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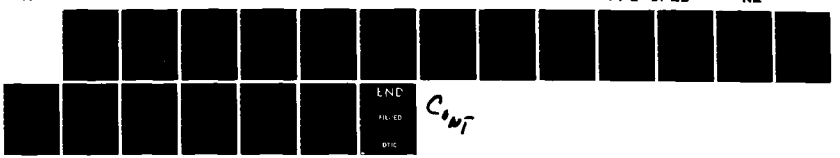

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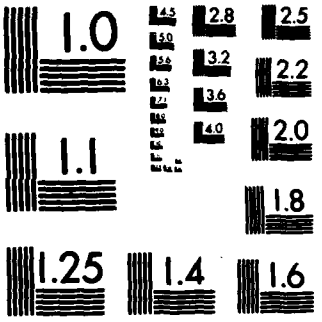

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Mechanisms of Bunyavirus Virulence: A Genetic Approach

Annual Report

Neal Nathanson, M.D.

February 1, 1982

Supported by

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FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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1. Introductory note: Scope and Duration

As negotiated, the budget for this contract for the 01 year, is summarized below.

Components of Budget	12 Months	14 Months
Total Direct Costs	46,753	54,545
Indirect Costs (62% TDC)	30,389	35,454
Total Contract	77,142	90,000

Since it was clear that only a part of the original proposal could be funded on a contract of this size, we have selected the development, characterization, and utilization of hybridomas as the focus for this project. Nevertheless, the original 3 investigators are collaborating on this aspect of the program.

Neal Nathanson	Professor	Pathogenesis, immunology
Jon Gentsch	Assistant Professor	Genetics and protein chemistry
Francisco Gonzalez	Post-doctoral Fellow*	Hybridomas

*Assistant professor, 7-1-82.

Also, it should be noted that this report was written in January 1982, when the contract had run only 3 months of the 01 year.

2. Hybridomas against LaCrosse and Tahyna viruses

(a) Manuscript

Much of the work to date on our hybridomas is summarized in the manuscript which has been submitted for publication. This contains technical details which will not be repeated in the Progress Report.

Gonzalez-Scarano F, Shope RE, Calisher CH, and Nathanson N: Characterization of monoclonal antibodies against the G1 and N proteins of LaCrosse and Tahyna, two California serogroup bunyaviruses. Submitted, January, 1982.

(b) Uses of hybridomas

The central theme of our studies is to use the California virus system as a model to study the factors which determine the outcome of acute viral encephalitis. Among these factors are virus determinants (neuroinvasiveness and neurovirulence) and host determinants (recovery from infection and protection against subsequent infection). We plan to use monoclonal antibodies as a tool to investigate several aspects of this model.

(i) To make reassortant viruses from virulent and avirulent parent viruses, monoclones can be used to rapidly phenotype putative reassortants for both G1 and N proteins.

(ii) To make antigenic variants of parental virus, growth in the presence of monoclones will select efficiently for variant viruses. These variant viruses can be used for two distinct purposes: (a) To determine if variants show changes in their virulence, which could occur if the G1 protein is an important determinant of virulence as has been shown already by Shope et al (1981). (b) To group the monoclones themselves according to their reactivity with a panel of variant viruses.

(iii) To identify and map biological functions of the viral glycoproteins. Important functions associated with glycoproteins of enveloped viruses are: binding to erythrocytes (hemagglutination), binding to host cells (neutralization), fusion of membranes (hemolysis, cell fusion, infectivity), and neuraminidase (probably lacking in bunyaviruses).

(iv) To determine the protective role of antibodies directed against antigenic determinants of California encephalitis virus glycoproteins. In other enveloped viruses antibodies against certain sites on the glycoprotein will neutralize, but the efficiency may differ markedly, depending on whether the site is involved in attachment or in fusion. Furthermore, antibody against some sites may fail to neutralize and such antibodies may actually protect the virus against neutralization (blocking antibodies). Conversely, there may be a synergistic effect of neutralizing antibodies against two different antigenic sites. Finally, the properties of the antibody itself (avidity, complement fixation, ability to mediate virolysis and cytolysis, and the like) may influence its protective efficiency. Such questions can be studied with monoclonal antibodies much more precisely than could ever be accomplished with polyclonal antisera.

(c) Immunization of mice and construction of hybridomas

LaCrosse and Tahyna viruses were selected for this study because they represent antigenically distinct strains with relative differences in virulence in mice, LaCrosse being the more neuroinvasive (kills after ip injection) and Tahyna the more avirulent (fails to kill after ip injection above 2 weeks of age). Also, it had been shown that reassortants could readily be made between these two viruses.

