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ANTIBODIES TO NUCLEOPROTEIN AND TO HYDRAZIDE-ALTERED SOLUBLE NUCLEOPROTEIN IN TUBERCULOUS PATIENTS RECEIVING ISONIAZID

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SUMMARY

Antibodies to calf thymus nuclei, nucleoprotein, DNA, soluble nucleoprotein and hydrazide (hydrallazine and isoniazid)-altered nucleoprotein were investigated by a standard complement-fixation method in 214 tuberculous patients receiving isoniazid. Findings were compared to those on thirty-seven sera from lupus patients receiving neither steroids nor immunosuppressants and on sixty-six sera from normal controls.

The incidence of antibodies to all antigens studied except DNA was significantly higher in isoniazid-treated tuberculous patients than in the normal controls, but lower than in the lupus patients. Unlike lupus there were no detectable DNA antibodies in the tuberculous or in the control sera. Antibodies to nucleoprotein (soluble and insoluble) and particularly to hydrazide-altered nucleoprotein were the most frequently found in the isoniazid-treated tuberculous patients. In general, antinuclear antibodies were more frequent in the isoniazid-treated tuberculous female than in the male; in the adult than in the child.

It is suggested that hydrazides may cause *in vivo* similar alteration of nucleoprotein to that which they cause *in vitro*. Hydrazide-altered nucleoprotein probably elicits the production of antinuclear antibodies which in turn may activate systemic lupus erythematosus in otherwise predisposed individuals.

INTRODUCTION

It has been reported that approximately 20% of tuberculous patients on prolonged isoniazid (isonicotinic acid hydrazide, INH) treatment develop antinuclear antibodies (ANA), usually without concurrent clinical symptoms (Cannat & Seligmann, 1966). A small number of such patients may develop a clinical syndrome indistinguishable from systemic lupus erythematosus (SLE) (Bickers *et al.*, 1961; Zingale *et al.*, 1963; Lee, Rivero & Siegel, 1966; Siegel, Lee & Peress, 1967; Auquier *et al.*, 1967; Debeyre, Kahn & de Séze, 1967; Masel,

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1967; Doust & Moatamed, 1968). Prolonged administration of isoniazid or of the related anti-hypertensive drug hydrallazine to highly inbred strains of mice may also result in the development of ANA without apparent disease (Cannat & Seligmann, 1968).

The dichotomy between the incidence of ANA and that of actual clinical SLE following isoniazid treatment may indicate that the drug primarily induces the production of ANA, and that only otherwise predisposed individuals will, under these circumstances, develop SLE.

Recent information regarding physico-chemical alterations suffered by nucleoprotein when treated *in vitro* with hydrallazine (Tan, 1968) suggests that ANA which develop in hydrazide-treated patients may result from the action of such drugs on nuclear antigens *in vivo*. Evidence of a predisposition (lupus diathesis) is available for hydrallazine (Alarcón-Segovia *et al.*, 1965, 1967) and other drug-induced lupus syndromes (Holley, 1964; Lappat & Cawein, 1968). We have investigated the presence of antibodies to various calf thymus nuclear antigens in sera from isoniazid-treated tuberculous patients in order to determine their incidence and if, when present, ANA are primarily directed to nucleoprotein, as could be expected if the mechanism for their development is the alteration of this nuclear antigen by isoniazid. In addition, we have investigated in such sera the presence of ANA directed primarily to isoniazid and hydrallazine-altered soluble nucleoprotein which might further indicate that the triggering mechanism for ANA production in these patients is *in vivo* alteration of nucleoprotein by the drug.

MATERIALS AND METHODS

Sera

The sera were frozen immediately after collection, and were kept at −40°C until the time of the study. Repeated freezing and thawing was avoided. Sera studied included:

(a) Those from 214 hospitalized, isoniazid treated, bacteriologically proven, tuberculous patients. Thirty of these patients were children, twenty-one of whom were females. One hundred sera belonged to adult males and the rest to adult females. The known total dose of isoniazid was computed in each case. Thirty patients were known to have received less than 15 g of isoniazid; the rest had received from 15 g to over 2000 g. Patients studied had received a variety of other anti-tuberculous drugs, but isoniazid was the only one common to all of them. No patient had developed clinical evidence of SLE.

