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STUDIES ON DRUG INDUCED LUPUS ERYTHEMATOSUS IN MICE

I. DRUG INDUCED ANTINUCLEAR ANTIBODIES (ANA)

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SUMMARY

Hydralazin, isoniazid, procainamide, α-methyldopa and chlorthalidone were given orally to mice of three inbred strains: BALB/CJ, C57/BL/6J and A/JAX. All drugs significantly increased the frequency of antinuclear antibodies (ANA). The results were most marked after 8 months of treatment. Although a slight preponderance of females was observed for ANA frequencies in BALB/CJ and A/JAX mice, this was significant only for hydralazin- and procainamide-treated animals. C57/BL/6J males appeared to respond better than females. Genetical factors appear of importance in the induction of drug induced lupus erythematosus (DILE).

INTRODUCTION

Several drugs have been reported to induce syndromes similar to systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) after prolonged administration.

The first drug known to provoke SLE-like syndrome was hydralazin (Perry & Schroeder, 1954)—hence the term 'hydralazin syndrome'. However, since other drugs have subsequently been reported to induce similar syndromes which, in many cases, are identical with idiopathic SLE, it seems appropriate to suggest the name 'drug induced lupus erythematosus' (DILE).

The etiology of this syndrome is still obscure; several authors have postulated a genetical predisposition which is liable to be triggered off by the administration of a certain drug (Condemi *et al.*, 1967; Alarçon Segovia *et al.*, 1965; Holley, 1964). A few family studies support this hypothesis (Lappat & Cawein, 1968; Hift & Watson, 1968; Holley, 1964); however, a prospective study by Blomgren *et al.* (1969) produced no evidence to support this theory.

A small number of animal experiments on the induction of SLE by drugs have been performed (Harbinson *et al.*, 1962; Comens, 1956; Monier, Richard & Thivolet, 1968), but with contradictory results. This may be partly due to heterogeneity of the experimental

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animals used, since results were clearer in experiments in which larger numbers of genetically well defined animals were involved (Cannat & Seligmann, 1968). The present study was undertaken to investigate whether SLE-like symptoms can be induced in mice by the administration of the following drugs: hydralazin and procainamide which can produce a complete clinical picture of SLE in men; isoniazid which may evoke rheumatoid symptoms and can cause liver disease, usually without clinical SLE (Bickers *et al.*, 1961); α-methyldopa, which can evoke a positive direct antiglobulin test and antinuclear antibodies (ANA) (Alarçon Segovia, 1969; Breckenridge *et al.*, 1967); and chlorthalidone, which may produce ANA (Feltkamp, Dorhout Mees & Nieuwenhuis, 1970). By using different inbred strains it was possible to investigate the role of a genetical predisposition in the induction of SLE-like symptoms.

MATERIALS AND METHODS

Mice

1500 mice of the BALB/CJ, C57/BL/6J and A/JAX strains, comprising equal numbers of males and females, were obtained from Jackson Laboratories (Bar Harbor, U.S.A.). At the beginning of the experiments the animals were 6–8 weeks old, weighing 20–25 g. They were divided into 6 groups, each comprising 250 mice, and received the following drugs:

Drugs

Group 1. Hydralazin (Apresolin ®).
Group 2. Isoniazid (Nidaton ®).
Group 3. Procainamide HCl.
Group 4. α-Methyldopa (Aldomet ®).
Group 5. Chlorthalidone (Hygroton ®).
Group 6. Received no drugs and served as controls.

As the drugs were administered in the drinking water, quantitative records were maintained on 500 mice during the first 2 weeks in order to obtain information on average consumption. The dosages, so calculated for each drug are given in Table 1. These dosages, based on bodyweight, are about ten times the amounts normally given to human subjects for therapeutic purposes.

ANA determination

Before starting the experiment and thereafter once every two months, blood was obtained by orbital sinus puncture using a haematocrit capillary. After centrifugation the plasma was separated from the erythrocytes by sawing the tube, and tested for (ANA) in a 1 : 10 dilution in phosphate buffered saline (PBS) (0·01 M, pH 7·2); ANA determination was performed according to Ten Veen & Feltkamp (1969), using formalinized chicken red cell nuclei as nuclear antigen.

