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IMMUNOPHARMACOLOGIC EFFECTS OF CYCLOLEUCINE

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ABSTRACT

ROSENTHALE, MARVIN E., LOUIS J. DATKO, JACOB KASSARICH AND EUGENE I. ROSANOFF: Immunopharmacologic effects of cycloleucine. J. Pharmacol. Exp. Ther. **180:** 501–513, 1972.

Cycloleucine (1-aminocyclopentane carboxylic acid) was shown to be quite active in preventing the clinical and histologic signs of experimental allergic encephalomyelitis and adjuvant arthritis. In contrast, it was inactive in two *in vitro* models designed to detect various types of clinically effective anti-inflammatory, anti-immune agents: tissue culture growth and polymorphonuclear leukocyte migration. Its immunopharmacologic spectrum differs from that of other clinically active agents, suggesting that it may work *via* a different mechanism or at a different site.

Cycloleucine (1-aminocyclopentane carboxylic acid) is an unnatural, nonmetabolizable amino acid, inhibitory against various experimental malignancies but having little effect as an antitumor agent in humans (Johnson, 1963). The immunopharmacologic activity of cycloleucine first became evident in rodents through our observation of its effects on experimental allergic encephalomyelitis (EAE) (Rosenthale *et al.*, 1968; Rosenthale and Gluckman, 1968) and adjuvant arthritis (AA) (Rosenthale *et al.*, 1969a). These immunosuppressive effects were recently confirmed in several other systems (Frisch, 1969; Frisch and Wilson, 1969; Rubin and Tint, 1971). This paper is an extension of our early work and describes the effects of cycloleucine in several experimental analogs of immunoinflammatory disease.

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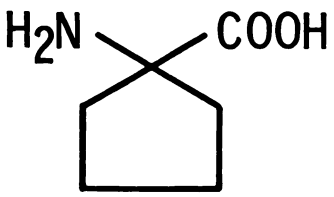
Methods

Cycloleucine. See structure I for composition of cycloleucine. It is soluble in water (5%), its molecular weight is 129 and it has the general properties of a neutral amino acid. Lacking an α-hydrogen, it is symmetrical in structure and has no optical isomers.

Animals. Male Lewis strain rats (Microbiological Associates, Bethesda, Md.) weighing 150 to 200 g were used in all experiments. Rats were housed four to a cage and allowed food (Purina Lab Chow on floor of cage) and water *ad libitum*. Temperature, humidity and hours of artificial lighting were kept constant.

EAE and AA. EAE and AA were induced by methods previously described (Rosenthale *et al.*, 1969b; Rosenthale and Nagra, 1967), *i.e.*, briefly, by subplantar administration on day 0 of 0.05 ml of either an encephalitogenic emulsion consisting of syngeneic spinal cord (10 mg) in complete Freund's adjuvant (0.1 mg of *Mycobacterium tuberculosis*) for production of EAE or a suspension of either *M. tuberculosis* or *Mycobacterium bu-*



1-AMINOCYCLOPENTANE-CARBOXYLIC ACID

STRUCTURE I

tyricum (0.75 mg) in mineral oil for the production of AA.

Tissue culture. Cycloleucine and several other drugs were tested on complete monolayer cultures of embryonic rat fibroblasts and embryonic WI-38 human lung fibroblasts by a modification of the methods described by Foley and Eagle (1958) and Eagle and Foley (1958). Cells were grown on Eagle's basal medium supplemented with 10% fetal calf serum and were maintained on Eagle's basal medium containing 2% fetal calf serum. Drugs at various dilutions were added at a volume of 0.2 ml to the cells being maintained in roller tubes and all tubes were microscopically evaluated after 24 and 48 hours incubation at 37°C. Five tubes were used for each drug concentration.

Polymorphonuclear (PMN) leukocyte migration. A modification of the method of Phelps and Stanislaw (1969) using the Boyden-type chamber described by Cornely (1966) was used to study the effects of the drugs on the migration of PMN leukocytes through a Millipore filter.

