NEUTRALIZING ANTIBODIES TO STREPTOCOCCAL DIPHOSPHOPYRIDINE NUCLEOTIDASE IN THE SERUM OF EXPERIMENTAL ANIMALS AND HUMAN BEINGS*

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(Received for publication, May 9, 1958)

Many strains of Group A streptococci elaborate into the medium in which they are grown an enzyme that catalyzes specifically the cleavage of diphosphopyridine nucleotide (DPN)[1] (1, 2). The studies now to be described demonstrate that this enzyme when injected into rabbits or guinea pigs stimulates the production of antibodies that neutralize the ability of the enzyme to destroy DPN. Moreover, similar neutralizing antibodies have been detected in the blood serum of a high percentage of randomly selected hospital patients, and the titer of these antibodies was found to rise sharply following known streptococcal infections.

Materials and Methods

Antibodies directed against streptococcal DPNase, hereafter referred to as ASDA, were assayed by measuring the ability of serial dilutions of serum to inhibit the DPN-destroying activity of a known concentration of the enzyme. Optimal conditions for the assay procedure were determined, and the test was applied to blood serum obtained from experimental animals before and after specific immunization, and to serum from a large number of human beings.

Streptococcal DPNase.—Streptococcal preparations containing DPNase were generously supplied by Dr. Alan W. Bernheimer of the Department of Microbiology, New York University College of Medicine. These were for the most part fractionated culture supernates prepared from 15 liter cultures of the C203S strain of Group A streptococcus according to the method previously described (3). The streptococcal preparations were dialyzed overnight against tap water prior to use. The dialyzed solutions maintained their DPNase activity for as long as 1 month when stored at 4°C. Dilutions of the dialyzed material were made in a

* These studies were supported in part by research grants from the National Heart Institute of the United States Public Health Service and the New York Heart Association.

The authors wish to acknowledge the helpful assistance of Miss Helen Sammet throughout these studies.

[1] DPN was obtained from Sigma Chemical Co., St. Louis; its purity ranged from 90 to 95 per cent.

potassium phosphate buffer at pH 7.3 containing 0.1 per cent bovine serum albumin (Armour). Crude culture filtrates and fractions separated by continuous flow paper electrophoresis were used on several occasions and found to be satisfactory.

Assay of DPNase Activity.—DPNase activity was measured by the method of Kaplan, Colowick, and Nason (4), slightly modified as described in a previous publication (1). The unit of DPNase activity was defined as the amount of enzyme that destroys 0.01 μM DPN in 7½ minutes at 37°C.

Measurement of Antibodies to Streptococcal DPNase (ASDA).—A dialyzed streptococcal preparation was assayed for DPNase activity and diluted with buffer solution to a concentration of about 350 DPNase units per ml. Serial twofold dilutions of the serum to be tested were made in buffer solution, and 0.5 ml. of each serum dilution was placed in a test tube together with 0.5 ml. of the appropriately diluted enzyme solution. Serum dilutions of 1–4 through 1–64 were adequate to span the range of antibody titers usually encountered. In those cases, however, in which the titers were very low, undiluted serum or a 1–2 dilution was employed, and higher dilutions than 1–64 were necessary in those instances in which the titer of antibody was elevated. Two control tubes each containing 0.5 ml. of DPNase solution and 0.5 ml. of buffer were included in each run. The mixtures of serum or buffer solution and DPNase were incubated for 30 minutes in a water bath at 37°C. The amount of residual DPNase activity was then determined by taking 0.2 ml. of each mixture and adding it to a tube containing 0.4 mg. DPN (0.3 ml. of a DPN solution containing 1.33 mg. per ml.). In practice it was found expedient to pipette the DPN into tubes kept in an ice water bath while the serum and DPNase mixtures were incubating, and then rapidly to transfer 0.2 ml. of each mixture into the tube containing the DPN solution. These tubes were incubated for 7½ minutes in a water bath at 37°C. The reaction was stopped by plunging the tubes into an ice water bath, and 3.0 ml. of 1 M sodium cyanide was added immediately to each tube. Optical density was measured in a Beckman DU spectrophotometer at 340 mμ using 1.0 cm. corex cuvettes. Suitable blanks and DPN standards were included in all determinations.

