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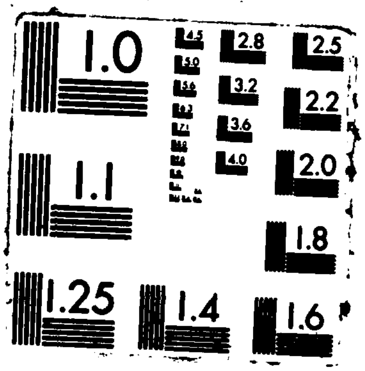
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Peripheral blood lymphocytes (PBL) from humans without antibodies to dengue 2 virus lysed dengue virus-infected Raji cells to a significantly greater degree than uninfected Raji cells. Addition of mouse anti-dengue antibody increased the lysis of dengue-infected Raji cells by PBL. These results indicate that both PBL-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity (ADCC) can cause significant lysis of dengue-infected cells.

The effector cells responsible for lysis of dengue virus-infected Raji cells in the NK and ADCC assays were analyzed. The PBL active in lysing dengue virus-infected Raji cells were characterized using monoclonal antibodies and were compared to lymphocytes that lysed K562 cells. Leu11+ cells lysed dengue virus-infected cells and K562 cells. Leu11$^-$ cells lysed dengue virus-infected cells, but not K562 cells. In the Leu11$^+$ fraction, Leu11$^+$ Leu7$^-$
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cells were more active than Leu11+Leu7+ cells in lysing dengue virus-infected cells. T3+ cells also lysed dengue virus-infected cells, but they did not lyse K562 cells. $T3^-$ cells lysed both target cells. These results, along with the observation that Leu11+ cells and T3+ cells are different subsets of PBL, indicate that the PBL that are active in lysing dengue virus-infected cells are heterogeneous and are contained in Leu11+ and T3+ subsets. Leu11+ cells are more active than T3+ cells. Leu11+ cells are active in lysing virus-infected cells by ADCC, whereas T3+ cells are not active.

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We have initiated studies concerning interferon production by peripheral blood mononuclear cells (PBMC) after infection with dengue virus. Dengue virus-infected monocytes produced IFNα. Dengue virus-infected monocytes induced IFNα from autologous PBL. To determine whether the levels of IFN which were detected could prevent dengue virus infection, monocytes were treated with 400 U/ml of IFNα before infection. Treatment of monocytes with IFNα decreased the yield of infectious virus more than 99% and the percentage of dengue-antigen positive cells by 98%. These results suggest that IFN produced by dengue virus-infected monocytes and PBL may have an important role in controlling dengue virus infection.

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HUMAN IMMUNE RESPONSES TO DENGUE VIRUSES

ANNUAL REPORT

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Foreword: This year of the research contract began on September 1, 1984.

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 virus is important in attempts to prevent disease by successful immunization.

Dengue virus infection is a major health problem in tropical and subtropical areas because of its severe complications, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (1). Primary dengue virus infection generally occurs without these complications (1). DHF/DSS are more often observed in patients suffering from secondary dengue virus infection with another serotype of dengue virus than that which caused the primary infection (2). Anti-dengue virus antibodies which can enhance dengue virus infection of Fc receptor bearing cells have been thought to play an important role in the pathogenesis of DHF/DSS (2,3). It has been postulated that these enhancing antibodies increase the number of infected monocytes, which are the major source of virus production, and that the immune-mediated destruction of the dengue virus-infected monocytes leads to the complications (2,4). The immune mechanisms which are responsible for the destruction of dengue virus-infected monocytes have not been defined. Effector mechanisms which should be considered include natural killer (NK) cell-mediated lysis, antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent complement-mediated lysis, and cytotoxic T lymphocytes.

In this report we focus on two important subjects: (i) cytolysis of dengue virus-infected cells by human PBL and (ii) IFN induction by dengue virus from human PBMC.

We recently reported that dengue virus type 2-infected cells were lysed by human peripheral blood mononuclear cells (PBMC) to a greater degree than uninfected cells in natural killer (NK) assays, and that the predominant effector cells were contained in the non-adherent fraction of PBMC (5). These observations raised questions which are addressed in this report. What are the characteristics of the lymphocytes which lyse dengue virus-infected cells? Are they similar to or different from NK cells which lyse K562 cells, or NK cells which lyse target cells infected with viruses other than dengue?

We also reported that PBL of non-immune adults lyse dengue virus-infected cells by ADCC (5). It is conceivable that anti-dengue virus antibodies in collaboration with antibody dependent killer (K) cells destroy dengue-virus infected monocytes and cause DHF/DSS, but it is also possible that ADCC is an effective mechanism for eliminating dengue virus-infected cells and helps to prevent these complications. It is important, therefore, to further analyze ADCC of dengue virus-infected cells in order to better understand the pathogenesis of DHF/DSS and immune defense mechanisms against dengue virus infections.

We have initiated studies concerning the role of human monocytes in dengue infections. It is known that monocytes are the major subpopulation of PBMC which support dengue virus infection and immune-mediated destruction of dengue virus-infected monocytes are thought to lead to DHF/DSS. Monocytes also initiate immune responses by presenting antigens and by secreting IL-1. In this phase of our work, we focused on the production of IFN by dengue virus-infected monocytes. Dengue virus infected monocytes, are also used as inducer cells of IFN from PBL, because in other virus systems, virus-infected cells induce IFN from PBL of non-immune donors.

II. Cytolysis of dengue virus-infected cells

II-A. Preparation of Raji cells persistently infected with dengue viruses type 1, 2, 3 and 4

Raji cells were infected with dengue 2 virus at a multiplicity of infection of 0.05 pfu per cell at 37°C for 2 hours, resuspended at the concentration of 2×10^5/ml in RPMI/10% FCS and cultured at 37°C in 5% CO_2. Nine days after infection, 90 percent of the cells were positive for dengue membrane and cytoplasmic antigens.

Raji cells were also infected with dengue 4 virus in the presence of anti-dengue 4 antibody diluted 1:100, because in preliminary experiments Raji cells were not infected with dengue 4 virus alone. The cells were resuspended 1×10^5/ml in RPMI/20% FCS. Five days after infection 25% of the cells contained dengue 4 viral antigen. Raji cells were also infected with dengue virus types 1 and 3 in the presence of antiserum to dengue 1 and 3, respectively.

Using limiting dilution techniques we have established Raji cell lines persistently infected with each type of dengue virus. These cells are split every three days at a ratio of 1:10 and have remained infected over 1 year, with more than 98% of cells expressing membrane and cytoplasmic dengue antigens. The cells in these persistently-infected culture lines are more than 95 percent viable. They have been cryopreserved and are available for distribution to interested scientists.

II-B. Natural killing (NK) and antibody-dependent cell-mediated cytotoxicity (ADCC) of dengue virus-infected cells.

II-B-1. Lysis of dengue virus-infected Raji cells by PBL

Dengue virus-infected Raji cells and uninfected Raji cells were used as target cells with human PBL of non-immune donors as effector cells. Table 1 shows the results of an 18 hour ^{51}Cr release assay. Dengue virus type 1, 2, 3 and 4-infected Raji cells were lysed to a greater degree than uninfected Raji cells (p=0.01).

