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Lysis of Dengue Virus-Infected Cells by Natural Cell-Mediated Cytotoxicity and Antibody-Dependent Cell-Mediated Cytotoxicity

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Peripheral blood mononuclear cells (PBMC) from humans without antibodies to dengue 2 virus lysed dengue 2 virus-infected Raji cells to a significantly greater degree than uninfected Raji cells. The addition of mouse anti-dengue antibody increased the lysis of dengue-infected Raji cells by PBMC. Dengue 2 immune human sera also increased lysis of dengue-infected Raji cells by PBMC. These results indicate that both PBMC-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity (ADCC) can cause significant lysis of dengue-infected Raji cells. The lysis of infected Raji cells in the ADCC assay correlated with the dilution of dengue-specific antibody which was added, indicating the dengue virus specificity of the lysis of dengue virus-infected Raji cells. Alpha interferon (IFNα) was detected in the culture supernatant of PBMC and dengue-infected Raji cells. However, enhanced lysis of dengue-infected Raji cells by PBMC may not be due to the IFN produced, because neutralization of all IFN activity with anti-IFNα antibody did not decrease the lysis of dengue-infected cells, and effector cells pretreated with exogenous IFNα also lysed dengue-infected cells to a greater degree than uninfected cells. The effector cells responsible for lysis of dengue virus-infected Raji cells in the natural killer and ADCC assays were analyzed. Nonadherent PBMC caused more lysis than did adherent cells. Characterization of nonadherent cells with monoclonal antibodies showed that the predominant responsible effector cells were contained in OKM1$^+$ and OKT3$^-$ fraction in the natural killer and ADCC assays.

Dengue virus infection is a major worldwide cause of morbidity (15), and hemorrhagic fever and shock are severe and, at times, fatal complications of dengue infections (15). These complications are more commonly observed in individuals undergoing a secondary dengue infection with a virus of another dengue subtype than that which they had experienced in their primary infection (16). These observations indicate that the immune response of the host may play an important role in the complications of dengue infection.

The mechanism of this apparent sensitization to subsequent dengue infections is not defined. One theory is that antibody formed during the secondary response forms complexes with viral antigen and that massive complement activation follows the formation of this immune complex, resulting in increased vascular permeability and initiating a shock syndrome (26). Another theory postulates that immune-mediated destruction of dengue-infected monocytes, which are the most productive source of replicating virus (17), is responsible for releasing various substances, including C3b and unidentified vascular permeability factor(s), from such monocytes (15). The complication of hemorrhagic fever and shock make it important to understand the mechanism of immunological sensitization and of elimination of dengue-infected cells.

Dengue virus infection induces type-specific and cross-type-reactive antibody responses (30). Infection results in lifelong resistance to challenge with homologous virus; however, protection against heterologous strains is short-lived (27, 28), and secondary infection may be more severe than primary infection. At subneutralizing levels of antibody, enhanced infection of cells with Fc receptors can be detected in vitro (10). This effect has also been studied in vivo by the passive transfer of antibody to infected monkeys which resulted in enhanced replication of virus in circulating monocytes (14). The role, however, of this antibody-enhanced replication of dengue virus infection is uncertain in the dengue hemorrhage fever and shock syndrome. On the other hand, antibody may help to eliminate dengue-infected cells. Cytolysis of dengue-infected Vero cells by antibody-dependent, complement-mediated cytolysis has been reported, although the percent lysis of infected cells was low (8).

Antibody may also eliminate virus-infected cells by antibody-dependent, cell-mediated cytotoxicity (ADCC). ADCC has been reported to lyse virus-infected cells in vitro (18, 23), but its role in eliminating virus-infected cells in vivo has not been defined. Other cell-mediated immune responses have been found to be effective for the lysis of virus-infected cells in vitro and may aid in eliminating viral infection in vivo. These include macrophages (32), natural killer (NK) cells (25), and cytotoxic T cells (11). These cellular effector functions have not been analyzed in dengue virus infections. The illness caused by dengue virus infections and its complications stimulated us to examine the cellular immune responses to dengue infection. In this paper, we describe the lysis of dengue-infected Raji cells by PBMC active in the NK and ADCC assays.

MATERIALS AND METHODS

Virus. Dengue virus type 2 (dengue 2 virus) New Guinea C strain was supplied by the Walter Reed Army Institute of Research. This virus was then propagated in mosquito cells (c6/36). Briefly, mosquito cells were infected with dengue 2 virus at a multiplicity of infection of 0.1 PFU per cell and incubated in Eagle minimal essential medium containing 2% heat-inactivated fetal calf serum (FCS) and 0.8% bovine serum albumin at 28°C for 7 days. The culture fluid was stored as virus stock at −85°C until use. The infectious virus titer was determined to be 1×10^7 PFU/ml by plaquing on Vero cells with standard techniques (33).

Anti-dengue 2 antibody. Ascitic fluid from mice hyperimmunized with dengue 2 virus was used for antibody (4). The titer of this antibody was 1:256 by complement fixation test

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and 1:1,024 by neutralization tests. Hyperimmune ascitic fluid was heated at 56°C for 30 min to destroy complement activity before use.