To immunize mice, advantage was taken of the fact that LaCrosse and Tahyna viruses produce active infections in mice; intraperitoneal or intraceretoral injection was used, to initiate a severe infection with some deaths, and survivors were used. A booster injection of virus was given and 2-4 days later mice were sacrificed and spleen cells preprepared.

Spleen cells from LAC or TAH immunized mice were fused with a BALB/c myeloma line (P3 x 63 clone 653) which is a nonsecretor. In HAT medium this line will be killed (HPRT-) since it cannot utilize the pruine salvage pathway; this function is provided by the lymphocyte partner in the hybrid cell. A mixture of spleen:myeloma cells at a 10:1 ratio was made and PEG 1000 used as fusing agent. The mixture was plated in micro wells, 5×10^5 cells per well.

After 2-3 weeks of incubation, wells with visible colonies were tested for anti-viral antibody in ELISA assay, using partially purified virus as antigen. Positive cultures were cloned in 0.25% agarose and individual colonies were transferred to flasks and again tested for antibody. Hybridoma cells were maintained in 15% serum and supernates collected as a cell culture source of monoclonal antibody. For high titer preparations, 10^7 hybridoma cells were injected ip into Pristane-primed BALB/c mice and ascitic fluids collected 1-2 weeks later. Antibody titers of ascitic fluids were usually about 100-fold higher than titers of tissue cultures supernates. For neutralization, HI, CF, and ELISA the ascitic fluid served well, but tissue culture supernate was required for clear immunoprecipitations. Cells stored well in a serum-DMSO mixture in liquid N2.

(d) Characterization of hybridomas

To characterize the LAC and TAH hybridomas, each was tested in ELISA, neutralization (N), and hemagglutination (HI) systems, against each of 11 California serogroup viruses.

In addition, each monoclone was used to immunoprecipitate virus proteins from a 3H-amino acid labelled lysate of infected cells, and each was typed as to immunoglobulin class by an RIA.

The essential results are set forth in the Table 1 and may be summarized:

(i) Of 23 monoclones, 15 were directed against the G1 glycoprotein and 8 against the N nucleoprotein, while none were against the G1 glycoprotein or the L polypeptide.

(ii) Of the 15 G1 clones, 11 both neutralized and had HI activity, one had HI activity only, and 3 were neutralization and HI negative. From this it was inferred that the G1 glycoprotein had at least two antigenic sites. One site is postulated to bind to receptors on both erythrocytes and substrate cells, accounting for the concordance of neutralization and HI results. The other site appears uninvolved in attachment to receptors.

(iii) Of the 15 G1 clones 4 were type-specific (group A/1) i.e., reacted with the immunizing virus only, 3 were almost type-specific (group A/2 or C), while 8 were cross-reactive (group B or D).

(iv) None of the 8 nucleocapsid clones showed neutralizing or HI activity, as expected.

(v) Of the 8 nucleocapsid clones, only one was type-specific i.e., was a group C clone, while 7 were cross-reactive (group C/D or D).

(vi) The 15 G1 clones were isotyped as IgG1 (7 clones), IgG2a (6 clones), or IgG2b (2 clones). By contrast, the 8 nucleocapsid clones were IgM (5 clones), Ig2a (2 clones), or undetermined (1 clone).

(e) Phenotyping of reassortant viruses with monoclones

A major use for the monoclonal antibodies was to phenotype reassortant viruses. Using authenticated reassortants (courtesy of D. Bishop), prototype tests were conducted by ELISA, neutralization, HI, and CF methods. These different tests produced congruent results and a subset of the data are shown in Table 2. Clearly, selected monoclones are capable of distinguishing LAC and TAH G1 and LAC and TAH nucleocapsid proteins, and therefore can be used to phenotype reassortants for the products of the M RNA and S RNA genes.

