PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 160, 254–257 (1979)

Administration of α-Methyldopa and Autoimmune Hemolytic Anemia in Mice[1] (40429)

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An estimated 6–26% of patients treated with the antihypertensive drug, α-methyldopa, develop a positive direct antiglobulin test (1, 2). In general, a positive test occurs after several months or years of methyldopa therapy and is associated with a high daily dose of the drug (1, 3, 4). In spite of the high incidence of positive antiglobulin tests, overt hemolysis and anemia occur in less than 1% of methyldopa treated individuals (5, 6). The anemia is generally moderate, gradual in onset, and accompanied by reticulocytosis (3). Severe cases and fatalities have been reported (3, 5). Methyldopa anemia closely resembles idiopathic warm type autoimmune hemolytic anemia, but it can be differentiated on the basis of prompt remission following withdrawal of methyldopa (5, 7). The direct antiglobulin test, however, may remain positive for several months to years after discontinuing the drug (5, 7, 8). Even though the first cases of methyldopa associated hemolytic anemia were reported in 1966 (5) and this type of anemia is the most frequently occurring immune type of drug-induced hemolytic anemia (3), the mechanism by which methyldopa fosters autoantibody formation still remains obscure.

We report here experiments to develop an animal model of methyldopa associated hemolytic anemia. Mice of three strains were subjected to long term treatment with methyldopate and evaluated for the development of positive direct antiglobulin tests and anemia. Two of the strains studied, NZB and A/J, are considered genetically susceptible to autoimmune disease (9, 10, 12). The NZB strain spontaneously develops an autoimmune syndrome including hemolytic anemia (9), while an autoimmune hemolytic anemia can be induced in the A/J strain by neonatal thymectomy (12, 13), and this strain shows a marked propensity to develop antinuclear antibodies (11, 12). No autoimmune phenomena have been described in CBA mice, the third strain examined in this study (10, 12, 14).

Materials and methods. Treatment. A/J, CBA/H-T6 and NZB/B1 strains of mice were purchased from the University of Minnesota Mouse Colony, Minneapolis, MN. Mice were treated either orally or by injection with methyldopate hydrochloride (Aldomet, Merck and Company, Inc., West Point, PA) beginning at 2–3 months of age. Animals treated by injection received one daily dose of five milligrams of methyldopate subcutaneously for periods of 3 weeks in CBA mice, 4 months in NZB mice and 8 months in A/J mice. This dosage was continued in each strain for an additional 2.7 months, but on a schedule of 5 consecutive daily injections per week. On a body weight basis, this dose is equivalent to 200 mg/kg for a 25 g mouse and is comparable to a dose of 14 g/day for a 70 kg man.

CBA mice treated orally received 0.1 mg of methyldopate per ml of drinking water for 6.6 months; the dosage was then increased to 0.4 mg/ml. NZB mice received a constant dose of 0.4 mg methyldopate per ml of drinking water. Fresh solutions of the drug were prepared twice weekly and were offered to the mice *ad libitum*. Oral treatment was continued for the life of the animal. Measurements of water consumption indicated that each mouse received 0.1–1.2 mg of methyldopate per day or 4–48 mg methyldopate per kg body weight for a 25 g mouse. This is equivalent to a dose of 0.28–3.5 g/day for a 70 kg man. Recommended therapeutic dosages in man range from 0.5 to 2.0 g/day (15).

Deaths were recorded daily, and occasional moribund animals were sacrificed. All remaining mice were sacrificed at 753 days of age when the study was terminated. Age at death or at the time of sacrifice is taken as survival time in all calculations.

[1] Supported in part by grants from the Veterans Administration.

0037-9727/79/020254-04$01.00/0

Evaluation of treatment. Experimental and control mice were bled before initiation of treatment with methyldopate and at periodic intervals during the course of treatment. Samples of 200 μl were collected from the retro-orbital plexus at each bleeding using two heparinized capillary tubes for each mouse (Fisher Scientific Company, Philadelphia, PA). Percent packed erythrocyte volumes were measured by a standard microhematocrit method. Erythrocytes from treated and control mice were tested for agglutination with antisera to mouse immunoglobulins and C3 as previously described (16). Antisera to total immunoglobulins, IgG_1, IgG_2, IgM, IgA and C3 were used.

Student's *t*-test was used to compare the difference between means. Frequencies of positive antiglobulin reactions were compared by Chi square analysis. Probability values of less than 0.01 were considered significant.