Fluorescent reagent

A rabbit anti-mouse IgG conjugated with fluorescein isothiocyanate according to The & Feltkamp (1970) was used at a dilution of 1 : 25 in PBS. This reagent was characterized by a protein concentration of 9·2 mg/ml, an agar block titration titre (Feltkamp, 1970) of 1 : 32, and a molar F/P ratio of 2·5, freed from proteins with F/P ratios <1 and >4.

After 10 months the mice were bled. Both serum and heparinized plasma was obtained from fifty mice in order to exclude any discrepancies that might occur through the use of serum instead of plasma as during the experiment in the previous months. Liver tissue was frozen from fifty mice in liquid nitrogen and used with the corresponding serum in order to establish the true auto-antibody character of the ANA.

Direct anti-globulin test

This test was performed on the cells obtained by centrifugation of the capillaries used for the orbital sinus puncture; the technique used was that of Hijmans *et al.* (1969).

The blood obtained at the 10th month (the mice being 12 months old) was also used for the determination of antibodies to native DNA, by means of a radio-immuno-assay, performed (with minor alterations) according to Wold *et al.* (1968). In addition the rheumatoid factor was determined using human O red cells sensitized with rabbit immunoglobulins (Van Loghem-Langereis, 1952; Feltkamp & Van Rossum, 1968).

TABLE 1. Daily drug intake of the various groups of mice

Drug	Dose (mg)
Hydralazin	1
Isoniazid	2
Procainamide	20
α-Methyldopa	5*
Chlorthalidone	0·2

* The α-methyldopa dosage was lowered to 1·25 mg after 6 weeks as the mice refused to drink water containing high concentrations of this drug.

RESULTS

A. Test for autoantibody activity

No discrepancies were revealed on testing the sera from fifty mice simultaneously on formalinized chicken red cell nuclei and on cryostat sections of their own livers. We therefore considered the demonstrated ANA to be true autoantibodies. The nuclear fluorescence was mostly of the homogeneous type.

B. Testing for ANA activity

No discrepancies were found between heparinized plasma or serum from the same mouse. We found no indications for the existence of artefacts as suggested by Johnson & Bencze (1965).

C. Spontaneous ANA incidence

As shown in Table 2 the A/JAX mice showed the highest ANA incidence (8%) at the beginning of the experiment, followed by BALB/CJ (3%) and C57/BL/6J (1%). In contrast to

BALB/CJ and C57/BL/6J mice, which showed a higher, although not significant, ANA incidence in females than in males, A/JAX mice showed no such trend. These differences between the three strains remained constant; a rise in ANA frequencies was observed with increasing age.

D. Effect of drug administration on ANA frequencies

During the course of the experiment all the drugs induced a significant increase of ANA frequencies in all three strains (Table 3, Fig. 1). There were, however, marked differences

TABLE 2. Spontaneous ANA frequencies in control mice

Age (months)	BALB/CJ		C57/BL/6J		A/JAX	
	Male	Female	Male	Female	Male	Female
2	4/205 = 2%	8/182 = 4%	3/192 = 2%	2/199 = 1%	14/204 = 7%	18/196 = 9%
12	5/ 21 = 25%	12/ 32 = 37·5%	4/ 29 = 14%	7/ 28 = 25%	18/ 34 = 53%	15/ 29 = 52%

between the drugs as well as between the different strains. Whereas the graphs of Fig. 1 suggest that the drugs had a considerable effect, Fig. 2, representing the combined statistical evaluations of all the experimental groups, shows that not earlier than 8 months after the commencement of treatment significant differences in ANA frequencies were found between treated and control animals for all drugs and all strains. It is clear that A/JAX mice are the strongest responders, i.e. they are the first to show significantly elevated ANA frequencies and they reach also the highest values. C57/BL/6J mice appear to be slow responders.