(b) Sera from sixty-six normal volunteers studied as controls. These controls were representative as per decade distribution of the age groups of adult tuberculous patients studied. No controls for children were obtainable.

(c) Thirty-seven sera from patients with SLE who had not received steroid or immunosuppressive therapy for at least 2 months prior to collection. Three of these patients had anticonvulsant-activated SLE.

Antigens

(a) *Calf thymus nuclei* were prepared by the method of Allfrey, Littau & Mirsky (1964). The suspension of nuclei was found to have less than 5% non-nuclear material when stained with crystal violet.

(b) *Calf thymus nucleoprotein* (NP) kindly provided by Dr H. Mittenzwei, Hormon-Chemie, Munich, Germany.

(c) *Soluble nucleoprotein* (sNP) prepared from calf thymus nuclei as described by Tan (1967).

(d) *Calf thymus DNA* obtained commercially (Sigma Chemical Co., Saint Louis, Missouri, U.S.A.), and utilized in both native and heat denatured forms.

(e) *Soluble nucleoprotein altered with hydrallazine hydrochloride* (sNP/Hz) as done by Tan (1968) by adding 1 mg of hydrallazine/mg of protein as determined by the micro-Kjeldahl method, and stirring overnight at low temperature (Tan, personal communication).

(f) *Soluble nucleoprotein altered with isoniazid* (sNP/INH) prepared in a similar way to (e). Following treatment with INH there was an increase in viscosity of nucleoprotein similar to that which occurs after treatment with hydrallazine (Tan, 1968).

Antinuclear antibodies

These were investigated by a standard complement fixation test (Kabat & Mayer, 1961) adapted to a micromethod (Microtiter, Cooke Engineering Co., Alexandria, Virginia, U.S.A.), using each of the nuclear antigens and 2 units of 50% haemolytic complement obtained from fresh guinea-pig serum. Anticomplementary activity of each antigen was investigated and the antigens were used at the next higher dilution.

Sera were studied in large batches with adequate known positive and negative controls, as well as controls for anticomplementary activity of each serum. Titres were recorded as the reciprocal of the last dilution showing complement fixation as shown by lack of haemolysis of the sensitized sheep red blood cells.

Absorption studies

Absorption studies were conducted in selected tuberculous sera with the highest titres of antibodies to each of the antigens studied. Aliquots of each serum were absorbed with each antigen and tested after absorption by the same complement fixation method. Absorption with isoniazid was also performed.

Statistical analysis

The significance of the findings was determined by the χ^2 test.

RESULTS

Incidence of antinuclear antibodies to all antigens studied except DNA was significantly higher in isoniazid-treated tuberculous patients than in the normal controls (Fig. 1). Incidence (Fig. 1) and titres (Fig. 2) of antinuclear antibodies to all antigens were lower than in lupus patients similarly studied. Results with native and heat denatured DNA were uniformly negative in the tuberculous patients and the controls and will therefore be considered subsequently as DNA only. Since there was no significant difference between the findings with sNP/INH and sNP/Hz, the results will be given mainly for the findings with sNP/INH.

Antibodies were detectable in patients who had received less than 15 g of INH. Except for the fact that only one of eighteen patients who had received more than 270 g of isoniazid (equivalent to 18 months on 500 mg daily dose) had no antinuclear antibodies with sNP/INH as antigen, statistical analysis showed no correlation between total dose of isoniazid received by the patients and incidence of antibodies to any of the antigens studied. It is probable however, that some patients had previously received isoniazid without our knowledge.

Antibodies were considerably more frequent to sNP/INH than to untreated sNP in the tuberculous patients (Fig. 1). Also, the titres were higher more often (more than one dilution in thirty-eight sera) with sNP/INH than with sNP in these patients.

