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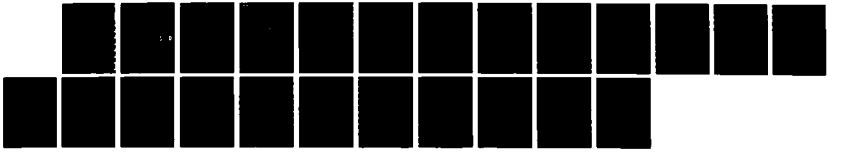

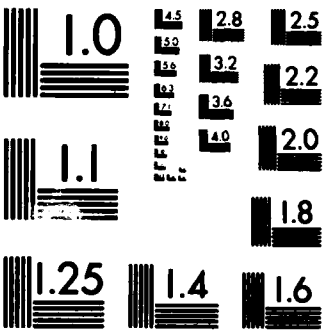

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Human Immune Responses to Dengue Viruses

Annual Report

(September 1982-July 1983)

Francis A. Ennis, M.D.

September 1983

Supported by
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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. During this first year we 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.

Polyclonal hyperimmune murine sera, to dengue types 2 or 4 which is type specific in neutralizing antibody assays, and convalescent human serum contain antibodies which lyse dengue-2 infected cells in the presence of normal human peripheral blood lymphocytes (HPBL) in this ADCC assay. Hyperimmune polyclonal antisera to dengue 2 virus plus complement lyse dengue 2 infected cells, however, antiserum to dengue 4 virus does not kill dengue 2 infected cells in the presence of complement. This apparent subtype specificity of the complement dependent lytic antibody, is unlike the cross-reactive killing of infected target cells in the ADCC assay in which dengue 2 infected cells are killed by hyperimmune antisera to dengue-4 as well as to dengue-2. The lymphocyte responsible for cell lysis in the ADCC assay appears to be $T3^-$ and $OKM1^+$.

Additional studies which have been performed include: (a) preliminary experiments in which adherent human peripheral blood leucocytes have been infected to provide a possible source of HLA defined target cells to use in an HLA restricted cytotoxic T lymphocyte (CTL) assay; (b) natural killer cell and serum interferon responses were measured on coded samples sent from WRAIR which had been obtained from volunteers before and after receiving attenuated Dengue-4 vaccine.

Plans for future studies contained in the accompanying proposal and very briefly include: 1) establishing satisfactory target cells for the other dengue serotypes: 1, 3 and 4, to use in determining the viral antigenic specifity of the ADCC and complement-dependent lytic antibody responses to (a) primary and secondary clinical cases of dengue, (b) pre and post dengue vaccination, (c) in type specific hyperimmune animal sera and d) with monoclonal antibodies supplied by WRAIR scientists; 2) the complement-dependent lytic antibodies, antibodies active in ADCC, virus neutralizing and enhancing antibody's activities will be analyzed using these various antisera. 3) We will attempt to develop suitable HLA defined target cells, using human mononuclear adherent cells to assess the presence of memory HLA restricted cytotoxic T lymphocytes following infection or vaccination and 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. 4) The activities of NK cells, K cells and HLA restricted CTL will be assessed sequentially in future Dengue vaccine clinical studies. Details regarding future experiments are contained in the accompanying application.

FORWARD

This research contract began on September 1, 1982 and this report covers the first 11 months of this contract.

TABLE OF CONTENTS

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.

We have used viral and serological reagents obtained from scientists at WRAIR. During the first several months we focused our efforts on developing a suitable human target cell which would express Dengue viral antigens on surface membranes and might be susceptible to cytolysis by host immune responses. Human peripheral blood leucocytes (HPBL) had been used earlier by McMichael et al[2] and us[3] as target cells to detect influenza specific HLA restricted cytotoxic T lymphocytes (CTL). Direct infection of HPBL cells with Dengue-2 virus did not result in expression of viral antigens on cell membranes antigen in our laboratory which confirmed an earlier study.[4] We tried to increase the number of cells expressing Dengue antigens on their surface (detected by using hyperimmune Dengue-2 mouse ascitic fluid as the antibody source and the $F(ab^1)^2$ fragment of sheep anti-murine IgG (Heavy and Light Chains) in an indirect FA assay) by adding HPBL to Vero cells which had been infected with Dengue-2 virus several days earlier. Although this increased the expression of Dengue virus antigens on the surface of the HPBL cells from 0 to 20%, these HPBL cells were not lysed significantly in a complement dependent assay using the same ascitic fluid as antibody, which we had used in the FA procedures and which had a neutralizing antibody titer of 1:400 to Dengue type 2.

