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POTENTIAL VACCINE FOR ANTHRAX

ANNUAL REPORT

R.J. DOYLE
JYOTI S. SINGH
F.S. EKWUNIFE

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

B. anthracis Sterne cells were treated with monoclonal antibodies (MAbs) to protective antigen and subjected to the indirect-fluorescent antibody assays. Fluorescence was discernible throughout the surface of the cells. Extraction of the cells with 8M urea or 1% SDS (1 hr, 37°C) brought about a total loss of fluorescence. Pretreatment of the same cells with trypsin (100 ug/ml, 37°C, 1 hr) seemed to render the fluorescence somewhat stronger, with distinct patterns of granularity. Preincubation of the cells with soy bean agglutinin seemed to bring about a patchiness in the fluorescence distribution.

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B. anthracis Sterne cells, when treated with MAbs to pCHO, fluoresced very brightly and this fluorescence was distributed evenly on the whole surface. Extraction of the cells with 1% SDS did not decrease their ability to fluoresce. Blocking of the pCHO-monoclone binding sites with surface array proteins EA-I and EA-II had no effect on the distribution of fluorescence on the surface of B. anthracis.

B. cereus 246 cells were employed in similar studies using MAbs to PA as well as MAbs to pCHO. This was done to ascertain that the results obtained were actually specific for B. anthracis and not mere artifacts. All reactions were negative, no fluorescence was recorded.

Electron microscope studies were performed to corroborate the data of the indirect fluorescent antibody assays. B. anthracis cells were first incubated with the desired MAb, followed by a gold-labelled antiIg. The pellets were then dehydrated in an alcohol series, embedded in Epon 812 and then sectioned. The sections were stained with uranyl acetate/ lead-citrate and observed under TEM. The distribution of gold particles was quite apparent on the surfaces of the cells that were exposed to MAbs to pCHO. But the low number of gold particles in these EM studies was perplexing and did not justify the high intensity of fluorescence that was recorded by the indirect fluorescence Ab-assays. In future studies, the Epon 812 resin will be replaced by LR-white. This compound allows the curing process to occur at temperatures lower than 60^0C, which may prove to be more favorable for the delicate Ag-Ab reactions. Embedding and sectioning will be done prior to the immunolabelling.

It was possible to extract cell surface proteins using the method of Ezzell and Abshire (Infect. Immun. 56, 349-356, 1988). The proteins were then subjected to electrophoresis and Western Blot analyses. Distinct bands were detected for PA, EA1 and LF (lethal factor).

Based on the results obtained from sodium periodate oxidation, Smith degradation, ^{13}C-NMR, methylation and partial acid hydrolysis, the following can be said about the pCHO structure:

(i) Since 75% of the galactose residues are sensitive to periodate oxidation they are probably located at the non-reducing terminus. If internal, then at a periodate-sensitive position of the polysaccharide. (ii) Since all amino sugars are resistant to periodate oxidation, they may be internally linked, probably by 1,3 or 1,4 linkages.

(iii) ^{13}C-NMR analyses showed the presence of 1,4 linkages as the primary glycosidic bonds. 1,6 linkage was also observed, but no 1,3 or 1,2.

(iv) Smith degradation studies indicate a linear chain of galactose, N-acetyl-glucosamine and N-acetyl-mannosamine linked via 1,3 or 1,4 bond and forming the backbone chain of the pCHO.

(v) ^{13}C-NMR analyses seem to suggest that the galactose residues are all linked by 1,4 linkages and not at all by 1,6 bonds.

Distribution of surface structures as determined by indirect fluorescent antibody assays

General Procedures:

A series of dilutions of the bacterial strains to be tested was prepared in PBS (pH 7.3). A 5 ul sample of an appropriate dilution was spotted onto each well of a red, Teflon-coated spot well slide, air-dried and fixed in methanol for 1 minute. The slides were labelled and frozen for future use. For the monoclones that were in ascites fluid or sera, a 1:300 dilution was made in PBSTG (PBS+ 0.6% Tween 20+ 1% gelatin). The spot slide wells were first washed with PBST, the excess vacuumed off and the slide blocked for 30 minutes with PBSTG at 37°C. All incubations were carried out in a closed container containing wet paper towels to ensure moisture at all times. After vacuuming off excess PBSTG, 25ul of the desired monoclonal antibody (MAb) (diluted in PBSTG) was added to each experimental well. The control received the same volume of PBSTG. The slide was incubated at 37°C for one hour. The wells were washed with PBST and 40ul goat anti-mouse IgG conjugate (capped) added as a 1:200 dilution in PBSTG. After incubating for 30 minutes at 37°C, the wells were given a final wash with PBST and the excess buffer vacuumed off. To reduce fading during fluorescence microscopy coverslips were added by using a drop of mountant containing 1,4-diazobicyclo-(2,2,2)-octane (Sigma) at 25 mg/ml in 10% PBS-90% glycerol (pH 7.4).

[A] Treatment of B. anthracis Sterne cells with MAbs to protective antigen (PA1-1G7-21, PA1-2D3-3-1, PA2-III-8C8-1-1, PA 3-3C5-1-1).

Observations:

The MAbs against PA imparted an overall glow or fluorescence[1] onto the surface of the cells. Though faint, the fluorescence was however discernible from the respective controls, which were treated with the FITC labelled antiIg and no MAb.

(ii) When the same cells were pretreated with trypsin (37°C, 1 hour, 100µg/ml), the fluorescence was somewhat stronger and displayed distinct patterns of granularity.

