

IMMUNOLOGICAL STUDIES ON A C POLYSACCHARIDE COMPLEX
OF GROUP A STREPTOCOCCI HAVING A DIRECT
TOXIC EFFECT ON CONNECTIVE TISSUE*

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Group A streptococci contain a toxic cellular component which produces chronic remittent and intermittent nodular lesions in the dermal connective tissue of rabbits following a single intradermal injection (1). The toxic material appears to have a direct toxic effect on the dermal connective tissue, producing areas of fibrinoid necrosis and an associated granulomatous reaction. After a single injection of this material recurrent acute lesions have been observed to occur as late as 53 days after the injection, suggesting that the toxic material is capable of remaining in the tissues and of producing repeated injury over a long period of time. The association of Group A streptococci with certain of the chronic diseases of connective tissue, such as rheumatic fever, combined with the nature of the experimental connective tissue lesion, described here and elsewhere (1, 2) warrant further investigation of the properties of this toxic substance.

Recent studies have revealed that the toxic material is a macromolecular complex of the group-specific C polysaccharide and a peptide; the polysaccharide moiety being an essential part of the toxic activity (3). Additional studies have demonstrated that the ability to produce the connective tissue lesion is related to the physical dimensions of the polysaccharide-peptide complex. The isolated polysaccharide hapten with a molecular weight of about 8,000 displays no toxic activity. At the other extreme, large cell wall fragments are toxic but are less active on a weight basis than fragments of intermediate size (4). This communication presents immunological data showing that this toxic cellular component can be neutralized by antibodies directed against the C polysaccharide moiety.

Materials and Methods

Extraction of Cells.—The streptococci were grown as previously described (1). Cells from 1 L of an 18 hour broth culture were collected by centrifugation, washed 2 times with equal

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volumes of cold saline, and resuspended in 15 ml. pH 7.0 phosphate buffer. This suspension was then subjected to 1 hour sonic oscillation in a Raytheon 9 kc. sonic oscillator. The sonicate was centrifuged at 36,000 *G* for 30 minutes in a Spinco No. 40 rotor, and the supernate filtered through a Selas 02 filter to yield the sterile crude extract.

Fractionation of the crude extract was carried out as before, using the same terminology (3). Thus, the toxic cellular component was partially purified by collecting the sediment from the crude extract after centrifugation at 75,000 *G* for 1 hour. This sediment was washed 2 times with pH 7.0 buffer and labeled 75p60-3. It was stored at -20°C . Further purification was achieved by subjecting the 75p60-3 suspension to moving boundary electrophoresis in a Tiselius cell. The slowest moving fraction was collected and labeled electrophoretic trailing fraction (3).

Immunization of Rabbits.—Rabbits were immunized according to the schedule and dosage of McCarty and Lancefield (5). The vaccine consisted of heat-killed, pepsin-treated streptococci of strain C203/29/4, Group A, Type 3, (avirulent). The animals were bled from the heart 7 days after the last injection and 2 days prior to challenge.

Organisms.—The following Group A streptococci were kindly provided by Dr. Rebecca Lancefield: Type 1, strains K43 original C and K43 variant C; Type 3 strain C203/29/4, original C, (no M-avirulent), strain C203/42/3, original C (good M-virulent), and strain B455, variant C. Strain K43, variant C was derived from K43 original C. Strain B455 was derived from the same stock as C203/29. All of these strains have Type 1 T antigen (5).

The rabbits were immunized with the Type 3, C203/29/4 strain, original C. The antiserum was absorbed with strain B455, variant C. The sonic extracts and fractions used for the challenge of immunized animals and *in vitro* neutralization studies were prepared from a Type 1 strain used in previous studies.

Immunochemical Analysis.—The quantitative precipitin determinations were carried out by the method described by Kabat and Mayer (6) and the nitrogen was determined by nesslerization.

Histological Studies.—Tissue sections were prepared from formalin-fixed tissues and stained with hematoxylin and eosin.