We next evaluated the suitability of using Dengue-infected lymphoblastoid cell lines on target cells. Although such cell lines are not readily usable to detect HLA restricted T cell lysis (unless they have been developed by transformation of HLA typed HPBL precursors), it had been reported that a Dengue infected human lymphoblastoid cell line expressed Dengue viral antigens.[4]

Infection of certain human lymphoblastoid cell lines resulted in an increased percentage of cells expressing Dengue viral antigens on their membranes than we had observed using Dengue-2 infected Vero cells to infect HPBL. Following infection with a higher titered Dengue-2 virus pool prepared in a mosquito cell line (C6/36) instead of Vero cells, we achieved a very high percentage of Raji cells with Dengue antigens on their surface and for the first time we observed immune specific lysis of Dengue virus infected cells vs. uninfected lymphoblastoid cells. The following is a summary of the procedures and results to date obtained using such Dengue infected target cells in the first phase of this contract.

II. CYTOLYSIS OF DENGUE INFECTED CELLS

A. Preparation of dengue-2 infected Target Cells for NK Cell and ADCC Assays

1. Infection of Vero Cells

Following infection of Vero cells with a high multiplicity of infection of dengue-2 virus (strain-New Guinea C), we noted CPE and lysis of the monolayer by day-6. Some of the Vero cells had Dengue antigens on their membranes, but many were not viable and specific lysis by immune serum and complement was not detected. Following infection with 0.01 MOI of virus, we noted a gradual increase in the percentage of cells positive for dengue virus membrane fluorescence from 2% on day 2 to 20% by day 10. These cells have remained infected over a period of several months and the percentage of Vero cells expressing dengue antigens remains relatively constant despite an increase in the number of total cells by several-fold before splitting cells into other flasks which is done weekly. Despite this persistent infection, we were unable to detect specific lysis of the dengue infected Vero cells with the hyperimmune anti-dengue-2 ascitic fluid and complement, presumably because the majority of the Vero cells did not express Dengue antigen on their surface.

2. Infection of Lymphoblastoid Cell Lines

We infected several human lymphoblastoid cell lines, and a human histiocytoma cell line with dengue-2 virus at a high MOI (5:1) using a 1:5 dilution (to avoid toxicity) of a mouse brain prepared virus stock supplied by Dr. Brandt. Cells were harvested and stained by an indirect FA technique for membrane antigens on day 4.

Table 1. Expression of Dengue Antigens on Membranes of Infected Human Cell Lines

Cell Line	% Positive Dengue Membrane Antigen
HSB-2 (T cell line)	31%
Jarkatt (T cell line)	37%
Raji (B cell line)	27%

We have focussed our subsequent efforts using Raji cells, because uninfected Raji cells had less non-specific flourescence than the other cell lines. We have analyzed their potential use as a candidate target cell for detecting cytolysis of Dengue infected cells.

IIA3. Raji Cells persistently infected with Dengue as infected target cells

Raji cells were then infected with a MOI of 0.05 of the C6/36 prepared dengue-2 virus and the infected cells were incubated at 37°C in RPMI and 10% FCS at a concentration of $2X10^5$ cells/ml. After seven days the cultures were examined and were found to contain have >95% viable cells. Forty percent of the Raji unfixed cells were surface antigen positive for Dengue antigens, and 0% of the uninfected Raji cells were positive. These cells have remained infected over several months and have been used as target cells in the following studies.

IIB. Human Peripheral Blood Leucocytes - "Natural Killer" Cells and Antibody Dependent-Cell Mediated Cytotoxicity

IIB1. Demonstration of NK and ADCC mediated lysis of Dengue-2 infected Cells

These dengue-2 Raji cells were then used as target cells with a panel of normal human PBL which had been cryopreserved, which had been obtained from ten donors who are seronegative for dengue virus. Four and 18 hour ^{51}chromium release assays were performed using dengue-2 infected, or uninfected Raji cells or target cells with or without hyperimmune anti Dengue 2 mouse ascitic fluid at a 1:10 dilution. The K562 cell line was used as a standard human cell for detecting NK cell mediated lysis.