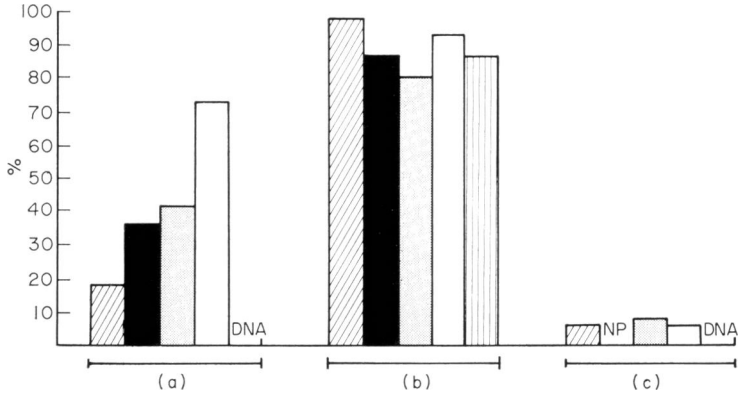

FIG. 1. Incidence of positive results of antinuclear antibodies to various calf thymus antigens in: (a) isoniazid-treated tuberculosis (214 sera), (b) untreated systemic lupus erythematosus (thirty-seven sera), and (c) normal controls (sixty-six sera). Calf thymus antigens: cross-hatched columns, nuclei; solid columns, nucleoprotein (NP); stippled columns, soluble nucleoprotein (SNP); open columns, INH-treated sNP; vertical hatched columns, DNA.

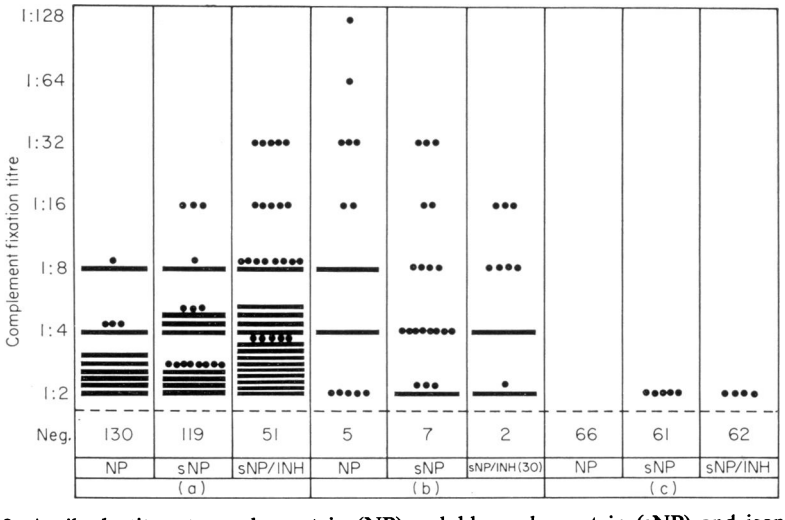

FIG. 2. Antibody titres to nucleoprotein (NP), soluble nucleoprotein (sNP) and isoniazid-altered soluble nucleoprotein (sNP/INH) in: (a) isoniazid treated tuberculosis (214 sera); (b) systemic lupus erythematosus (thirty-seven sera); and (c) normal controls (sixty-six sera). ——, ten sera; ●, one sera. Figures in parentheses indicate number of sera studied.

As seen in Table 1, antibodies to nucleoprotein (NP) and/or soluble nucleoprotein (sNP) were more frequent in isoniazid-treated females than in males (both antigens $P<0{\cdot}005$); in adults than in children (NP: $P<0{\cdot}005$), reaching an incidence of 65% with sNP in the adult female. Antinuclear antibodies detected with sNP/INH were also more frequent in INH-treated tuberculous adults than in children ($P<0{\cdot}005$), but there was no difference in their

incidence between sexes in the adult. Eight of the twenty-one girls with tuberculosis had antibodies to sNP/INH while these were absent in all nine boys. The presence of antibodies to sNP and sNP/INH was also related to age in the adult. All women over 40 years old had antibodies to these antigens while they were present in only 33% of those under 40 years of age ($P<0{\cdot}005$). There was no significant difference between the incidence of antinuclear

TABLE 1. Variations in the incidence of antibodies to various nuclear antigens in isoniazid treated tuberculous adults and children grouped by sex

		% positive				
		Nuclei	NP	sNP	sNP/IHN	DNA
Adults						
Female	(84)*	17	50	65	77	0
Male	(100)	19	29	27	78	0
Total	(184)	19	39	45	78	0
Children						
Female	(21)	29	14	33	38	0
Male	(9)	0	0	11	0	0
Total	(30)	20	10	27	27	0
Total						
Female	(105)	19	43	59	70	0
Male	(109)	17	27	26	72	0

* Parentheses indicate number of patients in each group.

antibodies to sNP and to sNP/INH in the adult INH-treated tuberculous female, but these were considerably more frequent with SNP/INH than with untreated sNP in the adult male.

