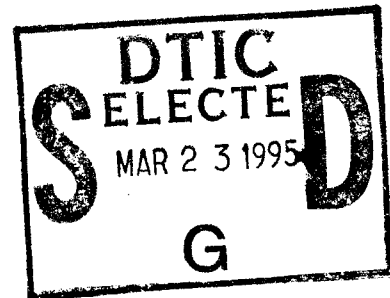


1 **Postmortem Antigenic Epitope Retention of Galactose-N-Acetylglucosamine**
2 **in *Bacillus anthracis*-Infected Rhesus Monkey (*Macaca mulatta*) Spleens**
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6 **Running title:** Immunohistochemical Staining of *Bacillus anthracis*

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Abstract

1
2 **BACKGROUND:** Anthrax bacilli can be identified by using a monoclonal
3 antibody to galactose-N-acetylglucosamine, a *Bacillus anthracis* cell-wall
4 polysaccharide. We characterized the retention of antigenicity for this
5 polysaccharide in prolonged postmortem autolysis of spleens from anthrax
6 infected monkeys.

7 **EXPERIMENTAL DESIGN:** Spleens from infected rhesus monkeys were allowed
8 to autolyze for up to 30 days. To visualize the availability and retention of the
9 polysaccharide at various days of postmortem autolysis, splenic tissue
10 impressions were immunostained by silver enhanced immunogold (IGSS) with a
11 monoclonal antibody against galactose-N-acetylglucosamine.

12 **RESULTS:** All tissues had bacilli and fragments of bacilli present from the day
13 of necropsy through 30 days of postmortem observation. Consistent
14 immunocytochemical staining of bacilli occurred from days 3 to 15. In some
15 cases, bacilli stained positively as late as 30 days. Extracellular stain reaction
16 product demonstrating specific immunoreactivity was heavy in day 0 samples and
17 gradually diminished over time to the day 15 samples.

18 **CONCLUSIONS:** The antigenic epitope recognized by the monoclonal antibody
19 against galactose-N-acetylglucosamine was preserved with consistency for 15
20 days postmortem in decaying splenic tissue. Membrane-bound polysaccharide did
21 not appear to be consistently available to antibody for reaction product formation
22 until 3 days postmortem. The polysaccharide also appeared to exist free of the
23 bacillus cell wall, and showed strong immunostaining properties during early
24 postmortem decay.

25 **Additional key words:** Monoclonal Antibody, Galactose-N-acetylglucosamine,
26 *Bacillus anthracis*

27

INTRODUCTION

1
2 Postmortem autolysis can hinder the definitive postmortem diagnosis of anthrax in humans, as
3 well as in domestic and wild animals. In a publication describing 42 human cases of inhalation
4 anthrax, anthrax bacilli were recovered by culture in only 20 cases. Twenty cases were culture
5 negative and 2 were not examined. Histology on 15 of the culture-negative cases revealed bacilli
6 (1). Theoretically, the availability of a reliable immunohistologic technique would have facilitated
7 a definitive diagnosis in those 15 cases.

8 Antibodies have been produced to a variety of *Bacillus anthracis* antigenic subunits. Antibody
9 to a *B. anthracis* cell-wall polysaccharide, galactose-N-acetylglucosamine, has demonstrated a high
10 level of specificity, suggesting it has immunodiagnostic potential (2). It demonstrated superior
11 immunohistochemical signal producing potential in a preliminary screen that also included
12 antibodies to a toxin protein, capsular peptide (poly-D-glutamic acid), and cell-wall protein.
13 However, preservation of the polysaccharide's antigenicity during autolysis has not been reported.
14 In this paper, we report the distribution, availability to antibody, and preservation of the antigenic
15 galactose-N-acetylglucosamine polysaccharide during autolysis of infected tissue.

EXPERIMENTAL DESIGN

16
17 Spleen samples from 5 rhesus monkeys infected with the Vollum 1B strain of *B. anthracis*,
18 which had previously served as controls in a drug and vaccine efficacy study (3), were allowed to
19 autolyze for up to 30 days at room temperature (20 to 22° C). Tissue impressions made at days 0,
20 1, 2, 3, 4, 5, 6, 9, 11, 13, 15, 18, 21, and 30 were Giemsa stained (Baxter, Deerfield, IL) and
21 immunostained by silver enhanced immunogold (IGSS) with a mouse monoclonal antibody
22 against the cell-wall polysaccharide, galactose-N-acetylglucosamine. Pattern and intensity of
23 positive immunostain reaction product were analyzed for all samples.

RESULTS AND DISCUSSION

24
25 Two distribution patterns of the IGSS reaction product were observed. The reaction product
26 was either closely bacillus-associated (b/a) or free in the extracellular tissue fluid (e/c) showing no

1 direct association with bacilli. Mean values for both Giemsa- and IGSS-stained impressions are
2 displayed in Figure 1.