TABLE 2. Percent Specific Lysis* of Dengue-2 Virus Infected Raji Cells by HPBL and DEN-2 HMAF Antibody

		TARGET CELLS			
EFFECTOR		RAJI INFECTED		RAJI UNINFECTED	
(E:T-50:1)	K562	HPBL	+AB	HPBL	+AB
1	14.6	7.7	30.5	3.3	1.7
2	37.6	8.6	29.4	5.8	7.3
3	47.6	18.1	16.5	1.9	7.1
4	31.6	4.4	10.0	0	4.0
5	33.6	13.4	ND	0.5	ND
6	45.1	6.1	14.6	0	7.3
7	38.8	0.1	10.2	1.1	4.1
8	72.3	37.4	55.1	26.8	31.9
9	33.1	2.4	16.5	0.3	5.5
10	ND	16.6	24.1	0.2	5.8
Mean	39±15	11±10	23±13	4±8	9±9
		p<0.005			p<0.001
		p<0.005			

*^{51}Cr release as determined (quadruplicate samples)

$$\text{Percent Specific Lysis} = \frac{\text{CPM EXP-CPM Medium}}{\text{CPM MAX-CPM MEDIUM}} \times 100$$

These results were our first demonstration of specific lysis of Dengue virus infected cells. The results of this experiment which have been confirmed in subsequent experiments indicate: (a) that of dengue virus infected cells are significantly (statistical analysis was performed using paired T tests) more susceptible to lysis by normal HPBL than cells not infected with dengue virus, and (b) the addition of specific antiserum, which had been heated at 56°C for 30 min. to destroy complement activity, significantly increased the level of lysis by the HPBL of dengue-2 virus infected Raji cells.

IIB2. Time Response Studies

An experiment was then performed to determine the time course of lysis of dengue virus infected Raji cells by HPBL ± antibody. The net increase in lysis of ^{51}Cr infected Raji by Effector "A" (no. 2 in Table 1) at an E:T ratio of 50:1, after subtraction of the lysis of uninfected Raji cells was:

TABLE 3 Time of Lysis by HPBL of Dengue-2 Infected Raji Cells with and Without Dengue-2 HMAF Antibody

Hrs. Incubation	-Ab (NK)	+Ab (ADCC)
2	0*	0
4	4	4
8	7	14
18	7	28
25	7	26

*Percent specific lysis

The level of killing showed a similar pattern but lower levels of lysis at the E:T ratio of 10:1 in NK and ADCC assays. We did not expect that the killing of NK was due to augmentation of NK activity in vitro by interferon, since Raji cells are reported to be poor producers of interferon[5], and the increase in lysis was detected by 4-8 hours. In addition, we found that these were only 3 units of interferon produced by the dengue type 2 Raji infected cells.

IIB3. Cold Target Inhibition of NK and ADCC

The lysis of dengue 2 infected Raji cells in the NK and ADCC assays was inhibited significantly greater by cold target competition with dengue 2 infected Raji cells than by uninfected Raji cells (Wilcoxon rank test).

Table 4. Percent Inhibition of ADCC and NK lysis of Dengue 2 infected Raji cells by Infected or Control Raji Cells

Ratio of Cells Cold:Labelled	ADCC Cold Cells Added D-2 Infected Raji	ADCC Cold Cells Added Uninf. Raji	NK Cold Cells Added D-2 Infected Raji	NK Cold Cells Added Uninf. Raji
32:1	99[x]	54	96	66
16:1	81	35	80	45
8:1	56	24	70	41
4:1	37	5	41	30
2:1	31	16	15	5
1:1	16	0	5	8
	p<0.025		p<0.05	

x Percent decrease in specific ^{51}Cr release observed in the absence of competing cold target cells.

These results indicate that unlabelled "cold" dengue-2 infected Raji cells competed significantly better ($p<0.001$) with ^{51}Cr labelled dengue-2 infected target cells than did uninfected Raji cells. The percent inhibition of labelled infected target cell lysis was consistently higher at all ratios by the cold dengue-2 infected Raji cells as in the ADCC assay even at ratios as low as 2:1 or 1:1. A similar pattern of increased inhibition by infected Raji cells was also seen in the NK assay at cold:labelled target cell ratios of 4:1 or greater.

IIB4. Effector Cell Dose Response Studies

The dose response effect of various E:T ratios was evaluated in detail using two different donors of effector cells, one of whom had HPBL which produced relatively low levels of killing of K562 cells (#2 in Table 2) and one who had HPBL which produced a higher level of lysis of K562 cells (#8 in Table 2), with immune serum added at a 1:10 dilution (ADCC) or without immune serum (NK).

TABLE 5 Percent Lysis in Dengue-2 infected Raji cells in ADCC and NK ASSAYS

E:T ratio	Effector "2" Infected Raji ADCC	Effector "2" Infected Raji NK	Effector "2" Control Raji NK	Effector "8" Infected Raji ADCC	Effector "8" Infected Raji NK	Effector "8" Control Raji NK
100	40	26	11	ND	ND	ND
50	33	29	11	64	48	38
25	26	22	7	59	40	33
12.5	17	15	7	46	34	25
6.3	12	8	6	34	28	18
3.2	6	2	2	19	17	9
1.6	4	3	4	27	19	6
	p<0.01		p<0.05	p<0.025		p<0.025

The results indicate that there is a clear dose-response effect observed between the number of HPBL added and percent specific lysis both of infected and uninfected targets in ADCC and NK assays. This experiment confirms the results shown above i.e. that dengue infected cells are more susceptible to lysis by both HPBL alone ("NK") and this lysis is further augmented by the addition of antibody in hyperimmune serum (ADCC).