(iii) Preincubation of the cells with soy bean agglutinin seemed to bring about a patchiness in the fluorescence distribution, and the graininess was not so apparent.

(iv) Extraction of the cells with urea or 1% SDS brought about an almost total loss of fluorescence. The cells acquired an overall yellowish hue, same as the untreated controls.

[B] Treatment of B. anthracis Sterne cells with MAbs to the polysaccharide.

Observations:

(i) The cells fluoresced very brightly and uniformly on the on the complete surface.

(ii) Treatment of the cells with 8M urea did reduce the intensity of the fluorescence, but not completely. No pattern or surface granularity was visible.

(iii) Extracting the cells with 1% SDS (37°C, 1 hr) did not eliminate their ability to fluorescence with MAbs to pCHO.

(iv) Boiling the cells (10 minutes) in either PBS or 1% SDS prior to MAb exposure, resulted an interesting phenomenon. The cells did not lose their ability to fluoresce. Instead, the fluorescence seemed to be more intense, diffuse, somewhat opaque and covered the complete cell surface. Conversely, the control, unboiled cells displayed fluorescence which was translucent and with distinct boundaries.

[C] Blocking of the PCHO-monoclone binding sites with surface array proteins EA-1, EA-II.

Observations:

Surface array protein blocking had no effects on fluorescence. The results obtained were the same as when the cells were not treated with surface array proteins. It appears that the polysaccharide structure is evenly distributed throughout the surface of B. anthracis.

[D] B. cereus 246 cells in conjunction with the various monoclones.

Observations:

In order to support the premise that the fluorescence patterns seen with B. anthracis were actually specific for that organism and not artifacts, it was necessary to carry out similar studies, using MAbs in conjunction with some other species of Bacillus. We employed B. cereus 246 cells. MAbs to pCHO as well as PA were utilized in studies resembling those above. All the reactions were negative. No fluorescence was recorded with any treatment.

Extraction of Cellular Proteins of B-anthracis

Cell surface proteins were extracted from B. anthracis sterne strain, using the method of Ezzell and Abshire (Infect. Immun. 56, 349-356, 1988). Concentrated protein samples were electrophoresed on polyacrylamide gels containing 10% (wt/vol) acrylamide (acrylamide-bisacrylamide [37.5:1]; pH 8.8) with 3.5% acrylamide stacking gels (pH 6.8). Electrophoresis was stopped when the bromophenol blue tracking dye had migrated 10 cm. The gels were routinely stained with the silver stain from Biorad. In order to identify the various bands, the Western blot technique of Ezzell and Abshire (1988) was employed, with some modifications. The desired protein sample was initially electrophoresed on a 10% SDS-page gel as a single sample on the surface, (no wells were formed in the stacking gel). The gel was cut into half longitudinally. One half was silver stained, whereas the other half was trans-blotted onto nitrocellulose (NC) paper. The NC was cut into 2.5 mm wide strips. Each strip was incubated in 1 ml of antiserum or MAb that was diluted 1:50 and 1:100 in PBSTG (0.5% gelatin) for 2 hr. at 37 ^{o}C. For mouse monoclones, the strips were subsequently incubated with 1 ml of goat-antimouse horseradish peroxidase (HRP) conjugate, diluted 1:400 in PBSTG. For human or guinea pig antibody, 1 ml of 1:5000 diluted Protein A-HRP was used. The strips were incubated in tetramyl-benzidine reagent to develop the color.

Observations:

A figure of the blot in which human sera and guinea pig sera

were used is enclosed. Note the band for PA in lane 1, for LF in lane 2, for EA1 in lane 3 and PA in lane 4.

Similarly processed blots, when tested with MAbs to PA, revealed distinct, sharp bands (Figs not enclosed) in the vicinity of 85 Kda. This confirms that PA can be removed from the cells with appropriate treatments.

Electron Microscope Studies

In order to confirm data on the fluorescence studies, it was necessary to conduct EM-studies.

Procedure:

Cells were grown in R-medium overnight, harvested and washed 2X with PBS. The cells were incubated for 1 hr ($37^{o}C$) with the desired MAb in small Eppendorf tubes, followed by a gold-labelled antiIg. The cells were washed 1X gently in PBS, preserved in 2% glutaraldehyde and later subjected to the following TEM procedure. The cells were washed 3X in 0.1 M cacodylic acid buffer (CAD) (pH 7.4), followed by post-fixation in 1% OsO_4 at room temp for 1 hr. The pellet was then dehydrated in a graded series of alcohol for 10 to 15 minutes, followed by embedding in Epon 8-12 at $60^{o}C$ for 24 to 48 hours. The samples were then sectioned, placed on grids and subjected to uranyl acetate staining (5 minutes, 2% in 50% ethanol). The sections were washed 3X in CAD buffer, stained 30 seconds in lead-citrate stain and observed under transmission electron microscope (TEM).

Observations:

The control specimens, which were treated with the Au-antiIg in the absence of any monoclone, showed no gold particles in the vicinity of the cells. The cells that were treated with MAbs to pCHO prior to the exposure of Au-antiIg showed the presence of clumps of gold particles distributed in the amorphous material on the outer surfaces of the cells. Though we are pleased to see a positive response, it is quite perplexing however to see the greatly reduced distribution of the

gold particles when we actually have data which shows that the corresponding fluorescence studies give strong reactions. Trypsin treatment of the cells prior to MAb to pCHO reduced the number of particles of gold somewhat.