Table 1. Lysis of dengue virus infected cells by PBL of non-immune donors

		% specific ^{51}Cr release[a]				
Donor	E/T ratio	Dengue 1 Raji*	Dengue 2 Raji*	Dengue 3 Raji*	Dengue 4 Raji*	Uninfected Raji*
H exp 1	40	26	28	32	25	9
2	20	13	25	23	18	4
3	40	24	33	34	29	10
4	20	11	24	16	17	7
U exp 1	30	30	39	28	29	17
2	30	48	49	41	39	18
3	30	39	40	41	39	26
4	30	36	41	44	46	14

[a]% specific ^{51}Cr release was determined after 18 hours of incubation. Significance was determined by Wilcoxon's rank sum test between the level of lysis of dengue 1, 2, 3, or 4-infected Raji cells and the level of lysis of uninfected Raji cells.

*statistically significant (p=0.01)

II-B-2. Lysis of dengue virus-infected cells by ADCC

To learn whether these dengue virus-infected cells are lysed by ADCC, anti-dengue antisera were added to the cytotoxicity assays. Addition of homologous anti-dengue antiserum significantly increased the lysis of dengue-virus infected cells, but did not increase the lysis of uninfected Raji cells (Table 2). Ascitic fluid from non-immune mice caused no augmentation of the level of lysis of dengue virus-infected or uninfected cells.

Table 2. Lysis of dengue virus-infected cells by antibody-dependent cell-mediated cytotoxicity

	Target Cells	Antibody Used	% specific ^{51}Cr release		% ADCC
			+Antibody[a]	-Antibody	
Exp 1	Dengue 1-Raji	Anti-dengue 1	26	13	13
	Uninf-Raji	Anti-dengue 1	4	4	0
Exp 2	Dengue 2-Raji	Anti-dengue 2	37	27	10
	Uninf-Raji	Anti-dengue 2	8	9	0
Exp 3	Dengue 3-Raji	Anti-dengue 3	54	34	20
	Uninf-Raji	Anti-dengue 3	7	10	0
Exp 4	Dengue 4-Raji	Anti-dengue 4	49	36	13
	Uninf-Raji	Anti-dengue 4	23	26	0

[a]Anti-dengue antiserum was used at 1:40 or at 1:80 dilution.

II-B-3. Serological specificity of ADCC-lysis of dengue virus-infected cells

We used anti-dengue virus type 1, 2, 3, and 4 antisera in ADCC assays. To determine the ADCC titer of each antiserum, sera were diluted and added to ADCC assays. The highest dilution which gave a percent specific ^{51}Cr release greater than that by PBL without antiserum plus 2 S.D. (standard deviation) was determined as ADCC titer of the antisera (Fig. 1).

Figure 1. Dose response relation between the dose of anti-dengue 2 antibody and lysis of dengue virus-infected cells by ADCC.

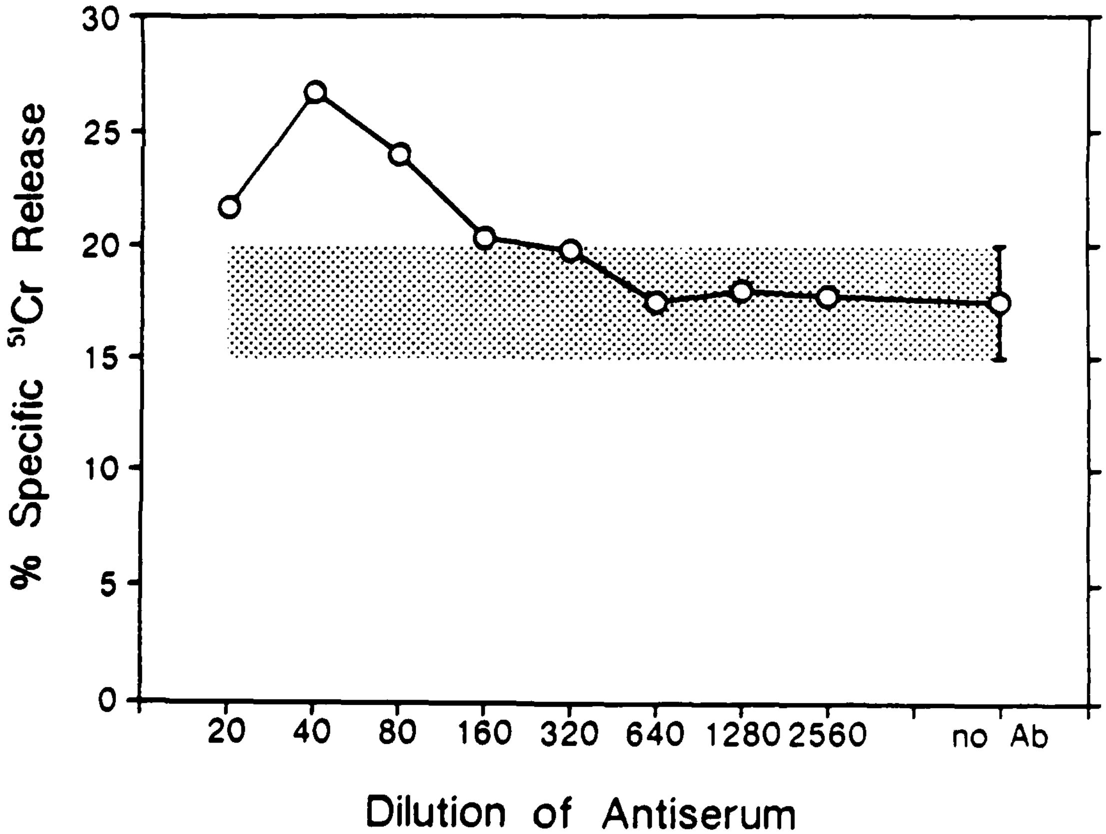


Table 3 shows the FA titer of these antisera. We found using these hyperimmunized murine ascitis fluids that the homologous antiserum was most active in augmenting the lysis of Raji cells infected with virus of the same serotype; however, each antiserum was also active to a lower level in lysing cells infected with other serotypes of dengue virus (Table 4). Therefore the lysis of dengue virus-infected cells by ADCC appears to be broadly cross-reactive using these polyclonal murine antisera.

Table 3. FA titers of polyclonal anti-dengue antisera detected on dengue virus infected cell lines

	FA titer				
Antisera	D1-inf	D2-inf	D3-inf	D4-inf	Uninf
Anti-dengue 1	320	40	160	80	<20
-dengue 2	1280	2560	1280	640	<20
-dengue 3	640	320	2560	320	<20
-dengue 4	320	320	320	1280	<20
NMAF	<20	<20	<20	<20	<20

Table 4. ADCC titer of anti-dengue antisera detected on dengue virus infected cell lines

	ADCC titer				
Antisera	D1-inf	D2-inf	D3-inf	D4-inf	Uninf
Anti-dengue 1	1280	40	160	160	<20
-dengue 2	1280	1280	640	320	<20
-dengue 3	320	40	640	320	<20
-dengue 4	320	320	320	640	<20
NMAF	<20	<20	<20	<20	<20

II-C. Characterization of effector cells

The above results indicate that human lymphocytes of non-immune donors lyse dengue virus-infected cells to a greater degree than uninfected cells. ADCC was also effective in lysing dengue virus-infected cells, and the hyperimmune mouse antisera were cross-reactive in ADCC of dengue virus-infected cells. We then characterized with monoclonal antibodies the human lymphocytes which are active in lysing dengue virus-infected cells, in comparison with lymphocytes which are active in lysing K562 cells and hepatitis A virus-infected cells.

