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LECTIN ENZYME ASSAY DETECTION OF VIRUSES, TISSUE CULTURE, AND A MYCOTOXIN SIMULANT

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September 1988

U.S. ARMY ARMAMENT MUNITIONS CHEMICAL COMMAND

Aberdeen Proving Ground, Maryland 21010-5423

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SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution is unlimited.
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S) CRDEC-TR-88148	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION CRDEC 6b. OFFICE SYMBOL (If applicable) SMCCR-RSB	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) Aberdeen Proving Ground, MD 21010-5423	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION CRDEC 8b. OFFICE SYMBOL (If applicable) SMCCR-RSB	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
8c. ADDRESS (City, State, and ZIP Code) Aberdeen Proving Ground, MD 21010-5423	10 SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. / PROJECT NO. 1L161101 / TASK NO. A91A / WORK UNIT ACCESSION NO.

11. TITLE (Include Security Classification)
Lectin Enzyme Assay Detection of Viruses, Tissue Culture, and a Mycotoxin Simulant

12. PERSONAL AUTHOR(S)
Kraybill, William H.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Technical	FROM 82 Feb TO 85 Aug	1988 September	18

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Lectin Enzyme Tissue Culture Detection Virus Mycotoxin Simulant Assay
06	13		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Wheat germ agglutinin (WGA) lectin has been used directly to detect rabies virus and indirectly to detect rotavirus; rabies by absorbance of large aggregates and rota by an ELISA (erythro-LIT) method where the antibody-antigen-antibody sandwich is revealed by lectin erythroadsorption. WGA has a specificity for binding N-acetyl glucosamine, epichlorohydrin (a mycotoxin simulant) and zearalenone mycotoxin. The cosmopolity and affinity of binding WGA to viruses and tissue culture was studied. WGA lectin, covalently bound to peroxidase, was shaken with virus, tissue culture, or mycotoxin suspensions in a microtiter tray for 10 min, then absorbed to the plastic tray by incubation for 20 min at 4 °C. Unreacted WGA peroxidase (HRP) was removed by washing six times with phosphate buffered saline, pH 7.4. Color reagent 2,2[1]-azino-di-(3-ethylbenzthiazoline sulfonic acid) (ABTS) containing hydrogen peroxide substrate, citric acid, and glycine was added to each well and incubated at 37 °C for 10-30 min on a shaker. (Continued on reverse)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	UNCLASSIFIED
22a. NAME OF RESPONSIBLE INDIVIDUAL SANDRA J. JOHNSON	22b. TELEPHONE (Include Area Code) (301) 671-2914 22c. OFFICE SYMBOL SMCCR-SPS-T

DD FORM 1473, 84 MAR 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE
UNCLASSIFIED

19. ABSTRACT (Continued)

Inactivated Venezuelan Equine Encephalitis was detected at less than 10^4 virions/mL, Escherichia coli bacteriophage at 10^7 virions/mL, HEP tissue culture at 10^6 cells/mL, and baby hamster kidney tissue culture at 10^5 cells/mL. Epichlorohydrin was detected by the lectin enzyme assay detection (LEAD) test, while zearalenone in concentrations from 5 pg to 50 mg/mL did not react with WGA-HRP. Thirty-six background air samples were negative to the LEAD test.

PREFACE

The work described in this report was authorized under Project No. 1L161101A91A, In-House Laboratory Independent Research. This work was started in February 1982 and completed in August 1985.

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Acknowledgment

The author appreciates the assistance of Dr. Cynthia LaDouceur, immunologist, in the preparation of Table 2 of this report.

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LECTIN ENZYME ASSAY DETECTION OF VIRUSES, TISSUE CULTURE, AND A MYCOTOXIN SIMULANT

1. INTRODUCTION

Nonspecific lectin detection of virus groups depends on the lectin binding with the specific virus protein polysaccharide.[1,2] Prevot and Guesdon[3] used wheat germ agglutinin (WGA) to detect rotavirus antigen in an erythro-lectin immunoassay. WGA has an avidity and specificity for N-acetyl glucosamine and a lesser specificity for sialic acid.[4] Delagneau, Perrin, and Atanasiu[5] found that the following five different lectins had an affinity for rabies virus:

- Phaseolus vulgaris (PHA) - red kidney bean agglutinin
- Triticum vulgaris (WGA) - wheat germ agglutinin
- Ricinus communis (RCA) - castor bean agglutinin
- Lens culinaris (LCA) - lentil agglutinin
- Concanavalia ensiformis (Con A) - jack bean agglutinin (to a lesser degree)

