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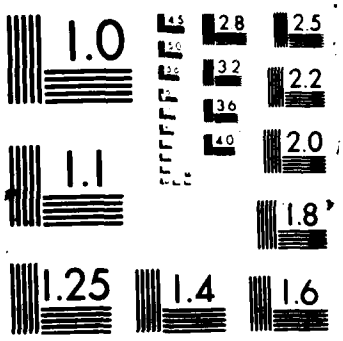
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HUMAN IMMUNE RESPONSE TO DENGUE INFECTIONS

ANNUAL REPORT

FRANCIS A. ENNIS

JULY 30, 1987

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We have analyzed human T cell responses to dengue virus. Peripheral blood lymphocytes from dengue-immune Thai donors and two American donors who were infected with low passaged CH53489 strain of dengue 3 virus were used. Sonicated dengue-infected Vero cells induced significant proliferative responses of PBL from dengue-immune donors, but did not induce specific proliferative responses of PBL from non-immune donors. IFNγ was detected in the culture fluid of dengue-immune PBL stimulated with dengue antigen.

A short term-cultured line was established using PBL from one American donor. Phenotypic analysis showed that they are T cell with CD4 phenotype ($CD3^+$, $CD4^+$, $CD8^-$, $CD16^-$, $CD20^-$). They responded to dengue 3 antigen and produced IFNγ. Then, 14 dengue-specific T cell clones/lines were established by a limiting diultion method using PBL from same American donor. All clones/lines responded to dengue 3 AG, but none of them responded to control Ag. Four lines were examined for phenotype. They were $CD3^+$, $CD4^+$, and $CD8^-$. These results indicate that PBL from dengue-immune donors contain dengue-specific T cells with CD4 phenotype, and that these $CD4^+$ cells produce IFNγ in response to dengue antigen.

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ABSTRACT

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A short term-cultured line was established using PBL from one American donor. Phenotypic analysis showed that they are T cell with CD4 phenotype ($CD3^+$, $CD4^+$, $CD8^-$, $CD16^-$, $CD20^-$). They responded to dengue 3 antigen, and produced IFN . Then, 14 dengue-specific T cell clones/lines were established by a limiting dilution method using PBL from same American donor. All clones/lines responded to dengue 3 (Ag), but none of them responded to control Ag. Four lines were examined for phenotype. They were $CD3^+$, $CD4^+$ and $CD8^-$. These results indicate that PBL from dengue-immune donors contain dengue-specific T cells with CD4 phenotype, and that these $CD4^+$ cells produce IFN in response to dengue antigen.

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TABLE OF CONTENTS

I. INTRODUCTION

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 (1). These complications are more commonly observed in individuals undergoing a secondary dengue infection with a different dengue serotype than they experienced as their primary infection (2). It has been speculated that dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) are mediated by host immune mechanisms. We hypothesize that destruction of dengue virus infected monocytes by dengue-specific, HLA-restricted cytotoxic T lymphocytes (CTL) may lead to DHF/DSS. To address this question, we have begun studies on the proliferative responses of memory T lymphocytes from dengue-immune donors. In this phase of research we are developing techniques to detect dengue virus-specific proliferative responses and to characterize the lymphocytes which respond to dengue virus antigens. We first analyze dengue-specific proliferative responses in bulk cultures. To characterize responding lymphocytes, we then establish a dengue-specific short term-cultured line and long term-cultured clones/lines. In addition to proliferative responses, IFN production by dengue-specific T lymphocytes were examined.

II. RESULTS

A. Analysis of dengue virus-specific immune responses in bulk culture.

A-1. Dengue virus-specific proliferative responses of peripheral blood lymphocytes (PBL) from dengue-immune donors.

We first attempted to induce dengue antigen (Ag)-specific proliferative responses of PBL from dengue-immune donors. PBL from 22 dengue-immune Thai donors and from two American donors were used. Five forms of dengue virus preparations were used as antigens. They were (a) sonicated, dengue-infected Vero cells; (b) sonicated, dengue-infected mosquito cells (C6/36); (c) culture fluids of dengue virus-infected C6/36 cells; (d) polyethyleneglycol-purified dengue virus; and (e) dengue virus-infected acetone-separated mouse brain. Proliferative responses of PBL were detected in tritiated thymidine (^{3}H-TdR) incorporation assays.

In preliminary experiments, sonicated, dengue-infected Vero cell Ag induced significant proliferative responses with PBL from dengue-immune donors, but not with PBL from non-immune donors. Table 1 shows representative results of proliferative responses. The proliferative response reached maximum levels on day 6, and showed a good correlation with the concentration of dengue antigen used. The other forms of Ag did not induce significant proliferative responses. Because of these results, we used sonicated, dengue-Vero Ag in the following experiments.