II-C-1. Effector cells active in NK-mediated lysis of dengue virus-infected cells

i. Characterization of effector cells using anti-Leu11 antibody

We first analyzed the effector cells using anti-Leu11 antibody because Leu11 antigen had been reported to be expressed on essentially all functional NK cells in the peripheral blood (6). PBL were treated with anti-Leu11b antibody and complement and were used as effector cells. Treatment of PBL with anti-Leu11b antibody and complement decreased the level of lysis of dengue virus-infected Raji cells, uninfected Raji cells and K562 cells. The percent decrease in the level of lysis of K562 cells (94% on the average) was significantly greater than that in the level of lysis of dengue virus-infected Raji cells (58% on the average) ($p<0.002$) (Table 5 and data not presented). PBL depleted of Leu11$^+$ cells from most donors did not lyse K562 cells or uninfected Raji cells, but did lyse dengue virus-infected cells to a low but significant level (Table 5). These results indicate that Leu11$^+$ PBL are the most active effector cells in lysing dengue virus-infected Raji cells, uninfected Raji cells and K562 cells, and that the Leu11$^-$ fraction also contains some effector cells which are active in lysing dengue virus-infected Raji cells but which are not active in lysing K562 cells or uninfected Raji cells.

PBL were sorted on a FACS using anti-Leu11b antibody and used as effector cells. Leu11$^+$ cells were active in lysing dengue virus-infected Raji cells and K562 cells or uninfected Raji cells. PBL contained in the Leu11$^-$ fraction did not lyse K562 or uninfected Raji cells, but did lyse dengue virus-infected Raji cells to some level (data not presented). This result is consistent with the results of the complement-mediated cell-depletion experiments.

Table 5. Lysis of dengue virus-infected cells by Leu11+ PBL and Leu11- PBL

Donor	Treatment of Effector Cells	E/T Ratio	% Specific ^{51}Cr release: Infected Raji	Uninfected Raji	K562
D	C'	40	64	11	37
		20	46	8	22
	Anti-Leu11 + C'	40	12*	0*	0*
		20	7*	0*	0*
F	C'	50	41	17	42
		25	27	9	27
	Anti-Leu11 + C'	50	28*	9*	1*
		25	14*	2*	1*
Z	C'	40	52	11	39
		20	40	7	17
	Anti-Leu11 + C'	40	17*	2*	2*
		20	12*	1*	0*

Significance was determined by Student's t test between the levels of lysis by PBL teated with C' alone and that by PBL treated with anti-Leu11b and C'.
*Statistically significant ($P<0.05$).

ii. Characterization of the most active Leu11+ cells using anti-Leu7 antibody

We then tried to further characterize the Leu11+ cells, which contained the most active effector cells, using anti-Leu7 antibody. We found using double staining analysis on a FACS that Leu11+ cells contain Leu7+ cells and Leu7- cells (Leu11+Leu7+ cells, 3.7% (1.0%-7.8%); Leu11+Leu7- cells, 11.1% (2.5%-21.2%)) as reported by Lanier et al (7) and Abo et al (8). We sorted PBL on a FACS using anti-Leu7 antibody and used them as effector cells. Both Leu7+ cells and Leu7-cells lysed K562 and dengue virus-infected cells (Table 6, Exp. 1). PBL were then sorted into Leu11+ Leu7+ and Leu11+ Leu7- fractions on a FACS. Although both Leu11+ Leu7+ and Leu11+ Leu7- cells lysed dengue virus-infected Raji cells, uninfected Raji cells and K562 cells, Leu11+ Leu7- cells were significantly more active in lysing these target cells than Leu11+ Leu7+ cells (Table 6, Exp. 2).

Table 6. Lysis of dengue virus-infected cells by PBL sorted with anti-Leu11 and anti-Leu7 antibodies

	Effector Cells	E/T Ratio	% Specific ^{51}Cr release[a] Infected Raji	Uninfected Raji	K562
Exp. 1 (Donor F)	Unfractioned	20	30	5	29
		10	22	2	21
	Leu7+	20	14	2	29
		10	ND	ND	ND
	Leu7-	20	31*	4*	32‖
		10	23	3	19
Exp. 2 (Donor B)	Unfractioned	20	21	9	77
		10	16	6	59
	Leu11+ Leu7+	20	ND	ND	ND
		10	18	8	87
	Leu11+ Leu7-	20	43	19	95
		10	36*	15*	94*

[a]Significance was determined by Student's t test between the level of lysis of target cells by Leu7+ cells and that by Leu7- cells, and between the level of lysis of the same target cell at the same E/T ratio by Leu11+ Leu7+ cells and that by Leu11+ Leu7- cells.
*Statistically significant ($p<0.05$), ‖ not significant.

iii. Lysis of dengue virus-infected cells by M1+ PBL

We also characterized the effector cells using OKM1 antibody. M1 antigen has also been reported to be expressed on NK cells (9). Treatment of PBL with OKM1 and complement decreased the level of lysis of dengue virus-infected Raji cells, uninfected Raji cells and K562 cells (Table 7, Exp. 1). FACS-sorted M1+ PBL lysed these three target cells, but M1- PBL did not lyse the target cells (Table 7, Exp. 2).

Table 7. Lysis of dengue virus-infected cells by MI^+ PBL

	Donor	Effector Cells	E/T Ratio	% Specific ^{51}Cr release: Infected Raji	Uninfected Raji	K562
Exp.1[a]	E	C'	35	23	14	45
		OKM1+C'	35	4*	2*	1*
	G	C'	40	18	4	40
		OKM1+C'	40	12*	2||	11*
Exp.2[b]	A	Unfractionated	10	11	3	4
			5	6	2	3
		$M1^+$	10	21	5	26
			5	12	4	13
		$M1^-$	10	3*	1*	1*
			5	2*	1*	1*
	F	Unfractionated	20	15	5	19
		$M1^+$	20	15	9	37
		$M1^-$	20	2*	0*	1*

[a]In experiment 1 PBL were treated with complement (C') alone or OKM1 and C', and then used as effector cells. Significance was determined by Student's t test between the level of lysis by PBL treated with C' alone and that by PBL treated with OKM1 and C'. *Statistically significant ($p<0.05$), || not significant.

[b]In experiment 2 PBL were sorted on a FACS after staining with OKM1 and FITC-labelled anti-mouse IgG. Significance was determined by Student's t test between the level of lysis at the same E/T ratio and on the same target cells by $M1^+$ cells and that by $M1^-$ cells. *Statistically significant ($p<0.05$).

iv. Lysis of dengue virus-infected cells by T3+ cells and T3- cells

In the previous experiments using anti-Leu11 and OKM1 monoclonal antibodies which have been reported to discriminate human NK cells, PBL treated with the anti-Leu11 antibody gave results which indicated heterogeneity of the cytotoxic lymphocytes depending on the target cells, i.e., Leu11+ cells lysed both K562 and dengue virus-infected Raji cells, and Leu11- cells were not active in lysing K562 cells but did lyse dengue virus-infected Raji cells (Table 5). These results stimulated us to further characterize the effector lymphocytes in the Leu11- subset.