Human sera. Human sera obtained from virus isolation-documented cases of dengue 2 illness in Puerto Rico in 1968 were used as sources of anti-dengue 2 human antibody. Human sera with or without antibody to Epstein-Barr virus were supplied by T. Sairenji of the University of Massachusetts Medical School. These sera did not contain antibodies to dengue virus. All human sera were heated at 56°C for 30 min before use.

Cell cultures. Raji cells (24), Raji cells persistently infected with dengue 2 virus, and K562 cells (22) were used as the targets in NK and ADCC assays. All cell lines were cultured in RPMI 1640 medium (Flow Laboratories, McLean, Va.) containing 10% FCS. Preparation of the dengue virus-infected Raji cells is described below. Theofilopoulos et al. reported that Raji cells were persistently infected with dengue 2 virus, and more than 70% of cells contained dengue antigens (34).

Human PBMC. Peripheral blood specimens were obtained from healthy donors who do not have antibodies to dengue virus. PBMC were separated by the Ficoll-Hypaque density gradient centrifugation method (3). Cells were suspended at a concentration of 1×10^7 to 3×10^7/ml in RPMI 1640 containing 10% FCS and 10% dimethylsulfoxide (Fisher Scientific Co., Pittsburgh, Pa.) and were cryo-preserved in liquid nitrogen until use.

IFN assay. Interferon (IFN) was assessed by a cytopathic effect reduction assay. Twofold serial dilution of IFN samples were incubated with human fibroblast cells for 20 h at 37°C and then challenged with vesicular stomatitis virus. An international IFN standard was included in each assay, and titers were read after 48 h at 37°C.

Neutralization of IFN. Neutralization of antiviral activity was carried out by using specific antisera to human alpha, beta, and gamma IFN. Antiserum to IFNα was purchased from Interferon Sciences, Inc., New Brunswick, N.J. Antisera to IFNβ and IFNγ were gifts from A. Meager, Division of Virology, National Institute of Biological Standards and Control, London. Diluted IFN samples containing 25 U/ml were incubated for 2 h at room temperature with serial dilutions of antiserum, and the residual IFN titers were determined by the cytopathic effect reduction assay.

Pretreatment of effector cells with actinomycin D. Effector cells (4×10^6/ml) were incubated with various concentrations of actinomycin D (Sigma Chemical Co., St. Louis, Mo.) at 37°C for 1 h. Cells were washed three times and then were used as effectors.

Pretreatment of effector cells with IFNα. Effector cells (2×10^6/ml) were incubated with 1×10^4 U/ml of human IFNα (Lee and Biomolecular Research Laboratories, San Diego, Calif.) at 37°C for 4 h. Cells were washed three times and then were used as effectors. Anti-IFNα antibody was added to the NK assay at a final dilution of 1:10.

Immunofluorescence staining. Dengue 2 virus membrane antigens were identified on infected Raji cells by indirect membrane fluorescence. Cells (2×10^6) in 0.05 ml of phosphate-buffered saline were incubated at 37°C for 30 min with 0.1 ml of dengue 2-hyperimmunized mouse ascitic fluid diluted to a concentration of 1:10. Cells were washed twice and reacted with fluorescein isothiocyanate-conjugated sheep anti-mouse immunoglobulin G (Cappel Laboratories, Cochransville, Pa.) at 37°C for 30 min. Cells were again washed twice, and the percentage of stained cells was assessed with a fluorescent microscope.

To identify intracellular dengue 2 viral antigens, cells were air-dried on slide glasses and fixed with acetone at −20°C for 10 min. The fixed cells were reacted with hyperimmunized mouse ascitic fluid and fluorescein isothiocyanate-conjugated anti-mouse immuoglobulin G as described above. Raji cells which were not infected with dengue 2 virus were always included as controls. They showed no fluorescence with the anti-dengue 2 sera in either the membrane or fixed-cell preparations.

Cell separation. Adherent cells were separated from PBMC with FCS-coated plastic petri dishes (Becton-Dickinson Labware, Oxnard, Calif.) as previously described (20). For separation of nonadherent cells, adherent cells were depleted from PBMC by adherence to plastic dishes (1). The percentage of PBMC contained in each fraction was 96% in the adherent fraction and 3% in the nonadherent fraction as determined by yeast ingestion (20).

Nonadherent cells were stained at 4°C by indirect membrane immunofluorescence with monoclonal OKM1 and OKT3 antibodies (Ortho Pharmaceutical Corp., Raritan, N.J.) and fluorescein isothiocyanate-conjungated sheep anti-mouse immunoglobulin G was used as secondary antibody. Stained lymphocytes were sorted with a fluorescence-activated cell sorter (FACS 440; Becton-Dickinson Co., Mountain View, Calif.).