Estimation of Toxic Activity.—Quantitation of toxicity was performed by dividing the maximum area, in millimeters, of the dermal lesion by the time required, in days, for the gross appearance of the lesion (3). This is referred to as the index value. The index values recorded in this paper represent the average of at least 6 rabbits.

RESULTS

In Vivo Studies.—

Six rabbits, hyperimmunized to yield a high anti-C titer, were injected intradermally with a 75p60-3 fraction of the sonic extract of a Type 1 organism. The partially purified fraction was used to reduce the complications of hypersensitivity, by eliminating several antigenic components of the crude extract. The animals received 0.2 ml. of the undiluted material on one flank and a 1/10 dilution in buffer on the opposite side. Six control rabbits were injected similarly.

The gross and microscopic features of the reaction in normal rabbits have been described in detail elsewhere (1, 2). The initial erythema and edema were similar in the immunized and control groups during the first 24 hours. In the non-immunized control group this initial response subsided to a negative or very minimal reaction within 2 to 3 days, and then evolved into the typical multinodular lesion in 4 to 8 days following the injection. The total area of involve-

ment was approximately 70 X 40 mm. The immunized animals developed single small nodules averaging 20 X 15 mm. at the sites of injection which subsided over a period of 10 to 18 days. The multinodular lesion did not occur in these immune animals nor were any exacerbations observed during daily inspection over a period of 50 days. Data comparing the index values for these two groups, seen in Table I, demonstrate the development of a definite active immunity. It should be pointed out that the rabbits were immunized with the C203/29/4 strain of a Type 3, Group A organism. This strain possessed no M protein and was avirulent. In addition the vaccine had been treated with pepsin. The challenge material was extracted from a Type 1, Group A organism.

The following experiment provides information pertinent to subsequent immunity studies.

TABLE I
*Immunization of Rabbits with a Group A Streptococcal Vaccine. Index Values Following Challenge with a 75p60-3 Fraction**

	Undiluted	1/10 dilution
Immunized †	83	25
Control †	428	215

* Index refers to maximum area of lesion in millimeters divided by time in days required for gross appearance.

† 6 rabbits per group.

A comparison was made between the lesion-producing properties of Group A organisms possessing the normal antigenic form of C polysaccharide and strains derived from these organisms which produced an antigenic variant of the polysaccharide (5). Cultures and sonic extracts of each organism were treated identically and a comparable amount of each extract, based on total nitrogen values, was injected intradermally.

As may be seen in Table II, there was no significant difference between strains possessing the original C polysaccharide and those with the variant form. It may be noted also that there is no difference between strains possessing or lacking the M protein.

According to McCarty (5) immunization of rabbits with organisms possessing the original polysaccharide yields antiserum giving very little cross-reaction with the variant polysaccharide. In view of this one would expect that if the resistance displayed in the first experiment was due solely to the antibodies against C polysaccharide, there should be no immune resistance displayed against the toxic component obtained from a strain producing the variant polysaccharide. Therefore, groups of immunized and control rabbits were challenged on one flank with an intradermal injection of the crude sonic extract (36s30-1) of a Type 1 organism possessing the original polysaccharide, and on the opposite

flank with a Type 1 organism possessing the variant strain. The animals had been immunized with the Type 3 organism as described in the first experiment. Table III shows that there is a 19-fold difference in the index between the im-

TABLE II
Comparison of Group A Streptococcal Strains Producing the Original and Variant C Polysaccharide

Strain*	Type	Produce M antigen	Index
K-43, original C	1	Yes	428
K-43, variant C	1	Yes	517
C203/42/3, original C	3	Yes	267
C203/29/4, original C	3	No	280
B455, variant C (derived from C203/29 stock)	3	No	447

* All strains have Type 1 T antigen.

