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USE OF PROTEIN A AS THE PRIMARY LAYER IN FLUORESCENT MICROSPHERE TECHNOLOGY

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Six unique monoclonal antibodies (mAb) to the galactose-binding adherence lectin of Entamoeba histolytica were assessed for their abilities to bind protein A, using BIAcore. Of the six antibodies tested, mAb H85, specific for pathogenic strains, and mAb 3F4, reactive with both pathogenic and nonpathogenic strains, showed the greatest degree of binding to Protein A. Therefore, protein A was used as a surface layer on microspheres to which mAbs H85 and 3F4 were attached. Conversion of this method to microsphere agglutination and enzyme linked immunabsorbent assay (ELISA) formats proved successful. Instrumented reads of agglutination reactions in the ELISA format achieved a lower limit of detection equivalent to 500 amoeba when performed on solubilized amebic antigen.

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PREFACE

The work described in this report was authorized under Contract No. DAAD05-91-P4347. This work was started in June 1991 and completed in September 1991. The experimental data are recorded in laboratory notebook 91-0035.

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USE OF PROTEIN A AS THE PRIMARY LAYER IN FLUORESCENT MICROSPHERE TECHNOLOGY

1. INTRODUCTION

Entamoeba histolytica causes amebic colitis worldwide with the highest incidence occurring in Central and South America, Africa, India, and in AIDS patients in the United States. This intestinal parasite is responsible for 480 million infections per year, and 110,000 deaths. It is spread by the ingestion of the organism and may progress to liver abscess or invasive colitis.[1]

Pathogenic determinants, found on the outer surface of Entamoeba histolytica, determine their ability to adhere to and/or invade mammalian tissues. The lectin produced by E. histolytica provides the basis for detection of adherence and invasive properties harbored by this potential threat agent. The galactose-binding lectin, a 260 kilodalton (kDa) heterodimeric glycoprotein, consists of a 170 kDa heavy subunit linked by disulfide bonds to a 35 kDa light subunit. This lectin mediates in vitro adherence to human colonic mucin glycoproteins suggesting its involvement in colonization and invasion of the colon. Six antigenically and functionally distinct epitopes have been mapped on the heavy subunit with monoclonal antibodies. The epitope specificities and designations of the mAbs, respectively are as follows: epitope 1, 3F4; epitope 2, 8A3; epitope 3, 7F4; epitope 4, 8C12; epitope 5, IG7; epitope 6, H85. Epitopes 1 through 6 are present on pathogenic strains (as determined by isoenzyme patterns), but only epitopes 1 and 2 are found on nonpathogenic strains. This specificity provides the basis for distinguishing pathogenic from nonpathogenic strains.[1,2]

BIAcore (Pharmacia Biosensor, Piscataway, N.J.) uses surface plasmon resonance to detect changes in a sensor chip surface as biomolecular reactions occur. Changes in light reflection are a direct result of changes in mass occurring at the sensor surface. The reaction surface can be made biospecific by immobilizing the ligand of choice onto a derivatized dextran layer. Controlled sequential injections of reactants makes it possible to measure each binding event using no molecular labels.[3]

Microsphere technology has been used to carry out antigen/antibody reactions, making them visible to the naked eye. These reactions can detect microorganisms or antigens in crude samples. Fluorescence impregnated microspheres provide additional use in instrumentally driven systems for detection of small amounts of agglutinating particles and, thus, greater sensitivity.[4]

This study uses BIAcore to assist in the optimal use of microsphere technology for methods which distinguish pathogenic from nonpathogenic strains of Entamoeba histolytica. The propensity of monoclonal antibodies to bind to protein A is assessed prior to incorporation of protein A as a reaction base. Protein A, derived from outer surface components of Staphylococcus aureus, binds the Fc portion of antibodies, thus allowing Fab antibody appendages to have unlimited access to their respective antigen counterparts.[5]

2. MATERIALS AND METHODS

2.1 Material Sources.

Purified amebic lectin, and purified monoclonal antibodies to six unique epitopes on the adherence lectin of E. histolytica, were provided by Dr. William Petri, Jr., University of Virginia, Charlottesville, VA. Purified protein A was obtained from Chemicon International, Los Angeles, CA. Fluorescence impregnated latex microspheres, Fluoresbrite PC-RED, with an excitation wavelength of 591 nm and an emission wavelength of 657 nm (referred to hereafter as bead A) were obtained from Polysciences, Inc., Warrington, PA. These microspheres were 1.0 micron in diameter. In addition, microspheres containing fluorescent dye with an excitation wavelength of 530 nm and an emission wavelength of 590 nm (referred to hereafter as bead B) were obtained from Seradyn, Indianapolis, IN. The diameter was 1.24 microns.