We demonstrated that Leu11+ cells did not possess a pan T antigen (Leu1) by double staining analysis Leu11+ Leu1+ cells, 0.6%; Leu11+ Leu1- cells, 6.7%; Leu11- Leu1+ cells, 76.4%; Leu11- Leu1- cells, 16.3%) as reported earlier by Lanier et al (7). These results indicate that Leu11- cells contained all of the T cells which may have contributed in the lysis of dengue virus-infected cells by Leu11-cells. We sorted PBL on a FACS using OKT3 antibody, which also recognizes a pan T antigen, and used them as effector cells (Table 8). As expected T3- cells which contain Leu11+ cells were active in lysing these three target cells. T3+ cells did not lyse K562 cells or uninfected Raji cells, however, they lysed dengue virus-infected cells to a low but significant degree ($p<0.02$), and the level of lysis of dengue virus-infected cells by T3+ cells varied somewhat depending on the donors (Table 8 and data not presented). The results shown in Table 8 indicate that T3+ PBL which do not lyse K562 cells are active in lysing dengue virus-infected cells, although they are not as active as Leu11+ PBL, and that T3+ cells contribute to the lysis of dengue virus-infected cells by Leu11- cells shown in Table 5.

Table 8. Lysis of dengue virus-infected cells by T3$^+$ cells and T3$^-$ cells[a]

Donor	Effector Cells	E/T Ratio	% Specific ^{51}Cr release: Infected Raji	Uninfected Raji	K562
A	Unfractionated	10	20	5	9
		5	15	2	3
	T3+[b]	10	16•	1	1•
		5	7•	1	1•
	T3-	10	18||	5*	33*
		5	14*	3||	21*
F	Unfractionated	20	20	9	34
		10	16	4	27
	T3+[b]	20	8•	4	0•
		10	6	1	0
	T3-	20	33*	18*	74*
		10	ND	ND	ND

[a]PBL were sorted on a FACS after staining with OKT3 and FITC labelled anti-mouse IgG. Significance was determined by Student's t test between the level of lysis by T3$^+$ cell and that by T3$^-$ cells, at the same E/T ration on the same target cell. *Statistically significant ($p<0.05$), || not significant.

[b]The level of lysis of dengue-infected Raji cells by T3$^+$ cells was compared with that of K562 cells by T3$^+$ cells. Significance was determined by Fisher's exact probability test. • Statistically significant ($p<0.02$).

II-C-2. Comparison with the effector cells which are active in lysing hepatitis A virus-infected cells

To learn whether the effector cells active in lysing dengue virus-infected cells are same subsets of cells as the effector cells active in lysing target cells infected with other viruses than dengue virus, the nature of the PBL responsible for killing the hepatitis A-infected BS-C-1 cells was then analyzed using anti-Leu11b, OKM1, and OKT3 monoclonal antibodies. PBL were reacted with anti-Leu11b or OKT3 antibody and sorted on the fluorescent activated cell sorter for use as effector cells (Table 9). $Leu11^+$ cells lysed hepatitis A-infected cells and K562 cells; however, $Leu11^-$ cells did not lyse either hepatitis A-infected cells or K562 cells (Exp. 1). $T3^+$ cells, which did not lyse K562 cells, did not lyse hepatitis A-infected cells. In contrast, $T3^-$ cells, which contain $Leu11^+$ cells (data not shown), lysed hepatitis A-infected cells as well as K562 cells (Exp. 2).

Table 9. Lysis of hepatitis A virus-infected BS-C-1 cells by PBL after sorting with anti-Leu11 or OKT3 antibody

	% specific ^{51}Cr release[b]		
Effector Cells[a]	Hepatitis A BS-C-1	Uninfected BS-C-1	K562
Exp. 1			
Unfractionated	28.1	12.4	30.5
$Leu11^+$	45.0	22.0	62.4
Leu11-	1.1*	0.5*	0.6*
Exp. 2			
Unfractionated	18.7	11.0	29.4
$T3^+$	0.3	0	1.1
T3-	36.4*	34.8*	59.7*

[a]The purity of the cells after sorting was more than 96%.

[b]Percent specific ^{51}Cr release was determined after 16 hours incubation. The E/T ratio was 20. Significance was determined by Student's t test between the level of specific lysis of target cells by $Leu11^+$ cells and that by $Leu11^-$ cells and between the lysis by $T3^+$ cells and that by $T3^-$ cells. *Statistically significant ($p<0.001$).

We then pretreated PBL with anti-Leu11b or OKM1 antibody and complement, and used them as effector cells (Table 10). Pretreatment of effector cells with anti-Leu11b antibody and complement, and OKM1 and complement removed their ability to lyse hepatitis A-infected BS-C-1 cells and K562 cells.

Table 10. Effect of treatment of PBL with anti-Leu11b or OKM1 antibody and complement on lysis of hepatitis A virus-infected BS-C-1 cells

Treatment of effector cells[a] with C' and antibody to	E/T ratio	% specific ^{51}Cr release[b]		
		Infected BS-C-1	Uninfected BS-C-1	K562
Exp. 1				
-	50	47.4	31.2	41.5
	25	28.1	16.9	26.8
	12.5	15.3	9.2	11.6
Leu11	50	6.2*	1.9*	0.4*
	25	1.3*	0.5*	0.8*
	12.5	1.3*	0.9*	1.1*
Exp. 2				
-	40	39.0	27.1	35.5
M1	40	6.2*	6.0*	5.1*

[a]Effector cells pretreated with C' and anti-Leu11b or OKM1 contained less than 1% of $Leu11^+$ or $M1^+$ cells, respectively.

[b]Percent specific ^{51}Cr release was determined after 16 hours incubation. Significance was determined by Student's t test, between the specific lysis of the same target cells at the same E/T ratio by PBL treated with C' alone and that by the PBL treated with anti-Leu11 antibody and C' (Exp. 1) and between the lysis by PBL treated with C' alone and that by the PBL treated with OKM1 and C' (Exp. 2) * Statistically significant ($p<0.001$).

The results shown in tables 9 and 10 indicate that the effector cells responsible for lysis of hepatitis A-infected BS-C-1 cells are contained in $Leu11^+$, $M1^+$, and $T3^-$ subsets, and that they are contained in the same subsets as the effector lymphocytes which lyse dengue virus-infected Raji cells and K562 cells. $Leu11^-$ and $T3^+$ cells which are active in lysing dengue virus-infected Raji cells are not active in lysing hepatitis A virus-infected cells.

II-C-3. Effector cells active in ADCC of dengue virus-infected cells

i. Characterization of active PBL using anti-Leu11 antibody

We first analyzed the effector cells of ADCC using anti-Leu11 antibody. PBL were treated with anti-Leu11b antibody and complement and were used as effector cells. Treatment of PBL with anti-Leu11b and complement decreased the level of lysis by ADCC; however, PBL depleted of Leu11$^+$ cells lysed dengue virus-infected cells to a low but significant level by ADCC (Table 11, Exp. 1). These results indicate that Leu11$^+$ PBL are the predominant effector cells in ADCC and that the Leu11$^-$ fraction also contain some effector cells active in lysing dengue virus-infected cells by ADCC.

PBL were then sorted on a FACS with anti-Leu11b antibody and used as effector cells. Leu11$^+$ cells were active in ADCC. PBL contained in the Leu11$^-$ fraction were also active (Table 11, Exp. 2). This result is consistent with the results of the complement-mediated cell-depletion experiments.