TABLE III
Homologous and Heterologous Challenge of Rabbits Immunized with Group A Streptococcal Vaccine

		Challenge	
		Homologous (Group A polysaccharide)	Heterologous (variant polysaccharide)
Immunized	Index	44	145
	$\frac{\text{No. with relapse}}{\text{Total}}$	0/6	1/6
Control	Index	835	504
	$\frac{\text{No. with relapse}}{\text{Total}}$	4/6	2/6
Ratio	$\frac{\text{Control index}}{\text{Immune index}}$	19	3.5

mune and control groups in the case of the homologous challenge. There is also a difference in the relapses occurring in the two groups. On the other hand there is a 3.5-fold difference between the index of the immunized and control animals at the sites injected with the heterologous (variant polysaccharide) material. This difference is also significant at the 0.01 level when the data is subjected to statistical analysis. One relapse also occurred with this heterologous challenge

in the immunized group. Since the toxic component is a very complex molecule it is possible that other directive groups besides the C polysaccharide are antigenic. If such directive groups are sufficiently similar in the three strains of organisms involved in this experiment, this may explain the small but significant degree of resistance against the extract of the variant strain.

While there is no question that the process of immunization vastly modifies the reaction to this toxic material, there remains some doubt regarding the completeness of the neutralization possibly due to the fact that the minimal reaction seen in the immunized group resulted from the hypersensitive state developed by the immunizing procedure. To help resolve this problem a group of 15 rabbits, immunized in the same manner, and 15 control rabbits, were in-

TABLE IV
*Immunization of Rabbits with Group A Vaccine and with Group D Vaccine (Streptococcus durans)**

	Index	$\frac{\text{No. with relapse}}{\text{Total}}$
Immunized (Group A vaccine).....	34	0/4
Control.....	474	2/5
Immunized (Group D vaccine).....	396	Persistent plaques

* Animals were killed at intervals from 3 hours to 35 days after challenge for histological comparison of lesions. These figures are from animals kept more than 10 days.

jected intradermally with 0.2 ml. of the same Type 1 crude sonic extract (36s30-1) as was used in the previous experiment. The features of the lesion observed in the gross in the two groups were comparable to those described in the above experiment. Two animals from each group were killed at the following intervals after injection and the lesions examined microscopically: 3 hours, 12 hours, 24 hours, 5 days, 10 days, 21 days, 35 days. Table IV compares the index values for those immune and control animals which were kept long enough to observe the development of lesions. Here again, there is a large difference (14 X) in the extent of the lesions in the two groups, as well as the absence of exacerbations in the immune rabbits. In this experiment another group of animals was included which had been immunized, by the same technique, with a vaccine of a Group D organism, *Streptococcus durans*. This was included as an additional control on the specificity of the immunization process. It can be seen in Table IV that there is no difference between the lesions in normal animals and those immunized with *S. durans*.

The comparative histological studies were in keeping with the gross features. Sections of the lesions which developed in the control animals revealed the histological changes previously described as being associated with the various

stages of this reaction (1, 2). Sections of the lesions which developed in the immunized animals and which were fixed 3, 12, and 24 hours after challenge with the toxic material, revealed changes comparable to those observed in the control animals. It should be noted that the reaction in the immunized group was restricted to a small area of the skin as indicated by the size of the lesions as they appeared in the gross.

Sections fixed 5, 10, 21, and 35 days after challenge revealed a marked difference between the control and immunized groups. In the immunized groups only moderate evidence of a chronic reaction was noted. This consisted of focal accumulations of macrophages and lymphocytes. This reaction appeared to gradually subside. In the sections fixed 35 days after challenge only scattered single lymphocytes and macrophages were noted in the dermal connective tissue which in other respects appeared normal. The sections from the control animals which were killed 5, 10, 21, and 35 days after injection revealed the combined acute and chronic inflammatory reaction, the histologic features of which have been described in detail elsewhere (1, 2). In none of the sections studied was there evidence of vascular necrosis or thrombosis which would suggest the possibility of a hypersensitivity reaction playing a part in the changes observed.

In Vitro Studies.—Additional evidence that the resistance of immunized animals is the result of a specific immune response directed primarily against the C polysaccharide moiety of the toxin, is provided by *in vitro* studies.