PMN cells were harvested from the glycogen-stimulated peritoneal cavity of the rabbit. To induce cell migration, *Escherichia coli* (4.7 mg) was added to the test compartment as the chemotactic agent. A 13-mm Millipore filter with a 3 μ pore size was placed between the upper drug plus cell and test compartments. The chambers then were incubated for 1.5 to 2 hours at 37°C and the filters were removed, stained and cleared in xylene. The cells migrating through the filter of each chamber were counted in 10 adjacent high power fields, starting at the center of each filter. Three chambers were run per dose level of drug.

Results

Effects on clinical manifestations of EAE and AA. Clinical manifestations of EAE began with severe weight loss 9 to 10 days after administration of the encephalitogenic emulsion. The dose of antigen was selected so that clinical signs were maximal at days 13 to 16 in 90 to 100% of the animals injected and consisted primarily of flaccid hindlimb paralysis, urinary incontinence, fecal impaction and abdominal flaccidity. For purposes of evaluation, animals showing any of these signs were considered positive. The protective effects of cycloleucine against these clinical signs of EAE are shown in figure 1.

Cycloleucine prevented the appearance of EAE in a dose-related manner on all three dosage schedules. The drug-treated animals appeared quite healthy when compared to the EAE-afflicted controls. When compared on an alternate-day dosing schedule in EAE, cycloleucine ranks in potency between the alkylating agent cyclophosphamide and the purine antagonist 6-mercaptopurine (Rosenthale and Gluckman, 1968).

Drug treatment of AA in rats can be evaluated in three phases: on the initial swelling of the injected paw occurring by days 3 to 6, followed by a slight remission lasting several days; on the large secondary increase in paw volume beginning on day 9 or 10 and accompanied by a swelling of the opposite, noninjected hind limb

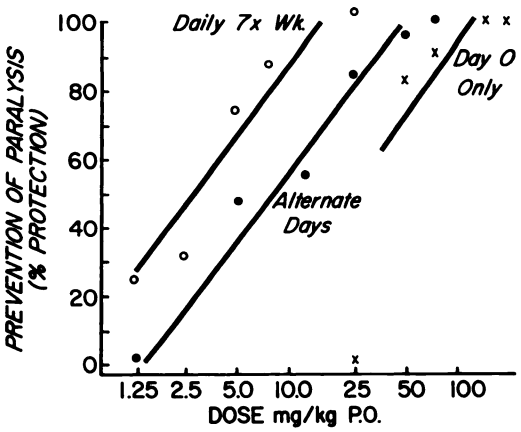


FIG. 1. Protective effects of cycloleucine on appearance of clinical signs of experimental allergic encephalomyelitis in the rat when administered by various dosage schedules beginning the day of antigen (day 0). Animals on the daily schedule received a total of 14 doses; those on the alternate weekday schedule, a total of six doses; and those on the day 0 only schedule, a single dose on day of antigen administration. Results were calculated by comparison with control groups examined simultaneously for maximal paralytic signs on days 13 to 16. Each point represents results from 12 to 36 rats.

and forepaws, urethritis and secondary polyarthritic lesions consisting of nodules on the ears and tail; and finally, the established disease beginning on day 14 can be treated. To evaluate AA, paw volumes were measured with a mercury plethysmograph and secondary signs were evaluated by assigning one point for the appearance of each up to a maximum of six points, as previously described (Rosenthale, 1970).

Cycloleucine was ineffective in preventing the primary inflammatory response of the injected right paw regardless of the dose or schedule used. Conversely, it was extremely effective in preventing the appearance of secondary swelling in both the injected right and noninjected left paw (fig. 2). Both severity and incidence of secondary polyarthritic signs also were inhibited by all three dosage schedules. Both steroidal and nonsteroidal anti-inflammatory agents reportedly are effective against both the primary and secondary phases of AA, whereas immunosuppressive-type drugs act only against the secondary phase (Graeme *et al.*, 1966).

The dose-response curves shown in figures 1 and 2 indicate that cycloleucine is effective against EAE and AA. The clinical manifestations of either of these two immunopathologic models of inflammation were prevented to a similar extent regardless of the dosage schedule, *i.e.*, a total dose of 100 mg/kg gave at least a 50% protective effect regardless of whether given as a single dose or distributed over several days.

