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Extraction of Group A Streptococcal M Protein with Nitrous Acid

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Specific precipitating and opsonic neutralizing antigens were identified in dilute-nitrous acid extracts of group A streptococci. This was done by decreasing the temperature and time of exposure of the streptococcal cells to the nitrous acid. Ninety percent of 31 strains of group A streptococcal dilute-nitrous acid extracts formed precipitin lines of identity with conventional Lancefield extracts of the same group A streptococci.

Several procedures have been used to extract the M protein antigens of group A streptococci (1, 2, 5-11, 13, 15, 17, 18, 21). Some of these procedures have been proven to extract both the precipitating and opsonic neutralizing activities of the M protein (2, 9, 21), but others have not (5, 6, 15). The Lancefield hot-acid extraction technique extracts both the precipitating and opsonic neutralizing activity (22). It is the only procedure employed in the precipitin test that is used in epidemiological typing of group A streptococci because the rabbit antisera are absorbed to be specific for the Lancefield extracts.

We have previously reported on the use of nitrous acid for extraction of group carbohydrate (3, 4) and the precipitating portion of the M protein (5) of group A streptococci. A further modification of the nitrous acid extraction is described in this report. This modification allows for the extraction of both the precipitating and opsonic neutralizing activities of M protein. A comparison of the modified dilute-nitrous acid and Lancefield hot-HCl extracts used in capillary precipitin typing is also reported.

MATERIALS AND METHODS

Strains and sera. Group A, type-specific streptococci were obtained from R. L. Lancefield, Rockefeller University, New York. Group A, M type-specific antisera were obtained either from the Centers for Disease Control, Atlanta, Ga., or from the Biomedical Research Center for Infectious Diseases.

Modified dilute-nitrous acid extraction. The method for extracting M proteins with HNO_2 is as follows: streptococcal cells from 250-ml Todd-Hewitt broth overnight cultures were collected, washed two times in saline, and then suspended in 0.5 ml of saline. The cell suspension was cooled to 0°C, and 200 μl of 4 M sodium nitrite was also precooled to 0°C, added, and mixed thoroughly. Then 100 μl of glacial acetic acid (room temperature) was added and mixed immediately. The HNO_2 reaction was allowed to occur for 5 min in an ice bath at 0°C. Nitrous acid reaction was stopped by raising the pH to 7 to 7.4 with 5 M sodium hydroxide. The reaction time was calculated from the time the last drop of acetic acid was added and promptly mixed.

Supernatants represented the extracts and were collected at 3,000 rpm for 30 min and tested for precipitation. The extracts to be tested for antiopsonic properties were dialyzed against 0.01 M phosphate buffer (pH 7 to 7.4) overnight at 4°C by using Spectrapor membranes (6,000 to 8,000 molecular weight cut-off; Spectrum Medical Inc., Los Angeles, Calif.).

Effect of temperature and time on HNO_2 extraction of M proteins. HNO_2 extractions were also performed at 25 and 37°C and in a boiling-water bath at 100°C. The effect of exposure time to the acid was also studied. In such cases, all reagents and cell suspensions were kept at 25°C before extraction procedures, which were carried out for 2, 5, 15, and 60 min and 20 h.

Streptococcal cell walls. Cell walls were prepared from whole heat-killed cells according to the procedure described by Salton and Horne (16) by using a Mickle disintegrator and Ballotini beads number 12. Tri-butyl phosphate prevented foaming during the disintegration process. Cell walls were lyophilized and stored at 4°C until used.

Extraction of M proteins from cell walls by HNO_2. A 50-mg sample of lyophilized cell walls was suspended in 0.5 ml of saline, and the extractions were performed by the same procedures used for whole cells.

Extraction of M proteins by hot-HCl procedure. M proteins were extracted from whole cells or cell walls according to the procedure described by Lancefield (11); cells were collected from 50 ml of overnight cultures and boiled in an HCl solution at pH 2 for 10 min.

Indirect bactericidal neutralization tests. Bactericidal neutralization tests were performed as previously described (5).

Whole cells were trypsinized by using 1% trypsin (Worthington) in 0.1 M phosphate buffer (pH 7.8) for 2 h at 37°C. Capillary precipitin tests were carried out by the method of Swift et al. (20), and the double gel diffusion was performed by the procedure described by Rotta et al. (14).