Table 11. Lysis by ADCC of dengue virus-infected cells by Leu11$^+$ cells and Leu11$^-$ cells

	Donor	Effector Cells[a]	E/T Ratio	% Specific ^{51}Cr Release[b]		
				+Antibody[c]	-Antibody	Δx[d]
Exp.1	E	C'	50	29*	19	10
		Anti-Leu11+C'	50	10*	5	5
	F	C'	25	49*	27	22
		Anti-Leu11+C'	25	28*	14	14
	G	C'	40	54*	27	27
		Anti-Leu11+C'	40	34*	13	21
Exp. 2	F	Unfractionated	20	32*	21	11
		Leu11+	20	23*	16	7
		Leu11-	40	19*	10	9
			20	13*	8	5

[a] In experiment 1 PBL were treated with complement alone or anti-Leu11b antibody and complement, and then used as effector cells. In experiment 2 PBL were sorted on a FACS after staining with anti-Leu11b and FITC labelled anti-mouse IgM.

[b] Significance was determined by student's t test between the lysis of target cells by PBL with anti-dengue 2 antibody and that by PBL without anti-dengue 2 antibody. *statistically significant ($p<0.05$).

[c] Hyperimmune mouse ascitis fluid was used as a source of anti-dengue 2 antibody at a 1:20 dilution.

[d] Δx, percent specific lysis by PBL with anti-dengue 2 antibody minus percent specific lysis by PBL without anti-dengue 2 antibody.

ii. PBL active in lysing dengue virus-infected cells by ADCC do not have T3 antigen

We then characterized the effector cells contained in Leu11- fraction. Experiments were performed to know whether T3+ cells are the effector cells or not. As we stated above, Leu11+ cells and T3+ cells are completely different subpopulations of PBL. PBL were sorted on the FACS using OKT3 antibody and were used as effector cells. T3+ cells were not active in killing dengue virus-infected cells by ADCC but PBL contained in the T3- fraction were active (Table 12).

As expected, T4+ cells and T8+ cells which are contained in T3+ fractions were not active in ADCC (data not presented). Therefore, PBL which have T cell surface antigens are not actve in killing dengue virus-infected cells by ADCC.

Table 12. Lysis by ADCC of dengue virus-infected cells by PBL contained in T3- fractions, but not by T3+ PBL

Donor	Effector Cells[a]	E/T Ratio	% Specific ^{51}Cr Release[a] +Antibody[b]	-Antibody	Δx[c]
A	Unfractionated	10	25*	20	5
	T3+	10	14‖	16	0
	T3-	10	26*	18	8
F	Unfractionated	20	30*	20	10
	T3+	20	5‖	7	0
	T3-	20	48*	33	15

[a]Significance was determined by student's t test between the lysis of the target cells by PBL with anti-dengue 2 antibody and that by PBL without anti-dengue 2 antibody. *statistically significant ($p<0.05$), ‖not significant.

[b]Hyperimmune mouse ascitis fluid was used as a source of anti-dengue 2 antibody at a 1:20 dilution.

[c]Δx, percent specific lysis of target cells by PBL with anti-dengue 2 antibody minus percent specific lysis by PBL without anti-dengue 2 antibody.

D. Discussion

In the first part of this annual report we have described the human lymphocytes which are active in lysing dengue virus-infected target cells (NK(DV)), in comparison with the lytic activity of the same effector cells for K562 tumor cells. Effector cells contained in Leu11$^+$, M1$^+$ and T3$^-$ fractions are the most active in lysing both dengue virus-infected cells and K562 cells. In the Leu11$^+$ subset, Leu11$^+$Leu7$^-$ cells are more active than Leu11$^+$Leu7$^+$ cells in lysing target cells. In addition to these effector cells, cells in the T3$^+$ fraction, which do not lyse K562 cells, contains some effector cells which are active in lysing dengue virus-infected Raji cells.

These results indicate the heterogeneity of human PBL which lyse dengue virus-infected cells. Heterogeneity of NK cells has been reported previously using herpes simplex virus-infected target cells. Fitzgerald 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 cells (NK(K562)) and that NK (HSV) were Leu7$^{+/-}$, Leu1$^-$ and Leu4$^-$ (10,11). Hendricks et al. reported that NK (HSV) were Leu7$^+$ and T3$^+$ (12). Since we may regard Leu1 and Leu4 as T3, the phenotypes of the predominant NK (DV) are generally consistent with those of the NK (HSV) reported by Fitzgerald et al. However, we found that T3$^+$ cells were also active in lysing dengue virus-infected cells using highly purified lymphocyte subsets sorted on a FACS. In addition we observed that the most active NK (DV) are Leu11$^+$ and M1$^+$ cells, and using a double-staining technique we found that Leu11$^+$Leu7$^-$ cells are more lytic to dengue virus-infected cells than Leu11$^+$Leu7$^+$ cells. This result is consistent with the reports by Lanier et al. and Abo et al. that Leu11$^+$Leu7$^-$ NK cells are more active than Leu11$^+$ Leu7$^+$ NK cells in lysing K562 cells (7,8).

We also describe the human PBL active in lysing cells persistently infected with hepatitis A virus in the absence of detectable viral antigens on the cell membrne (13). The lysis was due to effector cells in the Leu11$^+$ subset, and T3$^+$ cells did not contribute to the lysis of the hepatitis A virus-infected cells. Thus the lysis was accomplished by a homogeneous subset of Leu11$^+$ cells similar to the subset of the lymphocytes which kill K562 cells, and unlike the heterogeneous subsets of lymphocytes which are responsible for lysis of dengue virus-infected cells.

T3$^+$ lymphocytes which have NK-like activity have been reported by several investigators using other experimental systems. T3$^+$ effector cells have been generated in mixed lymphocyte cultures (MLC) (14-16), and in lymphocytes cultured with interleukin 2 (17,18). They have been called anomalous killer cells, activated lymphocyte killer cells or lymphokine activated killer cells. These cells have been reported to be able to lyse target cells which were not lysed by NK cells. Recently, Alsheikhly et al. reported that T3$^+$ cells acquire cytolytic activity after treatment with UV-inactivated mumps virions (19). These reports suggest that some T3$^+$ cells have the ability to lyse target cells in an NK-like fashion.

The mechanisms of increased lysis of dengue virus-infected cells remains to be elucidated. We reported results which indicated that IFN did not appear to mediate increased killing of dengue virus-infected cells by PBL (5). It has been reported by Casali et al. that glycoproteins of measles virus and lymphocytic choriomeningitis virus induce nonspecific cell-mediated cytotoxicity without induction of IFN (20,21). The HN glycoprotein of Sendai virus (gp 71) and the hemagglutinin and neuraminidase glycoproteins of influenza virus have also been reported to induce cell-mediated cytotoxicity (22,23). These results suggest the possibility that a dengue virus glycoprotein expressed on the dengue virus-infected cells may be the reason that $T3^+$ cells which are not active in killing K562 cells or uninfected Raji cells are active in killing dengue virus-infected cells.

There have been a few previous reports describing cells active in ADCC of virus-infected target cells. Several of these studies were performed before the availability of monoclonal antibodies to aid in characterizing the phenotypes of the active cells (24-26). There have been two more recent reports which stated that null cells and Tr cells possessing HNK-1 (Leu7) antigen were most active, although some killing of cells infected with respiratory syncytial virus and influenza virus was observed with $HNK\text{-}1^-$ cells (27,28). We have used anti-Leu11 antibody in addition to Leu7 antibody. Our results show that both $Leu7^+$ and $Leu7^-$ fractions contain ADCC effector cells and that the PBL contained in the $Leu7^-$ fraction are somewhat more active than those contained in the $Leu7^+$ fraction.