The effect of cycloleucine and various other drugs on the third phase of AA, the established disease, is shown in figure 3. In this test, AA first was allowed to develop before the effect of drug was tested. Here again, differential effects were observed between immunosuppressive agents and nonsteroidal or steroidal anti-inflammatory drugs. Immunosuppressive agents were without great therapeutic effect at doses previously shown to be extremely active prophylactically against EAE (Rosenthale *et al.*, 1969b) and the secondary phase of AA, whereas several nonsteroidal drugs and a glucocorticoid were quite effective in rapidly reversing the established inflammatory condition. The results seen with cycloleucine in the three phases of AA described here resemble those routinely obtained with immunosuppressive agents in this test (Glenn, 1966).

Cycloleucine and body weight. Cycloleu-

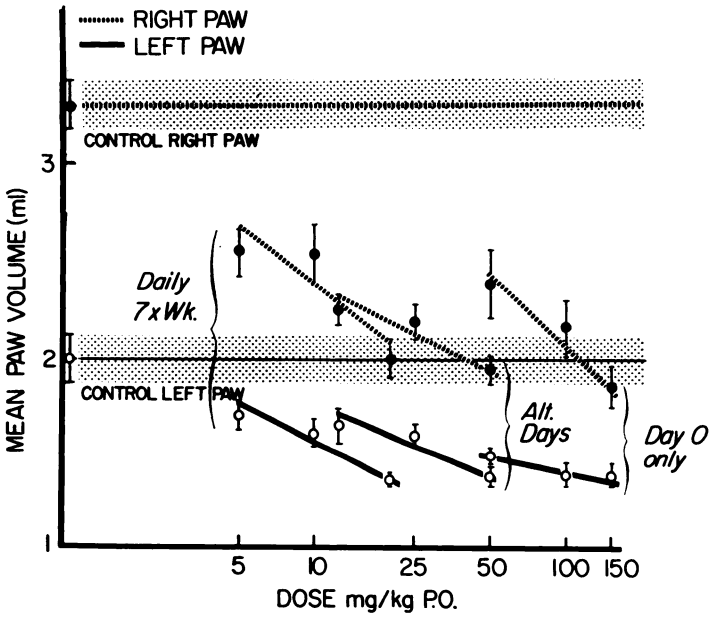


FIG. 2. Protective effects of cycloleucine on appearance of secondary paw swelling in rats with adjuvant-induced arthritis when administered by various dosage schedules beginning the day of antigen administration (day 0). Animals received the same total number of drug doses as described for figure 1. Paw volumes were evaluated on day 14. Each point represents mean paw volumes (±S.E.) of 10 rats for drug and 20 rats for controls. With the exception of the daily 5 mg/kg dose in the left paw, all points are significantly different from controls ($P < .05$).

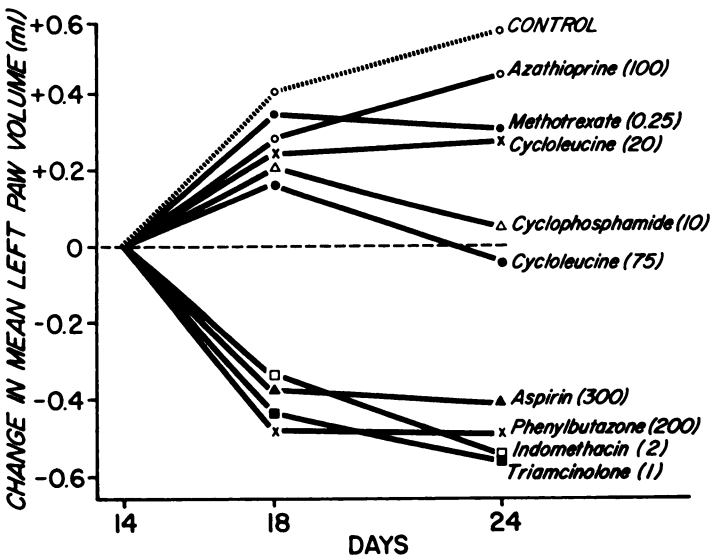


FIG. 3. Therapeutic effect of drugs on established adjuvant-induced arthritis. Methotrexate, azathioprine, cyclophosphamide and cycloleucine (75 mg/kg) were given orally on days 14, 16, 18, 21 and 23 (total of 5 doses). Aspirin, phenylbutazone, indomethacin, triamcinolone acetonide and cycloleucine (20 mg/kg) were administered daily on days 14 to 23 (total of 10 doses). The doses of drugs (milligram per kilogram orally) are shown in parentheses. Each point represents mean results of eight rats. The animals receiving cycloleucine were additionally force-fed 15 to 20 g per day of a basal diet to prevent body weight loss (Clark *et al.*, 1966). Of the rats administered cycloleucine at 20 mg/kg, one of eight died. Some weight gain (20 g compared with 30 g for normal rats) was obtained with cyclophosphamide; all other immunosuppressive agents caused either no change or slight losses in body weight.