It may be possible that the immunolabelling of the cells followed by an extensive regimen of post-fixing and cutting leads to a loss of the Au-particles or dissociation of the Ag-Ab reactions. Since we have proof of positive reactions with our fluorescence studies, it would be unwise not to pursue this further. We intend to make some modifications on the original TEM method. The cells will be grown and harvested as before. The cell-pellet will be graded up to 70% alcohol, after which it will be embedded in a resin called LR-white instead of epon 8-12. Sections will be made and attached to grids, and then followed up with the immunolabelling. The sections will be stained just prior to observing. The curing and embedding in LR-white can be done at $50^{o}C$ instead of 60^{o} as with the epon compound. This lowered temperature may prove favorable for the overall Ab-Ag reaction, in addition to the immunolabelling which will be done after the embedding and sectioning.

Polysaccharide sequencing:

Methods employed in the sequencing include: sodium periodate oxidation, Smith degradation, ^{13}C-NMR, and lately, methylation and partial acid hydrolysis. Based on the results obtained from these methods, we know the following with regard to the sequencing:

(i) galactose residues are probably at the non-reducing terminus or at the internal, but periodate-sensitive position of the polysaccharide. This is because 75% of the galactose residues are sensitive to periodate oxidation. The remaining 25% of galactose residues that are resistant to periodate oxidation may have been due to underoxidation caused by protection of galactose-sensitive residues by interresidue hemiacetal formation or hydrogen bonding.

(ii) All the amino-sugars of the pCHO are resistant to periodate-oxidation, thus implying that the amino sugars are internally linked, probably by 1,3 or 1,4 linkages.

(iii) ^{13}C-NMR analyses showed the presence of 1,4 linkages as the primary glycosidic bonds within the pCHO moiety. The presence of a 1,6 linkage was also observed from the NMR spectra. There are no 1,3 linkages involved and probably no 1,2 bonds. The spectra also showed the pCHO to contain 2 and 2 configurations.

(iv) Smith degradation, followed by reoxidation of the Smith degradation product, was not affected by the second periodate oxidation. This probably indicates a linear chain of galactose, N-acetyl-glucosamine and N-acetyl-mannosamine linked 1,3 or 1,4, as the backbone chain of the polysaccharide. The galacturonic acid residue may be linked as a side chain.

(v) All the galactose residues are probably 1,4 or 1,6 linked. This is due to the liberation of glycerol and threitol or erythritol from the Smith degradation product, following hydrolysis with 0.1M HCL at 100^{0} for 3 h. Glycerol is supposed to be liberated from the non-reducing terminal galactose residue, or from the 1,2, or 1,6--linked non-terminal or internal galactose residues. Threitol or erythritol on the other hand, results from the 1,4-linked non-terminal or internal galactose residues. From the ^{13}C-NMR spectra, 1,2 bonds are absent, therefore glycerol is liberated either from the 1,4 linked galactose at the non-reducing terminus, or from 1,6 linked non-terminal or internal galactose residue. Both 1,4 and 1,6 linkages have been observed from the ^{13}C-NMR spectra of the pCHO moiety. But threitol or erythritol results from the 1,4 linked internal galactose residue also. Since both glycerol and threitol were observed, it is likely that the galactose residues are all linked by 1,4 linkages and probably not all by 1,6 bond.

(vi) There are close serological relationships between the GlcNAC-galactose containing pCHO of B. anthracis and those of type XIV pneumococcus Forssman antigen, and various blood-group substances. The cross-reactions are known to be due to the terminal D-galactopyranosyl group linked to O-4 of the 2-acetamido-2-deoxy- D- glucopyranoyl residue.

(vii) Methylation analysis will finally tell the positions of these glycosidic bonds within the pCHO structure. Also, partial acid hydrolysis will yield small oligomers that can be better resolved on the NMR. We are currently using these two methods to determine the final pCHO structure.

Figure 1. B.anthracis Sterne cells treated with FITC labelled anti-Ig. No exposure to any monoclonal antibodies.

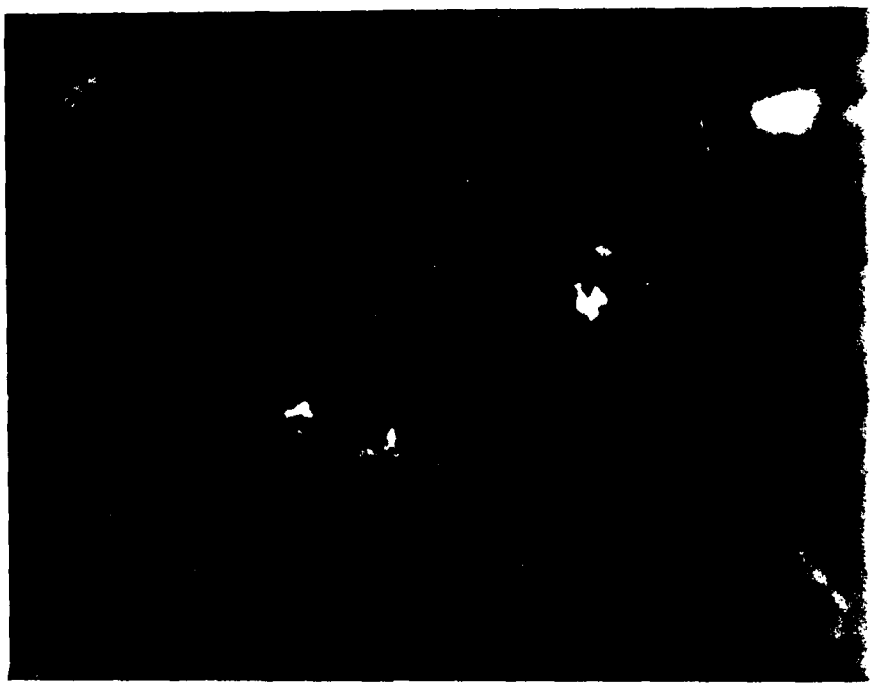


Figure 2. B.anthracis Sterne cells treated with monoclonal antibodies to PA, followed by FITC labelled anti-Ig.

