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THE RELATIONSHIP OF DESOXYRIBONUCLEASE INHIBITOR LEVELS IN HUMAN SERA TO THE OCCURRENCE OF ANTINUCLEAR ANTIBODIES

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SUMMARY

A group of sera from hospital patients and normal subjects has been tested for the presence of antinuclear antibodies, and the amount of labile inhibitor to desoxyribonuclease 1 present in each of the sera estimated. It has been found that the presence of antinuclear antibodies is associated with a high level of inhibitor to a statistically significant degree.

It has also been found that this inhibitor is formed during the clotting of whole blood and that it is derived from platelets. A possible relationship between the inhibitor, its formation during the clotting of blood, and the characteristic presence of antinuclear antibodies in systemic lupus erythematosus is discussed.

INTRODUCTION

First described in the crop gland of breeding pigeons (Dabrowska, Cooper & Laskowski, 1949), inhibitors to desoxyribonuclease (DNAase) 1* have been found to occur widely in tissues, including leucocyte and bone marrow extracts, and in serum. The inhibitor as found in serum is very heat labile, is trypsin sensitive, is maximally precipitated by 40% ammonium sulphate and is non-dialysable. Its optimal activity lies in the pH range of 6·75–7·00 which coincides with the optimal pH range of DNAase 1 (Johnson, Goger & Tillet, 1954).

This inhibitor of DNAase has previously been shown to occur in significantly larger quantity in the sera of a group of patients with systemic lupus erythematosus than in the sera of a group of controls (Lachmann, 1961). On this evidence it was suggested that the inhibitor might, by interfering with extracellular catabolism of DNA-nucleoprotein, allow this material to provide an antigenic stimulus not otherwise present and so be implicated in the production of the anti-nucleoprotein antibodies which are characteristic of this disease.

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* DNAase 1 is the desoxyribonuclease found in pancreas which is active at neutral pH and requires magnesium ions for activity.

In this study the relation of raised levels of serum DNAase inhibitor to the occurrence of autoantibodies to nuclei (which contain DNA-nucleoprotein) and also to thyroid cell cytoplasm (which does not) has been investigated to see if a specific association exists.

The origin of the DNAase inhibitor in serum has been a matter of some dispute. It has been both claimed (Henstell & Freedman, 1952) and denied (Johnson *et al.*, 1954) that it arises from breakdown of white cells during clotting. It is clearly important in considering any biological role for DNAase inhibitor to know if it occurs normally in plasma or is produced during clotting, and this question has been further investigated.

MATERIALS AND METHODS

Estimation of DNAase inhibitor

DNAase inhibitor has been estimated viscometrically using a simple viscometer designed to test small volumes of liquid (Lachmann, 1961). In this assay system, the activity of a standard DNAase solution is measured against a standard solution of fully polymerized DNA, in the presence of the test serum at 37°C. The system is complicated by the presence of a serum activator of DNAase (Feinstein & Green, 1956) as well as the inhibitor but the heat lability of the inhibitor allows the inhibitory activity alone to be measured. For each estimation, the enzyme activity of the standard DNAase was measured first in the presence of 0·2 ml of test serum, and then again in the presence of the same serum previously heated at 56°C for 5 min. The inhibitor is inactivated by this treatment (Johnson *et al.*, 1954) while the serum activator as previously described in rat plasma is heat stable (Feinstein & Green, 1956; Feinstein, 1960). It was found that 0·2 ml of test serum contained more than enough activator to maximally activate the DNAase used. The amount of inhibitor is expressed as the difference between the DNAase activity in the presence of active serum and in the presence of heated serum. Compared with the activity of the standard DNAase used, DNAase activity in the serum alone is small, and in any case does not interfere with the assay.

In each test 0·5 ml of DNA (at an approximate concentration of 3 mg/ml) and 0·7 ml of buffer (0·1 M-Tris, pH 7·2, containing 0·05 M-MgCl) were mixed in the viscometer and allowed to reach thermal equilibrium in a 37°C water bath. A volume of 0·2 ml of test serum was added, followed by 0·1 ml of standard DNAase solution (0·2–0·25 μg-DNAase/ml of Tris buffer as above but with the addition of a 0·1% bovine plasma albumin or gelatin to stabilize the enzyme). After a further 2 min mixing and equilibration, measurements of the specific viscosity were taken at 1-min intervals for up to 15 min or until the specific viscosity was reduced to one. As initially the enzyme's activity has a greater effect on the viscosity, and as at lower viscosities greater orientation of DNA molecules occurs in the capillary of the viscometer, such a limit has to be set on the period over which measurements are taken.