cine had a dual effect on weight gain; low therapeutic doses prevented the loss of body weight observed with either EAE or AA, whereas larger doses themselves caused decreased weight gain or even loss. The latter effect was probably due to a loss of appetite, resulting in a decreased food intake, and could be antagonized by force-feeding with 15 to 20 g per day of a basal diet (Clark *et al.*, 1966).

An example of these effects can be seen in figure 4. EAE controls (no drug) in this study showed the characteristic rapid and steep loss of body weight beginning on day 10. We have previously shown that 15 to 20 g per day of this basal diet is sufficient to promote normal body growth and that the loss of weight, but not the onset of paralytic signs of EAE, can be inhibited by force-feeding of this diet (Rosenthale *et al.*, 1969b). The acute weight loss was prevented to a great extent by administering cycloleucine at therapeutic doses of either 35 or 50 mg/kg but was enhanced by a larger, toxic dose (75 mg/kg). Force-feeding of another group of EAE rats with the basal diet in addition to the 35 or 50 mg/kg doses of cycloleucine resulted in virtually normal body weight gain, with no loss of the therapeutic effect of cycloleucine in EAE. The toxic effect observed at 75 mg/kg, however, was not affected by force-feeding.

Animals were force-fed only until day 14. Once this ceased, a severe drop in body weight ensued. The weight loss was accompanied by a delayed toxicity. By day 26, six out of eight rats given 35 mg/kg of drug plus basal diet had died, compared to none out of eight on this dose of drug alone. Similarly seven out of eight rats were dead in the 50 mg/kg plus diet group, compared with only one out of eight in the nondieted drug-treated group. All animals in both groups given 75 mg/kg were dead at day 16. This study indicates the following: 1) Cycloleucine at lower nontoxic doses can prevent the body weight loss which accompanies EAE. Similar effects also are obtained in AA. 2) Weight loss after larger doses appears to be due to a curtailed food intake, since force-feeding with a basal diet can completely reverse the body weight decrease. 3) Antagonism of weight loss by force-feeding does not interfere with either the therapeutic or toxic effects of cycloleucine. 4) Animals given cycloleucine plus diet appear to become dependent on