These results also demonstrated that uninfected Raji cells were killed to a variable degree by HPBL ("NK") alone, since donor "8" HPBL killed to a higher degree than donor "2". This variation between donor HPBL killing of uninfected Raji is reproducible (data not shown).

IIB5. NK and ADCC Responses following Infection

We then used several human sera as the source of antibody in the ADCC assay, and included known dengue 2 positive sera, supplied by WRAIR, and sera without antibodies to dengue virus.

TABLE 6 Virus Specificity Studies of ADCC using convalescent human sera obtained in Puerto Rico after dengue 2 outbreak in 1968

Dengue Sera	Percent specific lysis of Raji target cells: Infected	Uninfected
Humans-Puerto Rico		
#112	11.0*	3.6
#145	19.0	4.6
#110	7.5	5.5
#114	12.8	7.8
Control Human Sera		
A. Anti EBV ab		
1 +	0	0
2 -	0	0
B. Pooled human AB serum	-	-
C. F. Ennis serum	1.0	0

*Percent net specific lysis of dengue-2 infected Raji cells-after subtracting the level of lysis caused by pooled human AB serum obtained from dengue negative donors

These results with human serum confirmed earlier results using mouse serum and human effector cells. It is clear that HPBL cells (Effector "2" was used in this experiment at an E:T ratio of 50:1) lysed uninfected Raji cells to a low level in the presence of human serum from dengue endemic Puerto Rico or dengue-free areas (control sera). The level of killing by HPBL was higher of the dengue infected Raji target cells than of uninfected Raji cells ($p<0.025$ as determined by the Mann-Whitney U Test). Pooled human AB serum and sera with

or without antibodies to Ebstein-Barr virus (EBV), and without dengue virus antibodies did not lyse Dengue infected cells. These control sera were tested because Rayji cells possess EBV genome and we wanted to rule out the possibility that antibodies to EBV might be contributing to this lysis. Sera from individuals in Dengue free areas did not augment lysis of dengue 2 infected target cells by HPBL in this ADCC assay, unlike the augmented lysis using sera with dengue antibodies from Puerto Rico ($p<0.001$).

IIB6. Cells responsible for lysis in ADCC and NK

We initially assessed the nature of the cell(s) responsible for killing in these assays by testing the ability of adherent HPBL and supernatant (non-adherent) cells to lyse Dengue-2 infected Raji cells with and without immune serum. The results at an illustrative E:T ratio of 10:1 are shown in Table 7.

TABLE 7 Percent Lysis of Target Cells

Effector Cells	E:T	Infected Raji +ab	Infected Raji -ab	Uninfected Raji -ab	K562 -ab
Unfractionated			$p<0.03$	$p<0.02$	
HPBL	10	46.3	31.6	19.1	33.0
			$p<0.02$	$p<0.03$	
Adherent[x]	10	25.5	16.4	7.4	10.0
			$p<0.03$	$p<0.03$	
Non-adherent[xx]	10	46.6	37.0	18.4	38.5

x >95% phagocytose yeast bodies
xx < 5% phagocytose yeast bodies

The results suggested that the predominant cell type responsible for killing of dengue infected cells in the NK and ADCC assays were non-adherent cells, although some lysis was associated with the adherent cell fraction. We analyzed in more detail the nature of this non-adherent cell responsible for lysis of dengue infected cells in these assays. We treated normal human HPBL (effector "2") with monoclonal antibody to T cells (OK T3-a monoclonal antibody to T3, a pan T cell antigen) and with the monoclonal antibody to OKM1 which reacts with null lymphocytes and monocytic cells. Adherent cells were first removed and supernatant cells were reacted with these antibodies, then analyzed on a Becton-Dickinson FACS III, and were then sorted for use as effector cells in our assay.