Sera were stored in the liquid state for several weeks and in the frozen state for several months without loss of ASDA activity. Heating the serum at 63°C. for 5 minutes to inactivate complement had no appreciable effect on the ASDA activity.

Numerous control studies revealed that serum from rabbits, guinea pigs, or human beings had no appreciable DPNase activity under the conditions employed. In other control studies, the mixtures of serum and DPNase solution were incubated at different temperatures from 4°–56°C. and for periods of time ranging from 5 minutes to 24 hours. At temperatures above 37°C. the enzyme, known to be quite heat-labile, was inactivated even in the absence of serum. At temperatures below 37°C., the reaction between serum and enzyme often failed to go to completion. Incubation for 30 minutes at 37°C. was found to be optimal, and there was no significant change in the reaction between serum and enzyme on prolonging the incubation period beyond this point.

The anti-DPNase titer was calculated from the reduction in DPNase activity of the enzyme solution following incubation with an appropriate dilution of the serum. Since the estimation of DPNase by the cyanide method of Kaplan, Colowick, and Nason (4) is not linear beyond the point at which 60 per cent of the DPN substrate is split, ASDA activity was calculated only from those mixtures of diluted serum and enzyme which, after incubation with DPN, had optical density readings in the range of 0.400 to 0.600. The unit of ASDA activity was arbitrarily defined as that amount of antibody per ml. of serum that neutralized 100 units of streptococcal DPNase. This unit was adopted both for the sake of convenience and to make it more readily comparable with the widely employed units for measuring antibodies to streptolysin O (5). With undiluted serum, the test was capable of detecting as little as one unit of ASDA activity. When serial dilutions of serum were employed, the results were generally reproducible within a range of plus or minus 30 per cent.

EXPERIMENTAL

Immunization of Rabbits and Guinea Pigs to Streptococcal DPNase.—To learn whether antibodies to streptococcal DPNase could be produced in animals, an experiment was done in which a streptococcal preparation containing DPNase was injected into rabbits and guinea pigs, and serum obtained from these animals was tested for its ability to inhibit the enzyme.

Six normal hybrid rabbits weighing 2.5 to 3 kilos were each given an initial series of 5 intravenous injections. The injections were given every other day, and each injection consisted of 0.5 ml. of a streptococcal preparation containing 15,000 DPNase units. The animals were bled just prior to the first injection, and again 3 weeks and 10 weeks later. At the 10th week, each rabbit was given a single booster injection intravenously of 0.5 ml. of the same preparation containing 15,000 DPNase units, and the animals were bled again at the 11th and 14th week after the onset of the experiment. In another experiment, 6 normal male guinea pigs weighing 400 to 800 grams were each bled from the heart, and then given two intraperitoneal injections 2 weeks apart of 0.5 ml. of a partially purified streptococcal preparation containing 90,000 DPNase units per ml. Four weeks after the second injection they were again bled from the heart and the sera obtained before and after immunization were assayed for the presence of antibodies to DPNase.

TABLE I

Immunization of Rabbits to Streptococcal DPNase

Rabbit No.	0	3 wks.	10 wks.	11 wks.	14 wks.
	ASDA units/ml.				
1	<1	12	23	1350	235
2	<1	3	2	360	170
3	<1	<1	<1	25	21
4	<1	<1	5	33	23
5	<1	4	9	250	265
6	<1	<1	<1	10	10

Each rabbit was given initially an intravenous injection every other day for a total of 5 injections of 0.5 ml. of a streptococcal preparation containing 15,000 DPNase units, and a booster injection in the same dosage at the 10th week.

0, just prior to the first injection.

ASDA, antistreptococcal DPNase.