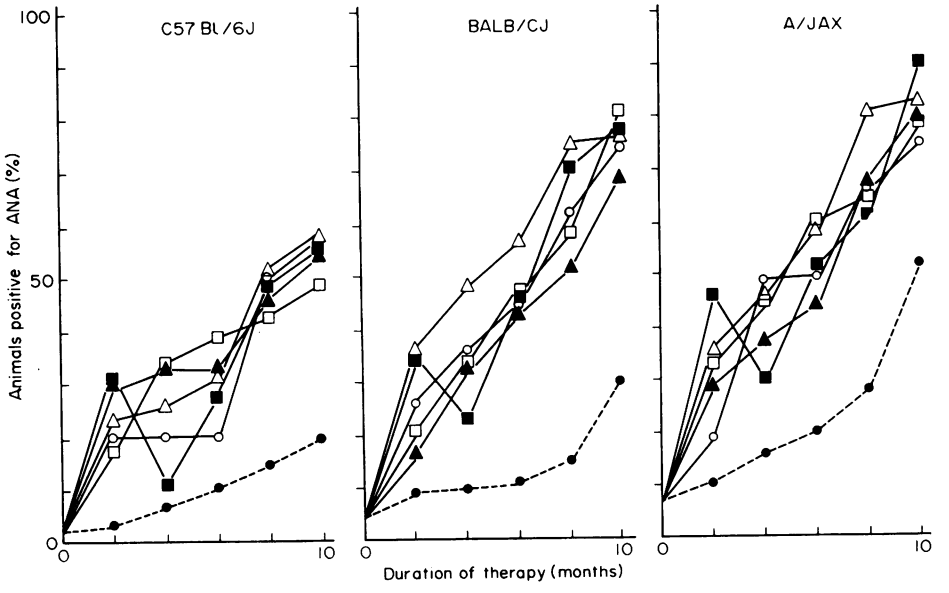

FIG. 1. ANA frequencies in drug treated and control mice. ▲, Hydralazin; ○, isoniazid; □, procainamide; ■, α-methyldopa; △, chlorthalidone; ●, controls.

TABLE 3. ANA frequencies in drug treated and control mice

Duration of therapy (months)	Age (months)	Drug	Females						Males					
			BALB/CJ		C57/BL/6J		A/JAX		BALB/CJ		C57/BL/6J		A/JAX	
			No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)
2	4	Hydralazin	4/28	14	6/33	18	9/38	24	5/27	18·5	7/33	21	12/35	**34**
		Isoniazid	8/37	22	9/40	22·5	8/39	21	10/31	31	8/41	19·5	7/40	17·5
		Procainamide	5/35	14	6/35	17	13/36	**57**	8/26	31	6/34	18	12/29	**41***
		α-Methyldopa	8/26	31	9/34	26	14/30	47	8/20	**40**	7/33	21	15/33	**45·5***
		Chlorthalidone	11/33	33	6/28	21	14/29	48	8/20	**40**	7/33	21	8/23	**35**
		Controls	2/25	8	2/29	7	4/32	12·5	2/32	6	1/32	3	3/32	9
4	6	Hydralazin	12/33	**36**	13/40	32	16/33	**48**	7/25	28	12/35	**34***	10/37	27
		Isoniazid	16/41	**39***	5/38	14	21/36	**58***	10/31	**32**	10/39	**26**	14/36	39
		Procainamide	11/37	**30**	12/37	32	18/34	**53**	11/30	**37**	12/34	**36***	10/27	37
		α-Methyldopa	5/24	21	4/33	12	8/27	30	5/17	29	4/30	13	9/29	31
		Chlorthalidone	11/24	**44***	5/22	22	15/24	**62·5***	8/16	**50***	6/20	**30**	13/24	**54***
		Controls	2/24	8	3/28	11	5/27	19	1/21	5	1/28	3·5	4/28	14
6	8	Hydralazin	11/26	**42**	14/42	33	16/39	41	12/28	**43**	12/39	**31**	19/39	**49**
		Isoniazid	12/32	**37·5**	6/30	20	19/35	**54**	16/33	**48***	6/31	19	12/29	41