2.2 Buffers Used.

Buffers used in the BIAcore procedures included 10mM sodium acetate buffer, pH 5.0, and HBS buffer consisting of 10mM HEPES, 150mM NaCl, 3.4mM EDTA, and 0.05% surfactant P20, pH 7.4. Amoeba were solubilized in 150mM NaCl, 50mM Tris, 0.5% Triton X100, 5mM EDTA, 2mM PMSF (poly-methyl-sulfonyl-fluoride), 2mM PHMB (parahydroxymercuribenzoic acid), pH 8.3. Latex microspheres were diluted and used in GBS containing 0.9% NaCl and 0.1 M glycine, pH 8.2.

2.3 Antibody/Protein A Interaction.

From a stock solution of 1 mg/mL protein A in Hepes buffer, pH 7.5, 30 ug was placed into 1 mL of Na acetate buffer at pH 5.0 for immobilization onto the dextran matrix in BIAcore. Thirty-five microliters of this dilution was used for the immobilization, resulting in a value of 4725 resonance units (RU). Approximately 1 ug total protein was injected and approximately 5 ng per mm^2 was bound to the matrix. Each of the monoclonal

antibodies were injected from concentrations of 20 ug/mL in HBS, both in 1 uL and 4 uL volumes. A regeneration step using 100mM HCl was performed between each injection of antibody to restore the protein A surface.

2.4 Bead and Antigen Preparation.

One mL of 2.5% solutions of microspheres in GBS were prepared. Protein A at a final concentration of 0.5 ug/mL was added to the microsphere preparations, and incubated at 37°C for 2 hrs. Microsphere preparations were incubated with an equal portion of antibody preparations to achieve a final concentration of 5 ug/mL, each at 37°C for 2 hrs. These preparations were diluted with 2 mLs GBS containing 0.1% BSA and 0.15% Na azide. Microspheres were placed in the refrigerator until use.

For use in the microtiter plate assays, pathogenic antigen and nonpathogenic antigen were solubilized from lyophilized preparations in 10 mL solubilization buffer. The resulting concentrations were approximately 5 x 10^4/mL to 1 x 10^5 /mL, equivalent amoeba. One hundred uL quantities were used in the microtiter assay. Controls were prepared from unlabeled microspheres by dilution in GBS to achieve 2.5% solutions.

2.5 Macroscopic Agglutination Assay.

MAb H85 and mAb 3F4 coated microspheres were tested with pathogenic and nonpathogenic purified lectin diluted 1:10, 1:20, and 1:40 in GBS.

2.6 Single Microsphere Assay: Fluorescent Read.

In a 96 well Dynatec microtiter plate, the following combinations of reactants were tested. Initially, 100 uL per well of microsphere B, ($530_{ex}/590_{em}$) coated with mAb 3F4, was placed in wells A1-12 and B1-12 and microsphere B, coated with mAb H85, was placed in wells C1-12 and D1-12. The plates were scanned on the Cytofluor 2300 (Bedford, MA.) using filters 530_{ex} and 590_{em} to obtain background reads. Coefficients of variation ranged from 2.6% to 4.7%. Solubilized pathogenic antigen was added to wells A,B,C,D, 1-6 and nonpathogenic solubilized antigen was added to wells A,B,C,D, 7-11 in 100 uL quantities. Solubilized preparations equaled 500-5000 equivalent amoeba. Column 12 served as control wells to which 100 uL GBS was added.

2.7 Two Microsphere Assay: Fluorescent Read.

To rows E,F,G,H, 1-12 was added 50 uL mAb 3F4 coated B microspheres. To E1-12 and G1-12, 50 uL of mAb H85 coated A ($591_{ex}/657_{em}$) microspheres was added. Wells F1-12 and H1-12 received 50 uL mAb 3F4 coated A microspheres. The plates were scanned on the Cytofluor 2300 using excitation filters 530_{ex} and

590_{em} at a sensitivity setting of one to obtain a background reading. Solubilized pathogenic antigen was added to wells E,F 1-11 in 100 uL quantity. Nonpathogenic antigen was added to wells G,H 1-11 at 100 uL. Solubilized preparations equaled 500-5000 equivalent amoeba. Column 12 served as the control wells to which 100 uL GBS was added.