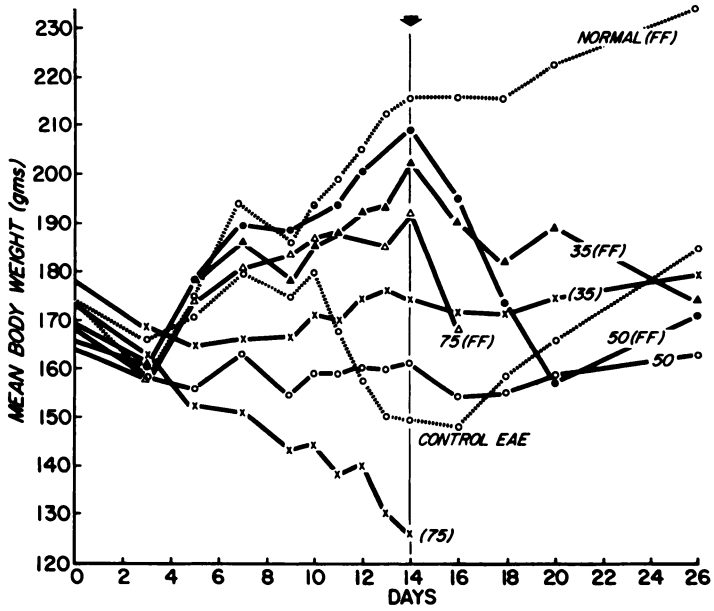


FIG. 4. Effect of force-feeding on therapeutic activity of cycloleucine in rats with EAE. FF, force-feeding (stomach tube) of basal diet (15–20 g/day in three or four divided doses on days 0 to 14). Numbers in parentheses refer to oral doses of cycloleucine, administered beginning on day of antigen (day 0) and then on alternate weekdays (total of six doses). All doses shown protected completely against the paralytic effects of EAE (zero of eight paralyzed in each group) for a 28-day period, whereas control animals had severe paralysis (eight of eight) on day 14. During the 14-day test period, three of eight rats died when given 75 mg/kg of cycloleucine alone, whereas five of eight died when this same dose was administered in combination with the force-fed diet.

the diet and subsequently die, apparently as the result of an inability to resume self-feeding.

Duration of effect. The duration of activity of a single dose of cycloleucine in rats afflicted with either AA or EAE is shown in table 1. Dose-related inhibition of AA paw swelling and of EAE paralysis was observed for all three doses of cycloleucine when evaluated on day 14 (table 1; figs. 1 and 2). A slow diminution of effect of cycloleucine on the right (injected) paw of AA rats occurred from day 14 to 56 with all three doses studied, although highly significant (greater than 25%) inhibition of swelling was still present on day 56 for the 100 and 50 mg/kg doses. A decrease in activity in the left (noninjected) paw was seen only at the lowest (50 mg/kg) dose.

In the EAE study, a similar decrease in activity over time was observed at the two lowest doses. A delayed onset of paralysis occurred by day 21 in one additional rat at the 100 mg/kg dose and in two additional rats at the 50 mg/kg dose. No animals became paralyzed at the highest dose during the 56-day observation period.

Delayed administration of cycloleucine. In EAE, the neurologic lesions appear initially as early as seven days after antigen injection (Waksman and Adams, 1962). Therapeutic agents drastically lose their effectiveness in EAE if given later than day 8 (Rosenthale *et al.*, 1969b). Similarly, removal of draining lymph nodes earlier than seven days after antigen inhibits development of AA (Newbould, 1964). These findings indicate that the delayed type of immunological mechanism responsible for the induction of either EAE or AA requires about seven days to mature and that elimination of immunologically committed lymphoid tissue after that time, either by immunosuppressive drugs or surgical extirpation, is of limited therapeutic usefulness.

Cycloleucine and several other immunosuppressive agents were ineffective in an established polyarthritis in rats (fig. 3). The temporal relationships between cycloleucine and the induction of either EAE or AA, therefore, were studied in greater detail (table 2). The results show that cycloleucine can still inhibit development of the clinical signs of either EAE or AA if given as a

TABLE 1

Percent inhibition of paw swelling in AA and of paralysis in EAE after a single oral dose of cycloleucine[a]

Day	50 mg/kg			100 mg/kg			150 mg/kg		
	Left paw	Right paw	EAE[b]	Left paw	Right paw	EAE[b]	Left paw	Right paw	EAE[b]
14	59	46	2/8	72	57	2/8	77	70	0/8
21	57	30	4/8	80	37	3/8	86	42	0/8
27	43	15	0/8	80	35	0/8	84	36	0/8
34	38	30	0/8	76	41	0/8	67	45	0/8
41	31	19	0/8	78	36	0/8	73	30	0/8
48	45	24	0/8	73	45	0/8	70	38	0/8
56	40	20	0/8	71	37	0/8	66	34	0/8

[a] Given on day of antigen (day 0) only; 10 rats per AA dose.

[b] Cumulative number of rats paralyzed/number tested; 8/8 rats were paralyzed by day 14 in control group.

TABLE 2

Effect of delayed administration of cycloleucine

Cycloleucine[a] Administration (Day)	EAE, % Inhibition of Paralysis[b]	AA, % Inhibition[b]			
		Paw swelling		Secondary score	No. rats with signs/no. tested
		Left	Right		
0	100	72[c]	57[c]	65	4/10
7	100				
8	100	57[c]	44[c]	92	1/10
9	83	33[d]	27[e]	12	8/10
10	0[d]	29[d]	19[d]	50	8/10

[a] Given as a single dose of 200 mg/kg to animals with EAE and 100 mg/kg to animals with AA.