Figure 3. B. anthracis Sterne cells pretreated with trypsin, followed by PA monoclonal Ab and FITC labelled anti-Ig.

Figure 4. B.anthracis Sterne cells pretreated with 1% SDS, followed by PA monoclonal Ab and FITC/anti-Ig.

Figure 5. B.anthracis Sterne cells treated with monoclonal Ab to polysaccharide (pCHO), followed by FITC labelled anti-Ig.
Notice the bright and uniform surface fluorescence.

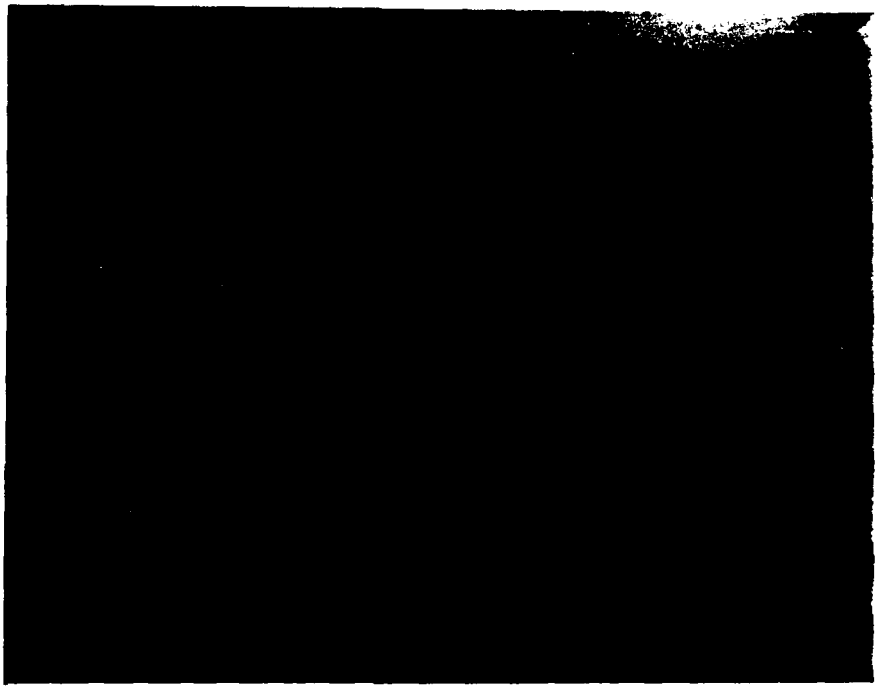


Figure 6. B. anthracis Sterne cells exposed to monoclonal Ab to pCHO, followed by FITC/anti-Ig.
Notice the translucent nature of fluorescence and the bright boundaries.

Figure 7. B. anthracis Sterne cells boiled in 1% SDS (10 min.), followed by monoclonal Ab to pCHO and the FITC/anti-Ig.

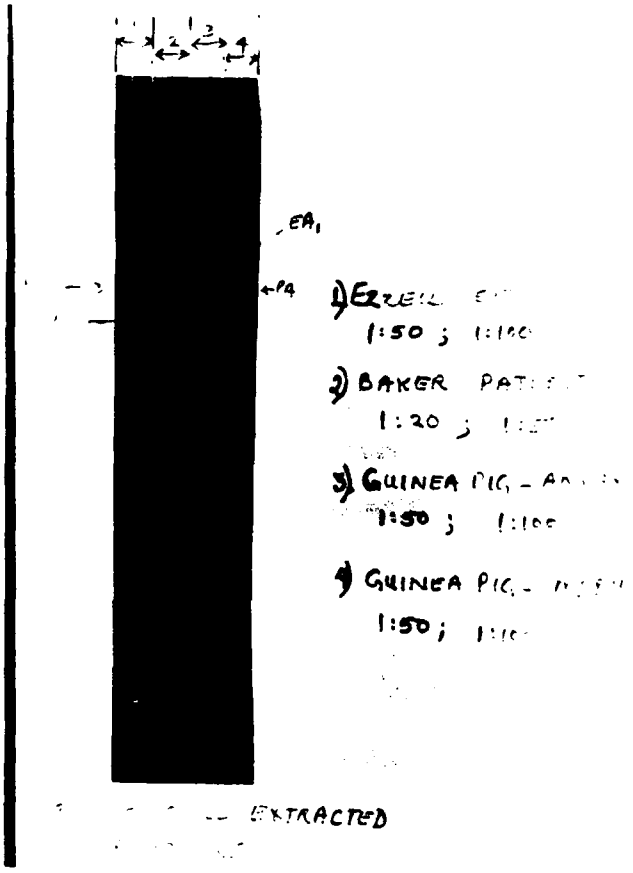


Figure 8. Western Blot analysis of a protein extracted from B. anthracis Sterne cells.

