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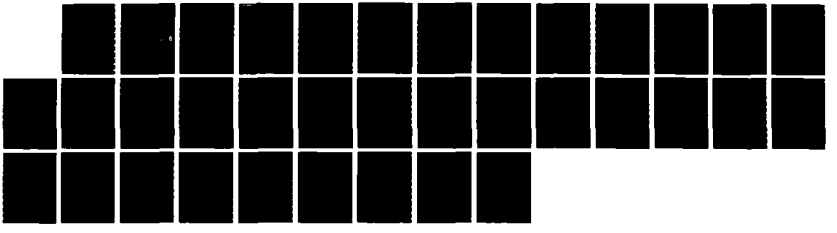

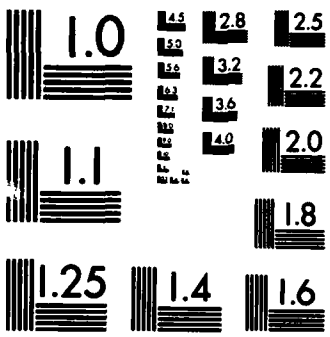

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Human Immune Responses to Dengue Viruses

Annual Report

(August 1983-July 1984)

Francis A. Ennis, M.D.

August 1984

Supported by
U.S. Army Medical Research and Development Command
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SUMMARY

The purpose of this contract is to analyse the immune responses to dengue virus infections. Dengue infections are a major cause of morbidity worldwide, and hemorrhagic fever and shock are very severe and frequently fatal complications of dengue infections. These complications are more commonly observed in individuals undergoing a secondary dengue infection with a different dengue virus serotype than they experienced as their primary infection (1). These observations make it important to understand the mechanism(s) of immunologic sensitization which appear to result in more severe subsequent dengue infections, and to minimize the potential for such adverse effects in developing vaccines to prevent dengue infections. In this phase of the research we are developing techniques for detecting viral-specific and non-specific lytic destruction of cells infected with dengue virus. We have developed a persistently infected human lymphoblastoid cell line which has been used as the target cell system for detecting antibody-dependent cell mediated cytotoxicity (ADCC), and to detect complement-dependent antibody-mediated lysis of dengue virus infected cells. These assays presently utilize dengue-type 2 virus infected Raji cells as the ^{51}Cr labelled target cells.

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. 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 NK and ADCC assays were analyzed. Non-adherent peripheral blood lymphocytes (PBL) caused more lysis than did adherent cells. Human PBL which lyse dengue virus-infected Raji cells in natural killing (NK) and ADCC assays were further characterized with several monoclonal antibodies. PBL were sorted by using a fluorescence activated cell sorter (FACS) or by pretreatment with monoclonal antibodies and complement, and were then used a effector cells.

The lymphocytes which kill dengue virus-infected cells can be divided into three groups: (i) PBL which lyse dengue-infected cells in the NK and ADCC assays and also lyse K562 cells (NK susceptible human tumor cells). These cells are contained in Leu11+, Leu7+/-, M1+, T3-,T4-and T8+/-fractions and are

the predominant effector cells in the NK and the ADCC assays; (ii) PBL which lyse dengue-infected cells in the NK and ADCC assays but do not lyse K562 cells. These cells are contained in the Leu11$^-$ fraction; (iii) PBL which lyse dengue-infected cells in the NK assay, but do not lyse dengue-infected cells in the ADCC assay or K562 cells in the NK assay. These cells are contained in T3+ and T4+ fractions. Leu11$^-$cells lyse dengue virus-infected Raji cells in the NK assay; however, they do not lyse hepatitis A-infected BS-C-1 cells which are lysed by Leu11$^+$ cells. These results suggest that NK cells which kill virus-infected cells are heterogeneous and vary depending upon the virus-infected target cells.

Plans for future studies are contained in the accompanying proposal and very briefly stated, they will include: 1) establishment of satisfactory target cells infected with the other dengue serotypes, Types 1, 3 and 4, to use in determining the viral antigenic specifity of the ADCC and antibody-dependent complement mediated lysis in (a) primary and secondary clinical cases of dengue, (b) pre and post dengue vaccination, and using (c) type specific hyperimmune animal sera and d) monoclonal antibodies supplied by WRAIR scientists; 2) analysis of the complement-mediated lytic antibodies, antibodies active in ADCC and virus neutralizing and enhancing antibodies using various antisera stated above; 3) attempt to develop suitable HLA defined target cells, using dengue-infected human mononuclear adherent cells, EB virus-transformed lymphoblastoid cells and mitogen-stimulated lymphoblasts to assess the presence of HLA-restricted cytotoxic T lymphocytes following infection or vaccination and study of the specificity of this response. A bank of HLA-typed buffy coat lymphocyte preparations has been stored, including samples obtained from volunteers pre and post dengue-4 vaccine administration. These will be used in assessing whether an HLA-restricted CTL responses to dengue virus occurs when suitable HLA defined target cell conditions are established and 4) sequential assessment of the activities of NK cells, K cells and HLA restricted CTL in dengue vaccine clinical studies. Details regarding future experiments are contained in the body of this report.

FORWARD

This research contract period began on September 1, 1983 and this report covers the first 11 months of this period of the contract.

BODY OF REPORT

I. INTRODUCTION

The purpose of this study is to define the immune responses of humans to dengue viruses. These studies should provide data which will be helpful in understanding the complex immune responses to dengue infections, which may be complicated by hemorrhagic fever and shock. An improved understanding of immune responses to dengue viruses should be helpful in attempts to prevent disease by successful immunization. In the past few years it has been demonstrated in several other virus infections that the virus- infected host develops several responses which can kill virus- infected cells. In the first portion of this study we have begun experiments to make it possible to assess these types of host responses to dengue infection or vaccination.

