Degradation of Streptococcal Cell Wall Antigens In Vivo

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Received for publication 31 July 1967

Specific chemical modification of group A polysaccharide antigen to the A-variant structure was demonstrated in the lymphoid organs of mice by autoradiography by use of radioantibodies specific for these structures. Both antigenic moieties persisted and were still discerned 10 weeks after injection of the group A cell wall. In rabbit skin, the group A specificity was altered after a prolonged period. Unlike the situation for the mouse, polysaccharide A was not converted to A-variant structure, but another specificity common to both polysaccharides persisted at the site of injection. Mucopentep, separated from the polysaccharide of group A cell wall, was eliminated from the site of injection in rabbit skin between 4 and 8 hr after injection. Group D streptococcal cell walls were also rapidly eliminated from tissue, and were no longer detectable 8 hr after injection into rabbit skin or 24 hr after injection into mice. The rapid degradation of these structures was correlated with their susceptibility to lysozyme in vitro and was in contrast to the prolonged persistence of group A cell walls, which were completely resistant to egg white lysozyme. This persistence in tissue correlated with the capacity of group A cell wall fragments to induce a chronic inflammatory process, whereas the isolated mucopentep or group D cell walls produced only an acute necrotic reaction.

The mucopentep-polysaccharide structure of group A streptococcal cell walls is an example of a compound common in the human environment, and which is only very slowly eliminated from tissue (7). We have been studying the significance to the host of accumulations of this poorly metabolized material which combines the properties of toxicity, persistence, and antigenicity (2, 9). One area of investigation is designed to obtain information on the nature of the degradation and specific chemical alterations of this material in the host.

This paper describes the antigenic modification of group-specific A polysaccharide in rabbit skin and various mouse tissues, as demonstrated by autoradiography with specific radioantibodies. Studies on the persistence of streptococcal cell walls and mucopentep are also presented in relation to the nature of the toxic reaction in the host.

MATERIALS AND METHODS

Details on the isolation and labeling of specific antibodies and autoradiographic procedures have been presented (7). Preparation of specific radioantibodies is given briefly here. New Zealand white rabbits were immunized intravenously with suspensions of streptococci of heat-killed group A type 3, strain D-58; group A-variant, type 1, strain K-43 variant; or group D, strain F-24. Specific antibodies against A and A-variant polysaccharides were purified by acid dissociation of antibody from specific immune precipitates. The antimucopentep was eluted from a precipitate of anti-group A-variant serum and group A mucopentep. Anti-group D cell wall was eluted from the homologous antiserum and group D cell wall precipitate. These preparations and a normal rabbit gamma-globulin isolated by diethylaminoethyl chromatography were labeled with 125I. No cross-reactive precipitating antibodies could be detected by immunodiffusion or the capillary tube technique between our antisera against group A or A-variant streptococci and the isolated polysaccharide antigens. Likewise, the purified antibody preparations isolated by acid elution of precipitates showed no precipitin reaction with the heterologous polysaccharide by these techniques. However, smears of group A and group A-variant cell walls stained with 125I-labeled antibody displayed extensive cross-reaction by autoradiography. The cross-reactivity could be removed by absorption of labeled antibody with the heterologous cell wall suspension. The absorption was followed quantitatively by measuring 125I in the supernatant fluids and washed sediments, by use of a gamma scintillation counter.
After exhaustive absorption of anti-A polysaccharide with A-variant cell walls, 56% of the 125I globulin remained in the supernatant fluid. Absorption of anti-A-variant with A cell walls left 71% of the labeled globulin unabsorbed. These absorbed globulins were used to demonstrate specific group A and A-variant antigenic structures in the tissue of injected animals by autoradiography.

Cell walls were isolated and treated with trypsin, ribonuclease, and dianapan as previously described (9). The isolation of mucopeptide and polysaccharide from hot formamide extracts of cell walls has been presented (1).