NK and ADCC assays. Target cells (1×10^6 to 5×10^6 cells) were labeled with 0.5 mCi of $^{51}Cr(Na_2CrO_4)$ (New England Nuclear Corp., Boston, Mass.) in 0.5 ml of RPMI–10% FCS at 37°C for 90 min. Labeled cells were washed twice and suspended in RPMI–10% FCS. Labeled cells (10^4) in 0.1 ml were added to each well in round-bottom microtiter plates (Limbro Scientific, Hamden, Conn.). Medium containing anti-dengue 2 antibody (for the ADCC assay) or lacking this antibody (for the NK assay) was added in 0.05-ml volumes to each well and cultured at 37°C for 30 min. Various concentrations of effector cells were added in 0.05 ml of medium to each well to give the described effector/target (E/T) ratios. After incubation at 37°C for 16 to 18 h, 0.1 ml of supernatant fluid was collected from each well and counted in an automatic gamma counter. Quadruplicate samples were tested. Spontaneous release from dengue-infected and uninfected Raji cells was ca. 20% of maximum release.

The percent specific ^{51}Cr release was calculated as follows: (counts per minute [cpm] experimental release − cpm spontaneous release/cpm maximal release − cpm spontaneous release) × 100.

Statistical analysis. The significance of differences between values was examined by the Student *t* test, paired *t* test, or Wilcoxon rank sum test. Differences yielding *P* values of <0.05 were regarded as significant.

RESULTS

Preparation of Raji cells persistently infected with dengue 2 virus as infected target cells. Raji cells were infected with dengue 2 virus at a multiplicity of infection of 0.05 PFU per cell at 37°C for 2 h. Infected cells were washed twice with RPMI, suspended at the concentration of 2×10^5 cells per ml in RPMI–10% FCS and cultured at 37°C in 5% CO_2. The cells were suspended in fresh medium (RPMI–10% FCS) at the concentration of 2×10^5 cells per ml every 3 days. At day 7 after infection, 40% of the cells were positive, and by day 9, 90% were positive for dengue membrane and cytoplasmic antigens. These cells are split every 3 days at a ratio of 1:10 and have remained infected over 9 months, with more than 90% expressing membrane and cytoplasmic dengue anti-

gens. The cells in this persistently infected culture line are more than 95% viable as determined by dye exclusion testing with trypan blue. The dengue virus titer of supernatant culture fluids is 3×10^3 PFU/ml, and the IFN titer is 3 U/ml. These persistently infected Raji cells were used as target cells in the following studies.

Lysis of dengue 2-infected Raji cells by PBMC with or without anti-dengue 2 antibody. Dengue 2-infected Raji cells and uninfected Raji cells were used as target cells with normal human PBMC as effector cells. These PBMC had been obtained from healthy donors who did not have antibodies to dengue virus. Table 1 shows the results of an 18-h ^{51}Cr release assay, with or without hyperimmune anti-dengue 2 ascitic fluid. Dengue-infected Raji cells were lysed by PBMC without antibody more than uninfected Raji cells ($P < 0.01$). The addition of anti-dengue 2 antibody significantly increased the lysis by PMBC of dengue 2-infected cells ($P < 0.0025$) but did not increase the lysis of uninfected Raji cells. Ascitic fluid from nonimmunized mice caused no augmentation of the lysis of dengue-infected or uninfected cells at a 1:20 dilution (data not shown).

PBMC also lysed acutely infected Raji cells to a greater degree than uninfected Raji cells on day 5 after infection (percent specific lysis was 18% from infected cells and 3% from uninfected cells with one donor, 10% from infected cells and 2% from uninfected cells with the other donor). These results indicate that PBMC lyse dengue-infected Raji cells significantly more than uninfected Raji cells and that the augmentation of PBMC-mediated killing by anti-dengue antibody was detected on dengue-infected target cells but not on the uninfected target cells. In general, there is a statistically significant correlation between the percent lysis of K562 cells and that of dengue-infected cells in the NK assay ($r = 0.77$; $P < 0.01$); however, the PBMC of some individuals which lyse K562 cells do not lyse dengue-infected cells very well.

Time course and effector-target dose-response studies of the lysis of dengue-infected Raji cells by PBMC and ADCC. Experiments were carried out to determine the time course of lysis of dengue-infected or uninfected Raji cells by PBMC with or without anti-dengue 2 antibody. PBMC from two donors were incubated with target cells at the E/T ratio of 50 (with effector no. 2) and 10 (with effector no. 8) for various numbers of hours (Fig. 1). The specific lysis by PBMC of dengue 2-infected Raji cells reached a maximum at 8 to 18 h of incubation, depending on the effector used. Specific lysis of infected cells by ADCC (percent ^{51}Cr release from infected cells by ADCC minus percent ^{51}Cr release from infected cells by PBMC without antibody) reached a maximum by 18 h of incubation.

The dose-response effect of various E/T ratios was evaluated with the same two donor cells as effectors (Table 2). There was an obvious dose-response relationship between the E/T ratio and specific ^{51}Cr release from the target in both the NK (PBMC alone) and the ADCC assays.

The results of these two experiments also confirmed the results described in Table 1; i.e., at any time of incubation, and at any E/T ratio studied, dengue-infected cells were lysed by PBMC to a greater degree than uninfected cells, and the addition of anti-dengue antibody augmented the lysis by PBMC of dengue-infected cells.

Dose-response relationship between the anti-dengue 2 antibody and the specific increase of lysis by ADCC. Hyperimmune ascitic fluid was used as antibody and was diluted from 1:10 to $1:1.5 \times 10^5$, and the PBMC from donors no. 2 and no. 8 were used as effector cells (Fig. 2). There was a dose-response relationship between the added antibody and the specific ^{51}Cr release by ADCC when hyperimmune ascitic fluid was used.