Dengue virus infection is a major worldwide cause of morbidity (2), and hemorrhagic fever and shock are severe and, at times, fatal complications of dengue infections (2). These complications are more commonly observed in individuals undergoing a secondary dengue infection with a virus of another dengue subtype than they had experienced in their primary infection (3). 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 these immune complex, resulting in increased vascular permeability, and initiating a shock syndrome (3). Another theory postulates that immune-mediated destruction of dengue-infected monocytes, which are the most productive source of replicating virus (4), is responsible for releasing various substances including C3b and unidentified vascular permeability factor(s) from such monocytes (2). The complication of hemorrhagic fever and shock make it important to understand the mechanism of immunologic sensitization and of elimination of dengue-infected cells.

Dengue virus infection induces type specific and cross-type reactive antibody responses (5). Infection results in life-long resistance to challenge with homologous virus but protection to heterologous strains is short-lived (6,7), 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 (8). 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 (9). 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 (10).

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 (11,12), 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 (13), natural killer cells (14) and cytotoxic T cells (15). These cellular effector functions have not been analyzed in dengue virus infections. The illness caused by dengue virus infections and its complication stimulated us to examine the cellular immune responses to dengue infection. In this annual report we describe the lysis of dengue type 2 virus infected Raji cells by PBMC active in the NK and ADCC assays, and define the specific lympbocytes responsible for lysing dengue infected cells in thes assays.

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II.A. Preparation of Raji cells persistently infected with dengue 2 virus as infected target cells.

Raji cells were infected with dengue 2 virus at an MOI of 0.05 PFU per cell at 37°C for 2 hours. Infected cells were washed twice with RPMI, resuspended at the concentration of 2 x 10^5 cells/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 x 10^5 cells/ml every three days. Seven days after infection, 40 percent of the cells were positive and by the ninth day 90 percent were positive for dengue membrane and cytoplasmic antigens. These cells are split every three days at a ratio of 1:10 and have remained infected over 1 year, with more than 90 percent of cells expressing membrane and cytoplasmic dengue antigens. The cells in this persistently-infected culture line are more than 95 percent viable as determined by dye exclusion testing with trypan blue. The dengue virus titer of supernatant culture fluids is 3 x 10^3 PFU/ml and the interferon titer is 3 IU/ml. These persistantly-infected Raji cells were used as target cells in the following studies.

II.B1. 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 do not have antibodies to dengue virus. Table 1 shows the results of an 18 hour ^{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 ($p<0.01$). 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 non-immunized mice caused no augmentation of the lysis of dengue-infected cells or uninfected cells at 1:20 dilution (data not shown).

PBMC also lysed acutely infected Raji cells to a greater degree than uninfected Raji cells on 5th day after infection (% 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.

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

Effector (E/T = 50)	% Specific ^{51}Cr release[a]				
	K562	Infected Raji[d]		Uninfected Raji[d]	
		-Ab[c]	+Ab[b]	-Ab[c]	+Ab
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 hour assay.

[b]Ascitic fluid from dengue 2-hyperimmunized mice was used as a source of anti-dengue 2 antibody at 1:20 dilution.

[c]Significance was determined by paired t test between the lysis of infected Raji cells without antibody and that of uninfected Raji cells without antibody ($p<0.01$).

[d]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).

II.B2. Time course and effector (E): target (T) 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 hours (Figure 1).

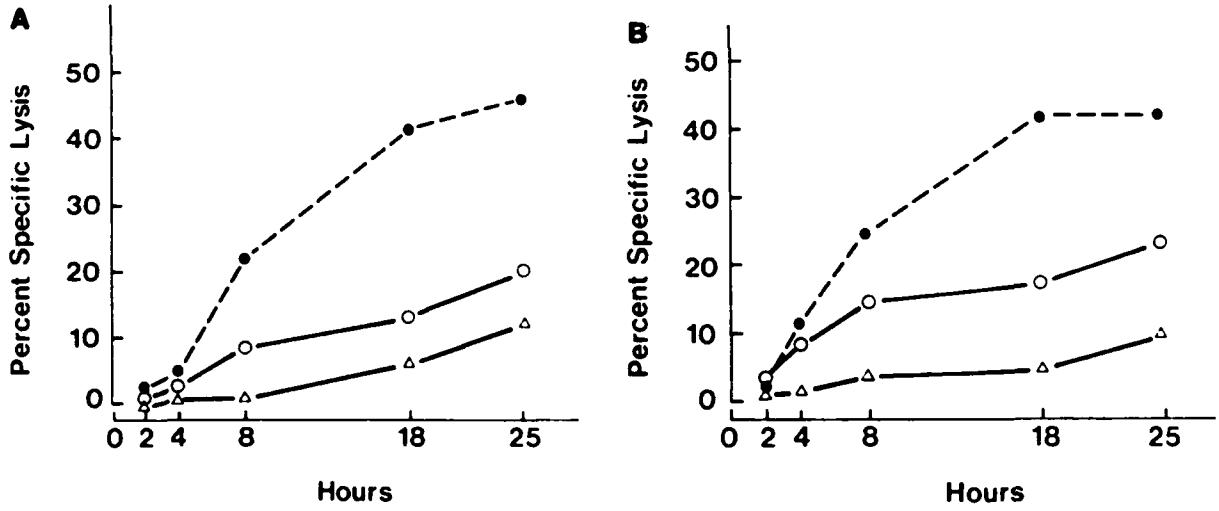

Figure 1. Time course study of the lysis of dengue 2-infected or uninfected Raji cells in NK and ADCC assays. The percent specific ^{51}Cr release from target cells in NK and ADCC assays were assessed after indicated hours of incubation. Hyperimmune mouse ascitic fluid was used at 1:20 dilution in the ADCC assay. Effector:Target ratio was 50:1 with donor No. 2 (Figure 1a) and 10:1 with donor No. 8 (Figure 1b)
O : infected Raji without Ab (NK assay), ● : infected Raji with Ab (ADCC assay), △ : Uninfected Raji without ab (NK assay).