The localization of antigens in tissue was demonstrated as follows. Rabbits were injected intradermally with 240 µg of group A cell wall fragments (9). Tissue was removed from the site of injection at intervals of 4 hr to 92 days and was frozen at −170 C. Samples were taken from either two or three animals at each time interval. Sections of 4 µ were cut in the cryostat, fixed in absolute methanol, and stained with labeled globulins. After washing, the slides were coated with Kodak NT B-2 nuclear track emulsion and exposed for 7 days in a Conrad-Jofres Cabinet. After developing, the sections were stained with Giemsa stain. Controls consisted of: (i) staining of section from normal tissue, (ii) blocking by prior exposure of tissue section to unlabeled antibody, and (iii) normal labeled rabbit globulin.

Mice of the C57 strain were injected intraperitoneally with 10.0 mg (lyophilized weight) of group A cell wall fragments. Three mice were killed at intervals of 24 hr, 4 days, and 2, 3, 4, 6, 8, and 10 weeks. Heart and mediastinal lymph nodes, thymus, spleen, liver, lung, and kidney were fixed in cold ethyl alcohol and embedded in paraffin, and 4-µ sections were prepared. Tissues from mice injected with group D streptococcal cell walls and buffer-injected mice were prepared in a similar manner.

Tissue sections from the above rabbit and mouse samples were stained with each of the following 125I-labeled globulins: anti-A polysaccharide, anti-A polysaccharide absorbed with A-variant cell walls, anti-A-variant polysaccharide, anti-A-variant polysaccharide absorbed with A cell walls, anti-A mucopeptide, and normal globulin.

Six rabbits were injected intradermally with group D cell wall fragments and tissue from the site of injection obtained at 8, 24, and 48 hr after injection. Two samples were obtained at each time interval, and cryostat sections were stained with the 125I-labeled anti-group D cell wall, anti-group A cell wall, anti-group A mucopeptide, and normal globulin. Four rabbits were injected intradermally with 200 µg of mucopeptide derived from group A streptococcal cell walls by formamide extraction (4). Injections were made on both flanks at 48, 24, 8, 4, and 1 hr and 10 min before killing the animal. Tissue samples were obtained from each of the injection sites, fixed in formalin, and embedded in paraffin. Sections of 4 µ were stained with 125I-labeled anti-mucopeptide or normal rabbit globulin and were examined by autoradiography as described above.

### RESULTS

Evidence for modification of group A polysaccharide in tissue of the mouse and rabbit. One day after intraperitoneal injection of group A cell wall fragments into the mouse, the cell walls were widely distributed in the phagocytic cells of the spleen, liver, mediastinal lymph nodes, and, to a much lesser extent, in the adrenal, lung, and peripheral lymph nodes, as shown by reaction with 125I anti-group A polysaccharide. The reaction of this material with labeled antibodies was similar to that of the cell wall suspension prior to injection; that is, the anti-A variant antibody absorbed with A cell walls did not react with the tissue sections or showed only a questionable reaction, by autoradiography (Table I). However, by 4 days after injection, and throughout the period of observation, autoradiographs of lymphatic tissues stained with this same anti-A-variant radioantibody showed heavy localization of antigen within macrophages in lymphatic tissue and liver. It is apparent that the mouse can convert the A polysaccharide to the variant structure. This reaction must proceed rather slowly, but, between 1 and 4 days, enough of the variant antigen has accumulated to be discerned in the same tissue areas in serial consecutive sections. Significant amounts of both antigenic forms are still present at 10 weeks. These results are illustrated in Fig. 1a-d.

The modification of antigenic structure of the polysaccharide in rabbit skin was substantially different from that observed in the mouse. Group A polysaccharide antigen could be found within macrophages for as long as 8 weeks in some animals (7). Two rabbits developed an exacerbation

### Table 1. Autoradiography of tissue sections stained with 125I-labeled antibodies

<table>
<thead>
<tr>
<th>Time after injection</th>
<th>No. of animals examined</th>
<th>Anti-A</th>
<th>Anti-A absorbed with A-variant cell wall</th>
<th>Anti-A variant absorbed with A cell wall</th>
<th>Anti-mucopeptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse tissue 0wk</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>1 day</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4 days to 10 wk</td>
<td>13</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rabbit skin injection sites</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 hr to 8 wk</td>
<td>12</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>13 wk</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

* Zero-time represents when smears of cell walls are made before injection.
FIG. 1. Autoradiographs of mediastinal node from mouse injected intraperitoneally with group A streptococcal cell walls 10 weeks previously. (a) Stained with $^{125}$I-labeled anti-A polysaccharide absorbed with A-variant cell walls. $\times 420$. (b) Serial section of same area of node stained with $^{125}$I-labeled anti-A-variant polysaccharide absorbed with group A cell walls. $\times 420$. (c) Serial section of same area of node stained with $^{125}$I anti-mucoprotein. $\times 420$. (d) Hematoxylin and eosin stain of serial section of same area of node to show type of macrophage with which the antigens are found. $\times 1,200$. 