We have examined proliferative responses of PBL from 22 dengue-immune Thai donors using dengue 1, 2, 3 and 4 antigens and control Vero antigen. PBL from dengue-immune Thai donors showed significant dengue-specific proliferative responses, which we considered as stimulation index of more than 2 (7/8 with dengue 1 Ag; 10/22 with dengue 2 Ag; 4/17 with dengue 3 Ag; 3/5 with dengue 4 Ag). PBL from only one non-immune donor showed proliferative response to a dengue antigen (Figure 1).

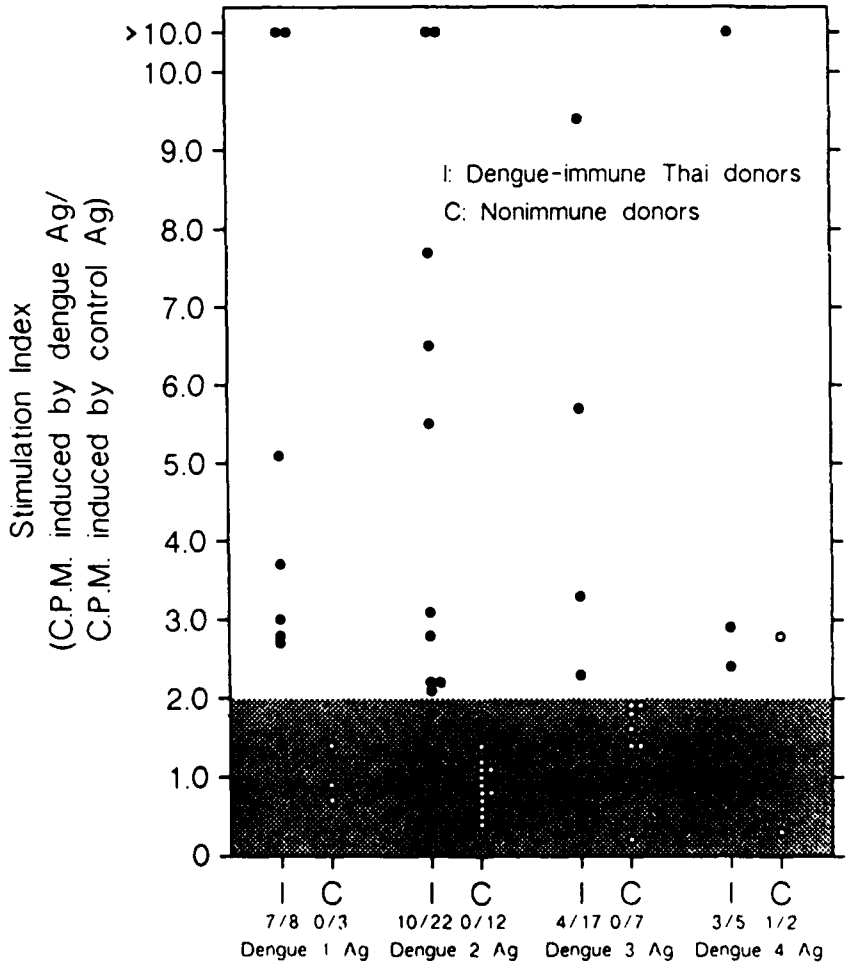
Table 1: Proliferative response of donor B PBL to dengue 3 Ag: dose-response study

Dilution of Ag	^{3}H-TdR incorporation (cpm)* Dengue 3 Ag	Control Ag
1:10	5648	273
1:20	2445	307
1:40	556	99
1:80	258	ND
1:160	137	ND
No Ag	57	57

*2 x 10^5 PBMC were cultured with serially diluted Ag for 6 days. 8 hours before harvest, cells were pulsed with 1.25 μCi ^{3}H-TdR.

Figure 1

Proliferative responses of PBL from Thai donors induced by dengue antigens



The PBL from one American donor, who had been infected with the low passaged CH53489 strain of dengue 3 virus, responded to both dengue 3 Ag and dengue 2 Ag to a similar level. The PBL from the other American donor, who had immunized with an attenuated CH53489 strain of dengue 3 virus, also responded to both dengue 3 and dengue 2 Ag (Table 2).