The specific lysis by PBMC of dengue 2-infected Raji cells reached a maximum at 8 to 18 hours 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 hours of incubation.

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

Table 2. Effector:target cell dose-response study in the lysis of dengue 2-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[c]		Uninfected Raji	Infected Raji[c]		Uninfected Raji
	ADCC[b]	NK	NK	ADCC[b]	NK	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 hour assay

[b] Hyperimmune mouse ascitic fluid was used at 1:10 dilution in ADCC assay

[c] Significance was determined by Wilcoxon's rank sum test between ADCC-lysis of infected Raji and NK-lysis of infected Raji ($P=0.025$ for both No. 2 and No. 8), and between NK-lysis of infected Raji and NK-lysis of uninfected Raji ($P=0.025$ for both No. 2 and No. 8).

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.

II.B3. Dose response relation 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 1:1.5x10^5 and the PBMC from donor No.2 and No.8 were used as effector cells (Figure 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.

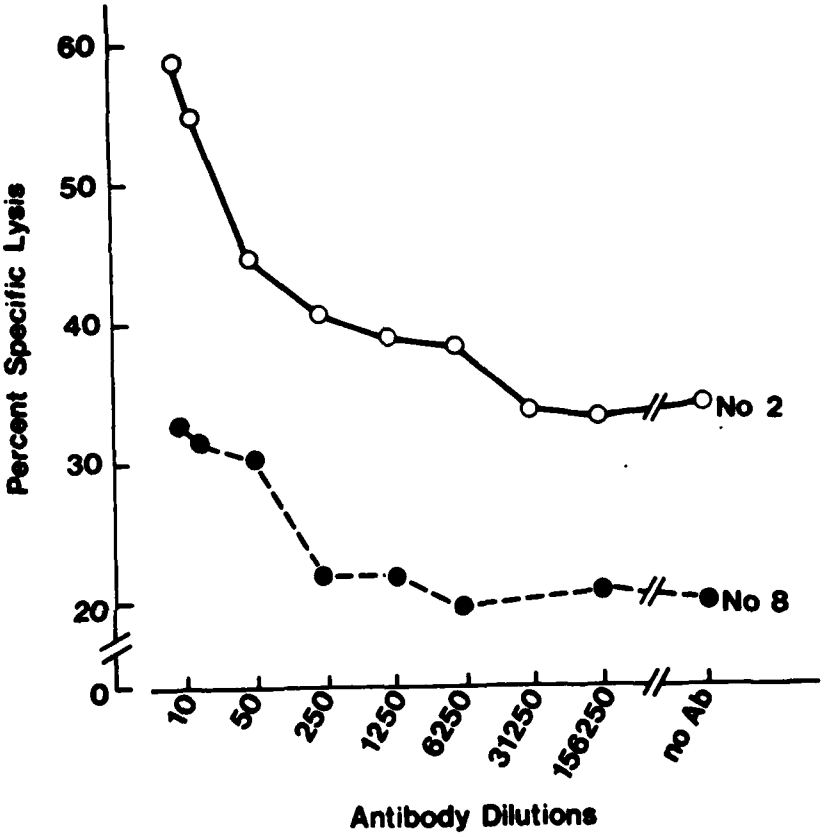

Figure 2. Dose-response relation between the dose of antibody added and the lysis of infected-Raji cells by ADCC. Percent specific ^{51}Cr release from dengue 2-infected Raji cells by ADCC was assessed after 18 hours incubation. Hyperimmune mouse ascitic fluid was used at 1:10 to 1:156250 dilution. Effector:Target ratio was 50:1 with donor No. 2 and 10:1 with No. 8.

II.B4. 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).

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

Human Sera[c]	Neutralizing antibody titer	% Specific ^{51}Cr release[a]			
		Infected Raji		Uninfected Raji	
		ADCC	Δx[b]	ADCC	Δx
(A) Anti-dengue 2 antibody positive					
1	(640)[e]	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
(B) 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	---	5.7	---
Anti-EBV antibody					
4	Ab+	13.6	-1.2	4.6	-1.1
5	Ab-	11.2	-3.6	2.8	-2.9

[a] Percent specific ^{51}Cr release from dengue 2-infected and uninfected Raji cells after 18 hour assay

[b] Δx means percent specific lysis of target minus percent specific lysis by ADCC with control AB serum

[c] Human sera were used at 1:10 dilution after inactivation of complement.

[d] Significance was determined by paired t test between Δx of infected Raji and Δx of uninfected Raji in the assay using sera positive for anti-dengue 2 antibody ($p < 0.025$).

[e] The neutralizing antibody titers in the human sera are described in parenthesis.

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 experments (data not shown). 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 ($P<0.025$). 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-EBV antibody, 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 EBV transformed cells.