PHA, WGA, and Con A all bind N-acetyl glucose amine, RCA[6] binds N-acetyl galactosamine; and LCA[7] absorbs to fucose in the mannose-containing oligosaccharide chain. Delagneau and associates also found that virus-lectin aggregates can be easily sedimented by allowing the container to stand at 4 °C for 1 hr.[5] At this point, no detectable absorbance (at 500 nm) remains in the supernatant. The aforementioned authors show that some viruses bind to sialic acid residues on tissue cultures and in the process may cover carbohydrate reactive sites to some lectins, but the reactive sites to WGA or PHA remain unmasked.[5] Table 1 lists some viruses that bind to some lectins. Lectins also may assay viruses indirectly by determining virus neurimmadase activity on erythrocytes,[8-10] using peanut agglutinin (PEA) and snail agglutinin (HPA) lectins; lympocyte activation,[11] Con A; or transforming Eukaryotic cells,[12,13] Con A, WGA, RCA, and Wisteria agglutinin (WFA).

Generally, lectins can be used in place of antibodies in most immunological reactions or immunoassays. Table 2 shows certain biological differences between lectins and antibodies. The lectin reactions in Table 2 are taken from Tom Pistole's article in the 1981 Annual Review of Microbiology.[9] The experimenter tried to match these reactions with comparable antibody reactions.

In the following experiments, an attempt was made to study the extent and affinity of binding WGA to viruses and/or tissue cells and culture constituents.

Table 1. Lectins That Bind to Viruses.

VIRUS	LECTIN					
	Wheat Germ (WGA) *Triticum vulgaris*	Red Kidney Bean (PHA) *Phaseolus vulgaris*	Lentil (LCA) *Lens culinaris*	Castor Bean (RCA) *Ricinus communis*	Jack Bean (Con A) *Concanavalis ensiformis*	Soybean (SBA)* *Glycine max*
Rabies	X[5]	X[5]	X[5]	X[5]	X[5]	
Lassa					X[14,15]	
Epstein-Barr					X[15]	
Japanese Encyphalitis					X[15]	
Bovine Leucosis				X[14]	X[14]	X[14]

*SBA - Soybean agglutinin from Glycine Max

NOTE: The methods used above were absorbance at 500 nm;[5] Enzyme-Lectin Immunoassay Test;[14,15] and fluorescence.[16]

Table 2. Comparison of Lectins and Antibodies.

LECTINS	ANTIBODIES
1. Protein	1. Protein
2. Derived from plants (those derived from animals are referred to as lectin-like or given a functionally descriptive name)	2. Derived from animals
3. Not a component of the vertebrate immune system	3. Component of the vertebrate immune system
4. Bind to glycoproteins and glycolipids	4. Bind to a variety of substances (proteins, carbohydrates, lipids nucleic acids, chemicals, etc.)
5. Act as opsonins	5. Act as opsonins
6. Capable of agglutination	6. Capable of agglutination
7. Multifunctional (act as storage proteins in seeds, control cell division, transport carbohydrates or calcium, stimulate pollen germination, aid in digestion, initiate disease by binding to host tissue sugars, etc.)	7. Multifunctional (activation of complement, neutralization of toxins and viruses, immobilization of bacteria, formation of precipitates, passage through placenta, antibody dependent cell-mediated cytotoxicity, etc.)

2. MATERIALS AND METHODS

Table 3 gives the principles of the Lectin Enzyme Assay Detection (LEAD) test, called LEAD to emphasize the use of lectin rather than antibody. The LEAD test is essentially different from the Enzyme-Linked Lectinobsorbent Assay® (ELLA) test[17] that uses centrifugation and hot air to absorb bacteria to the microtiter plate. The LEAD test includes binding of antigens to plastic; reacting antigen with lectin-peroxidase conjugate; removing excess conjugate by washing; and final color development with a substrate. The figure on page 10 diagrams lectin used in an Enzyme-Linked Immunosorbent Assay® (ELISA) test. The author found the sandwich type was unnecessary. WGA lectin had poor affinity for the plastic, while viruses bound very well. So initial binding of lectin to plastic was not done.

Table 3. Principles of LEAD Test.

1.	Cold adsorption of virus to plastic; bind lectin-peroxidase to virus at 4 °C for 10-30 min in polystyrene microtiter plate.
2.	Removal of unadsorbed virus and unreacted lectin-peroxidase. Wash five times with PBS, pH 6.8 and one time with PBS, pH 5.4.
3.	React substrate with enzyme in presence of color developer. Add mixed peroxidase-citrate with ABTS-glycine. Incubate at 37 °C for 10-120 min.