Results. Microhematocrits. As seen in Table I, no significant differences in microhematocrit levels were noted between methyldopate treated animals and untreated animals in either NZB or A/J strain mice. However, significant reductions in microhematocrits were observed with increasing age in all groups of mice. By 12–13 months of age, microhematocrits in NZB mice had decreased by approximately one third from the pretreatment values obtained at 2–3 months of age. Further decreases occurred only in the control and orally treated groups. Microhematocrit levels of the A/J mice remained constant during the first 12–13 months in both groups. Thereafter, progressive decreases of similar degree were observed in both treated and control groups. At 8–9 months of age, all groups of CBA mice showed slight decreases in microhematocrits from the pretreatment values. In both control and methyldopate injected CBA mice, but not in the orally treated group, microhematocrits continued to decrease with advancing age with maximum reductions at 2 years of age.

Antiglobulin reactions. There were no significant differences between experimental and control groups of any strain in the incidence (Table II) or intensity of antiglobulin reactions. The onset of positive reactions was similar in all NZB groups with 25–39% of the animals in each group reactive at 8 months of age. In 1 year old NZB mice, 11 of 21 controls, 4 of 7 injected and 5 of 10 orally treated animals were positive for red cell bound IgG_1 and/or IgG_2. Weakly positive reactions with antisera to IgM, IgA or C3 were observed with erythrocytes from only a few individuals.

With the exception of one control animal which was weakly reactive with polyvalent anti-mouse immunoglobulin antiserum at 12 months of age, both treated and control groups of A/J mice were negative in red cell antiglobulin tests through 16 months of age. At 21 months of age, erythrocytes from 27 to 29% of the surviving animals in both control and injected groups were positive for one or more immunoglobulins or C3. No positive antiglobulin reactions were observed at any time with the erythrocytes of methyldopate treated or control CBA mice.

Body weights and survival times. The dosages of methyldopate used in these experi-

TABLE I. MICROHEMATOCRIT LEVELS AND SURVIVAL TIMES IN MICE TREATED WITH METHYLDOPATE.

Mouse strain	Administration of methyldopate	Number of mice	Mean microhematocrit (±SE) at age: 2–3 months[a]	8–9 months	12–13 months	16.5–19.5 months	23–24 months	Mean survival time (±SE) Days
NZB	Injection	8		48.4 ± 0.6	33.9 ± 2.1[b]	34.3 ± 2.3[b]	—	508 ± 13
	Oral	13	49.6 ± 0.2	44.9 ± 0.8[b]	34.3 ± 1.2[b]	28.4 ± 2.7[b]	—	421 ± 45
	None	23		45.2 ± 1.5[b]	34.0 ± 0.9[b]	31.0 ± 0.8[b]	—	443 ± 16
A/J	Injection	19	49.1 ± 0.4	49.5 ± 0.3	48.9 ± 0.3	40.2 ± 1.7[b]	38.2 ± 0.9[b]	701 ± 20
	None	19		50.0 ± 0.3	48.8 ± 0.3	42.1 ± 0.7[b]	35.7 ± 1.9[b]	739 ± 6
CBA	Injection	10		44.4 ± 0.5[b]	41.2 ± 1.0[b]	39.7 ± 1.6[b]	36.4 ± 2.5[b]	655 ± 48
	Oral	10	49.7 ± 0.2	45.4 ± 0.4[b]	45.6 ± 0.6[b]	45.0 ± 0.9[b, c]	—	538 ± 42[d]
	None	10		45.0 ± 0.6[b]	42.5 ± 0.9[b]	39.6 ± 1.0[b]	39.4 ± 1.2[b]	706 ± 34

[a] Mean value of all mice of same strain before initiation of treatment.
[b] $p < 0.01$ in comparison to pretreatment microhematocrit of appropriate strain.
[c] $p < 0.01$ in comparison to control group of same age and strain.
[d] $p < 0.01$ in comparison to control group of same strain.

TABLE II. THE DEVELOPMENT OF POSITIVE ERYTHROCYTE ANTIGLOBULIN REACTIONS IN MICE TREATED WITH METHYLDOPATE.

Mouse strain	Administration of methyldopate	Number of mice with positive[a] direct antiglobulin reactions/number of mice tested at age: 8–9 months[b]	12–13 months	16.5–19.5 months	21–24 months
NZB	Injection	2/8	6/7	1/2	—
	Oral	5/13	6/10	3/7	—
	None	6/23	12/21	4/9	—
A/J	Injection	N.D.	0/19	0/17	3/11
	None	N.D.	1/9	0/17	4/14
CBA	Injection	0/10	0/10	0/10	0/6
	Oral	0/9	0/8	0/4	—
	None	0/8	0/8	0/7	0/6

[a] Microscopic reaction ≥ +1 (16) with at least one antiserum.
[b] All pretreatment tests at 2–3 months of age were negative. N.D. = not done.

ments had no apparent toxic effects on the mice. Body weights (not shown) were similar in treated and untreated groups of each strain throughout the course of study with one exception. At 12 months and 16.5 months of age, the body weights of orally treated CBA mice were significantly decreased ($p < 0.001$) from their previous weights at 8 months of age, and from the weights of the injected and control groups of the same age. A significantly decreased survival time was also noted in the orally treated but not in the injected CBA mice in comparison to control mice of the same strain (see Table I). Survival times of NZB mice were shorter than those of either the A/J or CBA control and injected mice. The survival times of treated and control mice of the A/J strain were not significantly different.