II.B5. Production of interferon during NK and ADCC assays of dengue-infected Raji Cells

We assessed whether interferon was produced during these assays to determine if interferon may have contributed to the enhanced lysis of dengue-infected Raji cells by PBMC (Table 4).

Table 4. Quantitation of IFN in culture supernatants of effector cells and dengue-infected Raji cells

Target Cells	Effector Cells	Anti-Dengue 2[b] Antibody	IFN[a] (u/ml)	%Specific Lysis
Dengue 2-infected Raji	+	+	1600	45.5
	+	-	1600	32.1
	-	-	<6	-
Uninfected Raji	+	-	6	18.8
	-	-	<6	-
None	+	-	<6	-

[a] Effector cells and target cells were cultured at an E/T ratio of 50:1 for 18 hours, and IFN in the culture supernatants was quantitated.

[b] Hyperimmune mouse ascitic fluid was used at 1:20 dilution

High titers of interferon (1600 u/ml) were detected only in the culture supernatants containing both PBMC and dengue-infected Raji cells. The same titer of interferon was also detected in the ADCC assay. However, only 6 u/ml of interferon was detected in the culture supernatant of PBMC and uninfected Raji cells. The interferon was characterized as alpha because it was neutralized by specific antisera to human IFNα, but not by antisera to IFNβ and IFN .

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 interferon titer in the culture supernatant at 18 hours. Although the addition of anti-IFNα antibody diluted 1:16 neutralized all the detectable IFN produced during the NK assay, the specific lysis of dengue-infected Raji cells did not decrease, suggesting that the presence of interferon throughout the assay was not required to demonstrate the increased lysis of dengue-infected cells.

Table 5. Effect of anti-IFNα antibody on the lysis of dengue-infected Raji cells by PBMC

Serum added	Dilution	% Specific Lysis[a]		IFN (u/ml)[b]	
		Infected Raji	Uninfected Raji	Infected Raji	Uninfected Raji
-	-	36.9	13.0	400	25
Anti-IFNα	1:40	31.5	13.0	<25	<25
	1:160	36.0	15.8	100	<25
Control Serum	1:40	35.1	15.0	200	<25
	1:160	31.8	13.7	400	50

[a]E/T ratio was 50.

[b]Dilutions of anti-IFNα antibody were added to wells in NK assay. IFN in the culture supernatant was quantitated after 18 hours culture.

In order to study the possible early contribution of interferon to the enhanced killing of dengue infected cells, we pretreated the effector cells with 0.02-0.16 μg/ml of actinomycin D (Table 6). Although pretreatment of effector cells with 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 0.02-0.16μg/ml of actinomycin D did not decrease the lysis of uninfected cells, either (data not shown).

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

Actinomycin D[a] (μg/ml)	% Specific[b] lysis	IFN[b] (U/ml)
0	31.8	1600
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 Materials and Methods.

[b] Effector cells and target cells were cultured at an E/T ratio of 50:1 for 18 hours.

In addition, we pretreated the effector cells with 10^4U/ml of exogenous human IFNα. 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 PBMC may not be due to the interferon produced during the assay.

II.C. Effector cells responsible for lysis of dengue-infected target cells in NK and ADCC assay.

II.C1. Introduction

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 cells and non-adherent 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 non-adherent cells, although some lysis was associated with the adherent cells.

Table 7. Lysis of dengue-infected Raji cells by adherent cells and non-adherent cells with or without anti-dengue 2 antibody

Effector Cells[c]	E/T ratio	% Specific ^{51}Cr release[a]			
		Infected Raji		Uninfected Raji	K562
		+Ab[b]	-Ab	-Ab	-Ab
Unfractionated	50	ND	55.4	43.9	73.7
PBMC	10	47.7	31.7	19.1	33.0
Adherent	50	ND	35.3**	16.7	15.0Σ
Cells	10	25.5*	16.4***	7.4	10.0ΣΣ
Non-adherent	50	ND	51.7**	26.6	75.5Σ
Cells	10	46.6*	37.0***	18.4	38.8ΣΣ

[a] Percent specific ^{51}Cr release from each target after 16 assay

[b] Hyperimmune mouse ascitic fluid was used at 1:10 dilution

[c] Percentage of phagocytic cells contained in each fraction is as follows: Unfractionated 15%; Adherent 96%; and Non-adherent 3%.

[d] Significance was determined for the difference in the level of lysis between groups with the same symbol

*$p<0.001$, **$p<0.0025$, ***$p<0.001$, Σ$p<0.001$, ΣΣ$p<0.001$.

II.C2. Lysis of target cells by PBL sorted with monoclonal antibody to Leu11, Leu7 or M1

PBL were sorted by FACS after reacting with antibody to Leu11, Leu7 or M1. Dengue 2-infected Raji cells, uninfected Raji cells and K562 cells were used as target cells in the NK and ADCC assays (Table 8 on the next page).

Leu11$^+$ cells lysed K562 cells and dengue-infected cells in the NK and ADCC assays. Although Leu11$^-$ cells did not lyse K562 cells in the NK assay, they lysed dengue-infected cells to a low but statistically significant degree in comparison to K562 cells ($p<0.001$)in the NK and ADCC assays (Exp. 1). Both Leu7$^+$ and Leu7$^-$ cells lysed K562 and dengue-infected cells in the NK and ADCC assays. Leu7$^-$ cells lysed target cells to a greater degree than Leu7$^+$ cells (Exp. 2). M1$^+$ cells lysed K562 and dengue-infected Raji cells in the NK and ADCC assays. M1$^-$ cells did not lyse K562 and dengue-infected cells in the NK assay; however, they lysed dengue-infected cells to some degree in the ADCC assay depending on the source of effector cells (Exp. 3).