From the data contained in Table I it is clear that following the injection of streptococcal DPNase the rabbits developed antibodies that inhibited the activity of the enzyme. Three animals responded promptly and had measurable ASDA activity in their sera 3 weeks after the initial injections; one animal had antibodies when tested on the 10th week; in the two remaining animals no antibodies were detectable either on the 3rd or 10th week after the injections. All six animals, however, had measurable levels of ASDA activity after the

booster injection. Similarly, five of the six guinea pigs had measurable ASDA activity in their serum following intraperitoneal injection of DPNase. It is of interest that none of the animals in these experiments had detectable ASDA activity in their serum prior to the injection of DPNase. In subsequent studies, sera from numerous normal rabbits, guinea pigs, and rats were tested for ASDA activity; these were uniformly negative. On the other hand, measurable amounts of ASDA were found in the serum of four rabbits injected with live or dead streptococci of a strain known to produce DPNase.[2] It is also noteworthy

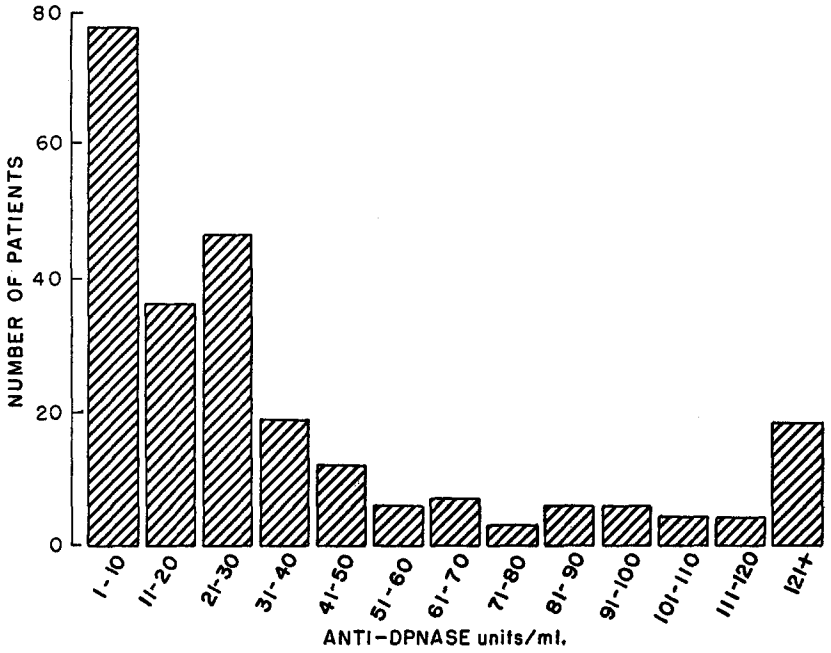

FIG. 1. Anti-DPNase titers of blood serum from 240 randomly selected hospital patients.

that in several experiments rabbit serum capable of neutralizing large amounts of streptococcal DPNase failed to inhibit the DPNase of *Neurospora crassa.*

Antibodies to Streptococcal DPNase in the Serum of Randomly Selected Hospital Patients.—The finding that streptococcal DPNase was antigenic and that antibodies capable of neutralizing the enzyme could be detected in the serum of animals following immunization suggested that similar antibodies might also be present in the serum of human beings, particularly since many strains of Group A streptococci isolated from human beings have been shown to produce DPNase when grown *in vitro* (2).

[2] For these sera the authors are indebted to Dr. Armine T. Wilson of the Alfred I. du Pont Institute, Wilmington.

Sera from 240 hospital patients were assayed for ASDA activity. The sera were selected at random from blood specimens sent to the Serology Laboratory of the New York Hospital, and virtually all were from adult male and female patients suffering from a wide variety of disease states. Without exception, all the sera examined had measurable ASDA activity. The titers covered a wide range, as illustrated in Fig. 1. In general, 80 per cent of the sera had ASDA titers of 50 units or less, 10 per cent were in the range between 50 and 100 units, and 10 per cent had 100 units or more. It is of interest, too, that two sera that were exceedingly active in neutralizing streptococcal DPNase failed to inhibit *Neurospora* DPNase.