All INH-treated tuberculous patients having antibodies detectable using whole nuclei as antigen in the complement fixation system, also had antibodies to NP and/or to sNP. Antibodies to whole nuclei were equally as frequent in the adult female as in the adult male. They were present in six of the twenty-one girls and absent in the nine boys (Table 1).

TABLE 2. Absorption studies in four positive tuberculous sera

Serum, antigen, titre	Absorption with:				
	Nuclei	NP	sNP	sNP/INH	INH
ERQ, Nuclei, 1:4	Neg.	1:4	1:4	1:4	1:4
JCA, NP, 1:8	1:4	Neg.	1:4	1:4	1:8
FBC, sNP, 1:8	1:4	Neg.	Neg.	1:4	1:8
JNN, sNP/INH, 1:32	1:16	1:8	1:16	Neg.	1:16

These relationships between the incidence of antinuclear antibodies in INH-treated tuberculous patients and their ages and sex could not be accounted for on the basis of total INH dose/body weight, nor by duration of active tuberculosis.

Antibodies to sNP/INH remained present despite previous absorption of sera with

isoniazid or sNP. Conversely antibodies to sNP were not absorbed out with sNP/INH. Table 2 shows results of absorption studies in four sera.

DISCUSSION

Occurrence of SLE has been reported following administration of a wide variety of drugs (Alarcón-Segovia, 1969). These drugs can be readily divided into two groups: (1) drugs which have only been sporadically reported as causative of SLE and which seem to do so by eliciting allergic reactions which in turn bring about lupus, and (2) drugs which have been reported to cause it in a larger number of cases. The drugs in the second groups often require prolonged administration and large doses and, when SLE is activated by them, it usually appears insidiously, without apparent allergic reaction. This suggests that their SLE activating potential lies on their pharmacologic properties and that these drugs do not act as haptens causing allergic reactions. Lupus inducing drugs with these characteristics include 1-hydrazinophthalazine hydrochloride (hydrallazine), isonicotinic acid hydrazide (isoniazid), procaine amide hydrochloride and various anticonvulsants of diverse chemical nature (Alarcón-Segovia, 1966).

It has also recently been shown that hydrallazine (Perry, Sakamoto & Tan, 1967), isoniazid (Cannat & Seligmann, 1966) and procaine amide (Dubois *et al.*, 1968; Russell & Ziff, 1968) are capable of eliciting antibodies in a much larger proportion of individuals than those who actually develop SLE. This suggests that these drugs may induce SLE by primarily eliciting the production of antinuclear antibodies. Two recent reports indicate that some of these drugs alter nuclear antigens *in vitro* or otherwise increase their antigenicity. This action, if occurring *in vivo*, could explain the development of antinuclear antibodies following drug intake. Tan (1968) has found that hydrallazine is capable of altering *in vitro* the soluble nucleoprotein he described, and Blomgren & Vaughan (1968) found that the photochemical complex of DNA with procaine amide is more antigenic than native or photo-oxidized DNA.

The findings of Cannat & Seligmann (1966) on the induction of antinuclear antibodies by isoniazid had not been previously confirmed. We felt that, if present, antinuclear antibodies might be primarily directed to nucleoprotein and particularly to hydrazide-altered nucleoprotein, since such alteration could be responsible for the development of ANA upon isoniazid administration. We sought ANA using both isoniazid and hydrallazine-altered sNP because the report by Tan (1968) dealt with the latter drug. The almost identical findings with both hydrazide-altered soluble nucleoproteins, provide some evidence that sNP may be altered by both hydrazides in a similar way. Both hydrazides caused similar increase in viscosity of sNP.