TABLE 1. CHARACTERISTICS OF 23 MONOCLONAL ANTIBODIES AGAINST LaCrosse (LAC) and Tahyna (TAH) VIRUSES

Clone No.	Immunizing Virus	Protein Precipitated	Ig Class	Group	Type-Specific Cross-Reactive	ELISA	N	HI
807-09	LAC	G1	IgG2a	A/1	S	+	+	+
807-15	LAC	G1	IgG2b	A/1	S	+	+	+
807-18	LAC	G1	IgG1	A/1	S	+	+	+
807-35	LAC	G1	IgG1	A/1	S	+	+	+
807-31	LAC	G1	IgG1	A/2	S	+	+	+
807-12	LAC	G1	IgG2a	B	C	+	+	+
807-22	LAC	G1	IgG2a	B	C	+	+	+
807-33	LAC	G1	IgG2a	B	C	+	+	+
807-25	LAC	G1	IgG2b	C	S	+		
807-26	LAC	G1	IgG2a	C	S	+		
807-21	LAC	G1	IgG2a	C/D	C	+		
807-13	TAH	G1	IgG1	B	C	+	+	+
813-48	TAH	G1	IgG1	B	C	+	+	+
813-77	TAH	G1	IgG1	B	C	+	+	+
814-443	TAH	G1	IgG1	C/D	C	+		+
820-374	LAC	N	IgM	C/D	C	+		
807-28	LAC	N	IgG2a	C/D	C	+		
807-32	LAC	N	IgM	C/D	C	+		
807-13	LAC	N	IgM	C/D	C	+		
807-02	TAH	N	IgG2a	C	S	+		
814-08	TAH	N	?	C/D	C	+		
814-48	TAH	N	IgM	D	C	+		
814-87	TAH	N	IgM	D	C	+		

TABLE 2. PHENOTYPING OF REASSORTANT VIRUSES USING SELECTED MONOCLONAL ANTIBODIES

Gene and Gene Products	Genotype of Virus	Serological Test	LAC		TAH	
			Clone	Titer	Clone	Titer
M RNA	LLL	Neutral-	807-31	>4.0	813-77	1.6
G1 protein	TLT	ization	807-31	>4.0	813-77	1.9
	LTL		807-31	<0.7	8.3-77	4.0
	TTT		807-31	<0.7	813-77	4.0
S RNA	LLL	ELISA	807-28	>4.6	814-02	1.6
N protein	LTL		807-28	>4.6	814-02	1.9
	TLT		807-28	2.2	814-02	>4.6
	TTT		807-28	2.2	814-02	>4.6

(f) Classification of California serogroup viruses with monoclones

Although not a major goal of our work, it was of interest to look at the 3 subgroups of the California serogroup, according to their reactivity with our panel of monoclonal antibodies. As shown in Table 3, the monoclonal panel (6 clones) suggested some inconsistencies with the conventional subgrouping of 11 viruses tested. The most striking exceptions were: (i) within the CE subgroup, LAC clones failed to react with CE virus; (ii) within the MEL subgroup both LAC and TAH clones reacted with JC virus; (iii) within the TVT subgroup, LAC clones reacted with TVT virus.

It is interesting to note that selected LAC and TAH clones are potentially useful reagents for identification of new field isolates. As shown in Table 4, the use of 5 selected clones could potentially distinguish between the 7 different California serogroup viruses found in North America. In fact, Dr. Calisher, one of our collaborators with responsibility for the CDC arbovirus references laboratory, is seriously interested in using and distributing selected clones for this purpose.

(g) Next questions

The data reported above represent solid progress in the characterization of LaCrosse and Tahyna hybridomas. However, there are major gaps which require further work. These include

(i) The production of G2 hybridomas.

(ii) Mapping and grouping G1 hybridomas to define the discrete antigenic sites against which they are directed.

(iii) Using hybridomas to localize the putative fusion function to G1 or G2.

(iv) Determination of the potential biological role of neutralizing and non-neutralizing G1 hybridomas.

Plans for such studies are set forth in the work proposed for the next year of this contract.

(h) Use of monoclones by other investigators

As a by-product of our work, several other investigators have requested and been sent selected monoclones or hybridoma cells. These include

R.E. Shope, YARU
C.H. Calisher, CDC
Fred Fuller (with D. Bishop), UAB

M. Bardos, Czechoslovak Academy of Sciences
L.J. Grady, NY State Department of Health
L. Kingsford, California State University

These workers wish to use the clones for a wide variety of purposes, from basic studies to practical applications. Thus, even in its early stages, this work has been supportive of a number of other laboratories.

TABLE 3. SUBGROUPS WITHIN THE CALIFORNIA SEROGROUP: REACTION PATTERN WITH CROSS-REACTIVE MONOCLONES

Subgroup	Virus		Neutralization LAC	Neutralization TAH
CE	LaCrosse	LAC	+	+
	Snowshoe Hare	SSH	+	+
	Tahyna	TAH	+(S)	+
	California encephalitis	CE	-	+
	San Angelo	SAN	-(S)	+
	Inkoo	INK	+	-(S)
MEL	Jamestown Canyon	JC	+	+
	Melao	MEL	-	-(S)
	Keystone	KEY	-	+
	Serro do Navio	SDN	-	-
TVT	Trivittatus	TVT	+	-(S)

* Neutralization tests with group B monoclones, of which there were 3 raised against LAC and 3 raised against TAH viruses. Split patterns (S) were 2/3 (-) or 2/3 (+).