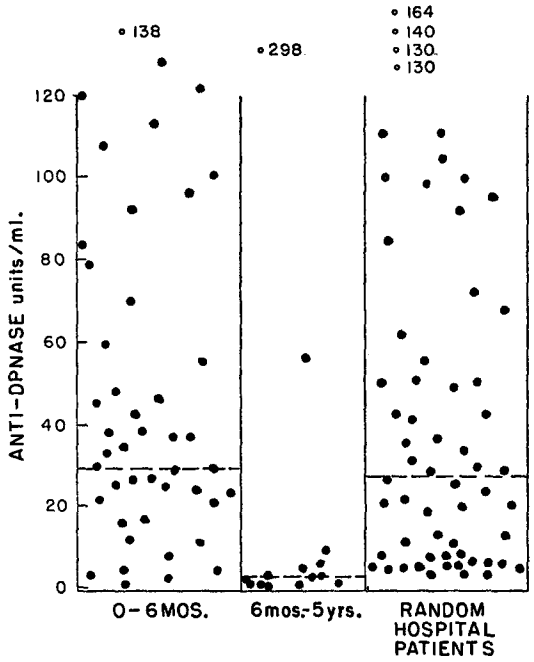

FIG. 2. Anti-DPNase titers of infants, young children, and randomly selected adult hospital patients. The horizontal interrupted lines represent the median titer in each group.

The presence of ASDA activity in all of the sera from adult human beings that were examined was not unexpected in view of the widespread prevalence of infections with Group A streptococci. The possibility was considered, however, that the ASDA activity might also be due, at least in part, to non-specific inhibitors of the enzyme rather than to antibodies resulting from exposure to DPNase-producing streptococci. It appeared of interest, therefore, to examine sera from infants and young children who because of their age were less likely to have had repeated streptococcal infections.

Serum was obtained from 45 specimens of blood from the umbilical cord of newborn infants and from infants under 6 months of age, and from 14 children 6 months to 5 years of age. Six months was arbitrarily selected as the dividing line because maternal antibodies may survive in the offspring up to this age.

Sera from 62 randomly selected adult hospital patients were included for purposes of comparison. The ASDA titers of these sera are illustrated in Fig. 2. The sera from umbilical cord blood and from infants under 6 months of age had a fairly wide range of ASDA activity, similar to that of the adult population. The ASDA levels in this age group presumably reflected those of the maternal serum. The sera from children between 6 months and 5 years, with only a few exceptions, had exceedingly low ASDA titers. In fact, 4 of these sera had no measurable ASDA activity at all. It is worthy of note that of all the sera examined from human beings these were the only ones in which ASDA activity could not be detected on repeated test. It is perhaps significant, too, that the serum with the highest level in the age group under 6 months (138 units) and the one with the highest level in the age group 6 months to 5 years (298 units)

TABLE II

Serum ASDA Levels of Normal Healthy Adults

Subject	1 wk.	2 wks.	3 wks.	4 wks.	5 wks.	6 wks.
	ASDA units/ml.					
E. B. F.	14	12	13	14	13	18
H. S.	9	10	9	11	—	13
E. P.	23	28	28	20	22	24
S. H. S.	—	15	14	15	15	13
A. K.	23	22	24	22	23	28
S. J. H.	27	21	26	26	21	24
R. L. H.	3	2	3	3	2	2

ASDA, antistreptococcal DPNase.

were both from children with fibrocystic disease of the pancreas who had had repeated severe respiratory tract infections. The findings in this study were consistent with the hypothesis that the anti-DPNase activity demonstrable in serum from human beings was due to the presence of antibodies resulting from exposure to streptococcal infections.

A study was then done of ASDA activity in the serum of normal human beings to learn whether it fluctuated significantly during a period of several weeks in the absence of overt upper respiratory tract infections.

Seven normal adult human beings, 3 males and 4 females, were bled at weekly intervals for 6 weeks and the sera assayed for ASDA activity. All the subjects had been free of upper respiratory tract infections for at least 1 month prior to the drawing of the first specimen of blood, and they had no clinically evident respiratory tract infections during the ensuing 6 week period. The ASDA levels of these individuals, listed in Table II, ranged from 2 to 28 units per ml. In any one individual, however, the ASDA levels varied only within relatively narrow limits during the period of observation.