II.C3. Lysis of target cells by PBL sorted with monoclonal antibody to T3, T4 or T8

PBL were sorted by FACS after reacting with antibody to T3, T4 or T8 and were then used as effector cells (Table 9).

T3$^-$ cells and T4$^-$ cells lysed dengue-infected cells and K562 cells in the NK and ADCC assays. T3$^+$ and T4$^+$ cells did not lyse K562 cells, although they lysed dengue-infected cells to a low but statistically significant degree ($p<0.001$ for T3$^+$ cells and $p<0.03$ for T4$^+$ cells) in the NK assay. The level of the lysis of dengue-infected Raji cells by T3$^+$ cells in the NK assay varied depending on the donors. T3$^+$ cells of one donor lysed these target cells to a similar level as T3$^-$ cells (Exp. 1b), however T3$^+$ cells of other donors were one-fifth as active as T3$^-$ cells (Exp. 1a and other data not shown). Addition of anti-dengue antibody did not increase the lysis of dengue-infected cells by T3$^+$ cells and T4$^+$ cells. Both T8$^+$ cells and T8$^-$ cells lysed K562 cells and dengue-infected cells in the NK assay, although the lysis was greater with T8$^-$ cells than T8$^+$ cells. Addition of anti-dengue antibody increased the lysis by T8$^-$ cells but not the lysis by T8$^+$ cells (Exp. 3).

These results indicate that the effector cells predominantly responsible for lysis of dengue-infected cells in the NK and ADCC assays are contained in Leu11$^+$, Leu7$^-$, M1$^+$, T3$^-$, T4$^-$ and T8$^-$ fractions which also lyse K562 cells in the NK assay, and that less active effector cells are contained in the Leu7$^+$ and T8$^+$ fractions which also lyse K562 cells, and in Leu 11$^-$, T3$^+$ and T4$^+$ fractions which do not lyse K562 cells.

Table 8. Lysis of dengue virus-infected cells by PBL after sorting with antibody to Leu11, Leu7, or M1.

	Effector Cells	E/T Ratio	% Specific Lysis*			
			Dengue-Infected Raji		Uninfected Raji	K562
			ADCC	NK	NK	NK
Exp1.	Unfractionated	20	32**	21	7	31
	Leu11+	20	23**	16	6	62
	Leu11-	20	13**	8	2	1
Exp2a.	Unfractionated	20	45**	30	5	29
	Leu7+	20	23**	14	2	29
	Leu7-	20	51**	33	4	32
Exp2b.	Unfractionated	10	27**	9	5	12
	Leu7+	10	19**	8	2	9
	Leu7-	10	29**	13	8	13
Exp3a.	Unfractionated	20	19‖	15	5	19
	M1+	20	20**	15	9	37
	M1-	20	4‖	2	0	1
Exp3b.	Unfractionated	10	17**	11	2	4
	M1+	10	25**	20	5	26
	M1-	10	9**	3	1	1

*Percent specific ^{51}Cr release from target cells after 16 hour culture. Hyperimmune mouse ascitic fluid was used at 1:20 dilution in the ADCC assay. Significance was determined by student's t test between the percent specific lysis of dengue-infected Raji cells in the ADCC assay and that in the NK assay. ‖-not significant, **-statistically significant ($p<0.05$).

Table 9. Lysis of dengue virus-infected cells by PBL after sorting with antibody to T3, T4 or T8.

	Effector Cells	E/T Ratio	% Specific Lysis*			
			Dengue-Infected Raji		Uninfected Raji	K562
			ADCC	NK	NK	NK
Expla.	Unfractionated	20	30**	20	9	34
	$T3^+$	20	5||	7	5	0
	$T3^-$	20	48**	33	18	74
Explb.	Unfractionated	10	25**	20	5	9
	$T3^+$	10	14||	16	1	1
	$T3^-$	10	26**	18	5	33
Exp2	Unfractionated	10	20||	17	3	11
	$T4^+$	10	7||	6	0	1
	$T4^-$	10	22**	14	4	21
Exp3	Unfractionated	20	32**	18	10	16
	$T8^+$	20	11||	8	3	8
	$T8^-$	20	37**	21	11	19

*Percent specific ^{51}Cr release from target cells after 16 hour culture. Hyperimmune mouse ascitic fluid was used at 1:20 dilution in the ADCC assay. Significance was determined by student's t test between the percent specific lysis of dengue-infected Raji cells in the ADCC assay and that in the NK assay. ||-Not significant. **-statistically significant ($p<0.05$).

IIC4. Pretreatment of effector cells with monoclonal antibody and complement

PBL pretreated with monoclonal antibody and complement were used as effector cells (Table 10).

Table 10. Lysis of dengue virus-infected cells by PBL pretreated with monoclonal antibody and complement.

Pretreatment of Effector Cells with C' and Antibody to	% Specific Lysis*			
	Dengue-Infected Raji		Uninfected Raji	K562
	ADCC	NK	NK	NK
Exp. 1 -	49	27	9	27
Leu11	28	14**	2	1**
Exp. 2 -	28	22	15	45
Leu7	22	15**	ND	28**
M1	9	4**	ND	1**
T3	23	15**	ND	41||
Exp. 3 -	29	20	12	16
T4	22	16**	8	15||
T8	23	15**	8	12**

*Percent specific ^{51}Cr release from target cells after 16 hour culture. E/T ratio was 20.
Hyperimmune mouse ascitic fluid was used at 1:20 dilution in the ADCC assay.
Significance was determined by student's t test between the NK-lysis of dengue-infected Raji cells and K562 cells by the effector cells pretreated with complement alone (indicated by -) and the NK-lysis by the effector cells pretreated with antibody and complement. ||-not significant. **-significant ($p<0.05$).