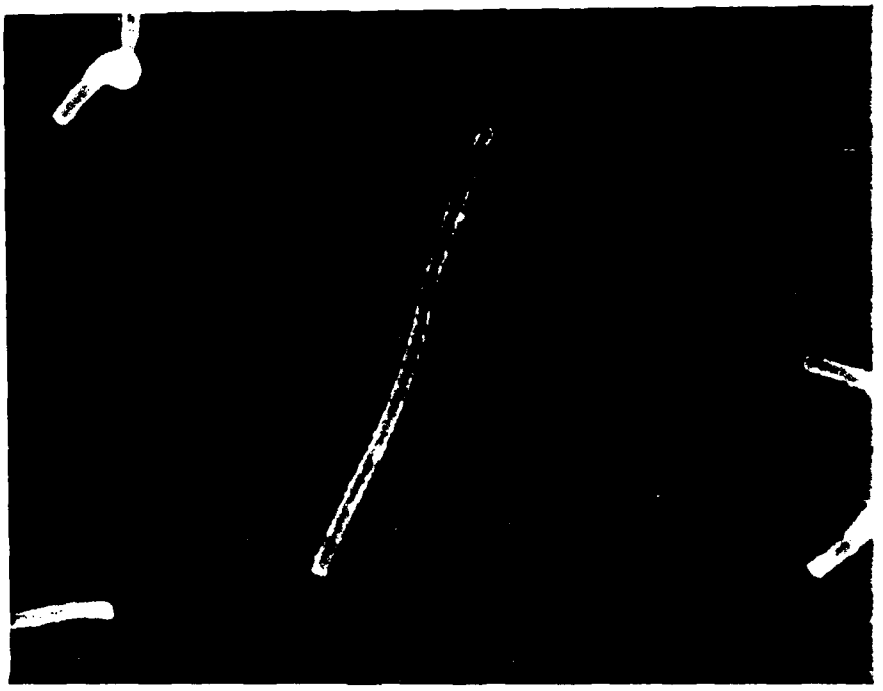


Figure 9. B. anthracis 4229 cells boiled for 15 min. prior to treatment with FITC labelled SBA.

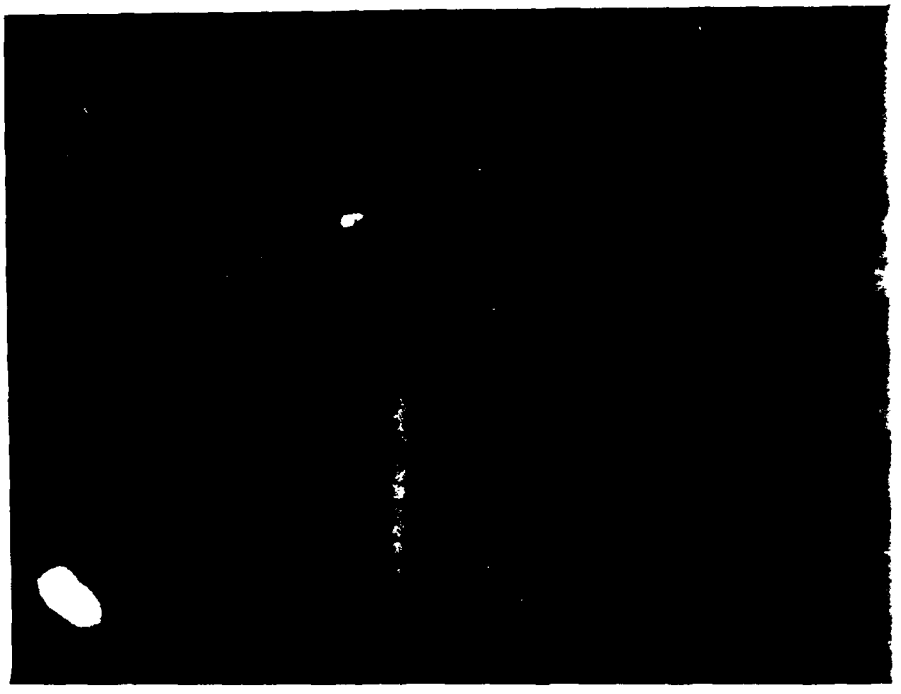


Figure 10. B. anthracis 4229 cells boiled for 15 minutes, followed by FITC labelled SBA.

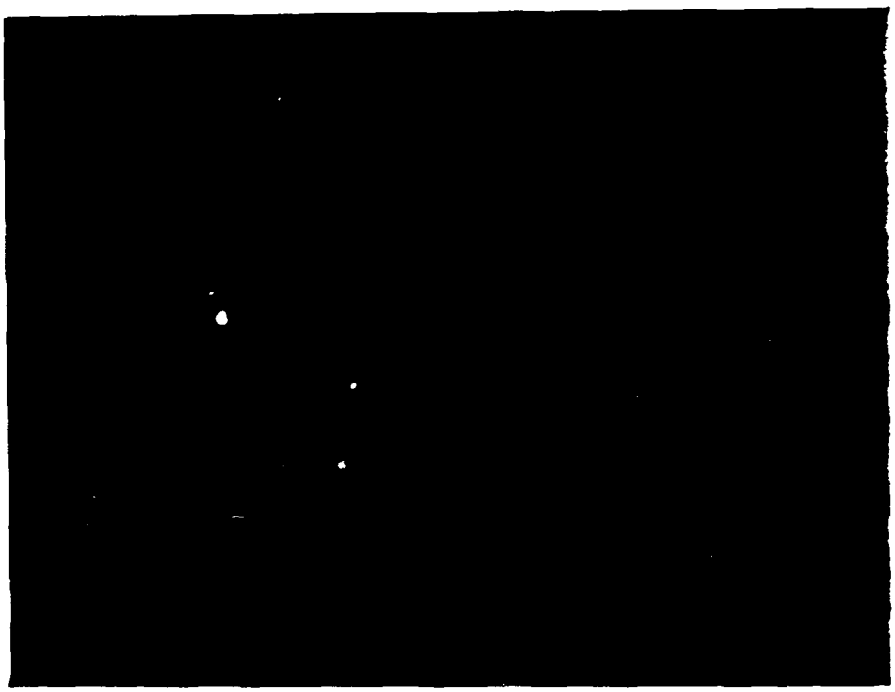


Figure 11. B. anthracis Δ-Sterne cells blocked with monoclonal Ab to pCHO, followed by FITC labelled SBA.