That antinuclear antibodies developing in isoniazid-treated tuberculous patients are primarily directed to nucleoprotein has already been suggested by the finding of homogeneous patterns with immunofluorescent techniques (Cannat & Seligmann, 1966). These authors also showed that such antibodies are related to isoniazid treatment rather than to the presence of tuberculosis. We were unable to obtain an adequate number of untreated tuberculous patients in order to confirm this. However, the sex and age differences found in the incidence of ANA irrespective of the activity or severity of tuberculosis, suggests that tuberculosis itself is not a factor in their development.

Using whole calf-thymus nuclei as an antigen in our complement fixation tests we found an incidence of antibodies similar to that reported by Cannat & Seligmann (1966) with

immunofluorescent methods. These antibodies seemed primarily directed to nucleoprotein (both soluble and insoluble). The use of these antigens in the complement fixation system greatly enhanced the incidence of positive findings and of higher titres in the isoniazid-treated tuberculous patients but did not change them significantly in the lupus patients or in the normal controls. The reason for the higher incidence of antibodies when sNP was used as antigen in the system may be that sNP is the antigen primarily altered *in vivo* by hydrazides in the same way as it is *in vitro* and that the positive results with NP may be due to cross-reactivity.

Adequate controls ruled out the possibility that the greater incidence of antibodies to hydrazide-altered nucleoprotein than to unaltered nucleoprotein was due to the anti-complementary effect of the former. Previous complement inactivation of the sera at 56°C for 30 min would have also destroyed any proteases present in them. It is, therefore, unlikely that the greater number of positive reactions obtained with sNP/INH or Hz was due to the known resistance of hydrazide-treated nucleoprotein to trypsin digestion (Tan, 1968). Reactivity of sNP/Hz with SLE antibody is destroyed by deoxyribonuclease in a manner similar to that of untreated sNP (Tan, 1968). It is therefore improbable that the presence of hydrazides would favour an inhibitor of deoxyribonuclease that might be present in the serum (Frost & Lachmann, 1968) and, thereby, allow for greater sensitivity of the test, independent of antibody specificity. Sex and age differences in the incidence of ANA in our isoniazid treated tuberculous patients seem particularly interesting. First, because age differences in the incidence of reactions with sNP/INH would be difficult to explain on the basis of factors other than antibody specificity. Second, because the lack of sex difference in the incidence of antibodies to sNP/INH despite a highly significant preponderance of those to unaltered sNP in the female may indicate that, once production of antibodies to altered sNP takes place, independent production of antibodies to unaltered sNP may follow particularly in the female.

Females develop SLE (Dubois & Tuffanelli, 1964) as well as clinically non-significant ANA (Svec & Veit, 1967) more frequently than men. Highly inbred female mice from two different strains develop ANA upon administration of either hydrallazine or isoniazid more frequently than male mice of the same strains given the same amounts of the drugs (Cannat & Seligmann, 1968). Antinuclear antibodies (Svec & Veit, 1967) as well as other autoantibodies (Goudie, Anderson & Gray, 1959; Hackett, Beech & Forbes, 1960; Heimer Levin & Rudd, 1963; Irvine *et al.*, 1965) are more frequent in elderly than in young control subjects. This might be due to differences in immunoglobulin class of such autoantibodies in different age groups (Svec & Veit, 1967) or to the gradual accumulation of environmental insults leading to alteration of cellular components which become antigenic and elicit the production of autoantibodies (Alarcón-Segovia, 1969). The higher incidence of autoantibodies in the normal elderly female than in the elderly male could indicate that either females are more prone to such antigen-altering environmental insults (Dameshek, 1960) or that reactivity of their immunological apparatus to such antigens is greater. Our finding of a higher incidence of ANA in human females than in males, when both are subjected to the same antigen-altering agent, suggest the latter. The higher incidence of antibodies to altered sNP in the tuberculous patients suggests that, as postulated by Tan (1968), there is probably *in vivo* complexing of these hydrazides with sNP. Hydrazide-altered sNP may then elicit the production of antibodies.

Variations in antibodies following hydrallazine or isoniazid administration is probably

also dependent on the rate of acetylation of the drug (Perry *et al.*, 1967). Investigation of this factor is currently being carried out in our laboratory.

In most cases antinuclear antibodies which develop upon administration of a drug such as isoniazid seem to be innocuous. Transition into SLE probably depends upon a predisposition (lupus diathesis) which may be genetically determined.

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