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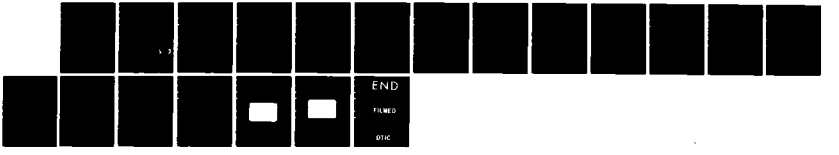

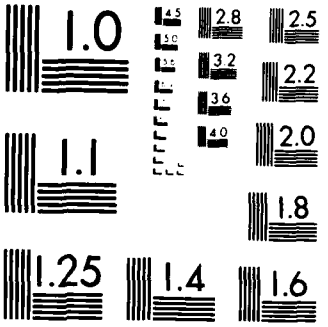

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Specificity of Monoclonal Antibodies Reactive With *Fusobacterium nucleatum*: Effect of Formalin Fixation

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20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

Monoclonal antibody specific for Fusobacterium nucleatum was reacted with untreated and formalin fixed F. nucleatum cells by an enzyme-linked immunosorbent assay (ELISA) and by indirect immunofluorescence. Treatment of bacterial cells with formalin destroyed the antigenic determinant responsible for reactivity with this monoclonal antibody in both assays. Formalin fixation had no effect on hemagglutination activity (HA) of F. nucleatum cells or reactivity with polyvalent rabbit antiserum by double diffusion in agar. Scanning electron

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microscopy demonstrated that formalin fixation did not affect binding of F. nucleatum cells to microtiter plates. When developing monoclonal antibodies that will be used as diagnostic reagents, the antigen form utilized for immunization should be identical to the antigen form which will eventually be used in the diagnostic assay.

Key Words: monoclonal antibodies, antigen specificity and *Fusobacterium nucleatum*.

ABSTRACT

Monoclonal antibody specific for Fusobacterium nucleatum was reacted with untreated and formalin fixed F.nucleatum cells by an enzyme-linked immunosorbent assay (ELISA) and by indirect immunofluorescence. Treatment of bacterial cells with formalin destroyed the antigenic determinant responsible for reactivity with this monoclonal antibody in both assays. Formalin fixation had no effect on hemagglutination activity (HA) of F. nucleatum cells or reactivity with polyvalent rabbit antiserum by double diffusion in agar. Scanning electron microscopy demonstrated that formalin fixation did not affect binding of F. nucleatum cells to microtiter plates. When developing monoclonal antibodies that will be used as diagnostic reagents, the antigen form utilized for immunization should be identical to the antigen form which will eventually be used in the diagnostic assay.

INTRODUCTION

Monoclonal antibody technology has been utilized for the manufacture of predefined antibodies of exquisite specificity (Kohler and Milstein, 1975). This specificity is due to the mono-specific reaction that occurs with a single antigenic determinant which may be limited in size to as little as four amino acid residues (Fudenberg, 1980). Highly specific antibody provides a powerful immunologic tool far superior to polyvalent sera. Antibody populations with this specificity allows selection of a monoclonal antibody reactive with a singular antigenic determinant. Most cross-reactivity that is observed when polyvalent sera are used as serological reagent is eliminated by the use of monoclonal antibody. Such specificity offers promise for development of antibodies that are monospecific for individual bacterial species. When reacting a polyvalent serum with a specific bacterial species, destruction or alteration of antigenic determinants will not necessarily reduce or eliminate reactivity. In contrast, reactivity with monoclonal antibody is likely to be reduced or eliminated if the specific antigenic site is altered. Observations in this study stress the importance of perserving antigenic sites reactive with monoclonal antibody and demonstrate how a common, seemingly innocuous procedure may destroy sites necessary for reactivity.