TABLE 8 FACS Separation of Effectors after Monoclonal Antibody Treatment

Monoclonal Ab Treatment	%OKT3$^+$ cells
OKT3$^+$ pre fractionation	78.0%
OKT3$^+$ post fractionation	98.7% (8124/8230)
OKT3$^-$ post fractionation	2.7% (96/3484)
	%OKM1$^+$ cells
OKM1$^+$ pre fractionation	16.0%
OKM1$^+$ post fractionation	95.9% (3890/4055)
OKM1$^-$ post fractionation	1.7% (191/11,175)

These sorted populations of cells were then used as effectors in our standard assays to determine the level of ADCC and NK lysis of Dengue infected Raji cells at 10, 5 and 2.5:1 E:T ratios. The data presented below indicate that the cell sorting was successful since the K562 target cells, which are sensitive to lysis by human NK cells, were lysed very well by cells from the OKM1$^+$, and OKT3$^-$ groups, and only low levels of K562 lysis were produced by cells in the OKM1$^-$, and OKT3$^+$ effector groups.

TABLE 9 Percent Lysis of Dengue Infected Cells by HPBL After Monoclonal Antibody Treatment and FACS Separations of HPBL

Monoclonal Antibody Treatment	D-2 Raji Ab+	D-2 Raji Ab-	Uninfected Raji	K562
OKM1 Ab				
Preseperation	17.0	11.3	2.4	4.2
OKMI$^+$	24.1	20.9	5.1	26.3
OKMI$^-$	9.1	3.1	1.1	1.1
OKT3 Ab				
preseparation	24.6	20.1	4.5	8.5
OKT3$^+$	14.2	15.5	1.2	0.8
OKT3$^-$	25.9	18.4	4.7	33.3

The results suggest that cells with OKM1$^+$ and without OKT3 surface antigens are responsible for lysis of the uninfected Raji and K562 cells. The increased level of lysis observed on Dengue infected cells also appear to be highest in the OKM1$^+$ enriched population ($p<0.03$). Effectors depleted of OKM1+ cells caused less lysis of Dengue 2 infected target cells, ($p<0.03$), and effector cells enriched for T3$^+$ cells were less lytic than T3$^-$cells in the ADCC assay ($p=0.05$). Results were similar at the other E:T ratios (5:1 and 2.5:1) which were studied and analysis of all the data indicate that OKMI$^+$ cells were more lytic to Dengue infected cells in the presence than in the absence of antibody ($p=0.01$).

II. C. Complement dependent lytic antibodies

After we succeeded in demonstrating the augmentation of specific lysis of dengue infected cells by antiserum added to HPBL we used the same antiserum (mouse hyperimmune anti-Dengue 2 ascitic fluid) with guinea pig serum as a source of complement and tested for lysis of ^{51} chromium labelled dengue 2 infected Raji cells using methods we reported earlier with influenza infected target cells. We had performed this assay earlier on dengue 2 infected Vero cells, and on dengue-2 infected HPBL and those results were negative (data not presented).

Table 10 Complement Dependent Antibody Lysis of Dengue-2 Infected Raji Cells

Complement Dilution (G.Pig Serum)	Antiserum Dilution 1:20	1:100	-
1:2	28.1*(0)**	26.3 (11.6)	24.4 (13.2)
		p<0.03	
1:4	25.5 (4.3)	22.0 (0) p<0.05	16.4 (5.7)
1:8	25.0 (5.9)	18.2 (0) p<0.05	8.5 (0)
		p<0.05	
-	0 (0)	0 (0)	0 (0)

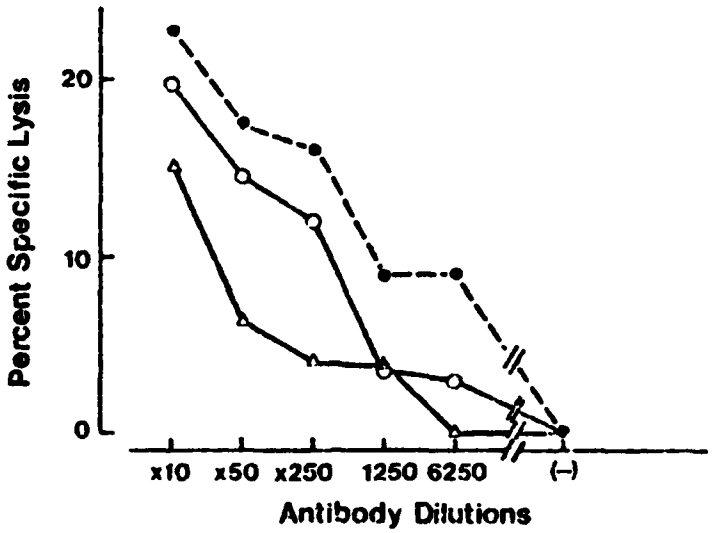
Percent specific lysis of dengue-2 infected *and uninfected ( )** Raji cells

Guinea pig serum alone at a 1:2 dilution without antibody lysed uninfected Raji cells. This confirms recently published detailed studies that indicated the alternative pathway of complement is active on uninfected Raji cell membranes (6). The level of lysis by the guinea pig serum alone at a 1:2 dilution was further increased on dengue-2 infected target cells. At a guinea pig serum dilution of 1:8, little non-specific lysis was observed, and antiserum dilutions of 1:20 and 1:100 both significantly lysed dengue 2 infected cells in the presence of the complement source, but not in its absence. The antisera are tested following heat inactivation at 56°C for 30 min. and the guinea pig sera represent pooled sera from 6 animals which were maintained at -70°C and not heated. Heating the guinea pig serum at 56° for 30 min. removes its ability to lyse dengue-2 infected Raji cells in the presence of antibody (data not presented).