The plate was covered and rotated slowly on a platform rotator at room temperature. Reads were obtained at 5 min, 30 min, 1 hr, and overnight. Wavelengths of 530_{ex} and 590_{em} were used for the reads. Values for each condition were averaged and the ratios of test signal / control signal were plotted to show change in signal ratios over time.

3. RESULTS

Resonance response units (RU) in BIAcore, obtained for attachment of each of the mAbs to protein A, can be seen in Table 1. Highest responders included H85, IG7, 7F4, and 3F4. Monoclonal antibodies 3F4 and H85 were chosen for use in the assay to differentiate pathogenic from nonpathogenic strains. The order of highest to lowest binding response was consistent with both the 1 uL and the 4 uL injection amount.

The macroscopic agglutination assay on purified antigen, using the prepared microspheres, showed positive results at 1:10, 1:20, and 1:40 with mAb 3F4 for both pathogenic and nonpathogenic strains, and at 1:10, 1:20 for H85 with pathogenic strains. Buffer controls of beads plus buffer and antigen plus buffer were negative.

Table 2 shows the change in signal ratio over time using the one microsphere system (B) for each condition. Ratios remained unchanged at one hr and slightly decreased overnight. Figure 1 further depicts the change. Table 3 shows the of change in signal ratio over time, using both microspheres A and B. Decreases in ratios began at 30 min, were more evident at 1 hr and dropped to approximately one/half of the control read overnight. Figure 2 shows that the decrease is most evident with the nonpathogenic strains.

Figure 3 compares decreases in reads of the one bead and the two bead systems, using nonpathogenic strains. The two bead system shows the greatest decrease in signal ratio. Figure 4 compares all conditions of the two bead system. Nonpathogenic strains show the greatest decreases in signal ratios.

Text continues on page 18.

Table 1. Resonance Units for Binding of Monoclonal Antibodies to Protein A Using BIAcore.

		Resonance Units	
Antibody	Epitope	1 uL injection	4 uL injection
3F4	1	263	759
8A3	2	16	72
8C12	4	26	105
H85	6	1286	4005
IG7	5	693	2375
7F4	3	698	1984

Table 2. Ratios of Test Signal to Control Signal Using One Fluorescence Impregnated Microsphere. Both Pathogenic and Nonpathogenic strains were tested at designated time intervals.

Microsphere		Excitation 530 nm, emission 590 nm				
mAb	Amoeba	5 min	30 min	60 min	Overnight	Expected Reaction
3F4	PZ	1.13*	1.10	0.96	0.64	positive
3F4	NPZ	1.20	1.16	1.01	0.67	positive
H85	PZ	1.19	1.16	0.99	0.74	positive
H85	NPZ	1.18	1.15	0.98	0.72	negative

*Mean test signal at 590 nm divided by mean control signal for each interval.