MATERIALS AND METHODS

The nonsecreting mouse myeloma cell line, p3 x 63Ag8.653, was grown in Dulbecco's Modified Eagles Medium (DMEM) (Flow Laboratories McLean, VA) supplemented with 10% heat inactivated fetal bovine serum and 2mM Lglutamine per ml. Cell viability of at least 90% was confirmed by the trypan blue exclusion of a cell sample immediately prior to fusion. Splenocytes were harvested from BALB/c BYJ mice (Jackson Laboratories, Bar Harbor, ME). These mice had been immunized with a 2% whole cell preparation of F. nucleatum (ATCC 25586) in phosphate buffered saline (PBS). A sensitizing injection (0.25ml) was delivered intraperitoneally followed by a booster injection (0.25ml) four weeks later (four days before the fusion procedure).

PREPARATION OF MONOCLONAL ANTIBODIES

Monoclonal antibody was obtained using a modification of techniques previously described (Kohler and Milstein, 1975 ; Hancock, 1982). Splenocytes (10^8) were exposed to 5 ml of 0.17M NH_4Cl for 10 minutes on ice. The splenocytes were then mixed with 10^8 mouse myeloma cells and washed 2X in serum free DMEM. Following centrifugation at 1000 X g, the disrupted cell pellet was suspended in 0.2 ml of 30% polyethylene glycol, (Sigma Chemical Co.,St. Louis, MO), in serum free DMEM with 5% dimethylsulfoxide (American Type Culture Collection, Rockville, MD), and subjected to centrifugation at 500 X g for 6 minutes. The fusion was stopped by the addition of 5 ml of DMEM and 5 ml of DMEM

supplemented with 20% fetal bovine serum. The cells were pelleted and resuspended with a feeder layer of mouse red blood cells in a selective medium (HAT) containing DMEM and supplemented with 20% fetal bovine serum, 2mM Lglutamine, 1% sodium pyruvate, 0.1mM hypoxanthine, 0.4mM aminopterin, and 0.015mM thymidine. The cells were suspended to a final concentration of 2×10^6 cells/ml, and plated 100ul in each well of 96 well tissue culture plates (M.A. Bioproducts, Walkersville, MD). Fresh HAT (100 ul) was added after 7 days. At 14 days, plates were visually observed for hybrid cell growth. The culture supernatant fluid from those wells demonstrating growth was screened for antibody reactive to F. nucleatum and the cells in positive wells were cloned 3X by limiting dilution in HAT medium with mouse red blood cell feeder layers. One clone, FN-18, was selected for characterization. The cloned cells were grown in HAT medium, suspended to a concentration of 1×10^7 cells /ml and injected, 1 ml intraperitoneally into pristane-primed (Sigma Chemical Co. St. Louis, MO) syngeneic mice. The ascites fluid contained the monoclonal antibody which was identified as IgG by double diffusion in agar.

Antigen Preparation for Serological Assays

Whole cell preparations of F. nucleatum were utilized as the antigen. One preparation consisted of untreated microorganisms suspended in 0.06 M carbonate buffer (pH 9.6) at a protein concentration of 0.92 mg/ml (Lowry, 1954). Similarly prepared concentrations of F. nucleatum were exposed to 1.0, 0.5, or 0.1% formalin for 1 hour at 37°C. The whole cells were then subjected to centrifugation at 2,500 X g and washed 3X in PBS and resuspended in 0.06 M carbonate buffer(pH 9.6) at a protein concentration of 0.92 mg/ml. An ELISA was performed (Vincent, 1983) using as reagents the monoclonal antibody obtained from ascites at a 1:700 dilution and a peroxidase conjugated goat anti-mouse gamma heavy chain serum (Cappell Laboratories Inc., Cochranville, PA) 1:800. The whole cell preparations (untreated and formalin fixed) in PBS at a protein concentration of 9.2 mg/ml were utilized for indirect immunofluorescence. The ascites fluid was used at dilutions of 1:5, 1:50, and 1:100. Fluorescein isothiocyanate labeled goat anti-mouse gamma heavy chain serum (Cappell Laboratories, Inc.) was utilized at a 1:100 dilution. Rabbit anti-F. nucleatum serum was tested by double diffusion in agar for reactivity with sonicated preparations of untreated and formalin-fixed F. nucleatum cells. Plates were incubated for four days at 37°C. in a humidor.