II. C.1 Dose response studies of complement dependent lytic antibodies

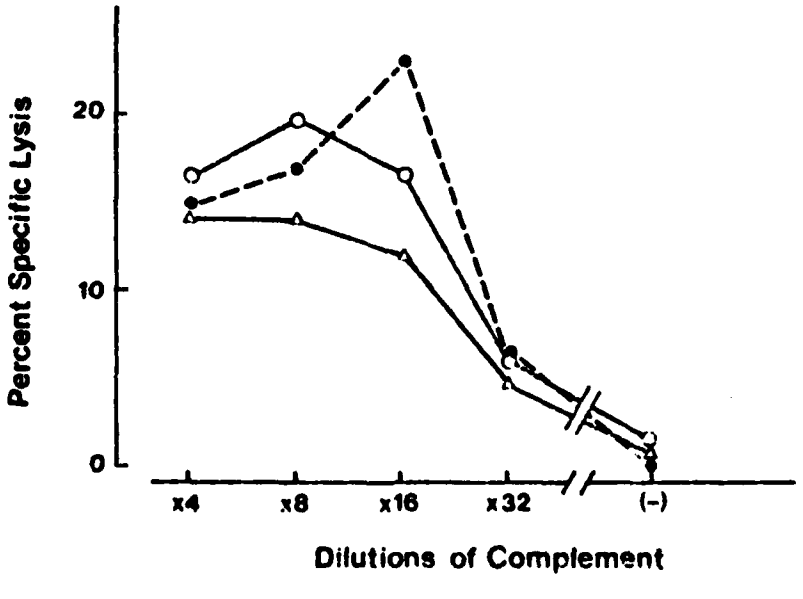
The dose response and kinetics of the complement-dependent antibody mediated lysis of dengue infected Raji cells were than examined using several dilutions of antiserum, and several dilutions of guinea pig serum as the source of complement and incubating for various time periods. Antisera dilutions at 1:10, 1:50 and 1:250 caused specific lysis, in the presence of guinea pig serum diluted 1:4-1:16, but not at 1:32. Most of the lysis occurred by 1 hour.

Fig. 1. Effect of antibody dilution on lysis of dengue-2 infected cells (-the lysis of uninfected Raji cells) in a complement-dependent assay



It is clear that the antiserum specifically lysed dengue-2 infected cells at dilutions up to ≥1:250, with complement dilutions of 1:8 or 1:16. Complement dilutions of 1:4 caused more non-specific lysis; (--- 1:4, o---o 1:8, and --- 1:16 complement dilutions).

Fig. 2. Effect of dilution of complement on antibody-mediated lysis of dengue-2 infected Raji cells. The net lysis is obtained by subtracting the level of lysis of the Raji cells not infected with dengue virus from the level of lysis of dengue infected cells and is presented in the figure. The antibody was to dengue-2, HMAF, which was diluted 1:10; --- , o---o and --- indicate incubation periods of 2, 4 and 18 hours, respectively.



The results shown in Fig. 2 demonstrate that in antibody excess specific lysis of dengue 2 infected Raji cells was observed with complement (pooled guinea pig sera) diluted from 1:4-1:16, but the level of lysis was reduced at the 1:32 dilution. Lysis was detected rapidly in the first few hours of incubation. The antibody required complement to mediate lysis in this and in other experiments not presented.

In a preliminary single experiment in which the degree of specific lysis caused by the immune mouse serum was lower than usual, sera from humans pre-vaccination with an attenuated dengue-2 virus and from 28 days post vaccination did not specifically lyse dengue-2 infected target cells in the assay in which 78% of the target cells had dengue antigens by immunofluorescence. We will be testing more of these vaccination sera, and Dr. Bancroft will also provide well defined sera from dengue cases in Thailand for these assays. We have recently demonstrated that non-immune human serum can be used as a very satisfactory source of complement instead of guinea pig sera in this lytic antibody assay. We will use human complement in our assays for lytic antibodies in human sera.