Table 2. Dengue Ag-induced proliferative responses of PBL from dengue 3 virus-infected donors

Donor	^{3}H-TdR incorporation (CPM)			
	Dengue 2 Ag	Dengue 3 Ag	Control Ag	No Ag
Donor A				
Exp. 1	ND	6857	2208	1481
Exp. 2	4477	ND	1284	1564
Donor B	21123	13762	1395	509

A-2. Augmentation of dengue-specific proliferative responses by exogenous interleukin 2 (IL2)

We then examined the effect of exogenous IL2 on the dengue-specific proliferative responses of the PBL. Delectinated human IL2 was added to the proliferation assays at 1:10 final dilution on day 6, and ^{3}H-TdR incorporation was assessed on day 9. IL2 augmented the specific-proliferative responses (Table 3).

Table 3. Augmentation of proliferative responses of PBL by addition of IL-2.

Donors	Dengue Ag used	^{3}H-TdR incorporation (cpm)					
		No IL-2			+IL-2*		
		Dengue Ag	Control Ag	No Ag	Dengue Ag	Control Ag	No Ag
Dengue-immune							
Donor B	Dengue 3	4163	211	130	15806	400	157
	Dengue 2	249	143	111	731	1145	740
1974	Dengue 2	513	473	123	4044	1257	669
1975	Dengue 2	1236	223	422	16345	2951	1260
3692	Dengue 4	2788	143	130	17553	1319	1004
Non-immune							
66493	Dengue 2	93	147	95	434	482	265
63641	Dengue 2	259	236	179	5361	2717	4304
66823	Dengue 2	494	723	344	4295	3360	3558

*Delectinated human Il-2 was added to the culture at 1:10 final dilution on day 6, and ^{3}H-TdR incorporation was assessed on day 9.

A-3. Interferon γ (IFNγ) production by PBL stimulated with dengue Ag.

We examined culture fluids of PBL stimulated with dengue Ag for IFNγ and IFNα using radioimmunoassay (RIA) (3). IFNγ was detected in the culture fluids of PBL stimulated with dengue Ag but not in the culture fluids of PBL stimulated with control Ag or cultured alone. IFNα was not detected in any culture conditions (Table 4). These results suggest that IFNγ is produced by dengue specific T lymphocytes stimulated with dengue Ag.

Table 4. IFNγ production by PBL from dengue-immune donors stimulated with dengue antigens.

Donors	Dengue Ag used	IFN (Units/mL)*					
		Dengue Ag		Control Ag		No Ag	
		IFNγ	IFNα	IFNγ	IFNα	IFNγ	IFNα
Dengue-immune							
Donor A	Dengue 3	23	<10	2	<10	<1	<10
Donor B	Dengue 3	180	<10	<1	<10	<1	<10
3692	Dengue 4	6	<10	<1	<10	<1	<10
Non-immune							
Donor K	Dengue 4	<1	<10	<1	<10	<1	<10

*4 x 10^5 PBL in 0.2 ml were cultured with Ag for 6 days. Culture fluids were collected and examined for IFNγ and IFNα by radioimmunoassay.

B. Establishment of a short term-cultured, dengue-specific T cell line.

We attempted to establish short term-cultured lines using PBL of a dengue 3 virus-infected donor B, and to characterize the proliferating cells. These PBL showed specific proliferative responses to dengue 3 Ag in the primary stimulation as shown in Table 1. On day 7, blast cells were enriched and restimulated with dengue 3 Ag in the rpesence of γ-irradiated autologous PBMC and IL2. They were restimulated every 7 days. Phenotypic analysis showed that proliferating lymphocytes were $CD3^+$, $CD4^+$, $CD8^-$, $CD16^-$ and $CD20^-$ (Table 5). Therefore, they are T cells with helper/inducer phenotype. They responded to dengue 3 Ag, but did not respond to control Ag (Table 6). They produced IFNγby the stimulation with dengue 3 Ag, but did not produce IFNγ by the stimulation with control Ag (Table 7). These results indicate that T cells with CD4 phenotype respond to dengue-Vero Ag and these cells produce IFNγ during proliferative responses.

Table 5. Phenotype of a short-term T cell line generated in response to dengue 3 Ag

Days after the beginning of culture	% positive cells*				
	CD3	CD4	CD8	CD16	CD20
14	89	84	2	2	<1
21	91	83	3	ND	ND

*Cells were stained with anti-Leu 4 (CD3), anti-Leu 3 (CD4), anti-Leu 2 (CD8), anti-Leu 11 (CD16) and anti-B1 (CD20) antibodies.

Table 6. Proliferative responses of a short-term T cell line to dengue 3 Ag.