ADCC lysis of dengue-infected Raji cells with human anti-dengue 2 sera. We used several human sera as the source of antibody in ADCC assays. These sera included four positive and four negative sera for anti-dengue 2 antibody (Table 3). Pooled human AB serum, which we used as a control, did not increase lysis by PBMC of dengue 2-infected and uninfected Raji cells in previous experiments (data not shown). The addition of antisera containing antibody to dengue 2 virus increased the lysis of dengue 2-infected cells significantly more than the lysis of uninfected cells ($P < 0.025$). The addition of sera without anti-dengue 2 antibodies, however, did not increase the lysis of infected cells. This experiment with human sera confirmed the results of earlier experiments with murine antibodies and human effector cells. Serum positive for anti-Epstein-Barr virus antibody

TABLE 1. Lysis of dengue 2 virus-infected Raji cells by PBMC and antibody

Effector (E/T = 50)	% Specific ^{51}Cr release[a]				
	K562	Infected Raji[c]		Uninfected Raji[c]	
		− Antibody[d]	+ Antibody[b]	− Antibody[d]	+ Antibody
1	14.6	7.7	30.5	5.3	1.7
2	37.6	8.6	29.4	7.8	7.3
3	47.6	18.1	16.5	4.0	7.1
4	31.6	4.4	10.0	0.4	4.0
5	33.6	13.4	ND	2.6	ND
6	45.1	6.1	14.6	1.6	7.3
7	38.8	0.1	10.2	3.2	3.2
8	72.3	37.4	55.1	28.3	29.5
9	33.1	2.4	16.5	1.3	3.6
10	ND	16.6	24.1	2.3	5.7
Mean	39.4	11.5	23.0	5.7	7.7

[a] Percent specific ^{51}Cr release from dengue 2-infected and uninfected cells after 18-h assay. ND, Not determined.
[b] Ascitic fluid from dengue 2-hyperimmunized mice was used as a source of anti-dengue 2 antibody at a 1:20 dilution.
[c] Significance was determined by paired t test between the lysis of target cells by PBMC with and without antibody ($P < 0.0025$ for infected Raji cells, not significant for uninfected Raji cells).
[d] Significance was determined by paired t test between the lysis of infected Raji cells without antibody and that of uninfected Raji cells without antibody ($P < 0.01$).

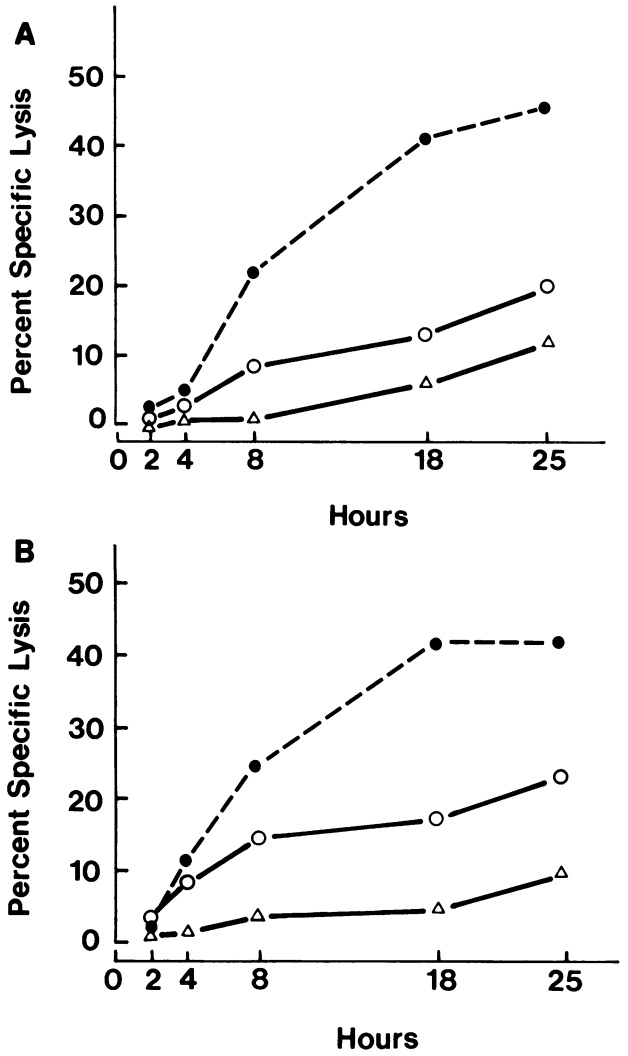

FIG. 1. Time course study of the lysis of dengue 2-infected or uninfected Raji cells in NK and ADCC assays. The percent specific ^{51}Cr release from target cells in NK and ADCC assays was assessed after the indicated hours of incubation. Hyperimmune mouse ascitic fluid was used at a 1:20 dilution in the ADCC assay. The E/T ratio was 50:1 with donor no. 2 (Fig. 1a) and 10:1 with donor no. 8 (Fig. 1b). Symbols: ○, infected Raji cells without antibody (NK assay); ●, infected Raji cells with antibody (ADCC assay); △, Uninfected Raji cells without antibody (NK assay).

but negative for anti-dengue 2 antibody did not augment the lysis of dengue-infected Raji cells. Therefore, the killing of dengue-infected Raji cells was not due to their being Epstein-Barr virus-transformed cells.