Hemagglutination (HA)

Whole cells of *F. nucleatum* have been shown to possess HA activity with sheep RBC (Falkler and Hawley, 1977). Serial twofold dilutions of the untreated whole cells and of each of the formalin-fixed preparations were performed using (total protein) 9.2 mg/ml in PBS *F. nucleatum* whole cell preparations. The titer was expressed as the reciprocal of the highest dilution resulting in 2^+ hemagglutination. All tests were performed in triplicate.

Scanning Electron Microscopy

Microtiter wells which had been coated with either untreated or formalin-fixed cells were fixed in 2% gluteraldehyde, dehydrated, and critical-point dried. The same site of each well was observed at the same magnification. The density of identifiable bacterial cells of both untreated and formalin-fixed cells was determined visually.

RESULTS

The results of the ELISA indicate that formalin fixation of the antigen preparation results in immunologic destruction of the antigenic determinant. Table I shows the optical density (490 nm) when a 1:700 dilution of the ascites fluid containing the monoclonal antibody was reacted with each of the various antigen preparations. Cells fixed with 1.0, 0.5, or 0.1% formalin showed very low reactivity. These results were verified by indirect immunofluorescence. Monoclonal antibody was reactive

at a 1:100 dilution with the untreated whole cells but was not reactive with formalin-fixed cells even at a 1:5 dilution. The polyvalent rabbit antiserum reacted by double diffusion in agar with the sonicated preparation of both untreated and formalin fixed cells. The HA activity of F. nucleatum was not affected by formalin fixation. As can be seen in Table II, HA activity with formalin fixed cells was identical to untreated cells. The antigenic site on the cell surface responsible for reactivity with the monoclonal antibody cannot be identical to the site imparting HA activity. In order to determine that formalin fixation did not adversely affect antigen binding to microtiter wells in the ELISA, scanning electron microscopy of selected wells was performed. Those wells receiving untreated cells and those receiving formalin-fixed cells were coated with approximately the same cellular density (Figures 1 and 2). This supports the concept that formalin fixation altered the structure of the antigenic determinant since it did not decrease cell binding to the microtiter plates.

DISCUSSION

The results of this study suggest the need for careful consideration of final needs when monoclonal antibodies are to be used. This study illustrates that formalin fixation can significantly alter antigenic determinants, thus eliminating monospecific activity directed against a given microorganism. Immunizing mice with formalin fixed cells will result in

monoclonal antibodies reactive with the formalin fixed immunizing antigen. However, the antibody produced may be directed toward a determinant that has been altered by formalin fixation and, therefore, would not react with naturally occurring bacterial cells, i.e., those from a clinical sample. Conversely, a mouse immunized with untreated cells may not produce monoclonal antibodies that will react with formalin-fixed cells. It thus would appear prudent for an investigator who is interested in monoclonal antibodies, to utilize the same form of microorganism for immunization as will be present in the preparation to be identified by their use.

ANIMAL STATEMENT

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" as prepared by the Committee on Care and Use of Laboratory Animals of The Institute of Laboratory Animal Resources, National Research Council.

MILITARY DISCLAIMER

Commercial materials and equipment are identified in this report to specify the investigative procedures. Such identification does not imply recommendation or endorsement or that the materials and equipment are necessarily the best available for the purpose. Furthermore, the opinions expressed herein are those of the authors and are not to be construed as those of the U. S. Army Medical Department.

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TABLE I. Optical Density Obtained by ELISA Utilizing Formalin-Fixed and Untreated F. nucleatum Whole Cells.