The relationship between K cells and NK cells is also a topic of interest. Most reports indicate that K cells are the same as NK cells (29,30) but another report has suggested that K cells are different from NK cells (31), using tumor cells as targets. We reported that the PBL which are most active in killing dengue virus-infected cells in the NK assay are $Leu11^+$ and $T3^-$cells; however, $T3^+$ PBL were also able to lyse dengue virus-infected cells to a lower level. We now report the PBL which are active in killing dengue virus-infected cells by ADCC are contained in $Leu11^+$ and $T3^-$fractions. These results indicate that most of the PBL which are active in the ADCC against dengue virus-infected cells are contained in the same subsets as PBL active in the NK assay. However, some PBL which are contained in $T3^+$ fraction and are active in the NK assay are not active in the ADCC assay.

The role of K cells in defense against or in the pathogenesis of dengue virus infection remains to be elucidated. It will be interesting to analyze the serotype specificity of the lysis of dengue-infected cells by ADCC, because it has been reported that DHF/DSS commonly occur in the patients who are secondarily infected with another serotype of dengue virus than the one which caused the primary infection (2), and that infection with one serotype of dengue virus induces type specific and type cross-reactive neutralizing antibodies (32). Using hyperimmunized murine antisera we have found that the level of lysis of the dengue virus-infected cells by ADCC was highest using homologous antibody; however, each antiserum was active to a lower level in lysing cells infected with another serotype of dengue virus. Therefore, the lysis of dengue virus-infected cells by ADCC appears to be broadly type-cross-reactive using polyclonal murine antisera. The specificity of the lysis of dengue virus-infected cells by ADCC and the antigens recognized by the antibodies in the ADCC assay remains to be defined. We plan to analyze the specificity in the lysis by ADCC, using monoclonal antibodies to dengue virus (33-35). The activity of K cells which lyse dengue virus-infected cells seems to vary somewhat between individuals (5). It will also be interesting to study the relationship between the K cell activity and the susceptibility to dengue virus infection or the occurrence of DHF/DSS using lymphocytes of patients with DHF/DSS from Thailand.

III. Studies initiated and in progress

III-A. IFN induction by dengue virus from human peripheral blood mononuclear cells

III-A-1. IFN production by dengue virus-infected human monocytes

Monocytes are reported to be the cells which support dengue virus infection in peripheral blood, and it has been postulated that enhancing antibody increases the number of infected monocytes. The hypothesis suggests that immune-mediated destruction of the dengue virus-infected monocytes leads to complications, dengue hemorrhagic fever and dengue shock syndrome, through the production of chemical mediators.

Monocytes are also known to regulate immune responses by producing interleukin 1 and by presenting antigens. Therefore, it is important to elucidate the response of monocytes to dengue virus infection in order to understand human immune responses to dengue virus. We first studied whether monocytes produce IFN when they are infected with dengue virus.

i. Detection of IFN activity in the culture fluids of dengue virus-infected monocytes

PBMC of non-immune donors were infected with dengue virus type 2 in the presence of anti-dengue antiserum diluted 1:2x10^4, and cultured at 37°C for 2 days. The culture supernatants were examined for IFN activity. The supernatant fluids of dengue 2 virus-infected PBMC contained IFN at titers from 100-400 U/mL, while those of *uninfected* PBMC contained no or low titers of IFN. Following infection of PBMC, 1-2% of the PBMC contained dengue antigens by immunofluorescent testing (Table 13).

Table 13. Induction of IFN by dengue virus from PBMC[a]

	IFN (units/mL)	
	Dengue 2-infected[b]	Uninfected[b]
A	200	50
R	100	<12
V	150	<12
K	150	<12
M	400	<12

[a] 2x10^6 PBMC were infected with dengue 2 virus at the m.o.i. of 2, in the presence of anti-dengue 2 Ab diluted 1:2x10^4 and cultured for 2 days in RPMI/10% FCS.

[b] % dengue Ag-positive cells by indirect FA testing; exposed to dengue 2 virus:1∿2%, not exposed to dengue 2 virus:0%.

We then characterized the IFN-producing cells using adherent and non-adherent fractions of PBMC. Table 14 shows the results of preliminary experiments that the adherent fraction contained dengue Ag-positive cells and produced IFN, and that the non-adherent fraction contained no Ag-positive cells and did not produce IFN. In more recent experiments the percentage of infected monocytes has increased. Table 15 shows that adherent cells from the PBMC of several donors produce IFN at titers from 100-400U/mL after infection with dengue virus.

Table 14. Characterization of IFN-producing cells

		Dengue 2-infected		Uninfected	
Donor	Cells	IFN (units/mL)	% Dengue Ag + cells	IFN (units/mL)	% Dengue Ag + cells
A	Unfractionated	200	1	25	0
	Adherent	400	10	<12	0
	Nonadherent	25	0	<6	0
W	Unfractionated	150	2	25	0
	Adherent	600	15	<12	0
	Nonadherent	9	0	18	0

Table 15. Production of IFN by dengue virus-infected human monocytes

		Dengue 2-infected		Uninfected	
Donor		IFN(units/mL)	% Ag-positive	IFN(units/mL)	%Ag positive
A	1	400	23.5	50	0
	2	400	52.6	<6	0
Y		100	22.3	<6	0
Z	Exp. 1	100	30.7	<6	0
	2	150	ND	<6	ND

ii. Time course study of IFN-production

We then studied the time course of IFN-production by dengue virus-infected monocytes. IFN was produced on the first day and reached a maximum on day 2 or 3, in correlation with the percentage of dengue Ag-positive cells (Fig. 2 and 3.)

Figure 2. Time course of IFN production by dengue virus-infected monocytes.

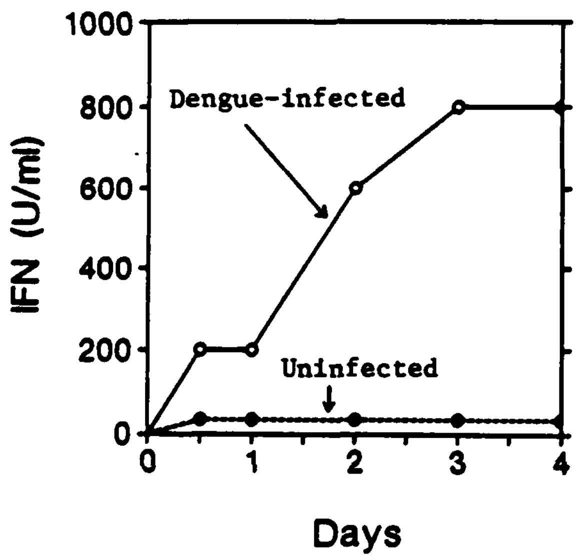
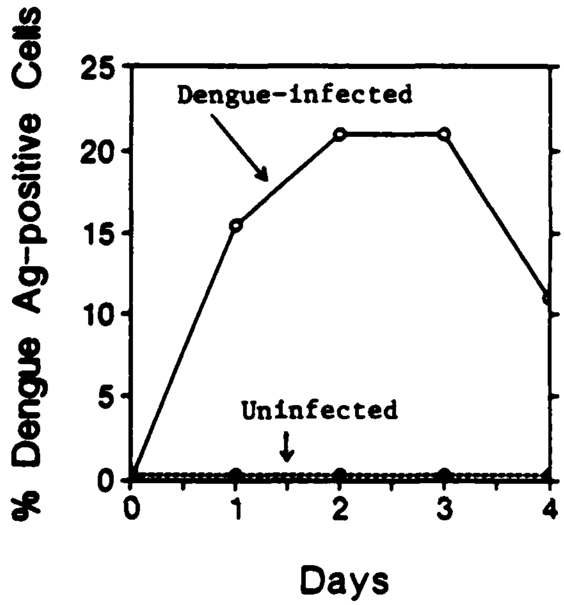