The specific viscosity is related to the DNAase activity, and the time for which it acts as follows:

$$\frac{1}{[\eta_t]} = \frac{1}{[\eta_0]} + \frac{kc^2t^2}{[\eta_0]}$$

where η is the specific viscosity of the solution (η_0 at time 0 and η_t at time t);
c is the concentration (or activity) of the enzyme; and
t is the time for which it acts;

k is a constant for the DNA solution which includes such factors as the molecular weight and the number of hydrogen bonds needed to hold two polynucleotides together. These factors affect the probability of a double-stranded break in the DNA molecule allowing two polynucleotides to separate. This relation is based upon the work of Thomas (1956).

Regression lines of $1/[\eta]$ against t^2 were calculated by computer which gave the regression coefficient (or slope) and the correlation coefficient r. Throughout, the enzyme activity has been expressed as $\sqrt{(\text{Slope})} \times 10^2$ units. The correlation coefficients, a measure not of the linearity of the points, but of the dependence of $1/[\eta]$ on t^2, was at least 0·96 and in most cases 0·99.

Fluorescent antibody tests

Tests for antibodies to nuclei and to thyroid cytoplasm, were performed on cryostat-cut sections of human thyroid by a complement fixation technique. After treating the sections sequentially with the test serum and a source of human complement, they were stained with a rabbit anti-human C'_3 serum conjugated with fluorescein.

RESULTS

Sera from thirty-one subjects were tested. The results of the assays and details of diagnoses are given in Table 1.

Of the thirty-one subjects tested, fourteen had antinuclear factors and five had antibodies to thyroid tissue. From the results a histogram has been constructed (Fig. 1). The amount of DNAase inhibitor is seen to show a bimodal distribution. Arbitrarily dividing the distribution at 11 units of inhibitor, twenty-three of the sera fall into a group with less inhibitor and eight into a group with more. Of the twenty-three in the lower group, seven are ANF-positive, and of the eight in the higher group, seven are ANF-positive. Of the seventeen ANF-negative sera all except one are in the lower group with respect to the amount of inhibitor. It thus appeared that a high amount of inhibitor is associated with the presence of antinuclear factors. However, whereas all but one patient with high inhibitor have positive ANF, only about half the patients with positive ANFs have high levels of inhibitor.

Treating the results statistically, using the exact treatment for a 2×2 table (Fisher, 1950), the probability of the association between a high level of inhibitor and antinuclear antibodies being due to chance is 0·01; whereas for the association of either a high or a low level of inhibitor with thyroid staining, the probability is 0·6.

Alternatively, the mean inhibitor value for ANF-positive sera is 9·85 and ANF-negative sera is 6·25. (The ratio of variances of the two groups is 1·411, which is insignificant at the 5% level.) These values give $t = 2{\cdot}920$ with 29 degrees of freedom, so that the probability that the difference in means is due to chance is less than 0·01.

The presence of antinuclear factors is thus associated with a high level of inhibitor to a significant degree. On the other hand, antibodies to thyroid tissue show no such association with the amount of inhibitor.

The high degree of significance for the association with ANF is the more significant in that some of the sera had been stored for a long period of time and some had been used as

ANF-positive controls in ANF tests and consequently undergone repeated thawing and freezing. As the inhibitor is quite unstable one would have expected it to have deteriorated, and possibly any association that might have been present could have been masked.

In some instances where, on the basis of its ANF test, a patient's serum would be expected to belong to the other group as regards the level of inhibitor, other explanations may be available. Thus the serum of K.C. (ANF-positive and low inhibitor) was taken after treatment by injection with DNAase and was 7 years old at the time of testing. Prior to treatment, estimates of the inhibitor in her sera had been made and found to be higher than normal