RESULTS

Effect of time and temperature on HNO_2 extractions. The temperature of HNO_2 extractions and the exposure time of cells to HNO_2 are crucial variables, affecting the resultant extract.

Precipitation reactions were observed in HNO_2 extracts when the extractions were performed at 0, 25, and 37°C for 15 min, but not in extracts obtained at 100°C for 15 min. However, a precipitin line of identity between HNO_2 and HCl extracts with homologous M6 antiserum formed in gel diffusion tests only when the HNO_2 extract had been obtained at 0°C. Lines of partial identity formed between homologous antiserum and HCl and HNO_2 extracts obtained at 25 and 37°C. The precipitin reactions of the HNO_2 extracts were specific, in that no precipitin reactions were observed with available heterologous M antisera (Table 1).

The length of time the cell walls were exposed to HNO_2 had less effect on the resultant extract than the temperature at which the extraction was performed.

Lines of complete identity formed in gel diffusion tests between HCl and HNO_2 extracts with homologous antiserum when the HNO_2 extracts were obtained by extraction at 0°C for 2, 5, 15, and 60 min. When the extraction was allowed to proceed for 20 h, a line of partial identity formed between the extracts. Lines of partial identity in gel diffusion tests formed between the HCl and HNO_2 extracts regardless of

TABLE 1. *Precipitation reactions of M6 proteins extracted from whole cells by HNO_2 for 15 min at various temperatures*

Extraction temp (°C)	Precipitation in capillaries		Precipitation in gel diffusion and identity with HCl extracts	
	Homologous M6 sera	Heterologous sera[a]	Homologous M6 sera	Heterologous sera[a]
0	+[b]	–	Complete identity	–
25	+	–	Partial identity	–
37	+/–	–	Partial identity	–
100[c]	–	ND	No reaction	ND

[a] Heterologous sera used were rabbit immune sera types 1, 3, 5, 11, 12, 13, 19, 24, 25, 26, 27, 28, 29, 30, 31, 33, 36, 39, 40, 46, 47, 49, 51, 52, 57, 59, 60, 62, 65, and 66.

[b] +, Positive; +/–, weakly positive; –, negative precipitation. ND, Not done.

[c] Boiling-water bath.

TABLE 2. *M6 indirect bactericidal neutralization test*[a]

Test mixture[b]	CFU per dilution of M6 streptococci[c]:			
	10^{-4}	1:4	1:16	1:64
M6 culture (inoculum)	340	110	21	5
M6 culture + HB	TNTC	TNTC	140	60
M6 culture + NRS + HB	TNTC	TNTC	560	200
M6 culture + M6 serum + HB	2	0	0	0
M6 culture + HB + supernatant of M6 5-min, 0°C HNO_2 extract + M6 serum	TNTC	TNTC	264	22

[a] Antiopsonic properties of M6 HNO_2 (0°C, 5 min) extract.

[b] HB, Human blood; NRS, normal rabbit sera.

[c] CFU, Colony-forming units; TNTC, too numerous to count.

extraction time of HNO_2 when the extractions were performed at 25°C.

The 5-min, 0°C HNO_2 extract was tested for antiopsonic activity in the indirect bactericidal test (Table 2). This extract contained antiopsonic activity. That is, the bactericidal activity of the homologous antiserum was blocked by adding the 5-min, 0°C HNO_2 extract to the antiserum before testing the antiserum for bactericidal antibody. Addition of a heterologous dilute nitrous acid extract did not block the bactericidal activity of the M6 antiserum (data not shown).

As with type M6 proteins extracted at 0°C for 5 min, types M1 and M24 extracted under the same conditions were found to be antiopsonic, i.e., they inhibited type-specific opsonization of homologous group A streptococci in the indirect bactericidal neutralization test.

M6 precipitating proteins extracted by HNO_2 at 0°C for 5 min were destroyed when lyophilized extracts were treated with trypsin. Moreover, whole cells or cell walls pretreated with trypsin did not yield M6 proteins when subsequently extracted by HNO_2 at 0°C.