TABLE III

ASDA Levels of Serum From Patients with Known Streptococcal Infections

Case No.	Streptococcus type	Acute illness	Convalescence
		ASDA units/ml.	
1	3	52	540
2	3	22	265
3	3	88	245
4	3	100	470
5	5	34	25
6	12	110	270
7	12	120	120
8	12	105	130
9	12	21	170
10	12	105	150
11	12	153	580
12	12	59	196
13	12	190	440
14	12	168	440
15	12	63	370
16	12	370	3000
17	14	135	155
18	14	57	140
19	14	15	140
20	14	530	930
21	14	55	1300
22	14	43	245
23	19	90	120
24	19	117	226
25	19	23	34
26	19	54	310
27	19	90	230
28	28	170	220
29	30	250	360
30	30	70	210
31	30	88	880
32	30	880	1710
33	30	115	400

ASDA, antistreptococcal DPNase.

Acute illness, serum obtained at the onset of clinical symptoms or 1 or 2 days thereafter.

Convalescence, serum obtained about 3 weeks after the initial specimen.

TABLE IV

Comparison of ASDA and ASO Titers of Patients with Known Streptococcal Infections

Case No.	Titer	1 day	21 days	35 days	60 days
1	ASDA	298	610	525	550
	ASO	100	250	—	—
2	ASDA	120	260	250	300
	ASO	250	833	—	—
3	ASDA	140	170	136	—
	ASO	1250	2500	—	—
4	ASDA	580	650	580	650
	ASO	250	500	—	—
5	ASDA	80	—	500	510
	ASO	166	—	625+	—
6	ASDA	290	220	—	—
	ASO	100	625	—	—
7	ASDA	63	110	—	170
	ASO	125	625	—	—
8	ASDA	1130	2450	3600	2890
	ASO	—	333	625+	—
9	ASDA	380	330	360	390
	ASO	250	625+	—	—
10	ASDA	7	430	—	94
	ASO	100	625+	—	—
11	ASDA	11	—	130	190
	ASO	125	625+	—	—
12	ASDA	59	2900	—	1600
	ASO	125	500	—	—
13	ASDA	189	260	240	217
	ASO	500	833	—	—

ASDA, antistreptococcal DPNase, units per ml.
ASO, antistreptolysin O, hemolytic units per ml.
—, ASDA or ASO not determined.

ASDA Levels Following Infection with Group A Streptococci.—To learn the effect of exposure to Group A streptococci on the anti-DPNase activity of human serum, a study was made of the ASDA levels of blood taken during the acute illness and again during convalescence from patients with known streptococcal infections.

Sera from 33 patients with Group A streptococcal infections of known type were obtained during the acute phase of the illness, and again approximately 3 weeks later. These sera were generously supplied by Dr. Charles H. Rammelkamp, Jr. The ASDA levels of these sera are listed in Table III. It is evident that in most cases there was a sharp rise in serum ASDA level following the streptococcal infection and that in the majority the increase in titer was twofold or more. It was unfortunately not possible to test the organisms isolated from these patients for ability to produce DPNase *in vitro*. A good many of the ASDA levels were high even during the acute phase of the illness; these probably represent an anamnestic response, since it is likely that the patients were harboring the streptococci for several days prior to the onset of clinical symptoms and the drawing of the initial blood specimen.

The findings of the foregoing study made it plain that serum ASDA levels increased sharply in most, though not in all, instances following a streptococcal infection. This is in keeping with the observation of Bernheimer, Lazarides, and Wilson that 58 per cent of strains of streptococci examined by them produced DPNase *in vitro* (2). The most thoroughly investigated of the streptococcal antibodies thus far is antistreptolysin O (ASO), and clinical studies have shown that some 80 per cent of patients develop significant increases in titer of ASO following streptococcal infections (6). It was of interest, therefore, to compare the changes in titer of ASDA with those of ASO in patients exposed to Group A streptococci.