Both the immunized and normal animals were bled from the heart 7 days after the last immunizing dose and 48 hours prior to the *in vivo* challenging injection, and the serum frozen at -20°C . Group A, Type 3, strain B455 cells were collected from 4 L of broth culture, washed 2 times, and subjected to 1 hour sonic vibration. The whole suspension was then dialyzed against distilled water for 2 days in the cold room and lyophilized. The B455 strain produces the antigenic variant of C polysaccharide and was derived from the same stock as C203/29/4, which was the strain used to immunize the rabbits. If we assume that the B455 strain is identical with the parent strain in all antigenic moieties except the C polysaccharide, then it should be possible to absorb the immune serum with the variant strain to yield antiserum reacting only with C polysaccharide. Thus, 50 mg. of the lyophilized extract was added to 30 ml. of pooled serum from 6 immunized rabbits. The precipitate developing after incubation at 37°C . for $\frac{1}{2}$ hour and 4°C . for 6 hours was removed by centrifugation at 2,000 R.P.M. This absorption was repeated 4 times with a total of 250 mg. of extract at which time the supernate gave no reaction in a ring precipitate test with the sonic extract of the B455 strain, but still showed a good precipitin reaction with the homologous extract as well as with C polysaccharide prepared by the formamide method. This absorbed serum was then centrifuged at 140,000 G in the Spinco No. 40 rotor for 1 hour to remove the lesion-producing component present in the B455 sonic extract (3). Merthiolate was added in a final concentration of 1:10,000 and the serum frozen at -20°C . Samples of the absorbed and unabsorbed sera were utilized in the Oakley agar gel diffusion technique as described by Deicher (7).

The data in Table V show that only antibodies against C polysaccharide can be detected by this method in absorbed antiserum. It can also be seen that the

purified electrophoretic trailing fraction showed a second line of precipitate with unabsorbed serum, demonstrating that this fraction contains at least 2 antigenic components although it is electrophoretically homogeneous.

Two ml. of the absorbed antiserum was mixed with an equal volume of the purified electrophoretic fraction containing 37 gamma N per ml. These amounts of antigen and antibody were

TABLE V
Comparison of Absorbed and Unabsorbed Antiserum by the Oakley Agar Diffusion Technique

Antigen sample	Serum sample	
	Anti C203/29/4	Anti C203/29/4 absorbed with B455
Sonic extract of C203/29/4.....	4*	1*
Sonic extract of B455.....	2	0
C polysaccharide (formamide).....	1	1
75p60-3 fraction.....	2	1
Electrophoretic trailing fraction.....	2	1

* No. of lines of precipitate developing during 3 weeks observation.

TABLE VI
Neutralization of Purified Toxin with Anti Streptococcal Serum Absorbed with Variant Cells

Sample injected	Index	$\frac{\text{Relapses}}{\text{Total animals}}$
Antiserum + toxin* - supernate.....	3.8	0/6
Normal rabbit serum + toxin - supernate.....	666	6/6
Saline + toxin - supernate.....	616	5/6
Antiserum + toxin - sediment resuspended in saline.....	1.8	0/6

* The purified electrophoretic trailing fraction of sonic extract.

known, from quantitative immunochemical studies, to yield a mixture in relatively great antibody excess (8). Following incubation at 25°C. for ½ hour and 4°C. for 24 hours, the resulting precipitate was collected at 2,000 r.p.m. for ½ hour at 2°C. The precipitate was resuspended in saline to the original volume. Similar samples of this electrophoretic fraction were incubated with normal rabbit serum and with saline and subjected to identical treatment. Supernate ring precipitin tests confirmed the fact that the antiserum-antigen mixture was in antibody excess. Each of 6 rabbits was injected intradermally in 4 sites with samples shown in Table VI. Each inoculum was calculated to contain 7.4 gamma of toxin N, if no precipitation had occurred. This was a dose known to consistently produce a good dermal lesion.

It may be seen that in terms of the index values and number of relapses, the anti C polysaccharide antibodies completely neutralized the connective tissue toxin. These rabbits were observed almost daily for 100 days and, although re-

lapses were observed to occur as late as 80 days after injection at the sites inoculated with normal serum or saline controls, nothing, except a small 10×10 mm. lesion at 3 sites, developed in areas inoculated with supernate or resuspended sediment from the antiserum-antigen mixture.