Treatment of effector cells with antibody to Leu11 and complement, or antibody to M1 and complement, abrogated their ability to lyse K562 cells and decreased the lysis of dengue-infected Raji cells in the NK assay by 80% (with OKM1) and 50% (with anti-Leu 11). Treatment with antibody to Leu 7 and complement or antibody to T8 and complement decreased the lysis of K562 and dengue-infected cells in the NK assay by 35% (with anti-Leu 7) and by 25% (with OKT 8). Although treatment of effector cells with antibody to T3 and complement or antibody to T4 and complement did not decrease the lysis of K562, it decreased the lysis of dengue-infected cells to some degree. Addition of anti-dengue antibody increased the lysis of dengue-infected cells by effector cells depleted of Leu11$^+$, Leu7$^+$, M1$^+$, T3$^+$, T4$^+$ and T8$^+$ cells. These results are consistent with those of the experiments using effector cells sorted by FACS (Table 8 and 9).

IIC5. Discussion of Results to Date Concerning Lysis of Dengue Infected Raji Cells by Human Lymphocytes

Table 11 contains a summary of our observations concerning the lymphocyte subsets active in lysing dengue virus-infected target cells in NK and ADCC assays, in the context of the lytic activity of the same effector cells using K562 cells. This summary was prepared using the results presented in Tables 8 and 9, and represents our concepts at present.

The predominant effector cells in the NK assay are contained in Leu11$^+$, Leu7$^-$, M1$^+$, T3$^-$, T4$^-$and T8$^-$ fractions, which also lyse K562 cells very well. Leu7$^+$ cells and T8$^+$ cells lyse dengue-infected cells to some degree as well as K562 cells in the NK assay. Besides these effector cells there are other cells which do not lyse K562 cells but lyse dengue-infected Raji cells to a low but significant degree. These cells are contained in Leu11$^-$, T3$^+$ and T4$^+$ fractions. These results indicate that most of the natural killer cells active in killing dengue-infected cells (NK(DV)) are contained in the same subpopulation as NK (K562) cells, but some NK (DV) cells are contained in subpopulations different from NK(K562) cells.

Characterization of human NK cells have been performed with K562 as target cells. They have been characterized as Leu11$^+$ (16), Leu7$^+$ (17), M1$^+$ (18,19,20), T3$^-$ (18,19,20) T8$^{+/-}$ (21), T11$^+$ (20) by using monoclonal antibodies. Heterogeneity of NK cells has been reported, especially with virus-infected target cells. Lopez et al. reported that NK cells which lyse HSV-1-infected target cells (NK(HSV)) have somewhat different characteristics from NK cells which lyse K562 (NK(K562)) (22). Hendricks et al. reported that NK(HSV) were Leu7$^+$, M1$^+$, T3$^+$, Leu2$^-$, while NK(K562) cells were Leu 7$^+$, M1$^+$, T3$^-$, Leu2$^{+/-}$ (23). Our results are consistent with these reports concerning the characteristics of NK(K562) cells and the heterogeneity of human NK cells; however, the characteristics of NK cells against dengue-infected Raji cells (NK(DV)) are somewhat different from those of NK(HSV) cells reported by Hendricks et al.

Table 11. Characterization of PBL which lyse dengue virus-infected Raji cells, and K562 cells.

Effector Cells		Dengue-Infected Raji		K562
		ADCC	NK	NK
Leu11	+	+++*	+++	+++
	-	++	+	-
Leu7	+	++	+	++
	-	+++	+++	+++
M1	+	+++	+++	+++
	-	+	-	-
T3	+	-	+	-
	-	+++	+++	+++
T4	+	-	+	-
	-	+++	+++	+++
T8	+	-	+	+
	-	+++	+++	+++

*Symbols are used to reflect the degree of lytic activity when FACS sorted populations of effector cells were placed on the respective target cells. +++indicates the predominant subset responsible for target cell lysis, ++and+indicate a subset which lysed targets but was not as active as the predominant subset, (++:>50% as active as the predominant subset, +:20-50% as active), and -indicates a subset which has no or little lytic activity (<20% as active as the predominant subset).

We also have shown that Leu11$^-$ cells which lyse dengue-infected cells do not lyse hepatitis A-infected BS-C-1 cells. T3$^+$ cells and T4$^+$ cells, which lyse dengue-infected cells to some degree, do not lyse hepatitis A-infected BS-C-1 cells (data not presented). These results also suggest that there is heterogeneity even in the NK cells which lyse virus-infected cells. The differences in the characteristics of NK cells which lyse dengue-infected cells, hepatitis A-infected cells or HSV-infected cells support the concept of the heterogeneity of NK(V) cells. Heterogeneity in the NK(V) cells and the mechanisms of increased lysis of virus-infected cells remains to be more precisely elucidated.