Table 3 contd—

Duration of therapy (months)	Age (months)	Drug	Females						Males					
			BALB/CJ		C57/BL/6J		A/JAX		BALB/CJ		C57/BL/6J		A/JAX	
			No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)
		Procainamide	18/35	**51***	13/35	37	25/37	**67·5†**	12/29	**41**	14/34	**41***	14/27	**52**
		α-Methyldopa	12/27	**44**	8/28	29	11/18	**61**	4/8	**50**	6/23	26	10/23	43
		Chlorthalidone	19/33	**58†**	14/34	**41**	16/34	47	7/13	**54**	8/35	23	16/35	**46**
		Controls	4/31	13	5/34	14	8/35	23	3/29	10	2/31	6·5	5/30	16·5
8	10	Hydralazin	16/32	**50***	16/39	**41**	17/23	**74***	14/26	**54***	20/38	**52***	16/26	**61·5**
		Isoniazid	23/38	**60†**	18/38	**47**	36/38	**95†**	16/25	**64†**	21/41	**51***	30/35	**86†**
		Procainamide	18/31	**58†**	17/42	**40·5**	27/37	**73***	17/30	**57***	18/41	**44**	18/33	**54·5**
		α-Methyldopa	16/23	**69·5†**	17/34	**50***	22/30	**73***	11/16	**69†**	15/34	**44**	16/32	50
		Chlorthalidone	23/30	**77†**	14/30	**47**	27/33	**82†**	14/20	**70***	19/34	**56†**	18/32	**56**
		Controls	5/31	16	6/35	17	10/31	32	4/27	15	5/35	14	8/32	25
10	12	Hydralazin	27/39	**69**	23/40	**57·5**	34/38	**89***	20/29	**69***	21/41	**51***	19/29	65·5
		Isoniazid	30/41	**73***	22/41	54	31/41	75	21/28	**75†**	24/41	**58·5†**	29/39	74
		Procainamide	30/36	**83·5†**	19/42	45	35/38	**92†**	24/30	**80†**	22/42	**52***	23/35	66
		α-Methyldopa	12/16	**75**	18/32	**56**	27/29	**93***	7/9	**78***	19/32	**59†**	30/33	**91***
		Chlorthalidone	24/33	**72†**	18/32	**56**	26/31	**84**	14/18	**78†**	20/35	**57†**	29/36	**80·5**
		Controls	12/32	37·5	7/28	25	15/29	52	5/21	25	4/29	14	18/34	53

Frequencies significantly higher in treated than in control mice are printed in **bold** type.
Bold = $P<0.05$.
* = $P<0.01$.
† = $P<0.001$.

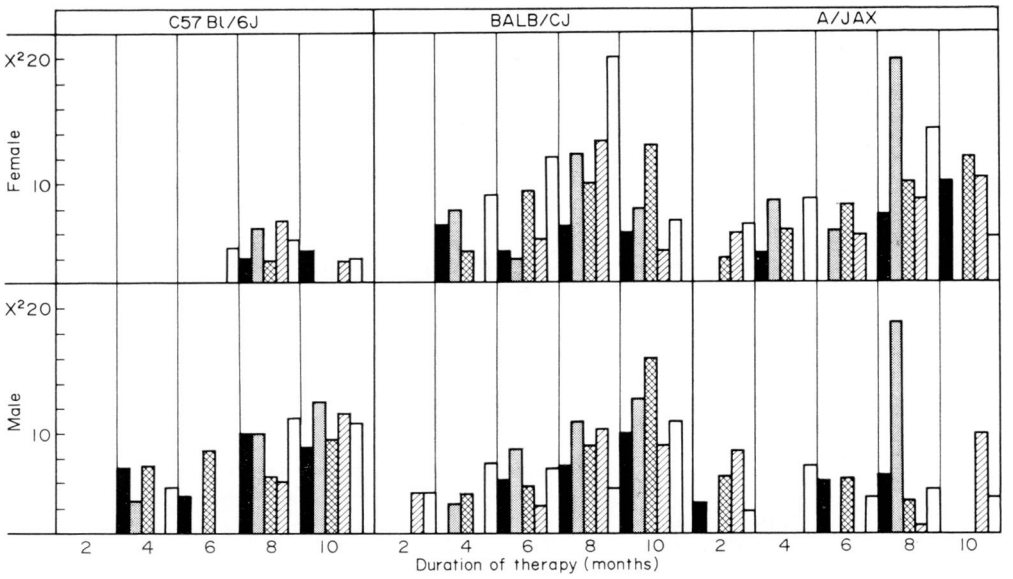

FIG. 2. χ Square values of the experimental groups, compared with the controls. Height of χ square is given on the vertical axis. Non-significant values, i.e. χ square below 3·84 ($= P>0·05$), are not shown. Solid bar, Hydralazin treated mice; stippled bar, isoniazid treated mice; diagonal cross hatched bar, procainamide treated mice; diagonal hatched bar, α-methyldopa treated mice; Open bar, chlorthalidone treated mice.

TABLE 4. Frequencies of antibodies to native DNA

Drugs	C57/BL/6J		BALB/CJ		A/JAX	
	No. positive	(%)	No. positive	(%)	No. positive	(%)
Hydralazin	0/16	0	0/16	0	12/59	20·5
Isoniazid	0/16	0	0/16	0	11/56	19·5
Procainamide	0/16	0	0/16	0	12/61	20
α-Methyldopa	n.d.		n.d.		4/20	20
Chlorthalidone	n.d.		n.d.		5/20	25
Controls	0/16	0	0/16	0	9/40	22·5

The mean frequency of antibodies to native DNA in drug treated animals is 20·4%. This differs not significantly from the anti-native-DNA frequency in control mice (22·5%).