II. C.2 Virus specificity of complement dependent antibody mediated lysis

In recent studies using hyperimmune murine anti-Dengue 2 and anti Dengue 4 sera on Raji target cells infected with Dengue-2 virus, we have observed the following pattern of lysis.

Table 11. Percent Specific Lysis of Dengue 2 Infected Cells

	Antisera to	ADCC	Ab+C
Expt. 1	Dengue 2	21.5	19.8
	Dengue 4	22.9	8.5
	Complement alone	ND	6.0
Expt. 2	Dengue 2	ND	13.7
	Dengue 4	ND	4.0
	Complement alone	ND	1.7
Expt. 3	Dengue 2	39.7	19.9
	Dengue 4	44.1	7.6
	Complement alone	ND	3.7

The results are preliminary because we need to test the immune sera on target cells infected with the other dengue types, in addition to dengue type 2. These results do, however, suggest that the antibody which augments NK lysis of dengue-2 infected cells recognizes cross-reactive antigen since dengue type 4 antibody also is active in the ADCC assay. The antibody which is lytic with complement appears to be type 2 specific, since type 4 antibodies with complement did not lyse dengue-2 infected cells.

III. STUDIES INITIATED TO DEVELOP HLA RESTRICTED DENGUE-INFECTED TARGET CELLS FOR USE IN CTL ASSAYS

IIIa. Cryopreservation of HLA typed HPBL

We have cryopreserved 35 normal donor HLA typed buffy coat preparations of HPBL with 30 vials of each, each vial contains 3 x 10^7 cells. These donors are non-immune to dengue. We have cryopreserved HPBL from two dengue-4 vaccine infected recipients, pre and post vaccination, and these are also HLA typed. We will receive a buffy coat from a HLA typed donor, known to be immune to Dengue-2, in the near future.

IIIb1. Lymphocytes

As stated above (please see Introduction and Section II A), the results of preliminary experiments suggested that HPBL could be infected more successfully by dengue-2 virus if they were incubated on dengue-infected Vero monolayers for 24 hours. However, this did not result in more than 20% of the HPBL expressing dengue virus antigens on their membranes, and immune cytolysis was not detected when these HPBL were treated with specific antibody and complement. The other sections of this report describe our subsequent successful development and utilization of a dengue-infected human lymphoblastoid cell line for detecting: complement-dependent antibody mediated lysis, preferential killing by natural killer cells of dengue infected cells and augmentation of this lysis by addition of antisera to dengue viruses. We have begun additional experiments to develop suitable HLA defined target dengue-infected target cells to detect virus specific cytotoxic T lymphocyte activity. This subset of lymphocytes which bear the OKT8 antigen on human T cells, are restricted in their killing of target cells infected with viruses by major histocompatability class I antigens e.g. the HLA A and B antigens of human cells, and the H-2 K and D region antigens on murine cells. This specificity of CTL for viral and self antigens has been defined for several virus infections in mice, including by the PI using influenza virus, and in humans by several laboratories, including in our laboratory for influenza, and by others for cytomegalovirus, and E-B virus infections. These lymphocytes appear to be necessary for recovery from and may contribute to the immunopathology of influenza pneumonia in mice.

IIIb2. Monocytes

We infected populations of HPBL following separation using an Elutriator controlled flow-rate centrifuge for separation of HPBL based on size and density. Successful fractionation occurred as confirmed by Wright-Giemsa staining staining of several fractions. The cells of some of these fractions were then placed on plastic dishes for 45 minutes at 36°C, and the supernatant fluids were removed and after two washes, they were infected with dengue-2 virus at an MOI of 1. A summary of the results of this preliminary experiment is shown below.

TABLE 11. Infection of HPBL with dengue-2 Virus after Separation by Flow-Rate Centrifugation

Sample		STAINING (Wright-Giemsa)	After Dengue-2 Virus Infection: Adherent Cells Memb. Ag*	Adherent Cells V.T.**	Supern. Cells Memb.Ag.	Supern. Cells V.T.
Original Buffy-Coat (Hypaque-Ficoll)		65% small lymphs 35% mono		10^4	3%	10^3
Flow Rate Fraction						
(12ml/min)	1	Ghost Cells	ND	ND	ND	ND
(20ml/min)	3	95% Small Lymphs	-	10^1	-	10^1
(28ml/min)	5	61% Monocytes 39% Lymphs	40%	10^5	14%	10^3
(40ml/min)	8	96% Lymphocytes	ND	ND	ND	ND

*Percent of unfixed cells expressing dengue viral antigens on their membranes by the indirect fluorescent antibody techniques.
**Virus Titer is expressed as $\log_{10}$ $TCID_{50}$ in Vero cells

The results of this experiment and other preliminary experiments indicate that HPBL fractions enriched for adherent monocytic cells will express dengue virus specific membrane antigens, and nonadherent lymphocytes will generally not, following infection with dengue-2 virus.