[b] Evaluated on day 14 (antigen given on day 0); 12 rats per EAE group; 10 per AA group.

[c] $P < .005$ compared to control AA group.

[d] No significant inhibition.

[e] $P < .05$ compared to control AA group.

single large dose eight to nine days after antigen but no later.

Toxicity due to drug was not observed in AA rats. In EAE rats, however, a delayed toxicity was evident beginning on about day 20 and deaths were observed as long as 42 days after drug. Administration of cycloleucine at 200 mg/kg on days 0, 7, 8, 9 and 10 resulted in delayed deaths of 1, 2, 9, 6 and 4 of 12 rats, respectively. Thus, it appears that the toxicity of a single, quite large dose of cycloleucine is increased if the drug is given at the time of onset of EAE. Similar results have been described for both methotrexate and cyclophosphamide (Rosenthale *et al.*, 1969b). No delayed paralysis (onset after day 14) was observed over a 53-day period in any of the groups studied. The increase in toxicity with an increased delay of drug administration may indicate that the animals were in a weakened condition at the time of onset of EAE.

Organ and hematologic changes. The various stress organ and hematologic changes which occur in EAE and AA previously have been documented (Rosenthale and Nagra, 1967). The effects of cycloleucine on these changes in normal (nondiseased) rats and in rats with either EAE or AA are shown in table 3.

In normal rats, a large, toxic dose of cycloleucine had no effects on the adrenals but caused involution of the thymus and spleen and induced a leukopenia, primarily by depressing the PMN leukocyte population. Smaller doses, previously shown to be therapeutically effective, were not toxic and had considerably less effect on these parameters.

In EAE, small doses of cycloleucine were capable of reversing the thymic and splenic involution, adrenal hypertrophy, neutrophilia and lymphopenia, whereas a larger, toxic dose itself caused involution of the thymus and spleen and a leukopenia, again primarily due to PMN leukocyte depression.

Similarly in AA, both doses of cycloleucine corrected the thymic involution, splenic and adrenal hypertrophy, leukocytosis, neutrophilia and lymphopenia seen in this experimental disease.

Histopathology. Neurologic lesions have been observed in rats administered encephalito-

TABLE 3

Effects of cycloleucine on normal and diseased rats[a]

Oral Dose	Frequency	Total No. Doses	Organ Weight			Hematology		
			Thymus	Spleen	Adrenals	White blood count	Poly	Lymph
	days			*mg*		$\times 10^3/mm^3$	%	%
Normal								
Water			452	520	33	12	18	80
50 mg/kg	0, 1, 2	3	382	445	35	10	16	81
50 mg/kg	Alternate weekdays	6	285	400	33	11	11	87
100 mg/kg[b]	Alternate weekdays	6	38	214	39	5	7	91
EAE								
Normal			442	519	35	12	14	84
Control EAE			167	331	45	12	44	53
50 mg/kg	0, 2, 4	3	358	475	28	15	35	60
50 mg/kg	Alternate weekdays	5	340	440	29	13	35	62
75 mg/kg[c]	Alternate weekdays	5	199	299	35	10	15	82
AA								
Normal			479	587	40	20	29	60
Control AA			184	806	61	69	57	32
25 mg/kg	Alternate weekdays	8	193	377	38	19	44	47
35 mg/kg	Alternate weekdays	8	183	373	38	16	27	58

[a] Observations made on day 14 for normal and EAE rats, 6 rats per group, and on day 20 for AA rats, 12 per group.

[b] 8/12 rats dead by day 14; the remaining 4 were autopsied.

[c] 4/12 rats dead by day 14.

genic antigen but showing no apparent clinical signs of EAE (Paterson, 1966). Accordingly, histopathologic studies were done to be certain that cycloleucine suppresses rather than masks EAE. All animals were sacrificed at the termination of the experimental period (day 14). The brain, spinal cord, thymus, spleen, adrenals and pituitary were removed, fixed and processed (Paterson, 1966; Waksman and Adams, 1962). Neural tissues were stained with cresyl violet, the thymus, spleen and adrenals with hematoxylin and eosin and the pituitary with Ponceau fuchsin. Specimens from the forebrain, midbrain, cerebellum, upper and lower medulla and spinal cord were examined for the inflammatory lesions typical of EAE (table 4).

