that atypical lymphocytes represent a response to nonspecific viral stimulation or to specific viral antigens due to recognition followed by transformation. The potential importance of this in DHF stems from studies which have demonstrated that the dengue virus in vitro invades and replicates poorly in resting lymphocytes but well in stimulated transformed B lymphoblast cells (16, 21, 31). It is conceivable that in secondary dengue infections, antigenic stimulation by the virus of cells primed to recognize the virus leads to blast transformation with subsequent enhanced viral invasion and replication

TABLE 2. *Changes in the concentration of peripheral leukocytes in children during acute and convalescent phases of DHF*

Phase of illness	Mean no. per mm³ ± standard error of the mean							
	Total leukocytes	Total lymphocytes	Normal lymphocytes	Atypical lymphocytes	Neutrophils	Basophils	Eosinophils	Monocytes
Acute day 1 (n = 16)[a]	9,218 ± 684	4,941 ± 485	3,973 ± 631	968 ± 309	3,752 ± 1,168	18 ± 4	46 ± 57	461 ± 55
Convalescent day 15 (n = 16)	10,250 ± 850 ($P < 0.01$)[b]	4,008 ± 390 ($P < 0.01$)	3,895 ± 408	113 ± 33 ($P < 0.001$)	5,474 ± 522 ($P < 0.01$)	41 ± 12 ($P < 0.01$)	379 ± 116 ($P < 0.001$)	348 ± 74
Convalescent day 30 (n = 15)	9,216 ± 528	4,184 ± 270 ($P < 0.01$)	4,128 ± 343	56 ± 20 ($P < 0.001$)	4,000 ± 310	37 ± 8 ($P < 0.01$)	580 ± 88 ($P < 0.001$)	415 ± 64

[a] n, Number of patients studied.
[b] Numbers within parentheses indicate the P value for convalescent value in comparison with acute illness (day 1) value.

within the atypical lymphocytes.

Alternatively, the atypical lymphocytes in secondary dengue infection could be part of the normal immune response directed at controlling dengue virus-infected cells. To clarify which of these possibilities might be occurring, it would be of interest to determine whether the atypical lymphocyte is infected with dengue virus and to identify the cell type from which the atypical lymphocyte is derived.

Differences between acute and convalescent samples were also found in the concentrations of eosinophils. Similar findings have been reported for other viral infections, where, in response to inflammation during the acute phase, eosinophil concentrations fell. During convalescence, the eosinophil concentrations rose to normal or supernormal levels (2). Eosinophilia during the convalescent phase of dengue fever was found by Carpenter and Sutton (7). In their cases the concentration of eosinophils during the convalescent phase was considerably higher than that seen in our patients.

An increase in the total number of basophils was also seen in the convalescent period. The increase in basophils may have represented a recovery from the depression of these cells due to a complement-mediated hypersensitivity reaction during the acute phase of illness (36).

The decrease in T cells in DHF correlates with the postmortem pathological findings of profound depression of lymphocytes in the thymus and T-dependent areas of the spleen and lymph nodes (1, 4). T-cell depression has also been observed in other viral infections, such as hepatitis B and measles (9, 32, 33). Destruction of infected T cells by complement-dependent cytotoxic antibody has been suggested as a cause for the depression seen in measles. This mechanism, however, seems unlikely for dengue as in vitro studies indicate that T-cell infection does not occur with this virus (31). Infection-induced serum factors or intrinsic alterations in the T cells themselves or both have been implicated as the cause for T-cell depression in hepatitis B, where they block the formation of E-cell rosettes (10). Similar phenomena may also occur in dengue, but we have no evidence for this at the present time. A further possibility suggested by studies of measles patients (25) is the shift of T cells from the peripheral blood to other areas rich in lymphoid tissues, but this theory is not compatible with the pathological findings in fatal dengue patients.