The experiments contained in the accompanying proposal indicate our plans for using monocytes from HLA typed donors to serve as dengue infected Chromium labelled target cells for detecting Dengue virus specific HLA restricted cytotoxic T lymphocytes in HPBL from individuals following natural infection or vaccination.

IV. EXPERIMENTAL VACCINE STUDIES

Five volunteers participated in two small dengue-4 attenuated vaccine studies which were performed by WRAIR investigators under isolation conditions. Peripheral blood samples were obtained with preservative-free heparin and were promptly shipped to us. We received the samples under code and separated the HPBL on Ficoll-Hypaque. Natural killer cell assays were performed on the freshly separated HPBL and the remaining HPBL were cryopreserved using a programmable freezer to maximize viability. The vaccinees had been HLA typed and these HPBL will be used as effector cells in later experiments after we develop a suitable HLA defined target cell.

The results of the natural killer cell assays indicated that four of the five volunteers had rises in NK cell activity on days 9-14, and one (MF) had no change from baseline. One of the volunteers (SG) had a high rise in serum interferon detected on samples from days 7-14 with a peak at day 7 (50 Intern. Units), and three others had lower rises detected (10 IU) during the same time period. The fifth volunteer had no rise in NK activity had no detectable level of serum interferon at any time (MF).

TABLE 12. Summary of dengue-4 Vaccine Volunteer NK Results

A. Dengue expt. #11			DAY				
Volunteer	NO.	E:T	0	9	21	52	63
J.B.	DP 1.	50	62.4	90.8	74.6	-	63.8
		25	43.7	80.1	55.6	-	57.8
		15	-		68.7	-	49.0
		10	21.3	83.3	55.0	-	-
B.S.	DP 2.	50	34.9	63.3	41.8	25.0	-
		25	21.4	46.4	32.7	16.3	-
		15	-	-	28.2	15.0	-
		10	10.6	27.1	18.5	5.8	-
B. Dengue expt. #14			DAY				
			0	7	9	14	21
M.F.	DP 6.	50	20.5	21.4	15.0	17.0	14.9
		25	9.5	13.4	13.3	14.4	5.3
		12.5	3.1	14.3	6.4	9.6	0
		6.3	-	-	1.8	-	-
S.G.	DP 7.	50	20.8	15.5	18.9	44.4	1.9
		25	11.8	11.3	9.3	32.9	0.2
		12.5	8.0	7.7	0	26.2	0
		6.3	-	-	0	-	-
P.W.	DP 7.	50	-	27.9	37.5	58.8	15.4
		25	22.9	17.9	27.2	51.2	9.5
		12.5	14.1	12.7	19.1	43.7	6.5
		6.3	10.7	-	11.1	-	-

The interferon in the sera obtained on day 7 and 9 from volunteer #4 (SG) was characterized by performing a neutralization with specific anti-human α and γ interferon as previously described (7).

TABLE 13 Characterization of Type of Interferon in Serum After Dengue-4 Vaccination

SAMPLES	
S.G. Sera Day 7, 9	INHIBITING DILUTION*
+ MEDIA	NONE
+ ANTI α IFN	1:27
+ ANTI γ IFN	NONE
STANDARD IFN	
α IFN	
+ MEDIA	NONE
+ ANTI γ	NONE
+ ANTI α	1:27
γ IFN	
+ MEDIA	NONE
+ ANTI γ	1:3
+ ANTI α	NONE

*Detection of specific antiserum which neutralizes 10 International Units of IFN in the sample

In a preliminary experiment, the sera from two subjects who had received dengue-4 vaccine and had a neutralizing antibody response to dengue-4 virus (3,4) have been tested on dengue-2 infected Raji target cells and their sera did not lyse the dengue-2 infected target cells in the presence of complement or normal HPBL. In the next phase of the research we plan to test these and the remaining sera from this clinical study on infected target cells, including human mononuclear cells infected with dengue-4 virus, which was the serotype of this vaccine. These preliminary results suggest that primary infection with an attenuated dengue-4 vaccine which induced serum interferon responses, enhanced natural killer cell activity, and neutralizing antibody responses did not induce antibodies which would be lytic in the presence of complement or normal HPBL, to cells infected with the type-2 virus. We must study other sera e.g. after primary and secondary natural infections on target cells infected with each of the dengue serotypes before this major question is satisfactorily addressed. These sera will be provided by Drs. Bancroft and Brandt of WRAIR. The reviewer is referred to our application for the detailed plans of our proposed studies.

V. REFERENCES

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