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of the nodular skin lesion 13 weeks after injection. Tissue sections of the lesions from these two animals showed no group A polysaccharide antigen, but reacted strongly in the autoradiographs with anti-mucopeptide and anti-A variant antibodies. However, the anti-A variant serum absorbed with A cell walls was negative in the tissue autoradiographs. This absorbed serum still had 71% of the initial 125I globulin and still reacted with A-variant cell walls. Therefore, the antigenic structure persisting after this long period in rabbit tissue was neither A nor A-variant antigen but a third specificity present in both structures. We must assume that although this proposed structure is present on the group A preparation (since it can absorb the antibody in the anti-variant serum), it was not immunogenic in the rabbits immunized with group A streptococcal vaccine.

Degradation of group D streptococcal cell walls and mucopeptide isolated from group A cell walls. Tissue sections from rabbit skin sites injected at various intervals with 200 μg of mucopeptide were stained with the 125I labeled antimucopeptide. Autoradiographs revealed the following sequence of events. Within 10 min after injection, the mucopeptide antigen was associated with a pale green amorphous interfibrillar material (Fig. 2a). Within 1 hr, the collagen bundles were separated by edema and the antigen was distributed over a larger area, but only associated with the amorphous interfibrillar material (Fig. 2b). At 4 hr, the mucopeptide antigen was accumulated into more dense areas, the tissue was greatly swollen, and polymorphonuclear leukocytes were appearing and seemed to aggregate around the collections of antigen (Fig. 2c). At 8 hr, there was an intense accumulation of polymorphonuclear neutrophils. No mucopeptide antigen could be detected at this time (Fig. 2d) or in the tissue sections removed at 24 and 48 hr after injection. The sequence of histological events was similar to that described previously (1). At no time could mucopeptide be seen intracellularly, nor was it associated with the collagen bundles.

In the rabbit skin sites injected with group D cell wall fragments, no cell wall material could be detected at 8 or 24 hr after injection, by use of either homologous anti-group D cell wall antibody or anti-group A mucopeptide. Likewise, in mice injected intraperitoneally with group D cell walls, no cell wall material could be detected at 24 hr or at any longer interval up to 6 weeks in any tissues studied.

**Discussion**

When group A streptococcal cell wall structures are injected into animals, two of the major antigenic components—mucopeptide and group-specific polysaccharide—persist together in the tissue for a considerable period. In the rabbit skin, these antigens can be demonstrated in the area of injection for at least 8 weeks, although this is highly variable between individual rabbits and is related to the inflammatory reaction of the animal (7). This is in contrast to the rapid degradation of the mucopeptide from which the polysaccharide has been removed. As this report shows, the isolated mucopeptide cannot be detected at the inoculation site 8 hr after injection. Schmidt (8) has shown that the isolated polysaccharide moiety is rapidly eliminated from tissue. The cell walls from group D streptococci are also rapidly removed or degraded in rabbit and mouse tissue.

Both the group D cell walls and the mucopeptide isolated from group A cell walls are readily lysed by egg white lysozyme, and neither material can invoke a chronic granulomatous inflammation comparable to that produced with group A cell walls, which are resistant to lysozyme. We have previously (1) that mucopeptide is the toxic moiety of the cell wall structure, since isolated mucopeptide induces a severe dermonecrotic reaction in rabbits. The polysaccharide functions to mask the mucopeptide and protect it from tissue lysozyme, so that the mucopeptide-polysaccharide complex produces a chronic irritation, whereas the mucopeptide alone produces an acute toxic reaction followed by complete healing. Our present studies support this hypothesis by demonstrating that mucopeptide is indeed rapidly eliminated from an injection site, presumably by tissue lysozyme. The rapid elimination of group D cell walls also emphasizes the importance of the polysaccharide structure and helps explain the unique role of group A cell wall structures as agents in experimental models of connective tissue disease (2, 9). Table 2 summarizes the relationships among lysozyme susceptibility, persistence in tissue, and nature of the toxic reaction.