Regardless of the mechanism, the decreases in T cells may lead to functional changes. If depression were due to the loss of specific subclasses, there could be alterations in the helper

TABLE 3. *Changes in the relative percentages of peripheral blood lymphocyte subpopulations in children during acute and convalescent phases of DHF*

Phase of illness	Mean % ± standard error of the mean					
	5-min E rosettes	1-h E rosettes	18-h E rosettes	EA rosettes	EAC rosettes	Null cells
Acute day 1 (n = 16)[a]	28.8 ± 1.3	33.3 ± 1.6	40.9 ± 0.5	8.6 ± 1.7	14.8 ± 1.8	35.7 ± 3.1[b]
Convalescent day 15 (n = 16)	44.1 ± 1.5 ($P < 0.001$)[c]	50.7 ± 1.4 ($P < 0.001$)	60.8 ± 1.2 ($P < 0.001$)	11.3 ± 2.3	16.9 ± 2.1	11.0 ± 1.8 ($P < 0.001$)
Convalescent day 30 (n = 15)	44.9 ± 1.6 ($P < 0.001$)	52.4 ± 1.7 ($P < 0.001$)	64.9 ± 0.8 ($P < 0.001$)	9.1 ± 1.7	17.6 ± 0.8	8.4 ± 0.8 ($P < 0.001$)

[a] n, Number of patients studied.

[b] Calculated percentage of non-T, non-B, non-Fc receptor-bearing cells obtained by subtracting the EA, EAC, and 18-h E-rosette values from 100%.

[c] Numbers within parentheses indicate the P value for convalescent value in comparison with acute illness (day 1) value.

TABLE 4. *Changes in the concentration of peripheral blood lymphocyte subpopulations in children during acute and convalescent phases of DHF*

Phase of illness	Mean no. per mm^3 ± standard error of the mean					
	5-min E rosettes	1-h E rosettes	18-h E rosettes	EA rosettes	EAC	Null cells
Acute day 1 (n = 16)[a]	1,406 ± 62	1,643 ± 191	2,022 ± 114	427 ± 57	730 ± 123	1,762 ± 359[b]
Convalescent day 15 (n = 16)	1,766 ± 182 ($P < 0.001$)[c]	2,034 ± 101 ($P < 0.01$)	2,438 ± 249 ($P < 0.01$)	454 ± 65	677 ± 68	439 ± 72
Convalescent day 30 (n = 15)	1,879 ± 177 ($P < 0.001$)	2,194 ± 259 ($P < 0.01$)	2,715 ± 104 ($P < 0.001$)	379 ± 31	738 ± 58	352 ± 46 ($P < 0.001$)

[a] n, Number of patients studied.

[b] Calculated concentration (cells per cubic millimeter) of non-T, non-B, non-Fc receptor-bearing cells obtained by subtracting the EA, EAC, and 18-h E-rosette values from the total number of lymphocytes.

[c] Numbers within parentheses indicate the P value for convalescent value in comparison with acute illness (day 1) value.

or suppressor functions mediated by these classes. Likewise, alterations might occur in any or all of the cell-mediated cytotoxicity mechanisms, as T cells are also responsible for these functions. Clearly, further investigation is needed to define functional changes and to determine the mechanism of T-cell depression.

Concomitant with decreased T cells in these patients was an increase in both the percentage and the total number of non-T, non-B, non-Fc receptor-bearing cells. Thus, there is an increase in what really is a subclass of "null" cells. As originally defined, null cells are non-T, non-B cells, and their percentage is determined by subtracting the number of T cells plus the number of B cells from 100 (34). However, it is now clear that the null cell population is heterogeneous (13, 20). These cells may develop into or be a subpopulation of B cells (8) or T cells.

ACKNOWLEDGMENTS

We thank the staffs of the Departments of Immunobiology and Virology of the Armed Forces Institute of Medical Sciences and the Children's Hospital for their excellent technical and clinical assistance. We are especially grateful to Air Vice Marshall Sithibun Purnaverja, Director General of the Armed Forces Research Institute of the Medical Sciences, and the Kingdom of Thailand for support to us during the conduct of this work. We thank June Macon for typing this manuscript.

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