Mild nitrous acid (5-min, 0°C extracts of M protein types 1, 3, 5, 6, 11, 12, 13, 19, 24, 25, 26, 27, 28, 30, 31, 36, 39, 40, 46, 47, 49, 50, 57, 59, 60, 62, 65, and 66) contained precipitating antigens homologous to those in the conventional HCl extracts. HNO_2 extracts of two types (M29 and M33) did not give precipitin reactions, and one type (M52) gave a line of partial identity to the HCl extracts in gel diffusion tests. When these strains were extracted at 25°C for 15 min, only 6 of these extracts were completely identical to Lancefield extracts, 17 were partially identical, and the rest did not show any precipitation with homologous type-specific rabbit immune sera. Seven different types extracted by the 5-min,

0°C procedure gave precipitation reactions with homologous sera (M types 1, 5, 6, 24, 25, 30, 40). Not one of these extracts gave any reaction with the other 30 heterologous type-specific rabbit immune sera.

DISCUSSION

The present work introduces HNO_2 as a reagent for extracting M proteins from whole cells or cell walls of group A streptococci. The M proteins extracted by HNO_2 at 0°C for 5 min were trypsin sensitive, antiopsonic, and homologous to M proteins extracted by the classical HCl method. We have demonstrated that the mild-HNO_2 extracts of types 1, 6, and 24 contained antiopsonic and type-specific precipitating antigens; it remains to be proven whether the M antigens of all group A strains can be extracted in the same manner.

Recent reports imply that the precipitating and antiopsonic properties of M proteins belong to two different antigenic determinants and give rise to two different antibody populations (5, 6). We believe that our work supports this differentiation.

Denaturation of proteins by HNO_2 occurs as a deamination process (a primary reaction), with formation of nitroso and diazo derivatives of phenolic groups as a secondary reaction (12). The effect of such chemical modification on the biological and immunological properties of M proteins was found to be related to the extent of denaturation. In the work reported here, we were able to manipulate the denaturation process by using different temperatures and times for extraction of M proteins by HNO_2, and we obtained various extracts with different immunological properties. The variable extent to which the immunological properties of M proteins resist denaturation suggests very strongly that the two properties (precipitating and antiopsonic) are not due to a single antigenic determinant but to different ones.

With mild conditions for extraction (0°C for 5 min of exposure to HNO_2), the extracts of M types 1, 6, and 24 demonstrated precipitating and antiopsonic properties. At higher temperatures and longer exposure time (25°C for 15 min), the antiopsonic property was lacking in the extracts which gave type-specific precipitation with their homologous type-specific rabbit immune sera. Such results can be explained on the basis of the differentiation of precipitating and antiopsonic determinants, and it can be said that precipitating determinants are more resistant to denaturation by HNO_2 than antiopsonic ones. At higher temperatures, the denaturation process continued further, and neither precipitating nor antiopsonic properties were detected.

These results were obtained by using cells collected from 250 ml of overnight cultures or 50 mg of lyophilized cell walls, and a change in these amounts of cells will be accompanied by a change in the immunological properties of the resultant HNO_2 extract. By increasing the amount of cells, we were able, using the same amount of reagents used in the dilute HNO_2 extract and performing the reaction at 25°C for 15 min, to prepare an extract which appears to be identical to M6 HCl extracts. Electron microscope studies on M proteins have been reported by Swanson et al. (19). These authors used HNO_2 to remove group A polysaccharide from streptococci. The details of the procedure for performing these extractions were not given, but the resultant extracts did not contain M proteins. According to our findings, such data can be interpreted as a complete denaturation of the extracted M proteins which was probably due to the cell/acid ratio used. Similar results were obtained in our case; HNO_2 extractions of M12 at 25°C for 15 min did not yield any detectable M proteins. However, when the nitrous acid extraction was carried out at 0°C for 5 min, the extracted M12 proteins were found to be identical to the HCl-extracted M12 proteins.

A type-to-type difference was also observed in response to the action of HNO_2 on the different extracts. Twenty-eight different types extracted at 0°C for 5 min yielded complete identity with their respective HCl extracts, one type was partially identical, and two types did not demonstrate any type-specific precipitating properties.

We believe that the results we obtained with the 5-min, 0°C HNO_2 extract indicate that the procedure may be a useful tool in the epidemiological typing of group A streptococci. Even though we did not achieve 100% agreement with the Lancefield typing system, future experiments may prove the HNO_2 procedure to be a very useful adjunct to the Lancefield system.

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