Figure 3. Time course of the appearance of dengue antigen



iii. Production of IFN by U937 cells infected with dengue virus

We infected the U937 human monocyte cell line with dengue virus at the m.o.i. of 2 in the presence of anti-dengue 2 antiserum diluted 1:2x10^4 and examined the culture supernatants for IFN activity. U937 cells infected with dengue virus contained dengue antigen positive cells and IFN was detected in culture supernatants (Table 16). No IFN activity was detected in the culture fluid of uninfected U937 cells.

Table 16. Production of IFN by dengue virus infected U937 cells

	Dengue-infected		Uninfected	
Days after infection	IFN (units/mL)	% Ag-positive cells	IFN (units/mL)	% Ag-positive cells
1	150	28.7	<8	0
2	200	30.9	<8	0
3	300	39.3	<8	0
4	300	ND	<8	ND

iv. Characterization of IFN produced by dengue virus type 2-infected monocytes

The IFN produced by dengue virus-infected human monocytes was characterized using specific antisera to human IFNs. Antiserum to IFNα neutralized all IFN activity in the supernatant fluids of dengue virus-infected PBMC and monocytes, but antiserum to IFNβ and antiserum to IFNγ did not neutralize IFN activity (Table 17). These results indicate that most if not all of the IFN produced by dengue virus-infected monocytes are IFNα. These results were confirmed by radioimmunoassay (RIA) of IFN. (data not presented)

Table 17. Characterization of Produced IFN

		IFN (units/mL)			
Donor	Sample from	Medium	antiα	antiβ	antiγ
A	PBMC	100	<10	100	100
R	PBMC	200	<6	200	150
V	PBMC	200	<6	200	150
A	Adherent	400	12	400	300
W	Adherent	400	<12	400	400
	Control IFNα	500	<12	ND	ND
	Control IFNβ	500	ND	<12	ND
	Control IFNγ	500	ND	ND	<12

III-A-2. Induction of IFN from non-immune PBL by autologous dengue virus-infected monocytes

We then studied the induction of IFN by dengue-infected monocytes from autologous non-immune PBL. Dengue 2 virus-infected monocytes and uninfected monocytes were treated with 0.025% glutaraldehyde for 10 minutes, and then cultured with PBL for 18 hours. We showed above that dengue infected monocytes produced IFN; therefore in these studies we use glutaraldehyde treated, dengue 2-infected monocytes which do not produce IFN. IFN activity at titers of 100-400U/mL were detected in the supernatants of the culture containing both PBL and dengue virus-infected monocytes. The supernatants of the culture containing PBL alone or PBL and uninfected monocytes contained very low titers of IFN activity (Table 18). These results indicate that dengue virus-infected monocytes induce IFN from PBL of non-immune donors.

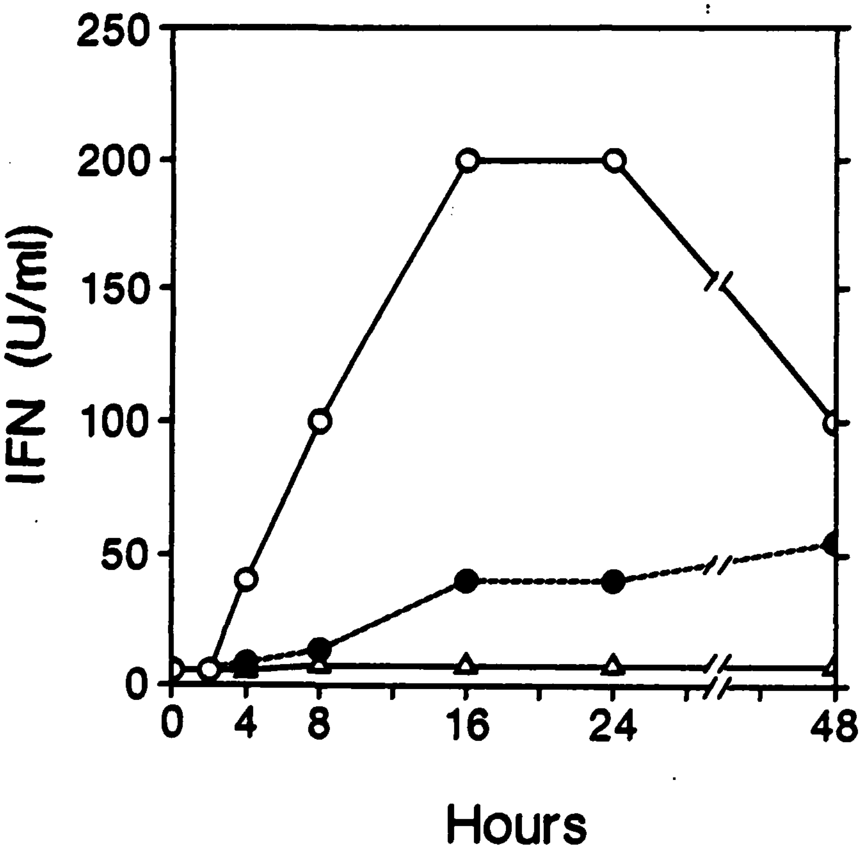
Table 18. Induction of IFN from PBL by dengue 2-infected autologous monocytes

		IFN (units/mL)		
Donor	PBL/inducer ratio	dengue-infected monocytes	uninfected monocytes	no inducer
A	10	200	38	12
	100	100	25	12
Y	10	400	25	18

When PBL were treated with actinomycin D at the concentration of 0.64 μg/mL, no IFN activity was detected in the supernatant fluid of the culture containing PBL and dengue virus-infected monocytes (data not presented). This result confirms that IFN detected in the fluid was produced by PBL in response to dengue virus-infected autologous monocytes.

Time course study showed that IFN was detected as early as 4 hours after the beginning of culture and reached the maximum after 16 hours (Fig. 4).

Figure 4. Time course of IFN induction from PBL by dengue virus-infected monocytes.



○ PBL + dengue virus-infected monocytes
● PBL + uninfected monocytes
△ PBL alone

III-A-3. Inhibition of dengue virus infection by IFN

We showed in the above sections that dengue virus induced IFN from PBMC of non-immune donors by two mechanisms (a) Human monocytes produce IFNα when they are infected with dengue virus (b) Dengue virus-infected monocytes induce IFN from PBL. The induced IFNs are primarily IFNα. We have begun studies to analyze whether the IFNs produced are actually effective in prevention of dengue virus infection.