Comparisons of ASDA and ASO Levels in Patients with Group A Streptococcal Infections.—

Serum was obtained from 13 patients with acute streptococcal infections at the onset of the illness and 21, 35, and 60 days thereafter. These sera were kindly provided by Dr. Gene H. Stollerman. Group A streptococci were cultured from the throat in each case, though more specific typing of the organisms was not possible. The ASO titers were performed in Dr. Stollerman's laboratory by a modification of the method of Rantz and Randall (5). The ASDA and corresponding ASO levels of these sera are contained in Table IV. In all 13 cases there was a substantial rise in ASO titer, and in 8 of these there was a concomitant increase in ASDA activity. In the remaining 5 cases (numbers 3, 4, 6, 9, and 13) the ASDA levels remained essentially unchanged despite the increase in ASO. In another series of cases, not listed in Table IV, ASDA and ASO levels were found, in general, to increase in parallel following streptococcal infections. In a few instances, however, both antibody levels remained essentially unchanged, and in several cases the ASDA titer rose and the ASO titer did not.

DISCUSSION

Antisera produced in rabbits and guinea pigs by the injection of partially purified streptococcal preparations containing DPNase inhibit selectively the

biological activity of the enzyme, as the studies here described have demonstrated. Similar inhibitory activity directed against streptococcal DPNase was found to be present in the serum of a very large percentage of human beings and to increase strikingly following streptococcal infections. Very low levels of ASDA activity were observed in the serum of young children between the ages of 6 months and 5 years. Indeed, the only human sera of the several hundred examined which failed to contain demonstrable ASDA activity were in this age group, perhaps because these individuals had fewer exposures to streptococcal infections. Furthermore, sera capable in high dilution of neutralizing streptococcal DPNase failed to inhibit a similar enzyme produced by *Neurospora crassa*. Taken together, the findings indicate that the ASDA activity of human serum is not a non-specific inhibition of DPNase, but rather, a specific antibody produced in response to exposure to appropriate strains of streptococci.

The precise relationship between the ASDA response following streptococcal infection and the ability of the offending organism to produce DPNase *in vitro* is not clear at the present time. Lazarides and Bernheimer have shown an association between DPNase production and serological type of Group A streptococci (7). Thus, they found that streptococci belonging to types 3, 4, 6, and 12 tend to produce DPNase with considerable regularity, and in keeping with this, most of the 15 patients from whom sera were obtained following infections with type 3 or type 12 streptococci showed a significant rise in ASDA titer (see Table III). On the other hand, Lazarides and Bernheimer found that not one of 13 strains of type 14 nor any of 38 strains of type 19 they studied produced DPNase *in vitro*. Despite this, a number of patients suffering from infections with these types of streptococci showed substantial rises in ASDA titer (see Table III). Whether this is due to production of DPNase by some as yet untested strains of these types, or to a discrepancy between DPNase production *in vivo* and *in vitro*, or to other causes, remains undetermined.

The observation that antibodies to streptococcal DPNase are present in the serum of a very high percentage of human beings and that the titer of these antibodies rises subsequent to streptococcal infection makes it reasonable to conclude that this enzyme is produced in man during the course of such infections. These studies, however, cast no further light on the question of what role, if any, DPNase may play in disease processes. The ability to measure antibodies to streptococcal DPNase may have some application in clinical medicine in the recognition of antecedent streptococcal infections, especially in cases of glomerulonephritis in which the strains most often associated with the disease (types 4, 12, and Red Lake) are, with only a few exceptions, known to be DPNase producers. Also the finding that changes in ASDA levels did not always parallel changes in ASO levels, and particularly the fact that in some cases ASDA titers were found to rise when ASO titers did not, may serve to broaden the usefulness of such antibody studies in investigations concerned with the epidemiology of streptococcal infections.

SUMMARY

Specific neutralizing antibodies directed against streptococcal DPNase were induced experimentally in rabbits and guinea pigs by the injection of partially purified preparations of the enzyme. Similar antibodies capable of inhibiting the biological activity of the enzyme were found to occur naturally in the serum of a very high percentage of human beings, and the titer of these antibodies often rose sharply following streptococcal infections. The antibody response to streptococcal DPNase in general paralleled that to streptolysin O, though in some instances antibodies to one increased when those to the other did not.

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