TABLE 4. DIFFERENTIATION OF NORTH AMERICAN MEMBERS OF THE CALIFORNIA SEROGROUP USING MONOCLONAL ANTIBODIES IN THE NEUTRALIZATION TEST

Clone No.	LAC	SSH	JC	TVT	SAN	CE	KEY
807-09	+	-	-	-	-	-	-
807-31	+	+	-	-	-	-	-
807-33	+	+	+	+	-	-	-
807-12	+	+	+	+	+	-	-
807-13	+	+	+	-	+	+	-

3. Virulence of LaCrosse and Tahyna isolates

NOTE: As indicated above, we have designated the hybridoma studies as the specific object of this contract. However, the hybridomas will be used in experiments which are supported by our NIH grant AI 18085. Therefore, a brief account of the status of these experiments is set forth below.

The salient goal of our virulence and genetic studies is to correlate virus genes and their products (proteins) with biological properties of the virus, particularly virulence in rodents. The strategy which we are employing is to select and clone virus strains which demonstrate maximal differences in their virulence. The role of specific genes/gene products will then be analyzed in two ways: (a) construction of reassortant viruses using the selected clones as parents; (b) selection of hybridoma variant viruses, to look for the possible role of particular regions of the G1 molecule.

A further element in our plan is the selection of two viruses, LaCrosse (LAC) and Tahyna (TAH), as representative of more virulent (LAC) and less virulent (TAH) members of the California serogroup. Also of importance was the demonstration by Gentsch, Bishop and colleagues that LAC-TAH reassortants could be readily made.

(a) Standard LAC and TAH strains

To confirm that the standard strains of LAC (original) and TAH (Bardos 92) would exhibit characteristics similar to those published by Shope and others, we did age-specific titrations with the results shown in Tables 5 and 6. These titrations were consistent with the published literature and confirmed that by the ip route, TAH virus is nonlethal in mice age 2 weeks or older. By contrast, LAC virus will kill older mice, although it requires 10,000 - 100,000 suckling mouse LD50 to kill 50 - 100% of 4-week-old mice.

These results reinforce our original presumption that it would be preferable to have a LAC clone which showed greater ip virulence in weanling or adult mice and a TAH clone which showed less ip virulence in sucklings.

(b) Selection of virulent and avirulent strains

To obtain an avirulent TAH clone, we have written Drs. Malkova and Bardos, in Prague, who have shipped TAH strain 180/52. Malkova reported that this strain fails to kill suckling mice by the ip route in any dose.

To develop a more virulent strain of LAC virus Dr. C. Calisher

screened about 6 isolates of LAC, by ip and ic routes, and we selected a strain (Ohio-77) with maximum ip titer (10^4 LD50 per ml in weanling mice). We have been passing this virus in two ways. (a) ip inoculation of 1 week mice, brain harvest, and reinoculation of 1 week mice. (b) ip inoculation of weanling mich which are given 2 doses of cyclophosphamide on days 2 and 7, to maximize mortality. Brains are harvested and a 10% suspension again passed into weanlings. After about 10 passages, each passage line will be tested.

TABLE 5. TITRATIONS OF LAC AND TAH VIRUSES IN MICE

Assay	Log_{10} Titer per ml	
	LAC	TAH
pfu	6.2	ND
ic LD50		
1-3 days	7.1	6.3
3-4 weeks	6.5	6.2
8-10 weeks	5.8	4.5
ip LD50		
1-3 days	6.2	6.1
1 week	ND	3.0
2 weeks	ND	<1.0
3-4 weeks	2.8	<1.0
8-10 weeks	2.0	<1.0

* LAC: LAC/original in BALB/c mice.
TAH: TAH/B92 in CD mice
Undiluted: supernate of a 10% brain homogenate.

TABLE 6. TITRATIONS OF LAC AND TAH VIRUSES IN RATS

Assay	Log_{10} Titer per ml	
	LAC	TAH
pfu	6.2	
ic LD50		
newborn	7.5	
1 week		7.6
2 weeks		7.5
3 weeks	5.8	6.7
4 weeks	6.5	
6 weeks	3.1**	<1.5**

* Outbred Wistar rats received .03 ml ic. Undiluted: supernate of a 10% homogenate of suckling mouse brain.

**An inoculum of .03 ml of undiluted virus stock killed 100% of rats (LAC) and 0% rats (TAH).

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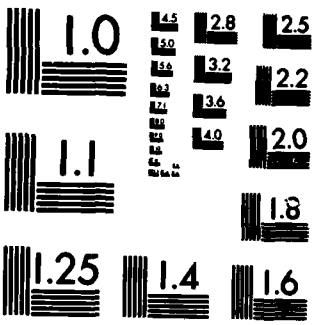

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