Discussion. In this study, anemia of varying degree developed in all three strains of mice studied, and positive erythrocyte antiglobulin reactions occurred in NZB and A/J mice. However, these conditions were observed in both experimental and control groups, and thus cannot be attributed to methyldopate treatment. The spontaneous occurrence of autoimmune hemolytic anemia in NZB mice (9, 17) was uninfluenced by the administration of methyldopate. Microhematocrit levels and antiglobulin reactions were similar in both of the methyldopate treated groups and in untreated controls of the NZB strain. Age at onset of anemia as well as incidence and immunoglobulin classes of anti-erythrocyte autoantibodies were also similar in all NZB groups.

Although spontaneous positive direct antiglobulin reactions have been observed in A/J mice, no spontaneous autoimmune hemolytic anemia has been described in this strain (12, 13). This type of anemia has been induced in A/J mice by neonatal thymectomy (12, 13). In addition, antinuclear antibodies that increase in incidence with advancing age are known to occur spontaneously in A/J mice (11, 12). We observed significantly decreased microhematocrits in mice of this strain at 16–19 months of age and older, and direct antiglobulin tests were positive with erythrocytes from 27 to 29% of these mice at 21–24 months of age. Since positive antiglobulin reactions and decreased hematocrits occurred equally in methyldopate treated and untreated A/J mice, the observed anemia appears to be a spontaneous age-associated phenomenon which may have an autoimmune component. Age related decreases in microhematocrit frequently associated with increasing incidence of anti-erythrocyte autoantibodies have also been reported in several other strains of mice (18).

In contrast to the results with NZB and A/J strain mice, we found no evidence of erythrocyte autoantibodies in either untreated or methyldopate treated CBA mice even at 24 months of age. Thus, the progressive decrease in microhematocrit levels observed with advancing age in both control and methyldopate injected CBA mice cannot be accounted for by autoantibody mediated red cell destruction. It is possible that the high mortality rate of the orally treated CBA groups resulted in selection for individuals with higher hematocrits. This could explain the failure of that group to develop a similar reduction in microhematocrit levels with age.

Thus far, only daily dosage and duration

of methyldopa therapy have been identified as factors promoting methyldopa associated positive antiglobulin reactions in man (1, 3, 4). In contrast, numerous factors have been shown to influence the development of autoimmune hemolytic anemia in mice (9, 12, 13, 18, 19) and idiopathic autoimmune hemolytic anemia in man (20). On the basis of our results, it appears that age and a genetic predisposition to autoimmune disorders are not sufficient for the induction of methyldopa associated autoimmune hemolytic anemia in mice even with prolonged high dose drug treatment. Thus, the factors determining the development of autoimmune hemolytic anemia during methyldopa therapy remain unknown. Because methyldopa polymer has been found to bind to erythrocytes and sensitize them to react with anti-IgG, it has been suggested that polymerized methyldopa reacts with serum proteins or erythrocyte antigens and renders them autoantigenic (6, 21). Thus, methyldopa induced autoantigens rather than an abnormality of the immune system would be responsible for methyldopa autoimmune hemolytic anemia (7, 21). Although our results provide no direct information on the mechanism whereby methyldopa treatment induces autoantibody formation, our finding that a genetic susceptibility to autoimmune disorders did not predispose mice to the development of autoimmune hemolytic anemia during methyldopa treatment would be consistent with this hypothesis.

Summary. Mice were subjected to prolonged high dose treatment with methyldopate hydrochloride (Aldomet), and evaluated for the development of positive direct antiglobulin reactions and anemia. NZB and A/J strains which are genetically susceptible to autoimmune disease and the nonsusceptible CBA/H-T6 strain were studied. Methyldopate was administered orally or subcutaneously at doses of 4–200 mg/kg body weight beginning at 2–3 months of age. Treatment by injection was carried out for 3.4–10.7 months while oral treatment was continued for the life of the animal. The time of onset and severity of the spontaneous autoimmune hemolytic anemia in NZB mice were not influenced by methyldopate treatment. Microhematocrit levels decreased significantly with advancing age in A/J and CBA mice, and positive direct erythrocyte antiglobulin reactions were noted in A/J mice at 21 months of age. These conditions occurred equally in experimental and control groups, however, and could not be attributed to methyldopate treatment. It is concluded that age and a genetic predisposition to autoimmune disorders are not sufficient to induce methyldopa associated autoimmune hemolytic anemia in mice even with long term high dose drug treatment.

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Received July 25, 1978. P.S.E.B.M. 1979, Vol. 160.