After 10 months the differences between experimental and control groups decreased, owing to the increase of spontaneous ANA frequencies in the control mice.

Sex differences were observed in the A/JAX strain after 10-months treatment. Both hydralazin- and procainamide-treated females showed significantly increased frequencies as com-

pared to the males ($P<0{\cdot}05$). Similar sex differences were noticed in the BALB/CJ strain. However, in the C57/BL/6J mice there was a preponderance of males.

After 6 weeks the α-methyldopa treatment had to be discontinued, as the mice refused to drink water containing this drug. This water abstinence led to a mortality rate which forced us to supply this group with normal water in order to prevent the group from dying. After 4 weeks of normal water treatment with α-methyldopa was restarted at a much lower dosage (1·25 mg/day). The dip in the α-methyldopa-induced ANA frequency line (Fig. 1) may therefore be due to this temporary withdrawal of the drug.

TABLE 5. Correlation between ANA and antibodies to native DNA

	All drug treated mice		Control mice	
	ANA positive	ANA negative	ANA positive	ANA negative
Anti-DNA positive	42	2	9	0
Anti-DNA negative	119	53	16	15
Total	216		40	
	$P<0{\cdot}001$		$P = 0{\cdot}02$	

In both drug treated and control mice the presence of antibodies to native DNA coincides with ANA.

E. Antibodies to native DNA

Table 4 shows the frequencies of antibodies to native DNA. These antibodies were found only in A/JAX mice. No significant differences were observed between males and females; the frequencies in both sexes were almost identical. There were also no significant differences between drug treated and control mice: in both drug treated and control mice anti-DNA antibodies occurred in equal frequencies. The presence of anti-DNA antibodies coincided with the presence of ANA (Table 5).

F. Results of other tests

Direct antiglobulin test: No positive reactions were found in any of the treated animals.
Rheumatoid factor: the Waaler Rose test was also negative in all the animals tested.

G. Mortality

Mortality was highest in the BALB/CJ strain, but did not differ significantly from that in the others. The α-methyldopa treated BALB/CJ mice alone had a death rate significantly above the controls ($P<0{\cdot}001$) (Table 6). Initially we attributed this death rate to the low water consumption but after lowering of the α-methyldopa dose BALB/CJ mice still appeared to die in larger numbers than mice of the other strains. Post-mortem examination revealed no special signs in these cases.

TABLE 6. Mortality of drug treated and control mice

Drug	Mortality								
	C57/BL/6J			BALB/CJ			A/JAX		
	No.		†	No.		†	No.		†
	Before drug administration	After 10 months		Before drug administration	After 10 months		Before drug administration	After 10 months	
Hydralazin	84	81	3 = 3·6%	78	68	10 = 13%	84	67	17 = 20%
Isoniazid	84	82	2 = 2·5%	78	69	9 = 11·5%	84	80	4 = 5%
Procainamide	84	84	0 = 0%	78	66	12 = 15%	84	73	11 = 13%
α-Methyldopa	72	70	2 = 2·8%	72	25	47 = 65%	72	62	10 = 14%
Chlorthalidone	71	67	4 = 5·6%	62	51	11 = 18%	72	67	5 = 7%
Controls	71	71	0 = 0%	72	54	18 = 25%	72	63	9 = 12·5%

BALB/CJ mice treated with α-methyldopa have a death rate significantly above the controls ($P < 0{\cdot}001$).