Neural lesions, consisting of multiple inflammatory foci and perivascular cuffing, were most severe in the lower medulla and spinal cord; as more rostral levels were reached, the intensity of inflammation decreased.

The oral administration of cycloleucine was effective in preventing the development of EAE lesions; there were no discernible changes in the thymus, spleen, adrenals or pituitary glands in any of the cycloleucine-treated animals. Treatment schedules of 50 mg/kg given two or three times allowed excellent body weight gain and completely suppressed central nervous system inflammation, whereas a single dose of 75 mg/kg allowed development of minimal to moderate lesions in two of six animals so treated. A delayed-onset paralysis also occurred at this dose. Since the administration of cycloleucine later than day nine apparently has little therapeutic effect on either EAE or AA (table 2) and since this drug has a prolonged duration of action (table 1), it appears that total doses of 75 mg/kg or greater given prior to day 9 are effective in the treatment of EAE and AA.

Antagonism with L-valine. It has been suggested that cycloleucine functions as a valine antagonist and that valine has been used to block the antitumor and toxic effects of cyclo-

TABLE 4

Effect of cycloleucine on the neuropathologic lesions of EAE

Oral Dose	Frequency	Total No. Doses	Clinical Signs	Neurologic Lesions[a]
	days			
Normal			0/12	Absent, 11/12
Control EAE			12/12	Severe, 12/12[b]
50 mg/kg	Alternate week-days	6[c]	0/12	Absent, 6/6
50 mg/kg	0, 2, 4	3	0/12	Absent, 6/6
50 mg/kg	0, 2	2	0/12	Absent, 6/6
75 mg/kg	0	1	1/12[d]	Moderate, 1/6[e] Very minimal, 1/6 Absent, 4/6

[a] 6/12 drug-treated animals autopsied on day 14; the remaining rats were observed until day 51.

[b] "Severe" indicates extensive perivascular cuffing, multiple inflammatory foci and in some cases meningitis observed in the five areas studied.

[c] Body organs not studied in this group.

[d] Delayed onset of paralysis in 4/6 remaining rats.

[e] "Moderate" indicates appearance of several perivascular cuffs and inflammatory foci in several slides. "Very minimal" showed only a slight meningitis of the basal area and cord.

leucine on *E. coli* in chickens and mice (Abshire and Pineau, 1967; Berlinguet *et al.*, 1962; Machlin *et al.*, 1963). Table 5 shows the effects of L-valine in EAE-afflicated rats treated with cycloleucine. A single 300 mg/kg dose completely suppressed EAE but was quite toxic by day 15. At a ratio of 80:1, L-valine completely prevented the toxicity of cycloleucine while only partially blocking its therapeutic effect (100% control of paralysis in the cycloleucine group compared with only 33% in the cycloleucine plus L-valine group). At a ratio of 106:1, virtually complete blockade of the therapeutic activity of cycloleucine was accomplished. These results were mirrored in the body weight changes; the decrease in body weight due to drug was blocked and decreases in body weight due to onset of EAE appeared in the L-valine-treated rats.

In an additional experiment, L-valine was administered at various times after cycloleucine to terminate its effects in AA rats (table 6). When a single large dose of cycloleucine (300 mg/kg)

TABLE 5

Antagonistic effects of L-valine and cycloleucine in rats with EAE

L-Valine was prepared as a slurry in water using an ultrasonic homogenizer (polytron) and given in two equal doses of 4 ml each, A.M. and P.M.

Treatment	Oral Dose	Frequency[a]	Total No. Doses	No. Paralyzed/ Total No. Rats	Total No. Dead/ Total No. Rats[b]
	per kg	*days*			
Control				8/8	0/8
Cycloleucine	300 mg	0	1	0/5	7/12
Cycloleucine +	300 mg	0	1	4/12	0/12
L-valine[c]	8 g	1, 2, 3	3		
Cycloleucine +	300 mg	0	1	9/12	0/12
L-valine[d]	8 g	1, 2, 3, 4	4		

[a] Antigen given on day 0.

[b] Total evaluation by day 15.

[c] 80:1 ratio of L-valine to cycloleucine.

[d] 106:1 ratio of L-valine to cycloleucine.