Human monocytes and U937 cells were cultured with human IFNα at a concentration of 400 IU/mL for 18 hours, and then infected with dengue 2 virus at the m.o.i. of 2 in the presence of anti-dengue 2 antibody. About 40% of the monocytes which were not pretreated with IFN were infected (determined by indirect FA testing) and high titers of IFN, and of dengue virus were detected in the culture supernatant on day 1 and 2. However, monocytes pretreated with IFNα contained no or a very low percentage of Ag-positive cells and no IFN activity. Yield of the infectious virus was reduced >99% below the levels of untreated cultures (Table 19). These results indicate that the levels of IFNα which were induced from PBMC by dengue virus can inhibit the further infection of dengue virus to human monocytes.

Table 19. Effect of IFN on infection of dengue 2 virus to human monocytes and U937 cells

		IFN pretreated			Not pretreated		
Cells Infected	Days after Infection	IFN (units/mL)	% Dengue Ag Positive Cells	Virus Titer	IFN (units/mL)	% Dengue Ag Positive Cells	Virus Titer
Monocytes	1	<8	0	ND	400	44	ND
	2	<8	0.8	$4.2x10^3$	1200	42	$3.5x10^6$
U937	1	<8	0	ND	100	15	ND
	2	<8	0.8	$4.0x10^3$	200	12	$1.2x10^6$

III-A-4. Discussion

In this section, we have described experiments which indicate that dengue virus induces IFN from human PBMC by two mechanisms. Monocytes produce IFN when they were infected with dengue virus. Dengue virus-infected monocytes in turn induce IFN from PBL. Dengue virus-infected lymphoblastoid cells (Raji) were also found to induce IFN from PBL of non-immune donors.

The role of IFN produced by PBL exposed to virus-infected cells is controversial. Augmented lysis of virus-infected cells by NK cells has been reported to be due to the produced IFN in the experiments using EBV-infected cells (36). However it has also been reported that the IFN produced did not contribute to the augmented lysis of HSV-infected cells (37,38), and of dengue-infected cells (5). IFNs have many biological activities, in addition to the augmentation of NK activity (39). IFN render cells resistant to viral infection (40), modulate the expression of Fc receptors (41), augment the expression of membrane antigens including HLA antigens (42,43), activate macrophages (44), suppress DTH (45), enhance the activity of CTL (46),and modulate the antibody production (47). Liu has reported that IFN and the IFN inducer, poly I:C were prophylactically and therapeutically effective against infection of mice with dengue 1 virus (48). Although very little is known about the role of IFN during dengue virus infection in vivo, it is possible that the IFNs produced by dengue-infected monocytes and by PBL in response to dengue virus-infected cells may play an important role in recovery from dengue virus infection or in the pathogenesis of DHF/DSS.

The mechanism of IFN-induction by dengue virus-infected cells remains to be elucidated. Glutaraldehyde-treated, dengue-infected cells which do not produce infectious dengue virus also induce IFN. In addition a dengue virus-infected cell line which does not produce detectable infectious dengue virus also induced IFN. These results indicate that infectivity of the dengue virus is not essential for the induction of IFN, and it is probable that some component expressed on the infected cells is responsible. Dengue virus has 3 structural proteins; V1, V2 and V3. V1 (MW 8000) is a nonglycosylated protein and is located between the envelope and core. V2 (MW 14000) is a nonglycosylated core protein. V3 (MW 51000-59000) is an envelope glycoprotein. Of these 3 proteins V3 may be the only exposed protein antigen on the virion and is responsible for hemagglutination (49). It has been reported that the hemagglutinin-neuraminidase glycoprotein of Sendai virus can induce IFN from mouse spleen cells (50). This suggests that some of the dengue viral proteins expressed on the infected cells may be responsible for the induction of IFN. It has been reported that nonvirion proteins are also present on the surface of dengue virus-infected cells (51). It is possible that these nonvirion proteins are responsible. The component responsible for inducing IFNs requires further analysis. Thus in this research area two interesting questions remain to be elucidated. i) Are proteins expressed on dengue virus-infected cells are responsible for the induction of IFN? ii) What role does the produced IFN play in the immune response to dengue virus? Answers to these questions will lead us to a better understanding of immune responses and their possible role in dengue virus infections. These questions will be addressed as part of our ongoing research in the immune responses to dengue virus, which will emphasize cellular, especially HLA restricted cytotoxic T lymphocyte responses, and antibody responses in the coming years.

III-B. Infection of human monocytes with dengue viruses

We have begun studies to develop methods for infecting purified populations of human monocytes a high proportion of which will be successfully infected with dengue virus and can be used for (a) antigen presenting cells to stimulate memory lymphocytes from dengue immune donors and (b) as ^{51}Chromium-labelled dengue infected target cells to use to detect HLA restricted dengue specific cytotoxic T lymphocytes. Preliminary experiments indicate that we can infect, with the prototype dengue type 2 New Guinea C strain, approximately 85% of human monocytes, following enrichment to 90% by adherence. We are now passing recent type 2 strains into C6/36 cells, in order to increase their titer, to determine if they are more infectious for human monocytes than the prototype highly mouse-adapted virus. After one passage in C6/36 cells in our laboratory, a dengue virus type 2 Thai strain infected 50% of monocytes as determined by indirect immunofluorescence. We will examine infectivity as plaque forming units in Vero cells, and by immunoflourescence and hope to obtain higher levels of infectivity on subsequent C6/36 passage for our studies. The level already obtained should be adequate for antigen presentation-stimulation experiments, but we hope to infect a higher percentage for use as target cells in CTL studies. We will also test the ability of enhancing antibodies to improve infectivity for monocytes.

III-C. Collection of PBL and sera from dengue-endemic or epidemic settings

1. Aruba - We obtained venous blood samples from 17 adults who had clinical dengue infections during an outbreak in early 1985. After discussions with the Aruba Department of Public Health who cooperated in this effort, Dr. Kurane flew to Aruba, and the Department of Public Health obtained the blood samples, which he diluted in medium and returned to laboratory within 24 hours. After separation, lymphocytes were crypreserved, and the plasma were sent to Dr. Bancroft at WRAIR for antibody testing. Twelve individuals apparently had recent dengue infection which was their first flavivirus infection, two did not have antibodies to dengue, and three were dengue immune. These plasma are available for our ADCC, complement dependent cytolysis, neutralizing and enhancing antibody analysis, and the lymphocytes will be used to help characterize the CTL response to primary dengue infection, as described in the current proposal.

2. Thailand - On July 18, 1985 we received a large shipment of cryopreserved HPBL from Thailand. The detailed plans for using these valuable reagents are described in the contract proposal. Twenty buffy coats separated on Ficoll-Hypaque were shipped in vials (from 20-40 vial/donor) containing 20×10^6 cells/vial. In addition, 50 mLs of plasma from these donors was shipped. These buffy coats are from Thai blood donors, over 80% should have antibody evidence of prior dengue infections based on sera-epidemiologic studies in Thailand. Their lymphocytes should contain memory T lymphocytes which we will stimulate to study CTL responses in detail as described in the proposal. Thai sera will be tested for antibodies to dengue types 1-4.

The shipment also contained buffy coat lymphocytes from children admitted recently to the Bangkok Children's Hospital with DHF and DSS. We will obtain convalescent samples from these patients in later shipments. These valuable specimens of lymphocytes and sera will be analyzed as described in the proposal, after we have established sensitive, standardized assays for measuring cell mediated immune responses to dengue virus.

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