The relationship between K cells and NK cells is also a topic of controversy. Some reports have stated that K cells were the same cells as NK cells (24,25), and others have stated that K cells were different from NK cells (22). The predominant effector cells in the ADCC assay against dengue virus-infected cells (K(DV)) were contained in Leu11$^+$, Leu7$^-$, M1$^+$, T3$^-$, T4$^-$and T8$^-$ fractions which lyse dengue-infected cells in the NK assay. Leu11$^-$ cells, which do not lyse K562 cells, are active in both the NK and ADCC assays against dengue-infected cells. These results indicate that most of the K(DV) cells and NK(DV) cells are contained in the same subpopulations as was previously reported using uninfected target cells (24). However, T3$^+$ and T4$^+$ cells which lyse dengue-infected cells in the NK assay are not active in the ADCC assay, indicating that some NK(DV) cells do not have ADCC activity. Since it has been reported that Leu11$^+$ cells do not contain Leu3$^+$ cells or Leu4$^+$ cells, which are considered as T4$^+$ cells and T3$^+$ cells respectively (16), our results suggest that Leu11$^-$, T3$^-$, T4$^-$ NK(DV) cells which do not lyse K562 cells have ADCC activity against dengue-infected cells, and that Leu11$^-$, T3$^+$, T4$^+$ NK(DV) cells which do not lyse K562 do not have ADCC activity.

It has been reported that null cells and T-cells were the effector cells in ADCC assays and that both cell types possessed HNK-1 (Leu7) antigen, using respiratory syncytial virus (26) or influenza virus-infected target cells (27). In our experiments both Leu7$^+$ cells and Leu7$^-$ cells have ADCC activity, and Leu7$^-$ cells are more active than Leu7$^+$ cells. These differences may be due to the differences in viruses used or may depend on donors. We also have shown heterogeneity of K(DV) cells. Most K(DV) cells lyse K562 cells in the NK assay; however, there are some K(DV) cells which do not lyse K562 cells. Heterogeneity of K(V) cells as well as that of NK(V) cells remains to be elucidated further. For these purposes, two color immunofluorescence investigations by FACS will be helpful, and should lead to a better understanding of the NK(V) and K(V) cells against virus-infected cells.

III. Studies Initiated and In Progress:

IIIA. Establishment of Raji cell lines persistently infected with dengue virus types 1.3, and 4 to determine dengue virus specificity of the lytic antibody (ADCC and Complement-dependent) immune response

We infected Raji cells with dengue 4 virus using murine antiserum to dengue 4 virus as enhancing antibody, because in preliminary experiments Raji cells were not infected with dengue 4 virus alone. Murine antiserum to dengue 4 virus was diluted from 1:50 to 1:156250. 0.1 ml of diluted antiserum and 0.1 ml of dengue 4 virus ($4x10^5$PFU/ml) were incubated together at 37°C for 30 minutes. Then $1x10^5$ Raji cells in 0.05 ml of RPMI were added to the mixture of antiserum and dengue 4 virus, and incubated at 37°C for 2 hours. The cells were washed twice and were resuspended at the concentration of $1x10^5$/ml in RPMI/20% FCS. After 5 days of culture, the cells were examined for the cytoplasmic dengue 4 antigen by indirect fluorescent staining using specific antiserum to dengue 4 virus (table 12). The highest percentage (25%) of positive cells was detected in Raji cells infected with virus using 1:50 diluted antiserum.

Table 12. FA staining of Raji cells infected[a] with dengue 4 virus using antiserum to dengue 4 virus.

Dilution of serum to dengue 4 virus	% dengue 4 Ag-positive[b] Raji cells
no serum	0
1:50	25
1:250	16
1:1250	7
1:6250	0
1:31250	0
1:156250	0

a) Infected cells were cultured for 5 days.
b) Indirect FA staining of cells fixed with acetone for 10 minutes at -20°C.

We then tried to obtain clones of Raji cells persistently infected with dengue 4 virus. We diluted these cells to the concentration of 2.5 cells/ml and added 0.2ml to the wells of flat bottom, 96 well tissue culture plates. After culture for two weeks, growing cells were detected in 50 out of 176 wells. These cells were examined by immunofluorescence for cytoplasmic dengue antigens. One cell line out of 50 lines tested was positive and 100% of cells of this line contained cytoplasmic dengue 4 antigen. These cells were propagated and cryopreserved in liquid nitrogen.

Using the same procedure we established 4 Raji cell lines persistently infected with dengue 1 virus and 3 Raji cell lines persistently infected with dengue 3 virus. We had already developed Raji cells, more than 90% of which have dengue 2 antigen, and by limiting dilution we obtained 11 Raji cell lines persistently infected with dengue 2 virus.

We have therefore Raji cell lines persistently infected with each of 4 subtypes of dengue virus. We are planning to use them for type-specificity analysis in ADCC assays and antibody-dependent complemented lysis.

IIIB. Preparation of HLA defined target cells to detect dengue virus specific HLA restricted cytotoxic T lymphocytes

We wish to develop HLA defined target cells for detecting HLA-restricted dengue virus-specific cytotoxic T lymphocytes. We considered HLA defined monocytes, lymphoblasts and EB virus-transformed lymphoblastoid cells as candidates because these cells can be infected with dengue virus.

IIIB1. Enrichment of monocytes and infection with dengue 2 virus

2.5×10^7 PBMC in 5 ml of RPMI/10% FCS were placed in plastic petri dishes (35 mm in diameter) containing a cover glass. After incubation for 1 hour, non-adherent cells were aspirated and the petri dishes were washed twice. The adherent cells were then infected with dengue 2 virus (1×10^7 PFU/ml) at dilutions of 1:1, 1:5 or 1:25. They were cultured for 2 days and examined for cytoplasmic dengue 2 antigens. During the 2 day culture period, some cells, which was adhering at the time of infection, began to float, and these cells (floating cells) were also examined for the dengue antigen. Table 13 shows that 31% of adherent cells have cytoplasmic dengue antigen, and that 15% of floating cells contained dengue antigens.