TABLE 1. Summary of data on the sera studied

Serum (hospital patients)		DNAase activity of 0·1 ml standard DNAase with 0·2 ml serum		Labile inhibitor (units)	Anti-nuclear antibodies	Anti-thyroid cytoplasm antibodies	LE cell test	Diagnosis
		Unheated	Heated					
D.B.1	15.2.67	526	1243	717	+++	—		SLE
F.M.		1230	1797	567	+++	—	+	Discoid LE
C.W.	29.12.64	265	1615	1350	—	—		Still's disease
E.P.	10.11.66	318	1566	1248	+++	++	++	Myxoedema
S.K.	25.10.65	293	1559	1266	+++	—		Juvenile RA
I.H.	7.1.66	285	1531	1248	+++	—	++	SLE
D.C.1	3.7.65	191	1675	1484	++	—	++	Probable SLE
D.B.2	14.9.63	239	1600	1361	+++	—	++	SLE
C.T.	8.7.64	273	1609	1336	+++	—	++	Lupoid hepatitis
G.B.	29.6.66	321	1416	1095	—	—		RA
J.J.		219	1546	1327	+++	—		Probable RA
L.F.	6.7.66	709	1585	876	—	—		Probable RA
K.C.	1959/60	897	1708	811	+++	—	+	SLE
E.K.	21.2.63	1007	1672	602	+++	—	(+)	Probable SLE
G.S.	24.1.64	675	1544	869	+++	—	+	SLE
I.P.	14.11.63	1105	1734	629	—	++		SLE
E.G.	'65	1328	1726	398	—	—		Sjögren's syndrome
L.H.	3.10.66	1122	1615	493	+++	—		Wilson's disease
A.D.	1.6.66	1110	1624	514	—	—		RA
M.S.	16.5.65	1172	1621	449	—	—		RA
A.C.		1606	1718	112	—	—		Pulmonary infarction
P.1	6.5.66	1488	1723	235	—	—		Probable RA
P.2	6.5.66	1407	1730	324	—	++		Probable RA
H.	13.1.67	587	1535	448	—	++		SLE
D.C.2	13.1.67	804	1398	594	—	++		SLE
A.K.	20.1.67	152	942	790	—	—		Probable SLE
M.C.	7.10.67	1095	1551	456	+++	—		RA
Normals								
R.G.G.	19.10.66	1203	1695	492	—	—		
P.G.F.	14.10.66	1124	1816	692	—	—		
J.U.	12.1.67	894	1418	524	—	—		
J.R.	12.1.67	859	1458	599	—	—		

(Lachmann, 1961). L.H. whose serum is ANF-positive and with a low level of inhibitor, was suffering from Wilson's disease, and being treated with penicillamine. The origin of the positive ANF in this case may be different from that applying in connective tissue disease.

The serum of C.W., a case of Still's disease, was the only serum found with ANF-negative with a high level of inhibitor.

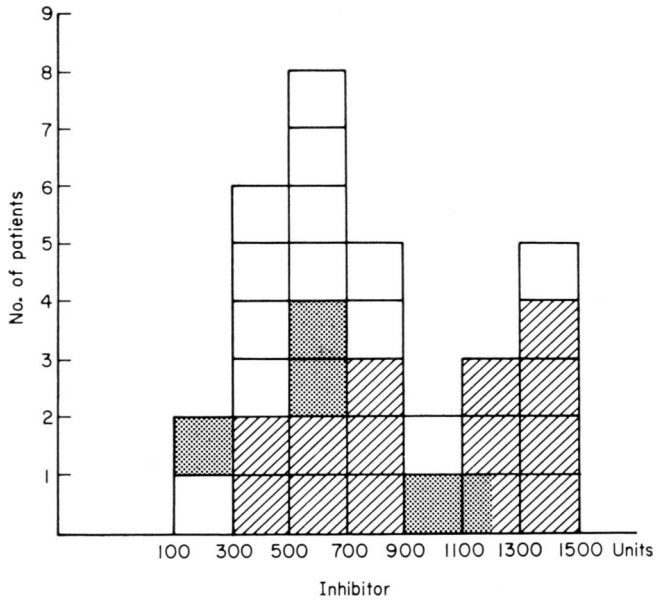

FIG. 1. Histogram of the amount of inhibition in the samples of sera studied. Hatched squares, antinuclear factor; stippled squares, anti-thyroid cytoplasm.

THE SOURCE OF THE INHIBITOR

(a) The effect of the presence of formed elements during clotting on the inhibitory activity of serum

The work of Henstell & Freedman (1952) and Gupta & Herriott (1963) supported the view that DNAase inhibitor could be formed during clotting. Johnson *et al.* (1954) and Kurnick *et al.* (1953) found that the DNAase inhibitory activity of serum did not increase in blood left standing.

In this experiment serum was prepared by centrifuging blood immediately after venepuncture and allowing plasma free of formed elements to clot. The inhibitory activity of this serum from clotted plasma was compared with that of serum from clotted whole blood and that of heparin plasma devoid of the formed blood elements. It was found that at the concentration used (5 units of heparin/ml of blood) heparin had no detectable effect on the DNAase activity in this test system.