3 The b/a signal, initially negligible, was strong by 3 days of tissue autolysis. This suggests that
4 the organism-bound polysaccharide was not accessible to antibody binding and/or reaction product
5 formation in fresh tissue, but became increasingly accessible with postmortem autolysis, possibly
6 due to the destruction of the organism's protective poly-D-glutamic acid capsule. By day 3, there
7 were 1 to 20 IGSS-stained organisms per high-power field (org/hpf) on impressions of tissues
8 from all animals. Consistent staining of bacilli was noted from days 3 to 15. Before day 3, IGSS-
9 stained bacilli had an irregular stain pattern (Fig. 2). From day 3 to 9, cell walls of the IGSS-
10 stained bacilli had a uniform stain pattern (Fig. 3), which suggested that antigen was easily
11 accessible to the primary antibody for reaction product formation. Again we noted an irregular cell
12 wall stain pattern in IGSS-stained organisms from tissues subjected to 11 or more days of
13 autolysis. From days 3 to 21, there were equal to or greater numbers of bacilli observed on IGSS-
14 stained impressions than on Giemsa-stained impressions, which suggested that immunostaining
15 with anti-galactose-N-acetylglucosamine enhanced recognition of anthrax bacilli over this period of
16 tissue autolysis. The number of Giemsa-stained microorganisms, < 1 org/hpf on day 18
17 impressions, gradually rose on days 21 and 30, surpassing the number of IGSS-stained
18 microorganisms on the day 30 impressions. This most likely represents proliferation of
19 contaminating bacterial growth, as significant additional sporulation and new growth of anthrax
20 bacilli would not be anticipated in the airtight microenvironment. In addition to specificity testing
21 described in prior work (2), we tested for and found no cross-reactivity of the primary antibody
22 with *Clostridium sporogenes*, *B. subtilis*, *Escherichia coli*, *Salmonella enteritidis*, *Pasteurella*
23 *multocida*, *Yersinia enterocolitica*, *Bordetella bronchiseptica*, *Brucella abortus*, *Brucella melitensis*,
24 and *Brucella suis*.

25 The amount of e/c reaction product, initially heavy, lessened with prolonged tissue autolysis.
26 The heavy concentration on day 0 impressions persisted with no remarkable change until day 3.

1 From day 3 to 15, reduced yet notable e/c reaction product persisted. With rare exception, there
2 was no remarkable e/c signal from day 18 to 30. This pattern of staining was never observed in
3 stain controls testing for nonspecific binding of the secondary antibody or on impressions prepared
4 from non-infected monkey spleens. This suggests that it was a positive indication of non-cellular
5 bound galactose-N-acetylglucosamine free in *B. anthracis*-infected tissue and fluids.

6 METHODS

7 SAMPLE COLLECTION AND SLIDE PREPARATION

8 Spleen samples for day 0 tissue impressions were taken from 5 untreated *B. anthracis*-infected
9 rhesus monkeys (*Macaca mulatta*) immediately postmortem (3). After gross necropsy
10 observations and splenic sampling, body cavities were closed and the carcasses were allowed to
11 remain at room temperature (20 to 22° C) for 24 hr. Using aseptic techniques, we harvested
12 spleens, diced them into approximate 0.5 cm cubes, and placed them in small capped containers to
13 allow room temperature autolysis for 2, 3, 4, 5, 6, 9, 11, 13, 15, 18, 21, and 30 days. Day 1
14 tissue impressions made at this time were placed on Superfrost/Plus (Fisher Scientific, St. Louis,
15 MO) microscope slides, air dried, and fixed for 1 hr in 10% buffered neutral formalin. One slide
16 from each group was Giemsa stained to evaluate the relative number of bacilli present.

17 IMMUNOSTAIN TECHNIQUE

18 After formalin fixation, the impressions were processed by the following method: a) reverse
19 osmosis (r/o) water, 2 x 5 min; b) Lugol's iodine (4), 1 x 5 min; c) r/o water, 1 x 5 min; d) 5%
20 sodium thiosulfate, 15 sec rinse (4); e) r/o water, 3 x 5 min; f) wash buffer consisting of 0.8%
21 bovine serum albumin (BSA) (Sigma Chemical Company, St. Louis, MO), 0.1% fish skin gelatin
22 (FSG) (Sigma Chemical Company), 0.5% triton-X 100 (Sigma Chemical Company), 0.02 M
23 glycine (Sigma Chemical Company), and 2 mM sodium azide (Sigma Chemical Company) in 0.05
24 M tris-buffered saline (TBS), 2 x 5 min (5); and g) blocking buffer consisting of 0.08% BSA,
25 0.01% FSG, 5% goat serum (Sigma Chemical Company), and 2 mM sodium azide in 0.05 M
26 TBS, 1 x 15 min. Impressions were drained without rinsing and treated with MA b EAII-6G6 (2)