As final proof that antibodies against the C polysaccharide moiety of the toxic molecule were responsible for neutralization, inhibition studies were carried out. For these experiments the partially purified 75p60-3 fraction was used since it was available in greater amounts than the more highly purified electrophoretic trailing fraction. The agar diffusion experiments indicate the

TABLE VII
*Titration of Neutralizing Capacity of Absorbed Antiserum**

Serum dil.	Absorbed antiserum 24 hrs. at 4°C. plus centrifugation			Normal serum 24 hrs. at 4°C. plus centrifugation			Absorbed antiserum, ½ hr. at 4° C. not centrifuged		
	Index	Av. time days†	Relapse	Index	Av. time days†	Relapse	Index	Av. time days†	Relapses
1/1	0	—	0	189	6.5	3/6	—	—	—
1/2	0	—	0	282	8.4	2/5	5	7	0
1/4	0	—	0	247	4.8	2/6	8	14	0
1/8	0	—	0	152	6.4	1/6	10	13	0
1/16	10	16	1/6	157	5.2	4/6	7	19.7	0
1/32	8	4	0	—	—	—	29	13.7	0
1/64	116	14.7	0	—	—	—	37	17.7	0
1/128	197	8.4	2/6	—	—	—	85	12.6	2/5
1/256	171	8.7	2/6	—	—	—	—	—	—
1/512	175	8.5	2/6	—	—	—	—	—	—

* Anti C203/29/4 absorbed with sonic extract of B455.

† Average time in days required for first gross appearance of lesion.

75p60 fraction plus absorbed antiserum also represents a homogeneous antigen-antibody system and should be suitable for these experiments. The activity of this crude preparation is more difficult to predict on a weight basis and therefore a titration of lesion-producing activity was carried out with an aliquot of the 75p60 preparation.

Each dilution was injected into 6 rabbits at 1 of 4 sites on each animal, and the highest dilution producing a consistent lesion within 7 days was determined. This dilution contained 15 gamma N per ml. Serial twofold dilutions of absorbed antiserum were mixed with an equal volume of the proper dilution of 75p60-2 fraction, incubated at 25°C. for ½ hour and at 4°C. for 24 hours, centrifuged at 2,000 R.P.M. at 2°C. and the supernate injected intradermally into 6 rabbits at 1 of 4 sites on each animal. Normal rabbit serum samples in dilutions of 1/1 to 1/16 were likewise mixed with the toxin and teated similarly. The order of injection of the dilutions of immune and control serum was rotated in order to avoid any possible effect an injection might have on an adjacent inoculum.

No difference in the development of a lesion was noted, regardless of whether antiserum undiluted or in a 1/512 dilution was injected on the same flank with a normal serum control site. Therefore, diffusion of antibody away from a site of injection did not occur in sufficient concentration to influence an adjacent site of inoculation.

As mentioned above the resuspended antigen-antibody precipitate was shown to have no lesion-producing capacity. From this fact it was concluded that the antibody was not merely removing the toxin by precipitation, but was actually neutralizing it. As a further test of this conclusion a second titration of antiserum was run concomitantly with that described above, with the difference

TABLE VIII
Inhibition of Neutralization by Anti-C Polysaccharide

Sample*	N in precipitate per ml. serum	Inhibition of precipitate	Supernate tests	Index
	$\mu\text{g.}$	<i>per cent</i>		
Antiserum	116	—	Antibody excess	8
Antiserum plus <i>N</i> -acetyl-D-glucosamine	69	40	Neg.-antibody and antigen	22
Antiserum plus C polysaccharide (formamide)	7.8	93	Neg.-antibody and antigen	124
Normal rabbit serum plus <i>N</i> -acetyl-D-glucosamine	0	—	Antigen excess	171

* Anti C203/29/4 absorbed with sonic extract of strain B455. Toxin was 75p60-3 fraction.