The results of inhibitor assays on the various preparations are shown in Table 2.

All of the heparin plasmas devoid of cells and all of the sera clotted from plasma, have less inhibitor than the sera from clotted whole blood. The sera from plasma, and the heparin plasma, were prepared by centrifuging at 5000 rev/min for 5 min. It is possible that this is inadequate to clear all the platelets from serum as subsequent observations suggest that more vigorous centrifugation results in even lower inhibitor levels in plasma. This evidence would suggest that the inhibitor is formed to some extent at least during clotting.

TABLE 2. Comparison of the labile inhibitor present in serum from clotted whole blood, in serum from clotted plasma (devoid of cellular elements) and in heparin plasma devoid of cellular elements

Source of serum or plasma		Labile inhibitor (units) Serum from:		
		Clotted whole blood	Clotted plasma	Heparin plasma
Normal subjects				
J.R.	13.1.67	700	141	406
J.U.	13.1.67	746	187	342
Hospital patients				
M.H.	14.1.67	948	545	380
D.C.	14.1.67	594	535	322

(b) The inhibitory activity of leucocyte and platelet lysates

Platelet rich and leucocyte rich suspensions were prepared by differential centrifugation following separation of white blood cells from red blood cells on a dextran gradient. The suspensions were washed to remove the EDTA added to prevent clotting, resuspended in Tris buffer, and lysed by freezing and thawing in liquid nitrogen and a 37°C waterbath, respectively. The supernates of the lysed suspensions were tested in the absence and presence of serum. One leucocyte lysate was derived from a cell suspension seven times concentrated compared with whole blood and the other leucocyte suspension. Two platelet suspensions were suspended in Tris buffer before freezing; one suspension, however, was frozen after resuspension in serum and incubation at room temperature for some hours.

The results are shown in Table 3.

In the absence of serum, the platelet lysates consistently gave a low level of labile inhibitor. The results with the leucocytes suspension were less consistent but little or no labile inhibitor was found.

In the presence of serum (with maximally activated DNAase), the platelet suspension supernate produced a considerable degree of inhibition, while the leucocyte lysates showed only negligible inhibitory activity. When the platelet suspension was allowed to stand in serum before freezing, 0·2 ml of the supernatant completely abolished the activity of the standard DNAase. Not all of this inhibitor was destroyed by heating at 56°C for 5 min.

In conclusion, the results indicate that the inhibitor is derived from platelets. It would appear either that the inhibitor itself does not occur in large amounts in platelets, but is

TABLE 3. Amount of labile inhibitor to DNAase 1 in platelet and leucocyte lysates

Tested lysate	DNAase activity of 0·1 ml standard DNAase 1 solution with 0·2 ml of lysate		Labile inhibitor (units)
	Unheated	Heated	
In the absence of serum			
Platelet lysate suspended in Tris buffer	291	451	160
	326		126
Leucocyte lysate suspended in Tris buffer (unconcentrated)	680	456	—
	651	731	80
In the presence of 0·2 ml heated serum			
Platelet lysate suspended in Tris buffer	872	1156	384
Platelet lysate suspended in fresh serum (from clotted plasma) lysed and incubated at room temperature	0	657	657*

* The DNAase activity of the standard enzyme solution in the presence of heated unlysed platelet suspension in fresh serum, and without incubation at room temperature, was 1180 units (see Table 4). This would suggest that approximately 500 units of stable inhibitor were formed in addition to the labile inhibitor as a result of lysing and incubating the suspension. The significance of this is under further investigation, but does not affect the present study.

TABLE 4. The effect of antigen–antibody complexes and aggregated human IgG on the production of inhibitor to DNAase from platelets

Suspending medium for platelets	Presence of platelets	BPA–anti-BPA complex on aggregated IgG	DNAase activity of 0·1 ml standard DNAase solution with 0·2 ml of suspension or suspending medium		Labile inhibitor (units)
			Unheated	Heated	
Heparin plasma (devoid of cellular elements)	–	—	1138	1233	95
	–	BPA–anti-BPA complex	1107	1295	188
	+	—	467	1210	733
	+	BPA–anti-BPA complex	437	1223	786
Fresh serum (from clotted plasma)	–	—	1095	1327	232
	+	—	439	*1180	741
	+	—	0	657	*657
		(lysed and incubated at room temperature)			(stable inhibitor)
	–	BPA–anti-BPA complex	1122	1263	141
	+	BPA–anti-BPA complex	555	1219	664
	+	Aggregated IgG	629	1134	505

* See note to Table 3.

formed or potentiated as a result of a platelet factor interacting with a serum factor; or that the inhibitor is fully active only on DNAase when it has been 'activated' by serum.