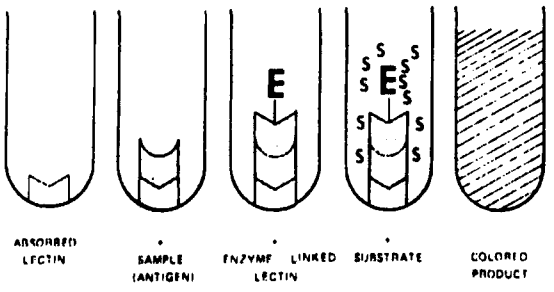

Figure. "ELISA" Method.

2.1 Lectins and Enzymes.

WGA lectin and a combination of WGA lectin and horseradish peroxidase (HRP) from E-Y Laboratories, Incorporated (San Mateo, CA) was used. Ten milliliters of a pH 6.8 mixture of equal volumes of 0.1 M mono- and dibasic sodium phosphate buffer was added to 2 mg of the freeze-dried WGA-HRP product for freezer storage in 1 mL of aliquotes. To make a working solution, these aliquotes were diluted in a 0.1 M dibasic sodium phosphate buffer (pH 7.4) containing 0.15 M of NaCl.

2.2 Viruses.

The following viruses were used: inactivated Venezuelan equine encaphalomyelities (VEE) vaccine, Escherichia coli (E. coli) B. Bacteriophage Type I, Vero virus with glutamine and fetal bovine serum, Rubella HA antigen from Orion Diagnostica (Helsinki, Finland) and Gilchrist type from Flow Laboratories (McLean, VA).

2.3 Tissue Culture (TC).

HEP, HELA, and baby hamster kidney TC antigen were obtained from Immuno-Mycologics (Oklahoma City, OK). Baby hamster kidney with glutamine and fetal bovine sera and minimal essential media with glutamine and fetal bovine sera were also used. Minimum essential medium with nonessential amino acids without glutamine were purchased from Microbiologist Associates Bioproducts (Walkersville, MD). Rubella (Gilchrist) tissue control was obtained from Flow Laboratories.

2.4 Color Reagent.

2,2¹-azino-di-(3-ethylbenzthiazoline sulfonic acid) (ABTS) was purchased from Sigma Chemical Company (St. Louis, MO). For substrate-color agent, equal amounts of mixture A and B were added. Mixture A contained 0.36 g of citric acid monohydrate and 0.04 mL of H_2O_2 in 100 mL of pH 6.8 phosphate buffered saline (PBS). Mixture B contained 0.3 g of ABTS and 4.58 g of glycine in 100 mL of PBS.

2.5 Modified ELISA (LEAD) Test.

Two procedures were used in a direct LEAD test. In procedure I (Table 4), the virus or TC were first absorbed to the microtiter plate by incubation in the refrigerator for 1 hr. Unabsorbed antigen is washed out, and peroxidase conjugated WGA was added and reacted in the cold. Unreacted peroxidase conjugated lectin was removed by washing, and the color agent substrate was added and incubated at 37 °C.

In procedure II (Table 5), WGA was used because of its low affinity for plastic. Here the antigen was reacted initially with the peroxidase lectin conjugate. Excess lectin conjugate did not bind to the plastic and could be removed by washing. Color was developed in the usual manner and read on the Biotek microtiter reader.

After July 16, 1985, background air samples were collected twice daily using an XM2 air sampler. This device removes particles ranging from 2 to 10 µm from the air at a rate of 1,000 mL/min for 45 min. These particles were placed in a solution of 40 mL of the above pH 7.4 dibasic sodium phosphate saline buffer. Air samples were evaluated in the direct mixing test (procedure II).

A possible mycotoxin simulant, epichlorohydrin, diluted from epoxy cement and zearalenone mycotoxin was also tested by the LEAD reaction (procedure II).

Table 4. LEAD Test (Procedure I).

1.	Add 0.1 mL of virus or TC concentrations to wells of the microtiter tray.
2.	Incubate at 4 °C for 1 hr.
3.	Wash six times with PBS.
4.	Add 0.1 mL of peroxidase-bound WGA at a concentration of 2 µg/mL.
5.	Incubate at 4 °C for 30 min.
6.	Wash six times with PBS.
7.	Add 0.1 mL of color agent-substrate (ABTS-H_2O_2).
8.	Incubate on a yankee rotator or micromix vibrator at 37 °C for 10-30 min.
9.	Read color development at 10, 20, and 30 min.

Table 5. LEAD Test (Procedure II).

1.	Add 0.1 mL of virus or TC concentrations to 0.1 mL of WGA-peroxidase in microtiter tray.
2.	Mix on yankee rotator or micromix vibrator at room temperature for 10 min.
3.	Incubate at 4 °C for 20 min.
4.	Wash six times with PBS.
5.	Add 0.1 mL of substrate-color agent.
6.	Incubate on a yankee rotator at 37 °C for 10-30 min.
7.	Read color development at 10, 20, and 30 min.