that the antibody-antigen mixture was incubated at 25°C. for ½ hour and 4°C. for ½ hour and the whole suspension injected without centrifugation of the precipitate. The results comparing the shorter incubation with long incubation plus removal of precipitate are shown in Table VII. It is evident that with dilutions of this serum above 1/32 the antiserum is much more effective if the precipitate is not removed before injection. It may also be seen that the index values increase in apparent proportion to the serum dilution when the precipitate is not removed. On the other hand, there is a precipitous drop in neutralizing capacity of the serum above 1/32 dilution when the precipitate is removed by centrifugation. This correlates very well with quantitative determinations of the precipitate (8). These results are interpreted to mean that some of the antibody which is removed in the precipitate after 24 hours incubation is still capable of reacting with more toxin; therefore, if the whole antigen-antibody suspension is injected, greater neutralizing activity of the antiserum is realized.

From this data, a 1/16 dilution of antiserum was selected for studies on inhibition of antibody neutralization. *N*-acetyl-D-glucosamine was added to one aliquot of absorbed serum to give a final molarity of 0.125. A second aliquot was absorbed with the C polysaccharide hapten

using the formamide extraction method described by Zittle and Harris (9). This relatively pure preparation contained 5.1 per cent N and showed only 1 line of precipitate with the unabsorbed antiserum in the Oakley technique. The antiserum was absorbed 3 times with this material by incubation at 37°C. for ½ hour followed by 6 hours at 4°C. and removal of the precipitate at 2,000 R.P.M. This absorbed serum showed neither excess C polysaccharide nor antibody by the ring precipitin test. A third aliquot of antiserum received no further treatment. The control consisted of normal rabbit serum plus 0.125 M *N*-acetyl-D-glucosamine. An equal volume of the same concentration of the 75p60-3 fraction used previously was added to each sample and incubated at 25°C. for ½ hour and 4°C. for 22 hours after which they were centrifuged at 2,000 R.P.M. The precipitates were washed 2 times with saline and total N determined. Each of these samples, with antigen and serum controls, was run in duplicate. These results and ring precipitin tests on the supernates are shown in Table VIII.

McCarty has shown that *N*-acetylglucosamine is the principal directive group of the C polysaccharide (10). The degree of inhibition of precipitation produced with this sugar, as well as with the C polysaccharide hapten, clearly demonstrates that the immune precipitation of the toxic material is due to antibodies reacting specifically with C polysaccharide.

Each of the supernates from these inhibition studies was injected into 6 rabbits. The inhibition of neutralizing capacity of the antiserum, while understandably not as clear cut as the quantitative precipitin data, shows that the specific neutralizing antibody is directed against the group specific C polysaccharide.

DISCUSSION

These studies clearly demonstrate that antibodies against C polysaccharide can specifically neutralize the capacity of the toxic cellular component of Group A streptococci to produce chronic remittent and intermittent lesions of dermal connective tissue. The unique histological features of the connective tissue lesion are greatly modified in rabbits actively immunized with a streptococcal vaccine. The antiserum from these rabbits, absorbed to contain only antibodies against the group-specific C polysaccharide, will precipitate and specifically neutralize the toxic material *in vitro*. Finally, this immune precipitation and neutralization can be inhibited by formamide-isolated C polysaccharide and by *N*-acetylglucosamine, the principal directive group of the polysaccharide antigen.

These findings provide further proof that the group-specific C polysaccharide is an important component of this toxic material. It should be noted, however that the isolated C polysaccharide hapten with a molecular weight of about 8,000, prepared by formamide extraction, has no toxic activity (4). As one would expect from the chemical composition of the streptococcal cell wall (10, 11), purified cell wall suspensions will produce the experimental connective tissue lesion, although they are less active on a weight basis than particles with dimensions intermediate between the hapten and recognizable cell walls (4). Thus, toxic activity is associated with a rather limited range of particle size of the polysaccharide-peptide complex.