Figure 12. B. anthracis Sterne cells blocked with surface-array protein (EA-11), followed by FITC labelled anti-Ig.

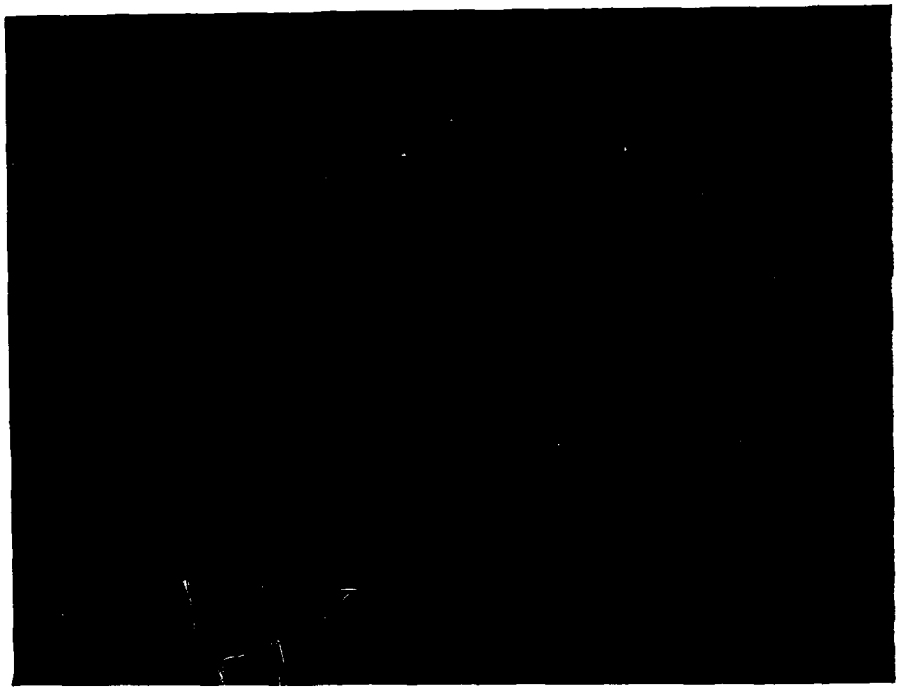


Figure 13. B. anthracis 4229 cells, blocked with surface-array protein (EA-11), followed by monoclonal Ab to pCHO and FITC labelled anti-Ig.

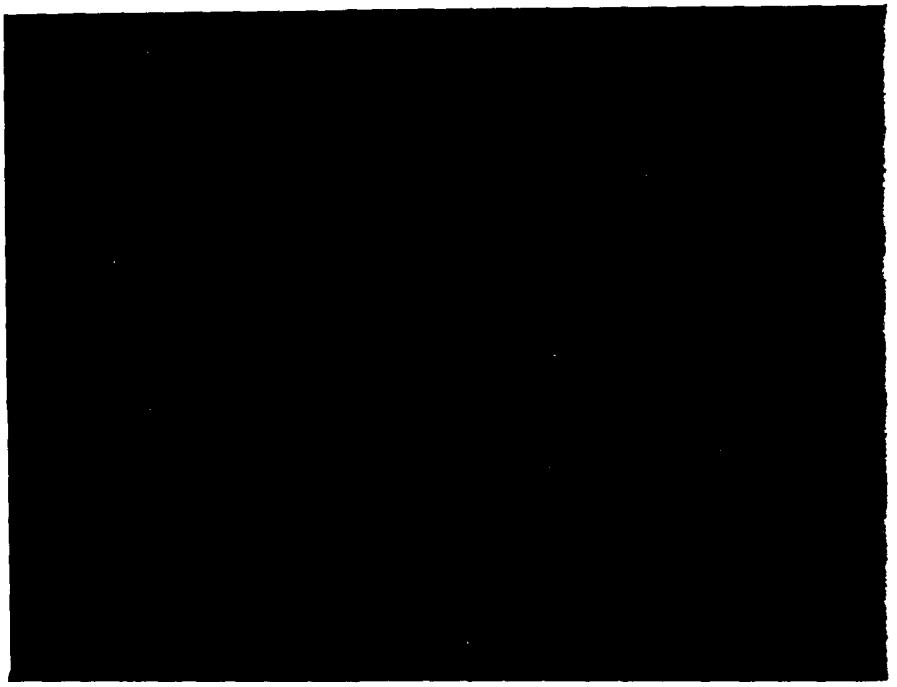


Figure 14. B. anthracis Sterne cells blocked with SBA, followed by monoclonal Ab to pCHO and FITC labelled anti-Ig.