By passage of group A streptococci through mice, variant strains have been obtained which have lost the group-specific C polysaccharide (10). The chemical basis for this alteration in antigenic specificity has been described by McCarty and Lancefield (5, 6). The specificity of the group A carbohydrate is largely dependent upon N-acetylgalactosamine residues which have a terminal position on rhamnose oligosaccharide side chains. In the A-variant carbohydrates the terminal N-acetylgalactosamine groups are absent, and antibody against the variant structure is specific for the rhamnose side chain.

Karakawa et al. (3) have shown that many human sera have antibodies against both group
Fig. 2. Autoradiographs of rabbit skin at intervals after intradermal injection of group A mucopeptide. Stained with 125I-labeled anti-mucopeptide and Giemsa. × 420. (a) At 10 min after injection, silver grains are associated with amorphous green interfibrillar material. (b) At 1 hr after injection, antigen is widely dispersed in amorphous interfibrillar material and does not appear associated with collagen bundles. (c) At 4 hr after injection, antigen is accumulating and infiltration of heterophils is pronounced. (d) At 8 hr after injection, mucopeptide antigen is completely removed and large numbers of heterophils are present.
A and A-variant polysaccharides, and the concentration of these antibodies correlated with the antistreptolysin O titers. Since isolation of A-variant streptococci from humans has not been reported, it would appear that the stimulus for antibodies against the A-variant is dependent upon an in vivo phenotypic conversion of group A carbohydrate to the A-variant structure. Presumably this would occur through the action of a β-N-acetylgalcosaminidase. The present studies provide a direct demonstration of the antigenic modification of group A to A-variant polysaccharide in the spleen, lymph nodes, and liver of C3H strain mice.

This modification of antigenic structure cannot be demonstrated in the skin of rabbits. The rabbit apparently slowly degrades this material by removing N-acetylgalactosamine and the rhamnose oligosaccharide side chains from A polysaccharide. After 13 weeks in situ, cell wall antigen could still be demonstrated in relapsing nodular skin lesions, but the persistent antigenic structure at this time had neither A nor A-variant specificity. It is proposed that this represents an antigenic linkage in the “backbone” structure which is common to both A and A-variant polysaccharides.

Subjective interpretation of the autoradiographs of rabbit skin tissue suggested a gradual decrease in relative concentration of the group A specificity through the first 8 weeks after injection, finally becoming negative by 92 days. A comparable change in relative intensity of silver grains could not be observed in the autoradiographs of mouse tissue. Quantitative determination of the modification and distribution of antigenic structures in mouse tissue is being pursued. The distribution of cell wall material relative to the rheumatic-like cardiac lesions of the mouse (2) will be presented in a separate communication.

E. Ayoub and L. Wannamaker (Federation Proc. 26:581, 1967) have recently demonstrated the presence of a β-N-acetylgalcosaminidase in human leukocytes and rabbit macrophages. In contrast to the enzyme of similar specificity from the soil Bacillus 2aSM, the tissue enzyme can only degrade the group A polysaccharide at a very high ratio of enzyme to substrate. The very prolonged period required to remove group A specifically in the rabbit skin is perhaps related to this apparent inaccessibility of the group A polysaccharide for the tissue enzyme.

The validity of these conclusions obviously depends upon the fact that the antibodies are specific for structures on the A and A-variant polysaccharides. This appears a reasonable assumption since the antigens were derived by formamide extraction of cell walls treated with enzymes and dapsone. The polysaccharide was reprecipitated three times with acetone, and it gave a quantitative analysis of rhamnose, total reducing sugar, and nitrogen which would allow for very little contamination. The antibodies eluted from immune precipitates formed with these antigens showed only single precipitin lines in immunodiffusion.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grant AI-00949 from the National Institute of Allergy and Infectious Diseases.

S. H. Ohanian received Public Health Service Training Grant GM-01138.

LITERATURE CITED