3F4, mAb to epitope 1; H85, mAb to epitope 6

PZ - pathogenic; NPZ - nonpathogenic

Test n = 10 (12) values; controls n = 1 (2) values

Equivalent concentration of amoeba 500 - 5000

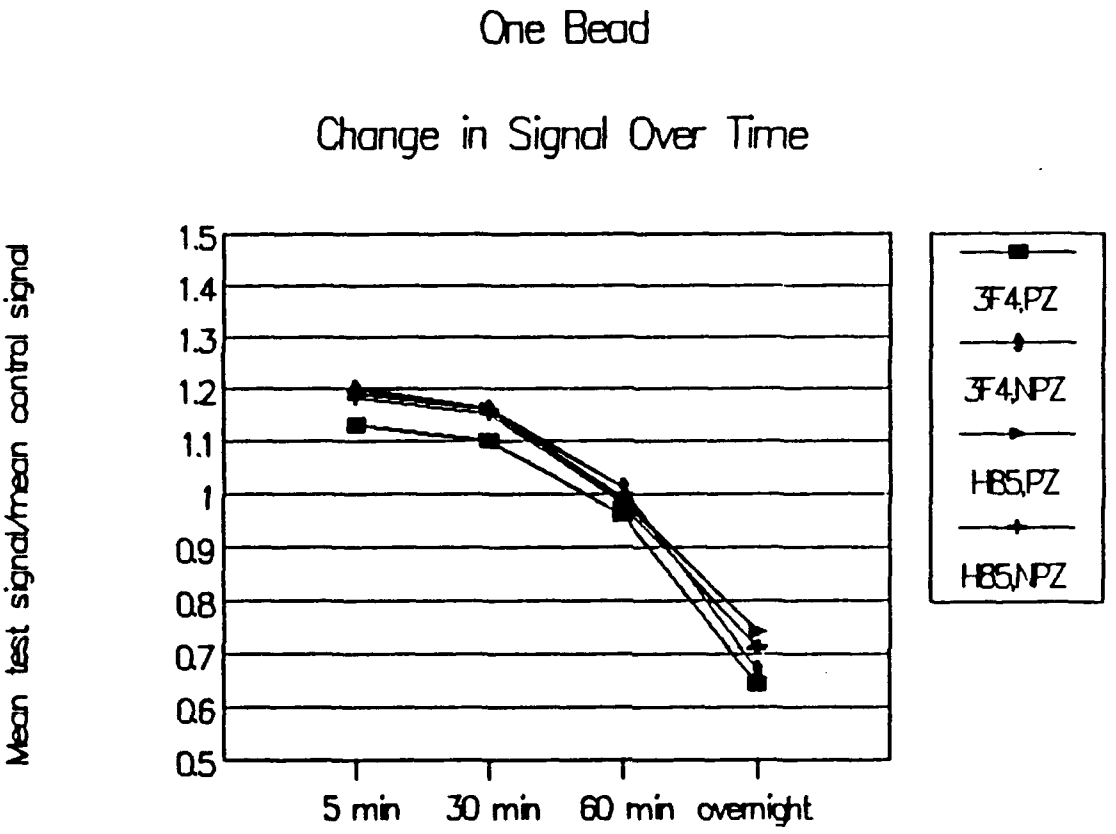

Figure 1. Change in Signal Ratios Over Time Using One Microsphere.

Table 3. Ratios of Test Signal to Control Signal Using Two Fluorescence Impregnated Microsphere.

Microspheres		Excitation 530 nm, emission 590 nm Excitation 592 nm, emission 620 nm				
mAb	Amoeba	5 min	30 min	60 min	Overnight	Expected Reaction
3F4 H85	PZ	1.01*	1.00	0.91	0.69	unknown
3F4 3F4	PZ	0.96	0.96	0.87	0.62	negative
3F4 H85	NPZ	0.95	0.94	0.83	0.47	unknown
3F4 3F4	NPZ	0.99	0.99	0.90	0.50	positive