(c) The effect of antibody–antigen interaction on the inhibitory activity of platelet suspensions

It has been reported that antibody–antigen interaction in the presence of complement causes the release of histamine and serotonin from platelets (Humphrey & Jacques, 1955). It was therefore of interest to see whether antigen–antibody interaction gave rise to the liberation of DNAase inhibitor.

Washed suspensions of platelets were added to heparin plasma or fresh serum, and tubes set up as shown in Table 4, with either bovine plasma albumin–anti-bovine plasma albumin precipitates or heat aggregated human IgG.

The results (shown in Table 4) fail to show any significant effect of these materials on the amount of inhibitor produced.

DISCUSSION

The results of the survey of the coincidence of antibodies to nuclei and the level of DNAase 1 inhibitor, in the sera of hospital patients and normal patients, show a bimodal distribution. The group of subjects, whose sera have less than 11 units of DNAase inhibitor, included normals and hospital patients, whereas the group which had more than 11 units of DNAase 1 inhibitor were all hospital patients. This suggests that a level of serum DNAase 1 inhibitor above 11 units is abnormal. This is further supported by the finding that a high level of DNAase inhibitor is associated with antibodies to nuclei to a highly significant degree. In contrast it was found that it is most unlikely that the level of inhibitor has any association with anti-thyroid cytoplasm antibodies. The association with antibodies to nuclei is thus at least to some degree specific. This is of interest as the DNAase inhibitor, by the very nature of its activity, is likely to be a factor in the catabolism of DNA-nucleoprotein, the antigen provoking the associated antibodies.

It should be noted that the association of antinuclei antibodies with a high level of serum DNAase inhibitor was found by examining serum and not plasma. Subsequently it was also found that much though not all DNAase inhibitor (in normal serum) is formed during clotting, i.e. *in vitro*. Further experiments are in progress to determine whether patients with antinuclear factors have high plasma levels of inhibitor and/or whether they have a raised capacity to generate inhibitor on clotting.

The association of antinuclear antibodies with a high level of DNAase inhibitor has a direction of association, in that seven out of eight of the subjects with an abnormal level of inhibitor have antinuclear antibodies, but only seven out of fourteen of the sera containing such antibodies have an abnormal level of DNAase 1 inhibitor. If this phenomenon is real, and not due to decomposition of the labile inhibitor during the storage of serum samples (in the case of one of the samples this was for over 7 years), it precludes a high level of DNAase inhibitor being the sole cause of the autoantibodies to DNA-nucleoprotein, i.e. a high level of inhibitor could be a sufficient but not a necessary cause.

The direction of association may alternatively be an indication that the causation is the other way round, i.e. that the presence of antibodies to DNA-nucleoprotein is a factor in the formation of DNAase inhibitor. However, preliminary experiments suggest that the

addition of antinuclear antibodies to normal blood before clotting does not increase the amount of inhibitor generated.

Finally, it is of course quite possible that there is no causal relationship between antinuclear antibodies and high levels of DNAase inhibitor but that both are consequences of some other abnormality.

However, it is attractive to postulate that the DNAase inhibitor, by retarding the breakdown of DNA-nucleoprotein, renders this material more antigenic and so is instrumental in the production of antinuclear antibodies. The question then arises whether the patients who develop antinuclear antibodies have an underlying abnormality, either in serum or platelet factors, which causes the generation of more inhibitor on clotting, or whether it is increased *in vivo* 'clotting', associated with the widespread inflammation of diseases like systemic lupus erythematosus or rheumatoid arthritis, that produces more inhibitor. In the latter event high inhibitor levels should be found in the plasma of these patients and study of plasma levels of inhibitor should help in distinguishing these possibilities.

As it is believed that the inflammatory process in systemic lupus erythematosus is at least in part a Type III allergic reaction involving circulating nuclear antigen–antibody complexes (Lachmann, 1967), the antinuclear antibodies would contribute to the formation of DNAase inhibitor. The inhibitor in turn could play a pathogenic role by making DNA-nucleoprotein more antigenic and so it would help to perpetuate the inflammation which gives rise to it.

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