Production of IFN during NK and ADCC assays of dengue-infected Raji cells. We assessed whether IFN was produced during these assays to determine whether IFN may have contributed to the enhanced lysis of dengue-infected Raji cells by PBMC (Table 4). High titers of IFN (1,600 U/ml) were detected only in the culture supernatants containing both PBMC and dengue-infected Raji cells. The same titer of IFN was also detected in the ADCC assay. However, only 6 U of IFN per ml was detected in the culture supernatant of PBMC and uninfected Raji cells. The IFN was characterized as alpha because it was neutralized by specific antisera to human IFNα but not by antisera to IFNβ and IFNγ.

Addition of anti-IFNα antibody and pretreatment of effector cells with actinomycin D or IFNα. We examined the effects of anti-IFNα antibody on the lysis of dengue-infected Raji cells in the NK assay (Table 5). We added various dilutions of anti-IFNα antibody to the NK assay and assessed the specific lysis and IFN titer in the culture supernatant at 18 h. Although the addition of anti-IFNα antibody diluted to a concentration of 1:40 neutralized all detectable IFN produced during the NK assay, the specific lysis of dengue-infected Raji cells did not decrease, suggesting that the presence of IFN throughout the assay was not required to demonstrate the increased lysis of dengue-infected cells.

To study the possible early contribution of IFN to the enhanced killing of dengue-infected cells, we pretreated the effector cells with 0.02 to 0.16 μg of actinomycin D per ml (Table 6). Although pretreatment of effector cells in actinomycin D decreased the production of IFN by 50 to 75%, the specific lysis of dengue-infected cells did not concomitantly decrease. Pretreatment of effector cells with 0.02 to 0.16 μg of actinomycin D per ml did not decrease the lysis of uninfected cells either (data not shown).

In addition, we pretreated the effector cells with 10^4 U of exogenous human IFNα per ml. Pretreatment of effector cells with IFNα increased proportionally the lysis of dengue-infected and uninfected Raji cells; i.e., 53% augmented lysis of dengue-infected Raji cells, and 60% augmented lysis of uninfected Raji cells. Effector cells pretreated with IFNα lysed dengue-infected cells to a greater degree than uninfected cells, as did untreated effector cells (data not shown). This result and those shown in Tables 5 and 6 appear to indicate that the enhanced lysis of dengue-infected cells by

TABLE 2. Effector-target cell dose-response study in the lysis of dengue 2 virus-infected cells in NK and ADCC assays

E/T ratio	% Specific ^{51}Cr release from target cells[a]					
	Effector no. 2			Effector no. 8		
	Infected Raji[b]		Uninfected Raji (NK)	Infected Raji[b]		Uninfected Raji (NK)
	ADCC[c]	NK		ADCC[c]	NK	
100	41.2	26.1	11.8	ND	ND	ND
50	32.5	26.9	10.7	64.2	48.3	38.4
25	25.9	19.7	7.1	58.7	39.6	32.9
12.5	18.9	14.5	6.6	45.9	33.7	25.1
6.3	11.8	7.9	6.0	33.6	26.8	17.5
3.2	6.1	1.7	1.6	19.4	17.2	8.9
1.6	ND	ND	ND	26.7	19.4	6.2

[a] Percent specific ^{51}Cr release from dengue 2-infected Raji cells after 18-h assay. ND, Not determined.
[b] Significance was determined by the Wilcoxon rank sum test between ADCC lysis of infected Raji cells and NK lysis of infected Raji cells ($P = 0.025$ for both no. 2 and no. 8) and between NK lysis of infected Raji cells and NK lysis of uninfected Raji cells ($P = 0.025$ for both no. 2 and no. 8).
[c] Hyperimmune mouse ascitic fluid was used at a 1:10 dilution in ADCC assay.

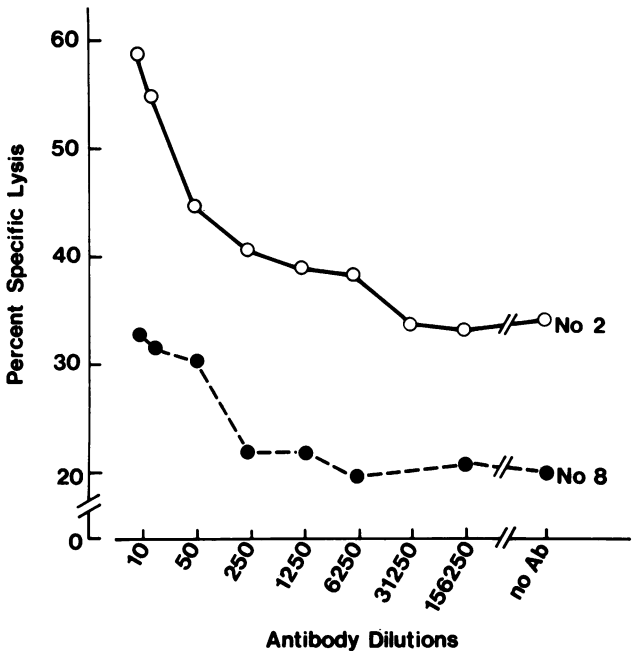

FIG. 2. Dose-response relation between the dose of antibody added and the lysis of infected Raji cells by ADCC. Percent specific ^{51}Cr release from dengue 2-infected Raji cells by ADCC was assessed after 18 h of incubation. Hyperimmune mouse ascitic fluid was used at 1:10 to 1:156,250 dilution. The E/T ratio was 50:1 with donor no. 2 and 10:1 with no. 8. Ab, Antibody.