DISCUSSION

In these experiments we showed that hydralazin, isoniazid, procainamide, α-methyldopa and chlorthalidone all produce an increase in ANA frequency in inbred strains of mice. As far as hydralazin and isoniazid are concerned these results partially confirm previous work by other investigators (Cannat & Seligmann, 1968; Braverman & Lerner, 1962). However, no experiments designed to induce SLE-like symptoms in mice with the other three drugs have yet been reported. Marked differences were observed between the three strains: A/JAX mice appeared to be the strongest responders; they were the first to show a significant increase in ANA frequency and also showed the highest frequencies, whereas C57/BL/6J mice showed less reaction. BALB/CJ mice were intermediate between the two; this correlates fairly well with the spontaneous ANA frequencies which are highest in A/JAX and lowest in C57/BL/6J. These findings both contradict and confirm the work of Cannat & Seligmann (1968), who demonstrated increased ANA frequencies in BALB/C and C57/BL mice under hydralazin and isoniazid treatment; frequencies in BALB/C were much lower than in C57/BL mice, again correlating with the spontaneous ANA frequencies of the controls. In spite of the differences in the figures the general conclusions to be drawn from the two experiments thus appear similar: namely that the spontaneous ANA frequencies in a given strain can provide an indication of the magnitude of response to be expected in an experimental situation. It might be supposed that exogenous stimuli such as the drugs used in this study will induce autoimmune phenomena such as ANA in individuals whose genetical predisposition to react to such stimuli is reflected by their spontaneous ANA frequency. Once this syndrome has been initiated, it is partially reversible as is illustrated by the temporary withdrawal of α-methyldopa; the ANA frequencies in the mice decreased but did not reach control levels. Resumption of administration rapidly increased ANA frequencies. This is in full agreement with the pattern in human DILE (Alarçon Segovia, 1969). In contrast to our results Cannat & Seligmann (1968) also reported no effect in BALB/C mice after 6-months treatment with isoniazid.

Whereas in idiopathic autoimmune disease females are more frequently affected than males, this is not the case in DILE (Molina *et al.*, 1969; Alarçon Segovia, Fishbein & Betancourt, 1969) and with two exceptions (female A/JAX mice treated for 10 months with hydralazin or procainamide) this was confirmed in our experiments. It was, however, surprising that C57/BL/6J males appeared to be stronger responders than females.

Although some drugs induced a significant increase in ANA frequency after as little as 2 months, 6–8 months were required before results were clear in all strains and with all drugs. A similar initial period of 2–8 months has also been reported for human DILE.

The results of the anti-DNA determination are compatible with the findings of Koffler *et al.* (1969) who reported negative tests for anti-native-DNA in patients with procainamide induced SLE. Also studies on hydralazin- and isoniazid-induced ANA in mice (Cannat & Seligmann, 1968) and men (Alarçon Segovia *et al.*, 1969) revealed no antibodies to native or single stranded DNA. But Molina *et al.* (1969) report the occurrence of antibodies to DNA in 36% of patients receiving procainamide, however, without indicating whether they used native or single stranded DNA. Spontaneous autoantibodies to double stranded DNA have so far been reported only in patients with SLE (60%) (Koffler *et al.*, 1969), and in NZB/W-F_1 mice (21–100%) and A/JAX mice (4%) (Thoburn, Koffler & Kunkel, 1971; Steinberg, Pincus & Talal, 1969). The anti-DNA antibody frequency in our A/JAX mice varies between 20 and

25%, which is considerably higher than the frequency reported by Steinberg *et al.* (1969). This discrepancy may be partly explained by the fact that our mice were all over 12-month old whereas those in the study of Steinberg were 8–12-month old.

We can offer no explanation for the persistently negative direct antiglobulin test on red cells, which is of particular interest with regard to the α-methyldopa used. It should be noted that no signs of overt haemolysis were observed in the α-methyldopa-treated group.

The reason for the increased death rate of BALB/CJ mice taking α-methyldopa is still obscure. Death occurred at random, i.e. ANA positive as well as ANA negative mice died. Therefore this phenomenon did not interfere with our statistics.

The present study gives no indication whether the drugs involved may have some effect on nuclear antigens, resulting in alterations which provoke the formation of antibodies cross-reacting with unaltered nuclei, or whether these drugs have a more direct influence on the behaviour of the immune system. Further work will be required to answer these fundamental questions.

The results of other serological and histopathological studies of the influence of these drugs on mice will be presented in the near future. Preliminary results indicate the possibility that antibodies to smooth muscle and skeletal muscle can also be evoked by these drugs.

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ADDED IN PROOF

In a similar experiment as described here, chlorothiazide (Chlotride ®) (5 mg/ml drinking water) was given to the same three inbred strains of mice. A control group and an α-methyldopa group were included. The α-methyldopa treated group showed reproducible results, compared with the first experiment. ANA frequencies in chlorothiazide treated and control mice did not show significant differences. It may be concluded that chlorothiazide does not have similar effects on the immune response as the other drugs used in this study.

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