Days after the beginning of culture	^{3}H-TdR incorporation (cpm)*		
	Dengue 3 Ag	Control Ag	No Ag
14	1325	250	270
21	1290	380	432

*5 x 10^4 cells were cultured with Ag in the presence of 2.5 x 10^5 γ-irradiated autologous PBMC for 72 hours. 8 hours before harvest, cells were pulsed with 1.5 μCi ^{3}H-TdR.

Table 7. IFNγ production by a short-term T cell line in response to dengue 3 Ag.

Days after the beginning of culture	IFN titer (Units/mL)*					
	Dengue 3 Ag		Control Ag		No Ag	
	IFNγ	IFNα	IFNγ	IFNα	IFNγ	IFNα
14	40	<10	<1	<10	<1	<10
21	280	<10	12	<10	10	<10

*5 x 10^4 cells were cultured with Ag in the presence of 2.5 x 10^5 γ-irradiated analogous PBMC for 72 hours. Culture fluids were collected and examined for IFNγ and IFNα by radioimmunoassay.

C. Establishment of dengue-specific T cell clones/lines using a limited dilution method.

We tried to establish dengue virus-specific T cell clones using PBL of donor B. PBMC were cultured with dengue 3 antigen. On day 7 blast cells were enriched, diluted to concentrations of 3, 10 or 30 cells/well and restimulated with dengue 3 antigen in the presence of γ-irradiated autologous PBMC and IL2. These cells were restimulated every 7 days. 14 cell lines were established. These cell lines were examined for their specificity for dengue 3 Ag by ^{3}H-TdR incorporation assay. All cell lines responded to dengue 3 Ag, but none of them responded to control Ag (Table 8). 4 cell lines (JK3, JK10, JK15, JK16) were further expanded and the phenotypes were examined using monoclonal antibodies. These four cell lines were $CD3^+$, $CD4^+$ and $CD8^-$ (Table 9). Therefore, they were T cells with helper/inducer phenotype.

Table 8. Proliferative responses of T cell clones/lines to dengue 3 antigen.

Clones	^{3}H-TdR incorporation (cpm)*		
	Dengue 3 Ag	Control Ag	No Ag
JK3	5876	543	662
JK4	7613	1957	1702
JK5**	6791	1917	2682
JK8	4578	1597	1575
JK9	3300	780	689
JK10	8147	719	556
JK12**	7319	2738	ND
JK13**	5826	1621	ND
JK15	6067	819	697
JK16	10197	1624	1301
JK17	12568	1800	1879
JK18	14744	3967	4597
JK19	2865	395	501
JK20	4245	890	1041

*2×10^4 cells were cultured with antigens diluted 1:30 in the presence of 2×10^5 γ-irradiated autologous PBMC for 72 hours. 8 hours before harvest, cells were pulsed with 1.25 μCi ^{3}H-TdR.
**1×10^4 cells were used for assay.

Table 9. Phenotype of T cell clones/lines

Clone	% positive cells*		
	CD3	CD4	CD8
JK3	99	99	<0.5
JK10	99	98	<0.5
JK15	97	97	<0.5
JK16	99	98	<0.5

*Cells were stained with anti-Leu 4 (CD3), anti-Leu 3 (CD4) and anti-Leu 2 (CD8) antibodies.

D. Establishment of dengue virus-infected, HLA-defined target cells.

To prepare target cells for dengue virus-specific, HLA-restricted cytotoxic T cells, we transformed PBL with Epstein-Barr Virus (EBV) and then infected them with dengue virus.

The peripheral blood lymphocyes (PBL) from some dengue-immune Thai donors, two dengue-immune American donors and many non-immune blood bank donors have been HLA-typed at the Tissue Typing Laboratory of the University of Massachusetts Medical Center. These PBL were cultured with the B95-8 strain of Epstein-Barr Virus (EBV) at the concentration of 2 x 10^6/ml in RPMI/20% FCS. Fifty percent of the medium was changed every 3 or 4 days. EBV-transformed B lymphoblastoid cell lines were established about one month after establishing the culture. These EBV-transformed cell lines were then infected with the Raji cell-passaged New Guinea C strain of dengue virus type 2 at an input multiplicity of about 50. The infected cells were cultured in RPMI/10% FCS and medium was changed every 3 or 4 days. One month after the beginning of culture, percentage of dengue antigen-positive cells was examined by indirect cytoplasmic immunofluorescent staining (FA staining) using anti-serum to dengue virus type 2. Table 10 shows that each cell line contains 40-80% of dengue antigen-positive cells. The high percentage of antigen-positive cells of these cell lines has been maintained for long periods, indicating that we have established persistent infection of dengue virus in the HLA-typed EBV-transformed cell lines.