Table 13. Percentage of Cells Containing Dengue Antigen After Infection of Adherent Cells

Dilution of virus inoculated	% dengue Ag - positive cells	
	Adherent cells	Floating cells
1:1	31%	15%
1:5	4%	2%
1:25	1%	0%

We also have separated PBMC into fractions 1 to 8 using an elutriator centrifuge to obtain monocyte-enriched fractions. The percentage of monocytes contained in each fraction was assessed by lysozomal staining. Fractions 2, 5 and 7 were chosen and further separated into adherent and non-adherent fraction by adhesion to plastic petri dishes. Each fraction was infected with undiluted dengue 2 virus fluid ($1x10^7$ PFU/ml), cultured for 2 days, and assessed for the percentage of dengue Ag-positive cells and virus titer in the supernatant fluid (Table 14). A high titer of virus (10^4PFU/ml) was detected only in the adherent fraction of fraction 5, which contained the highest percentage (55%) of monocytes. 40% of the adherent cells of fraction 5 have dengue 2 antigen. The viability of each fraction was more than 70%. The non-adherent fraction produced a low titer of dengue virus and contained a low percentage of dengue Ag-positive cells.

The results in table 14 and 15 show that adherent cells are infected with dengue virus and contain dengue antigens as previously reported (4), and indicate that we may be able to use them as target cells to detect HLA restricted dengue specific CTLs. We plan to try to obtain fractions which will contain higher percentages of monocytes using the elutriator centrifuge or other methods.

Table 14. Infection with dengue 2 PMBC fractions following Elutriator centrifugation

Fraction number	% monocytes	% dengue Ag-positive cells		Virus titer (PFU/ml)	
		Adherent	Non-adherent	Adherent	Non-adherent
Unfractionated	35%	ND	3%	10^3	10^2
2	35%	ND	0%	$<10^1$	$<10^1$
5	55%	40%	14%	10^4	10^1
7	25%	ND	ND	ND	ND

IIB2. Induction of lymphoblasts using pokeweed mitogen (PWM) and infection with dengue 2 virus

PBMC were cultured with various dilutions of PWM in RPMI/10% FCS at 37°C for 3 days, and the percentage of the lymphoblasts was assessed by detecting transferrin receptors (table 15).

Table 15. Induction of lymphoblast by Pokeweed Mitogen (PWM)

Concentration of PWM (%)	Culture day	% Lymphoblast (Transferrin receptor + cells
0.2	3	23%
1.0	3	30%
5.0	3	23%

When we used PWM at a concentration of 1.0% we detected the highest percentage of lymphoblasts (30%). Then we infected the lymphoblasts, which had been cultured with 1.0% of PWM for 3 days, with dengue 2 virus at a m.o.i. of 0.1. The percentage of dengue Ag-positive cells was assessed and the virus titer was assayed in the supernatant fluid every day for 7 days (Table 16, Exp. 1).

We detected 4.8×10^3 PFU/ml of virus as early as day 1, and the virus titers reached the maximum on day 2 and 3; however the percentage of the dengue Ag-positive cells was not more than 5%. Next, we infected the lymphoblasts at the m.o.i. of 30. On day 3, about 40% of cells have cytoplasmic dengue antigen (Table 16, Exp. 2 and Exp. 3). Since the percentage of the lymphoblasts in the cell population at the time of infection is about 30%, these results indicate that most of the lymphoblasts are being infected. We are now planning to enrich for lymphoblasts by using percoll gradients before infection with dengue virus. These procedures should provide lymphoblasts, more than 70% of which have dengue antigen, useful for detecting HLA restricted cytotoxic T lymphocytes which are dengue-virus specific.

Table 16. Infection of PWM-stimulated PBMC with dengue 2 virus

	M.O.I. (PFU/cell)	Days after infection	Viability (%)	% dengue Ag Positive cells	Virus titer (PFU/ml)
Exp. 1	0.1	1	86	2	4.8×10^3
	0.1	2	78	4	8.4×10^4
	0.1	3	76	5	8.0×10^4
	0.1	4	67	5	1.8×10^4
	0.1	5	64	2	5.2×10^3
	0.1	6	60	ND	3.2×10^3
	0.1	7	43	4	1.5×10^2
Exp. 2	30	3	ND	38	ND
Exp. 3	30	3	ND	38	ND

IIIB3. Transformation of HPBL by EB Virus

$2x10^6$ of PBMC from four different HLA-typed normal Blood Bank donors were infected with a 23 TD_{50} (50% transformation dose) of EBV-B95-8. They were resuspended at the concentration of $2x10^6$/ml in RPMI/20% FCS. Cultures were observed under the dissecting microscope for any morphologic changes and passaged every 3 or 4 days, at which time, fresh media (RPMI + 20% FCS + 1% pen/strep + 75 ug/ml gentamycin) was added. These cells were cultured at 37°C in 5% CO_2 for approximately one month before clusters of cells, which are indicative of transformed cell lines, were visible. After 2 weeks of further cell growth, aliquots of the most rapidly growing cell lines were frozen and stored in liquid nitrogen. To date, we have developed 2 or 3 transformed cell lines from each blood bank donor tested. We plan to definitively determine if these cell lines are transformed by staining the cells with anti-EBNA antibody, and after developing a variety of these HLA defined lines we will use them to detect dengue specific CTL activity.

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