1 primary antibody diluted 1:100 in incubating buffer consisting of 0.08% BSA, 0.1% FSG, 1%
2 goat serum, 0.1% triton-X 100, and 2 mM sodium azide in 0.05 M TBS for 90 min; washed with
3 buffer, 2 x 15 min; and then treated with blocking buffer, 1 x 10 min. Impressions were again
4 drained without rinsing and 5 nm gold conjugated goat anti-mouse secondary antibody (Amersham
5 Corporation, Arlington Heights, IL) diluted 1:40 in incubating buffer was applied for 60 min;
6 followed by wash buffer, 3 x 5 min; r/o water, 30 sec rinse; r/o water, 4 x 5 min; and silver
7 enhancement (Amersham Corporation), approximately 10 min.

8 If the silver autprecipitation time was < 20 min, the silver enhancement step was split into two
9 5 min treatments. The impressions were counterstained with working light green solution (6).

10 Nonspecific binding of the secondary antibody was tested for by eliminating the primary antibody
11 application and subsequent steps, proceeding immediately to application of the secondary
12 antibody. Nonspecific binding of the primary antibody was tested for by staining normal monkey
13 spleen impressions at 0, 5, 7, and 9 days of autolysis. A known anthrax positive spleen
14 impression control was also processed with this group. When a day group stained either negative
15 or variable, the group was restained along with a positive control to confirm that the results were
16 not due to a procedural error.

17 SLIDE INTERPRETATION

18 Stained impressions were evaluated by brightfield microscopy. The formation of a
19 brown-black reaction product indicated the presence of the target antigen. A subjective score of 0
20 (negligible) to 4 (heavy) was assigned for each impression, characterizing the amount of e/c
21 reaction product. Scores of 0 to 4 were also assigned for evaluating the number of Giemsa-stained
22 bacilli as well as evaluation of the b/a signal based on 0 < 1 org/hpf, 1 = 1-20 org/hpf, 2 = 21-60
23 org/hpf, 3 = 61-100 org/hpf, and 4 > 100 org/hpf. The final score assigned for each day of
24 sample evaluation was based on the average for the 5 animals. High-power field (hpf) is defined,
25 in this study, as microscopy with a 40X objective and 10X eyepieces (400X).

26 TEST FOR BACTERIAL CROSS-REACTIVITY

1 Using the same immunostain technique described above, we stained formalin-fixed spot slides
2 of *C. sporogenes*, and used formalin-fixed *B. anthracis* (Sterne strain) spot slides as positive
3 controls.

4 Using the monoclonal antibody labeled with fluorescein isothiocyanate (FITC), we tested for
5 additional cross-reactivity using formalin-fixed spot slides of the following bacteria: *B. subtilis*,
6 *C. sporogenes*, *E. coli*, *S. enteritidis*, *P. multocida*, *Y. enterocolitica*, *B. bronchiseptica*, *Br.*
7 *abortus*, *Br. melitensis*, and *Br. suis*. Impressions of day 3 postmortem *B. anthracis*-infected
8 spleens served as positive controls. Briefly, the impressions were rinsed in distilled water,
9 incubated at room temperature in a humidified chamber for 30 min with normal mouse serum
10 diluted 1:200 in phosphate-buffered saline (PBS), pH 7.4, followed by a 30-min incubation of
11 FITC-labeled monoclonal antibody to galactose-N-acetylglucosamine diluted 1:5 in PBS. The
12 impressions were rinsed in PBS, then in distilled water, mounted with Vectashield mounting
13 medium (Vector Laboratories, Burlingame, CA), and examined by fluorescence microscopy.

14

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1 FIG. 1. A comparison of mean score values for the relative numbers of Giemsa- and IGSS-
2 stained bacilli (b/a) and amount of extracellular (e/c) IGSS stain reaction product. A subjective
3 score of 0 (negligible) to 4 (heavy) was assigned for each impression. Scores of 0 to 4 were also
4 assigned for evaluating the number of Giemsa-stained bacilli as well as evaluation of the b/a signal
5 based on 0 < 1 org/hpf, 1 = 1-20 org/hpf, 2 = 21-60 org/hpf, 3 = 61-100 org/hpf, and 4 > 100
6 org/hpf.

7 FIG. 2. An IGSS-stained chain of bacilli from a spleen with 2 days of postmortem autolysis.
8 The stain pattern for bacilli was irregular and extracellular stain reaction product was moderately
9 heavy (score=3), X1,800.

10 FIG. 3. An IGSS-stained chain of bacilli from a spleen with 3 days of postmortem autolysis.
11 The stain pattern for bacilli was uniform and the average extracellular stain reaction product was
12 moderate (score=2). In this micrograph, extracellular stain reaction product is mild (score=1),
13 X1,400.

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