*Mean test signal at 590 nm divided by mean control signal for each interval

3F4, mAb to epitope 1; H85, mAb to epitope 6

PZ - pathogenic; NPZ - nonpathogenic

Test n = 10 (12) values; Control n = 1 (2) values

Equivalent concentration of amoeba 500 - 5000

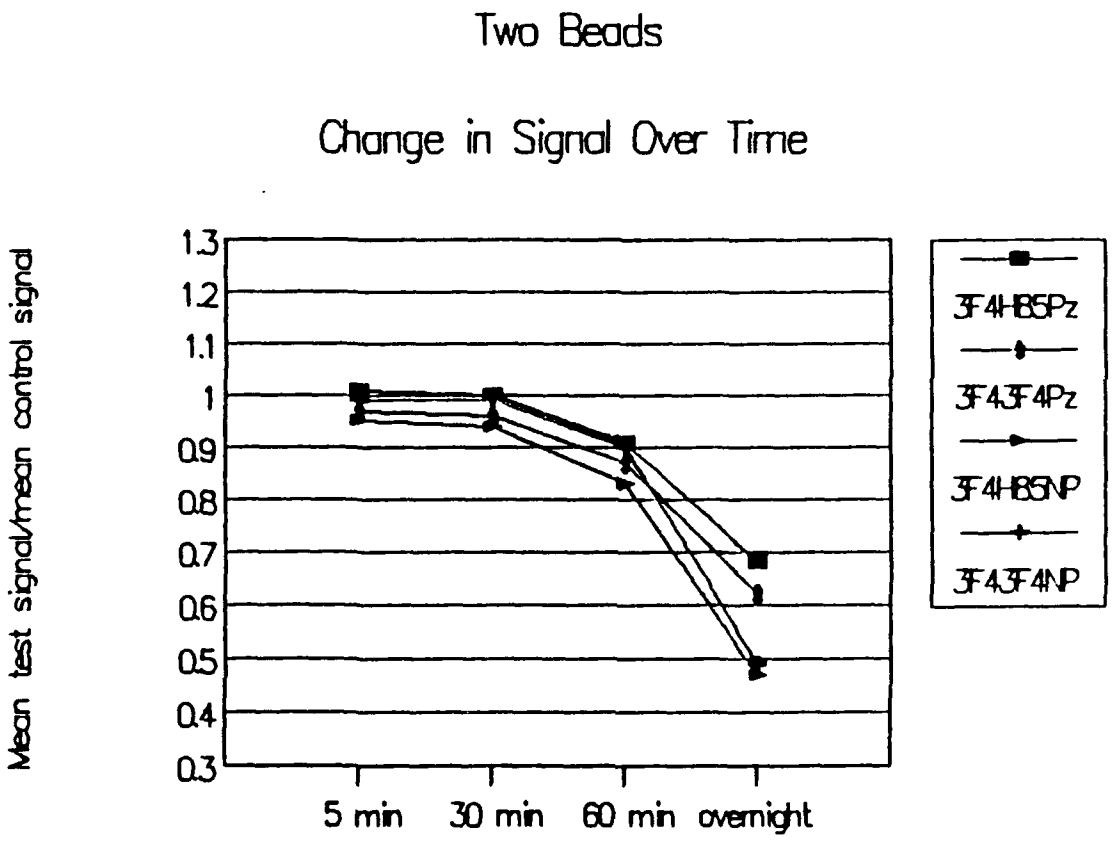

Figure 2. Change in Signal Ratio Over Time Using Two Microspheres.

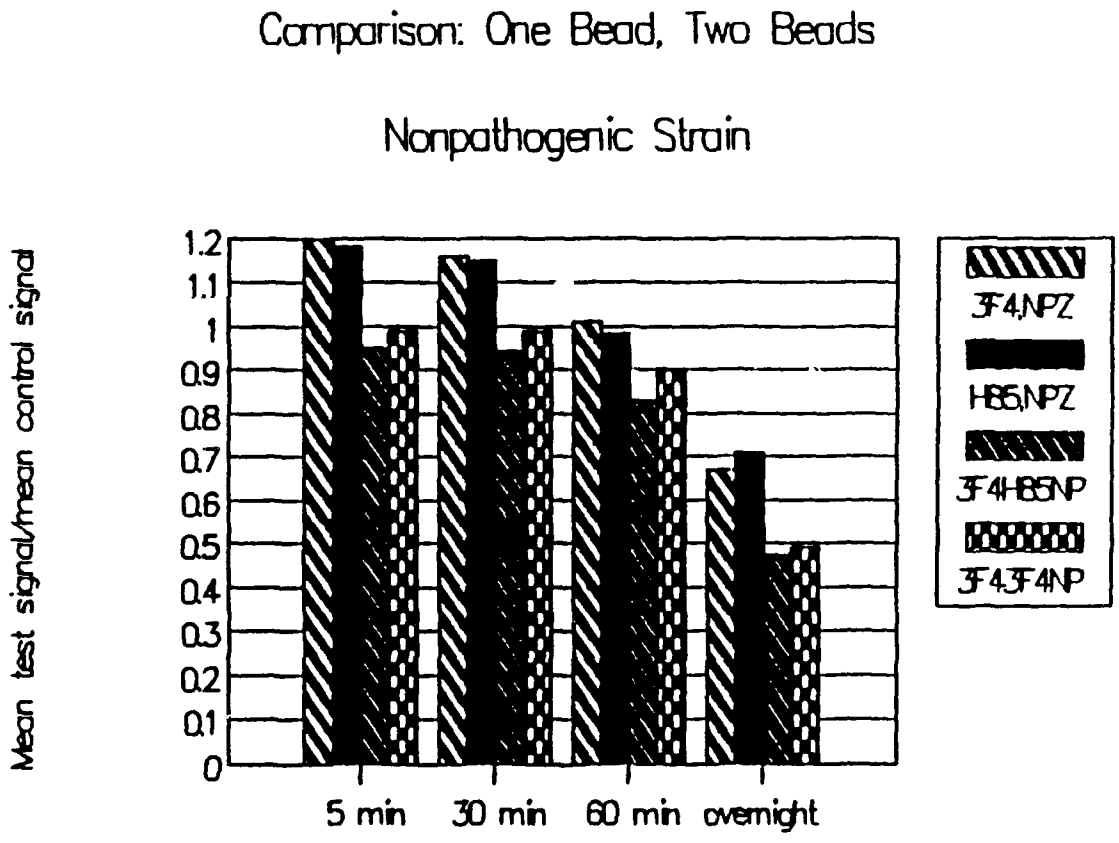

Figure 3. Comparison of the One Microsphere System to the Two Microsphere System Using Nonpathogenic Strains.