There is considerable evidence relating the Group A streptococci to certain disease states which are characterized by widespread involvement of connective tissue. The evidence relating these organisms to rheumatic fever is very impressive. However, the mechanism by which Group A streptococci produce the tissue changes of rheumatic fever observed in a small per cent of individuals infected with these organisms, has not been made clear in spite of a great deal of speculation. Some form of hypersensitivity has been most frequently invoked in an attempt to explain the most prominent features of rheumatic fever and other connective tissue diseases which include: (a) fibrinoid necrosis of connective tissue, (b) relapses associated with sterile lesions, and (c) a prolonged course (12). A critical review of the data supporting the hypersensitivity concept, such as that presented by MacLeod (13), emphasizes the fact that it is based wholly on indirect evidence.

The properties of the toxic C polysaccharide complex described here; the gross and microscopic features of the experimental lesion produced with this toxic material; and the observations on the course of the experimental lesion, indicate that this toxic component of Group A streptococci should be investigated as a possible factor in the pathogenesis of the non-suppurative sequelae associated with these organisms. This concept, that the lesions associated with the non-suppurative sequelae are the result of a Group A streptococcal component having a direct toxic action on connective tissue, is in distinct contrast to the concept of many investigators that hypersensitivity as a result of an immune response to streptococcal antigens is the primary cause of these tissue changes.

One of the properties of C polysaccharide relevant to this discussion is its poor antigenicity under conditions of natural infection (14-16). MacLeod (13) postulates that if a streptococcal toxin is involved in rheumatic fever it would have to be a poor antigen to account for the failure of one attack to confer immunity against subsequent attacks. Rantz and Randall (15) have shown that detectable antibodies against C polysaccharide developed in only 6 per cent of patients infected with Group A hemolytic streptococci. This was in contrast to a significant increase in antistreptolysin O in over 80 per cent of the group. Of the 298 cases which were included in that study, 44, or 15 per cent, had some non-suppurative complication and 30, or 63 per cent of these, displayed no anti-C antibodies. A most interesting observation was that of the 13 complications in their category of "late fever" none developed anti-C, and none of the 6 complications in the category of "carditis with late fever" showed any antibody against C polysaccharide.

In a similar study Rothbard *et al.* (16) reported that of 83 acute streptococcal infections occurring in 71 patients, 34 were followed by rheumatic fever. Only 4 of the rheumatic fever group had detectable anti-C antibodies.

If one assumes that the toxic C polysaccharide complex plays a role in rheumatic fever, then the studies of Rantz and Randall (15) and Rothbard *et al.* (16), along with the investigations reported here, could account for the fact

that remission of the rheumatic state is not associated with evidence of increased resistance to the disease. These data could also help explain the apparent ability of the toxic substance to remain in the tissue for relatively long periods of time and to continue to produce injury in the experimental lesion (1). Relative to this idea is the observation that repeated injection of the toxic material in rabbits, in the doses used to produce a lesion, has no effect on the evolution of the lesions (2). It is only when rabbits have been hyperimmunized with a vaccine to induce production of detectable C polysaccharide precipitins that modification of the lesion is observed.

There appears, therefore, to be sufficient similarity between the experimental model produced with the toxic complex of group-specific C polysaccharide and certain connective tissue diseases related to Group A streptococci, to warrant further investigations based on this experimental approach.

SUMMARY

A macromolecular complex of C polysaccharide and peptide derived from Group A streptococcal cells produces a multinodular, remittent, and intermittent lesion of dermal connective tissue following a single intradermal injection. This lesion is greatly modified, both in the gross and microscopically, in rabbits hyperimmunized with a Group A streptococcal vaccine.

The immune serum, absorbed to yield only antibodies against C polysaccharide as indicated by agar diffusion techniques, will precipitate and neutralize the toxic material. This neutralization and *in vitro* precipitation can be inhibited with formamide-isolated C polysaccharide hapten and with *N*-acetylglucosamine, the primary directive group of this antigen. This clearly demonstrates that antibodies against the group-specific C polysaccharide are responsible for resistance to this toxic material.

The immunological and other properties of the toxic C polysaccharide complex, the fact that it is a product of Group A streptococci, the gross and microscopic features of the experimental lesion produced with this substance, and the observations on the chronic course of the experimental lesion, indicate that this toxic material should be investigated as a possible factor in the pathogenesis of the non-suppurative sequelae associated with Group A streptococci.

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