PBMC may not be due to the IFN produced during the assay.

Effector cells responsible for lysis of dengue-infected target cells in NK and ADCC assay. We initially assessed the nature of the effector cells responsible for killing dengue-infected target cells with or without anti-dengue 2 antibody, using adherent and nonadherent cells from PBMC (Table 7). The results showed that the predominant cells responsible for the lysis of dengue-infected Raji cells in NK and ADCC assays were nonadherent cells, although some lysis was associated with the adherent cells.

We then analyzed in more detail the nature of the nonadherent cytotoxic cells, using monoclonal antibodies to the M1 and T3 antigens. Nonadherent PBMC were reacted with monoclonal antibodies to M1 or T3 antigens and sorted for use as effector cells in NK and ADCC assays (Table 8). In the NK assay, $OKM1^+$ cells caused higher lysis of dengue-infected Raji cells than $OKM1^-$ cells ($P < 0.001$). However, $OKT3^+$ and $OKT3^-$ cells lysed dengue-infected Raji cells to almost the same degree. In the ADCC assay, $OKM1^+$ cells also caused a significantly higher level of lysis ($P < 0.001$), although some level of lysis was observed with $OKM1^-$ cells. $OKT3^-$ cells caused a higher level of lysis than did $OKT3^+$ cells. K562 cells, which are used as target cells for assaying human NK cells, were lysed by $OKM1^+$ and $OKT3^-$ cells but not by $OKM1^-$ and $OKT3^+$ cells. These results indicated that the nonadherent effector cell(s) responsible for lysis of dengue-infected cells are contained in the $OKM1^+$ fraction and in both $OKT3^-$ and $OKT3^+$ fractions in the NK assay and are contained in $OKM1^+$ and $OKT3^-$ fractions in the ADCC assay. Therefore, some of the effector cells responsible for killing dengue-infected Raji cells include some cells of a different phenotye than the NK cells responsible for lysis of K562 cells, which are $OKM1^+$ and $OKT3^-$.

DISCUSSION

In these studies, we have shown that PBMC-mediated cytotoxicity and ADCC effectively kill dengue virus-infected cells in vitro. This conclusion is based on the following results. (i) PBMC from donors without antibodies to dengue 2 virus lysed dengue 2-infected Raji cells significantly better than uninfected Raji cells. (ii) The addition of murine or human anti-dengue 2 antibody into the NK assay increased the lysis of dengue-infected cells but did not increase the lysis of uninfected cells. The addition of ascitic fluid from unimmunized mice did not increase the lysis of dengue-infected cells. (iii) There was a dose-response relationship between the level of anti-dengue 2 antibody added and the specific ^{51}Cr release of ADCC, indicating the dengue virus specificity of the lysis of dengue-infected cells.

We have also detected increased lysis by PBMC of Raji cells infected with another subtype of dengue virus. PBMC lysed Raji cells persistently infected with dengue 4 virus to a higher level than uninfected Raji cells, and the addition of anti-dengue 4 antibody further increased the lysis of PBMC of dengue 4-infected Raji cells (unpublished data).

TABLE 3. Lysis of dengue 2 virus-infected Raji cells by ADCC, using human sera as antibody sources

Human sera (neutralizing antibody titer)[b]	% Specific ^{51}Cr release[a]			
	Infected Raji[c]		Uninfected Raji[c]	
	ADCC	Δx[d]	ADCC	Δx
Anti-dengue 2 antibody positive				
1 (640)	29.4	14.6	9.5	3.8
2 (640)	21.8	7.0	10.4	4.7
3 (160)	27.1	12.3	11.9	6.2
4 (80)	25.1	10.3	8.0	2.3
Anti-dengue 2 antibody negative				
1	13.8	−1.0	4.1	−1.6
2	15.3	0.5	4.5	−1.2
3 Control AB serum	14.8	0	5.7	0
Anti-EBV antibody				
4 Antibody +	13.6	−1.2	4.6	−1.1
5 Antibody −	11.2	−3.6	2.8	−2.9

[a] Percent specific ^{51}Cr release from dengue 2-infected and uninfected Raji cells after 18-h assay.
[b] Human sera were used at a 1:10 dilution after inactivation of complement. The neutralizing antibody titers in the human sera are described in parenthesis.
[c] Significance was determined by paired t test between Δx of infected Raji cells and Δx of uninfected Raji cells in the assay using sera positive for anti-dengue 2 antibody ($P < 0.025$).
[d] Δx, Percent specific lysis of target cells minus percent specific lysis by ADCC with control AB serum.