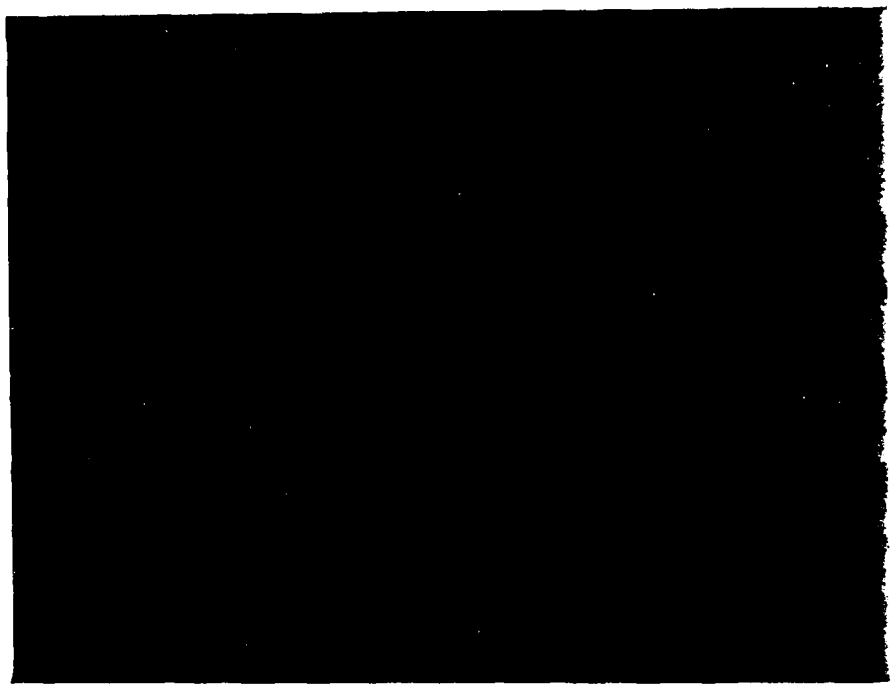


Figure 15. B. anthracis Sterne cells blocked with SBA prior to treatment with monoclonal Ab to pCHO and the FITC/anti-Ig.

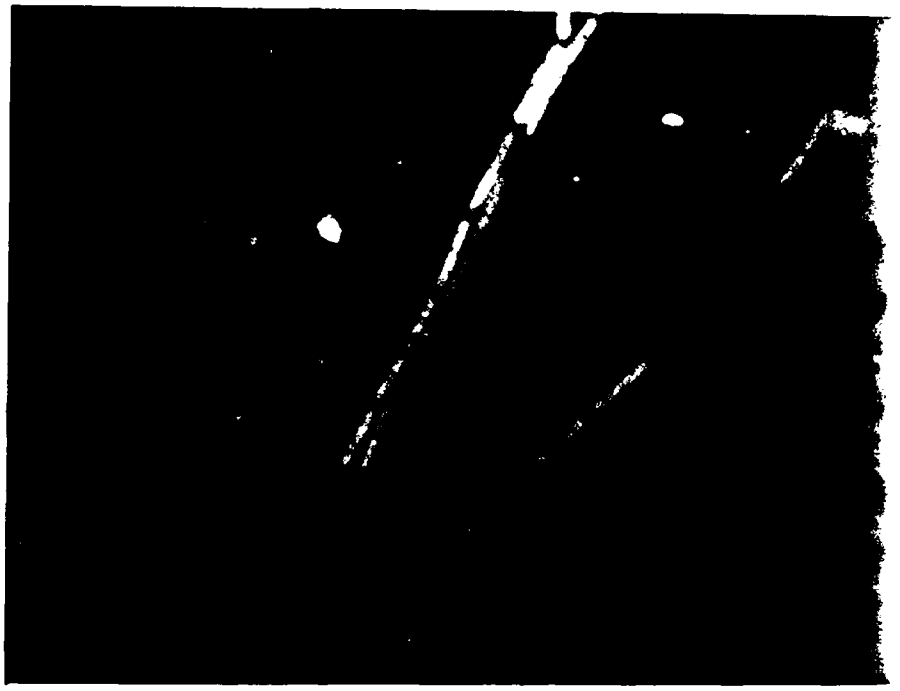


Figure 16. B. anthracis 4229 cells blocked with monoclonal Ab to pCHO, followed by FITC labelled SBA.

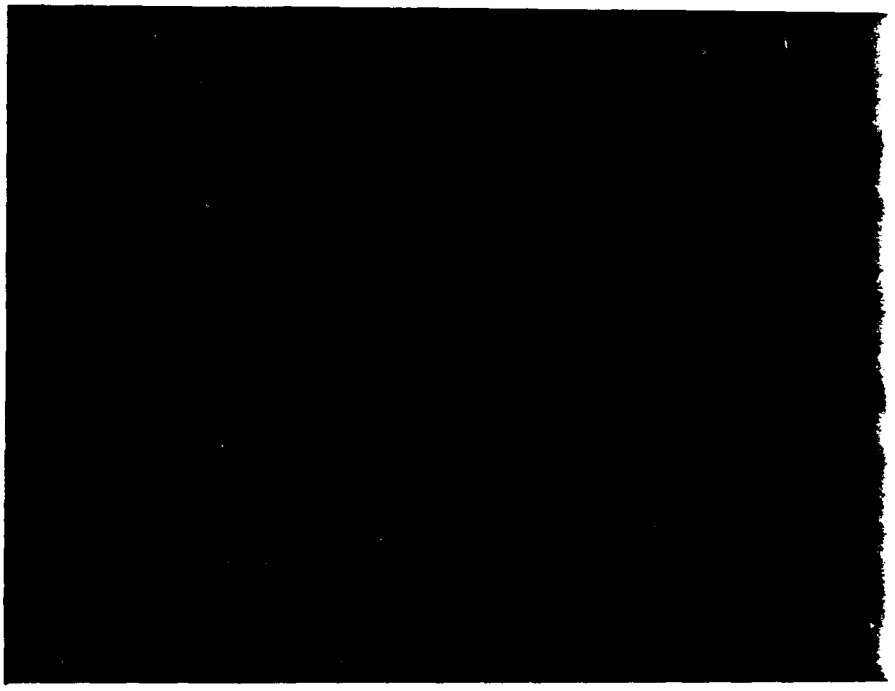


Figure 17. B. anthracis 4229 cells (controls). No exposure to monoclonal Abs, only FITC labelled anti-Ig.