Table 10. FA staining of dengue 2-infected, EBV-transformed cells.*

Cell lines	% dengue Ag-positive cells
66488 a	46
66488 b	66
1975	59
1986 a	43
1986 b	69
3693	87
3710	62
3723	72
4092 a	48
4092 b	76

*Hyperimmune mouse ascitic fluid was used as anti-dengue 2 Ab at 1:20 dilution.

III. DISCUSSION

We have detected dengue-specific human T cell responses to dengue virus in bulk culture, a short term-cultured line and long term cultured clones/lines. These analyses have been performed as a first step to detect dengue-specific, HLA-restricted CTL. We hypothesize that destruction of dengue-infected monocytes by dengue-specific, HLA-restricted CTL may lead to DHF/DSS.

Dengue virus-specific T cells showed proliferative responses by the stimulation with dengue antigen and produced IFNγ. We previously have reported statistically significant correlation between influenza-specific CTL activity generated and IFNγ activity detected in the culture fluids (4). Consequently, we expect a correlation between proliferative responses and CTL activity. However, all the viral antigens which can induce proliferation of T cells cannot induce CTL. Borysiewicz et al. reported that cell-free human cytomegalovirus (HCMV) induced proliferation of $CD4^+$ T cells which has no cytotoxic activity, but that autologous fibroblasts infected with HCMV induced HCMV-specific CTL with CD8 phenotype (5). Ndumbe et al. also reported that cell-free varicella zoster virus (VZV) induced proliferation of $CD4^+$ T cells which had no cytotoxic activity to VZV-infected cells, but that autologous fibroblasts infected with VZV induced proliferation of $CD8^+$ T cells which had cytotoxic activity to VZV-infected cells (6). Therefore, phenotypes and the function of T cells generated by the stimulation of viral Ag seem to depend on the form of viral Ag used. In the present experiments we have used sonicated, paraformaldehyde-fixed, dengue virus-infected Vero cells as dengue antigen. All the cell lines established in the short term culture and in the long term culture using limiting dilution were $CD4^+$ T cells. Therefore, it is possible that these cell lines are not cytotoxic to dengue virus-infected target cells. However, some CTL clones with CD4 phenotype have been reported and they are restricted by MHC Class II molecules (7-9). To address the cytolytic functions of dengue-specific T cells, we have established HLA-defined target cells using transformation by EBV. It has been reported that virus-infected, EBV-transformed lymphoblastoid cells are suitable target cells for virus-specific, HLA-restricted CTL (7-9). We have succeeded in transforming HLA-defined PBL by EBV and in infecting them with dengue virus. CTL assays are planned in the future.

Dengue virus-specific $CD4^+$ T cells produced IFNγ by the stimulation of dengue Ag. We have reported that both $CD4^+$ T cells and $CD8^+$ T cells produce IFNγ by the stimulation of influenza Ag (4). Thus, T cells which produce IFNγ in response to Ag may also be decided by the form of Ag used, although both $CD4^+$ T cells and $CD8^+$ T cells have ability to produce IFNγ. IFNγ has potent immunoregulatory effects. IFNγ activates monocytes/macrophages (10), increases expression of MHC Class I and Class II molecules (11) and activates expression of IL2 receptors (12). It has been reported that severe complications of dengue virus infections (DHF/DSS) are more commonly observed in secondary infections than in primary ones. We showed that dengue virus-specific T cells produce IFNγ which have potent immunoregulatory effects, and we can expect that IFNγ is produced in vivo in the secondary infections. Therefore, the role of IFNγ in the pathogenesis of DHF/DSS and in the recovery from secondary dengue infections is a subject to be analyzed to better understand the pathogenesis of dengue virus infections.

IV. Collection of PBL from dengue-immune Thai donors.

The co-PI, Ichiro Kurane, M.D., spent two weeks in Bangkok, Thailand, from November 29 to December 9, 1986. In collaboration with the Department of Virology, Armed Forces Research Institute of Medical Sciences (Chief, Bruce Innis, M.D.), he collected PBL from many Thai donors. The PBL collected are as follows: (a) PBL from 14 donors who were recently infected with dengue virus. He collected 7-19 vials and each vial contains $1x10^7$ PBL. (b) PBL from 10 Thai Red Cross donors. He collected 50-70 vials containing $1x10^7$ PBL each.

Anti-dengue antibody titers of the donors were assessed in AFRIMS. All the donors had anti-dengue antibody at the time of bleeding. The PBL were frozen in Bangkok, and were transported from Bangkok to Worcester, MA. The viability of the PBL after thawing are greater than 70%. They will be used for cytotoxic T cell assay in the future.

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