TABLE 4. Quantitation of IFN in culture superantants of effector cells and dengue virus-infected Raji cells

Target cells	Effector cells	Anti-dengue 2[a] antibody	IFN[b] (U/ml)	% Specific lysis
Dengue 2-infected Raji	+	+	1,600	45.5
	+	−	1,600	32.1
	−	−	<6	0
Uninfected Raji	+	−	6	18.8
	−	−	<6	0
None	+	−	<6	—[c]

[a] Hyperimmune mouse ascitic fluid was used at a 1:20 dilution.
[b] Effector cells and target cells were cultured at an E/T ratio of 50:1 for 18 h, and IFN in the culture supernatants was quantitated.
[c] —, No value due to lack of target cells.

There is a statistically significant correlation in the specific lysis by PBMC between K562 cells and dengue-infected cells. However, there is a relatively high heterogeneity as to the lytic activity of PBMC against dengue-infected cells, and some effector cells which lyse K562 to an average level lyse dengue-infected cells to a low level.

We have characterized the cells responsible for the lysis of dengue 2-infected Raji cells, using monoclonal antibodies to M1 and T3. Our results were as follows: effector cells responsible for the lysis of dengue-infected Raji cells in NK assay are contained in the $OKM1^+$ cells and in both $OKT3^+$ and $OKT3^-$ cells, effector cells in ADCC assay are contained in $OKM1^+$ and $OKT3^-$ cells. In the same experiments, K562 cells, which are usually used as target cells for human NK assay, were lysed very well by nonadherent cells contained in $OKM1^+$ and $OKT3^-$ fractions but not by cells contained in the $OKT3^+$ fraction. These results as well as the different level of lysis by PBMC of K562 and dengue-infected cells indicate that some of the effector cells responsible for lysis of dengue-infected cells are different cells from those responsible for lysis of K562. Heterogeneity of NK cells has been reported in human systems (12). Lopez has noted that the human NK cells killing K562 and herpes simplex virus type 1 (HSV-1)-infected target cells have somewhat different characteristics, although both cell types appear within the larger granular lymphocyte population (12). Our results with dengue virus-infected cells are consistent with these previous reports.

Little is known about the characteristics of effector cells responsible for ADCC to virus-infected cells. Some reports showed that null cells and T cells are effector cells in the ADCC assay for virus-infected cells, and both cell types have human NK (HNK-1) antigen, using respiratory syncytial virus- and influenza virus-infected targets (18, 23). In our experiments, T cells ($OKT3^+$ cells) were not responsible for ADCC of dengue-infected cells. We do not know whether this difference is due to the virus used, the methods of separating cells, or for some other reasons. Our studies with other monoclonal antibodies indicate that the effector cells responsible for lysis of dengue-infected cells in the NK and ADCC assays are contained in the $Leu11^+$ and $Leu7^-$ fraction, but some activity is in the $Leu11^-$ and $Leu7^+$ fraction (unpublished data). Further characterization of the effector cells is in progress.

There are reports with several other viruses that infected cells are lysed by NK cells to a greater degree than uninfected target cells (6, 9). However, the mechanism of enhanced lysis of virus-infected cells is not clearly known. In some cases, IFN is thought to be a prominent cause of enhanced lysis of infected cells. That is, NK cells which were activated by virus-induced IFN during the NK assay are thought to cause the enhanced killing of infected cells (29). We detected >400 U of IFNα per ml in the culture supernatants of PBMC and dengue-infected cells. However, the addition of anti-IFNα antibody, which neutralized all IFN produced during the assay, did not decrease the specific lysis of infected Raji cells by PBMC. Pretreatment of effector cells with actinomycin D, which decreased IFN production, did not decrease the lysis of dengue-infected cells. Dengue-infected cells were also lysed to a greater degree than uninfected cells by IFN-pretreated effector cells as when effector cells were not pretreated with IFN. These results suggest that the en-

TABLE 5. Effect of anti-IFN antibody on the lysis of Dengue virus-infected Raji cells by PBMC

Serum added	Dilution	IFN[a] (U/ml)	% Specific lysis[b]
None		400 (25)[c]	36.9 (13.0)[c]
Anti-IFNα	1:40	<25 (<25)	31.5 (13.0)
	1:160	100 (<25)	36.0 (15.8)
Control	1:40	200 (<25)	35.1 (15.0)
	1:160	400 (50)	31.8 (13.7)

[a] Dilutions of anti-IFN antibody were added to wells in the NK assay. IFN in the culture supernatant was quantitated after 18 h of culture.
[b] E/T ratio was 40:1.
[c] Numbers in parentheses indicate results obtained by using uninfected Raji cells as target cells.

TABLE 6. Effect of actinomycin D on the lysis of dengue virus-infected cells by PBMC and IFN production

Actinomycin D[a] (μg/ml)	% Specific[b] lysis	IFN[b] (U/ml)
0	31.8	1,600
0.02	31.8	800
0.04	31.1	800
0.08	27.7	800
0.16	27.7	400

[a] Pretreatment of effector cells with actinomycin D is described in the text.
[b] Effector and target cells were cultured at an E/T ratio of 50:1 for 18 h.