Figure 18.

B.anthracis Sterne cells (controls). No exposure to any monoclonal Abs, only gold labelled anti-Ig. MAG. 126,000.

Figure 19.

B. anthracis Sterne cells (controls). No exposure to monoclonal Abs, only gold labelled anti-Ig. MAG.126,000.

Figure 20.

B. anthracis Sterne Cells pretreated with trypsin, followed by monoclonal antibody to pCHO and gold labelled anti-Ig. MAG.126,000.

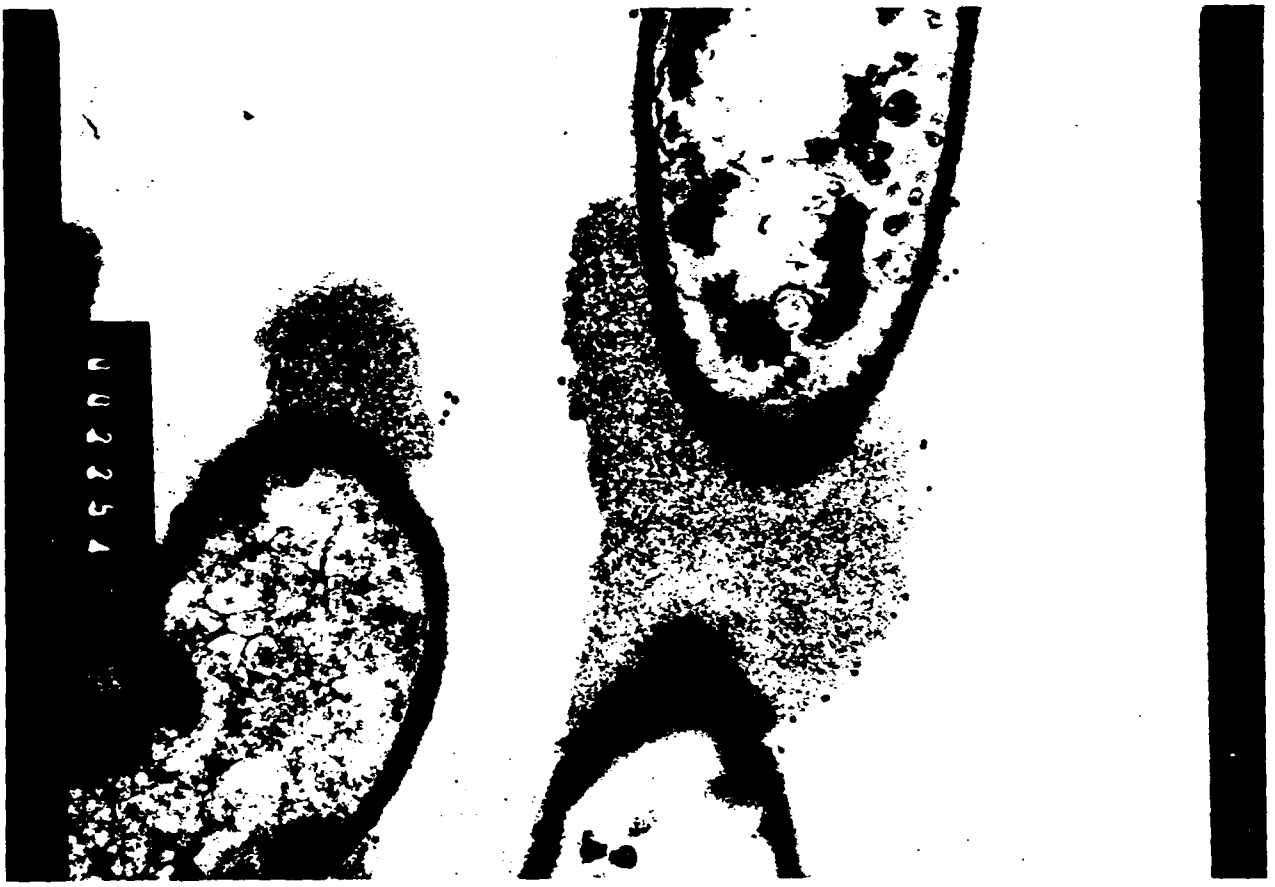
Figure 21.

B. anthracis Sterne cells pretreated with trypsin, followed by monoclonal Ab to pCHO and gold labelled anti-Ig. MAG.126,000.

Figure 22.

B. anthracis Sterne cells treated with monoclonal Ab to pCHO, followed gold labelled anti-Ig. MAG.126,000.

Figure 23.



B.anthracis Sterne cells treated with monoclonal Ab to pCHO(6G6-2-3), followed by gold labelled anti-Ig. MAG.84,000.

Figure 24.

B. anthracis Sterne cells treated with monoclonal Ab to pCHO(6G6-2-3), followed by gold labelled anti-Ig. MAG.56,000.

Figure 25.

B.anthracis Sterne cells treated with monoclonal Ab to pCHO(6G6-2-3), followed by gold labelled anti-Ig. MAG.84,000.

Figure 26.

B. anthracis Sterne cells treated with monoclonal Ab to pCHO(6G6-2-3), followed by gold labelled anti-Ig. MAG.126,000.

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