	ELISA		
Antigen Preparation	Optical Density	t-value	Level of Significance
Untreated Cells	1.128(0.174)*	—	—
Formalin Fixed (1%)	0.329(0.045)	28.7769	P<.00001†
Formalin Fixed (0.5%)	0.332(0.035)	29.0537	P<.00001
Formalin Fixed (0.1%)	0.431(0.069)	24.1156	P<.00001

*mean ± standard deviation (N=42)

†degrees of freedom = 82

TABLE II. Hemagglutinating Activity of Formalin-Fixed and Untreated F. nucleatum Whole Cells

	HEMAGGLUTINATION
Cell Preparations*	Titer† n=4
Untreated	1:256
Formalin Fixed (1%)	1:256
Formalin Fixed (0.5%)	1:256
Formalin Fixed (0.1%)	1:256

*All preparations contained 9.2 mg/ml of total protein.

†Highest dilution resulting in 1+ hemagglutination.

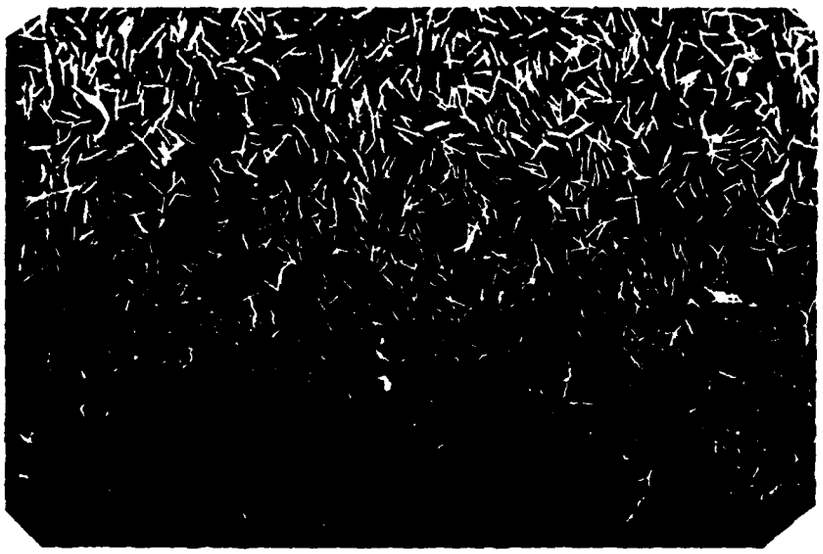

Figure I Scanning Electron Micrograph of Microtiter Well Coated With Untreated Whole Cells. Print Magnification is 880X.

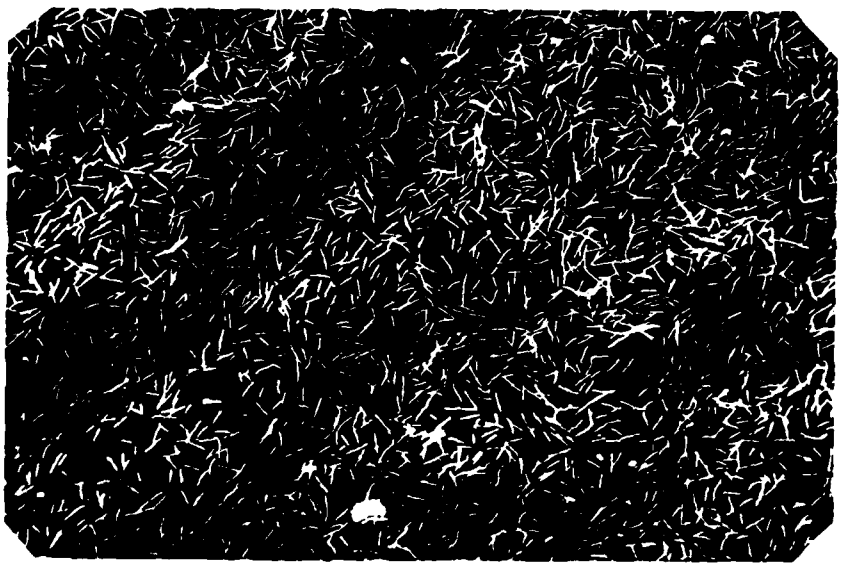

Figure 2 Scanning Electron Micrograph of Microtiter Well Coated With Formalin Fixed Whole Cells. Print Magnification is 880X.

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