3. RESULTS

3.1 Direct Stepwise Test, Procedure I.

When diluted in PBS, the WGA-peroxidase conjugate was titrated by a direct method. The viral or TC antigen or PBS control was absorbed to the wells of the microtiter plate by incubating at 4 °C for 1 hr. After washing with PBS, different enzyme-lectin conjugate concentrations were reacted in the adsorbed wells. Table 6 shows the results of the enzyme-lectin conjugate titration. The highest conjugate concentration able to detect a constant quantity of antigen without any background with PGS (e.g., 2 µg/mL) was retained. It took 2 hr to complete the above direct stepwise test.

Table 6. Enzyme-Lectin Conjugate Titration.

	WGA-Peroxidase				
	200	20	2	0.02	Virus* Control
PBS	4+	3+	±	-	3+
H_2O	4+	3+	-	-	

*10^4 plaque-forming units (PFU) VEE/mL with 2 µ of WGA-Peroxidase

3.2 Direct Mixing Test, Procedure II.

Preliminary tests with nonviral antigens showed that lectin conjugate and antigen could be reacted before and during adsorption to the microtiter plate. This could be accomplished because the antigen had a greater affinity for the microtiter plate than the lectin enzyme conjugate. Adsorption-reaction time was reduced to the time allowed for stepwise reaction with the WGA-peroxide conjugate. Total time to complete the direct mixing test was 1 hr. To find maximum sensitivity, a chessboard titration of lectin-enzyme conjugate was reacted against varying dilutions of antigen (Table 7).

Table 7. Lectin Titration. Horseradish Peroxidase and H_2O_2 with ABTS Reagent.

LECTIN (µg/mL)	ANTIGEN (VEE) DILUTIONS											
	10^5 PFU	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	PBS	H_2O
20.0	4	4	4	4	4	4	4	4	4	4	4	4
10.0	4	4	4	4	4	4	4	4	4	4	4	4
5.0	3	4	3	4	4	2	-	-	-	-	-	-
2.5	1	2	3	3	2	-	-	-	-	-	-	-
1.25	1	1	1	2	1	-	-	-	-	-	-	-
0.025	-	-	-	1	-	-	-	-	-	-	-	-
0.3125	-	-	-	-	-	-	-	-	-	-	-	-
0.15625	-	-	-	-	-	-	-	-	-	-	-	-

The minimum virus and tissue culture concentration that reacted with the LEAD test are found in Table 8. Maintenance media without tissue culture was unreactive. The maintenance media over the baby hamster kidney TC reacted the same as maintenance media with fetal bovine serum. However, a separate preparation of baby hamster kidney cells had some reaction by itself. The Rubella reaction could be accounted for by the Rubella control (TC without Rubella virus). Also, the vero-virus reaction was the same as the media and serum used to grow it. The VEE inactivated vaccine and the T-1 E. coli phage reaction stand by themselves. The T-1 phage was a purified preparation by high speed centrifugation. Thus, the phage represents virus alone, not TC or bacteria.

Table 8. Minimum Virus and Tissue Culture Concentration Reacting in LEAD Test.*

VEE (Inactivated) Vaccine	-10^4, 10^{3**} PFU
T-1 E. coli Phage	-10^7 PFU
Rubella HA Antigen (Inactivated) Orion	-10^{-6} Dilution
Vero Virus	-10^{-3} Dilution
Mem with Glutamate and Fetal Bovine Serum	-10^{-3} Dilution
Mem with Glutamate	-Neg
Hep Tissue Culture (TC)	-10^6 Cells/mL (10^{-2} Dilution)
Baby Hamster Kidney TC	-10^7 Cells/mL (10^{-1} Dilution)
Rubella HA Antigen Control (Flow Labs)	-10^{-6} Dilution
Baby Hamster Kidney TC (Fluid Medium) with Glutamate and Fetal Bovine Serum	-10^{-3} Dilution

*Attached 30-60 min, 4 °C
**Attached 3 hr, 4 °C; 30-60 min, 4 °C

All 36 background air samples collected in July and August 1985 were negative in the LEAD test. Epichlorohydrin was detected by the LEAD test, while zearalenone in concentrations from 5 pg to 50 µg/mL did not react with WGA-HRP.

If time had allowed, background air samples would have been tested at a 1:100 dilution to elminate the prozone effect (Table 7) where antigen excess may cause a false negative reaction. Aside from zearalenone, no other mycotoxins were tested.

4. CONCLUSION

Carbohydrate binding sites on the virus outer coat or on mammalian cells allowed detection of four viral and three TC antigens using WGA lectins conjugated to a peroxidase enzyme. A direct stepwise and direct mixing ELISA type LEAD test were demonstrated. The stepwise test required 2 hr, and the mixing test required 1 hr.

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