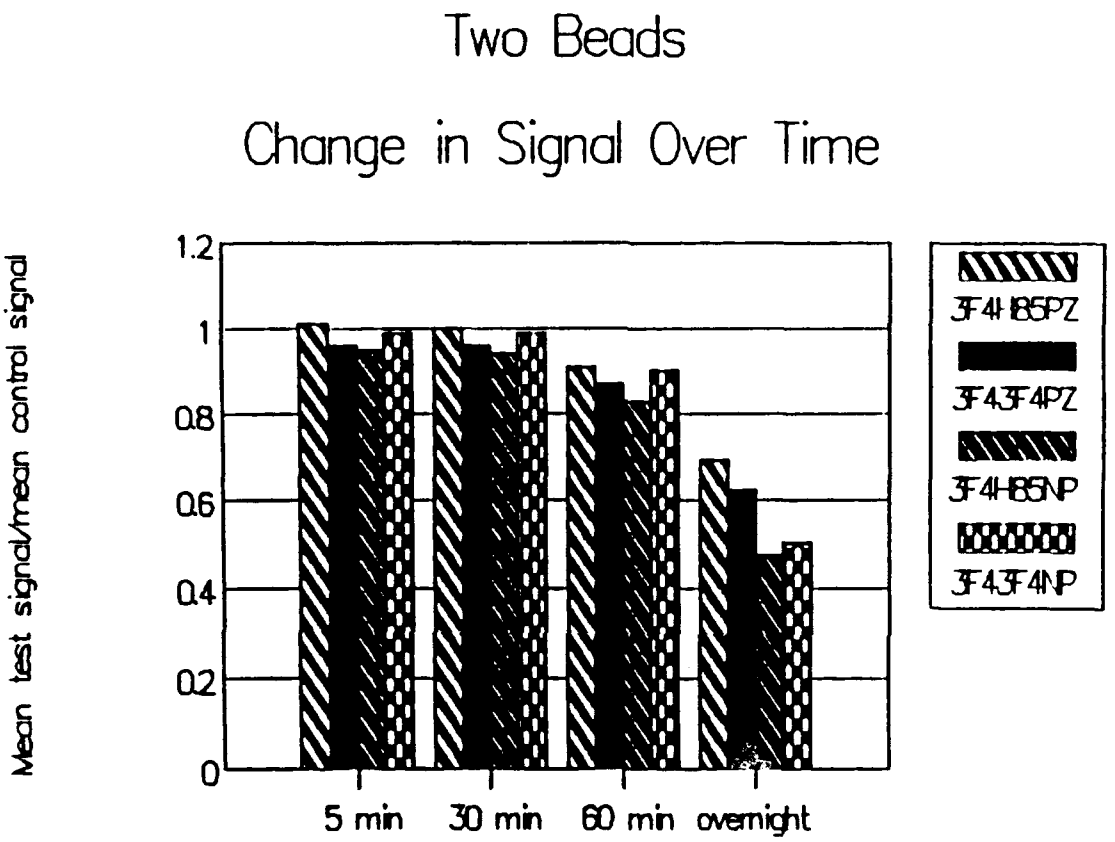

Figure 4. Comparison of the Two Microsphere System Using Pathogenic and Nonpathogenic Strains.

4. DISCUSSION

It has been known for some time that protein A binds monoclonal antibodies in varying degrees, depending on the isotype used.[6] The use of BIAcore in establishing the binding patterns of the monoclonals used was instrumental in establishing an enhanced method for performing agglutination reactions.

Comparison of the two fluorescent systems for measuring agglutination reactions showed that the two bead system with nonpathogenic strains gave the best indication of positivity. Conditions to be examined in follow-up experiments include establishment of a threshold signal for positivity, and elimination of the possibility that the amoeba carry Fc receptors on their surfaces. This preliminary study gives promising results for the use of fluorescence reads to detect antigen/antibody reactions with greater sensitivity than macroscopic agglutination.

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