TABLE 7. Lysis of dengue-infected Raji cells by adherent and nonadherent cells with or without anti-dengue 2 virus antibody

Effector cells[a]	E/T ratio	% Specific ^{51}Cr release[b]			
		Infected Raji		Uninfected Raji (−antibody)	K562 (−antibody)
		+Antibody[c]	−Antibody		
Unfractionated PBMC	50	ND	55.4	43.9	73.7
	10	47.7	31.7	19.1	33.0
Adherent cells	50	ND	35.3^{**}	16.7	$15.0^{\dagger}$
	10	25.5^{*}	16.4^{***}	7.4	$10.0^{\dagger\dagger}$
Nonadherent cells	50	ND	51.7^{**}	26.6	$75.5^{\dagger}$
	10	46.6^{*}	37.0^{***}	18.4	$38.8^{\dagger\dagger}$

[a] Percentage of phagocytic cells contained in each fraction: unfractionated, 15%; adherent, 96%; and nonadherent, 3%.
[b] Percent specific ^{51}Cr release from each target after 16 h of assay. Significance was determined for the difference in the level of lysis between groups with the same symbol: *, $P < 0.001$; **, $P < 0.0025$, ***, $P < 0.001$, †, $P < 0.001$, ††, $P < 0.001$.
[c] Hyperimmune mouse ascitic fluid was used at 1:10 dilution. ND, Not done.

TABLE 8. Lysis of dengue virus-infected Raji cells by effector cells sorted with OKM1 or OKT3 antibody

Effector cells[a]	% Specific ^{51}Cr release[b]			
	Infected Raji		Uninfected Raji (−antibody)	K562 (−antibody)
	+ Antibody[c]	− Antibody		
Expt 1				
Unfractionated PBL	17.0	11.3	2.4	4.2
$OKM1^+$	24.1^*	20.9^{**}	5.1	26.3^{***}
$OKM1^-$	9.1^*	3.1^{**}	1.1	1.1^{***}
Expt 2				
Unfractionated PBL	24.6	20.1	4.5	8.5
$OKT3^+$	$14.2^\dagger$	$15.5^{\dagger\dagger}$	1.2	$0.8^{\dagger\dagger\dagger}$
$OKT3^-$	$25.9^\dagger$	$18.4^{\dagger\dagger}$	4.7	$33.3^{\dagger\dagger\dagger}$

[a] Percentage of $OKM1^+$ cells was 16% in unfractionated, 96% in $OKM1^+$, and 2% in $OKM1^-$ fraction. Percentage of $OKT3^+$ cells was 78% in unfractionated, 99% in $OKT3^+$, and 2% in $OKT3^-$ fraction.

[b] Percent specific ^{51}Cr release after 16-h assay. The E/T ratio was 10. Significance was determined by Student's t test. *, $P < 0.001$; **, $P < 0.001$; ***, 0.0025; †, $P < 0.001$; ††, Not significant, †††, $P < 0.001$.

[c] Hyperimmune mouse ascitic fluid was used at a 1:20 dilution.

hanced lysis of dengue-infected Raji cells by PBMC may not be due to the INF produced during assay. These results are consistent with reports that natural killing of HSV-1-infected target cells is dissociated from the induction of IFN (2, 13). Other possible explanations of the enhanced lysis of virus-infected cells include: (i) enhanced adhesion of NK cells to virus-infected targets due to glycoproteins or to virus-induced cellular receptors (35), (ii) impairment of infected cells to repair damaged membranes (12), or (iii) an activation of NK cells via viral glycoproteins (7). The mechanism which is responsible for the enhanced lysis of dengue-infected cells by PBMC remains to be elucidated.

Despite the evidence that virus-infected targets are lysed more than uninfected targets by NK and ADCC in vitro (9, 23), there is relatively little information available on the in vivo significance of these cells in virus infection. Mice deprived of NK activity by the injection of anti-asialo GM1 antibody are more susceptible to murine cytomegalovirus, mouse hepatitis virus, and vaccinia virus (5). Beige mice, which have very low NK activity, are also very sensitive to murine cytomegalovirus infection (31). In a study designed to analyze the contribution of ADCC in HSV infection, mice were irradiated and then injected with human peripheral blood lymphocytes and anti-HSV antibody. These mice were reported to be more resistant to HSV infection than mice injected with only human peripheral blood lymphocytes or anti-HSV antibody (19).

This is the first report which demonstrates increased lysis of dengue-infected cells by PBMC in vitro in NK and ADCC assays. Many questions remain to be answered concerning the role of NK and ADCC in dengue infections. What is the role of NK and ADCC in the lysis of dengue-infected human monocytes? Monocytes are the source of replicating virus in humans and are hypothesized to release various mediators leading to dengue shock syndrome (15). What is the virus-antigenic specificity of killing by ADCC? Epidemiologically, it is reported that infection with a different subtype of virus from the primary one is more likely to cause the dengue shock syndrome (16); therefore, a cross-reactive antigen may be involved. These studies should lead us to a better understanding of dengue infection and eventually to prevent the dengue shock syndrome.

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