TABLE 6

Effects of cycloleucine when administered at specific times to rats with AA

Cycloleucine Administration (Day)[a]	L-Valine Administration (Days)[b]	Total No. Days Cycloleucine Present	% Inhibition[c]			No. with Arthritis Signs[c]	Toxicity[c]
			Left paw	Right paw	Secondary score		
0	2, 3, 4, 5	2	0	5	0	6/7	1/8
0	3, 4, 5, 6	3	0	0	0	7/7	1/8
0	4, 5, 6, 7	4	7	0	0	8/8	0/8
0	5, 6, 7, 8	5	68[d]	35[d]	35	8/8	0/8
5	6, 7, 8, 9	1	42[d]	23	8	8/8	0/8
7	8, 9, 10, 11	1	50[d]	29[d]	15	8/8	0/8
9	10, 11, 12, 13	1	47[d]	30[d]	29	7/7	1/8
	10, 11, 12, 13		20	14	0	8/8	0/8

[a] Given as a single dose of 300 mg/kg orally on day shown.

[b] Given as a divided daily oral dose of 8 g/kg on days shown.

[c] Results evaluated on day 16; antigen given on day 0.

[d] $P < .05$ compared to controls.

was given at the time of antigen administration (day 0), a minimal period of five days exposure to the drug (days 0–4) was necessary to demonstrate a therapeutic effect. However, the presence of a full dose of cycloleucine for only a single day during the period from day 5 to 9 afforded a therapeutic response. These results, when examined with those obtained after delayed drug administration when drug was ineffective after day 10 (table 2), suggest that there is a critical period (from days 5–9 after antigen administration) during which cycloleucine can selectively destroy the lymphoid elements responding to adjuvant stimulation.

Tissue culture studies. A comparison of the effects of cycloleucine, the steroid hydrocortisone, 6-mercaptopurine (6-MP), and the folic acid inhibitor methotrexate (MTX) on confluent sheets of rat and human (WI-38) embryonic lung fibroblast cells is shown in table 7. Cycloleucine was the least toxic of the immunosuppressants, exerting a partial cytopathic effect only at the highest concentrations. In contrast, hydrocortisone, 6-MP and MTX were toxic to the cells at lower concentrations. In other experiments comparing effects of cycloleucine and 6-MP on WI-38 cell growth, 6-MP completely inhibited cell growth at 60 μg/ml, whereas this effect could not be demonstrated with cycloleucine even at 60 times this concentration.

PMN leukocyte migration. Inhibition of leukocyte motility has been proposed as a possible mechanism in the anti-inflammatory activity of various compounds (Phelps and McCarty, 1967; Ward, 1968). Table 8 compares the effects of hydrocortisone and cycloleucine on PMN leukocyte migration in response to a chemotactic stimulus (*E. coli*). Hydrocortisone was quite effective in inhibiting migration at concentrations as low as 125 μg/ml, in close agreement with the literature (Ward, 1966). In contrast, cycloleucine at a concentration as large as 40 mg/ml, which approaches the limit of solubility (5%), did not inhibit PMN leukocyte motility. In these tests, cycloleucine was obviously nontoxic and had no effect on motility of the PMN cell.

Discussion

Immunologic and pathologic evidence indicates that EAE and AA are distinct, reproducible experimental diseases representing an immunologic reaction (primarily of the delayed or cellular type) to tissue autoantigen and thus can be

TABLE 7

Comparative cytotoxicity of cycloleucine and three other drugs on tissue culture cells

Tissue[a]	Drug Conc.[b]	Mean Cytotoxic Effect[c]							
		Hydrocortisone		MTX		6-MP		Cycloleucine	
		24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
	μg/ml	%		%		%		%	
Control		0	0	0	0	0	0	0	0
Embryonic human lung fibroblasts (WI-38)	3600	100	100	25	100	100	100	50	25
	1800	100	100	0	50	0	75	25	Trace
	900	0	50	0	0	0	50	0	0
	360	0	25	0	0	0	25	0	0
	180	0	0	0	0	0	25	0	0
Embryonic rat lung fibroblasts	3600	100	100	75	100	0	50	0	50
	1800	100	100	25	75	0	50	0	Trace
	900	0	50	0	25	0	50	0	0
	360	0	0	0	25	0	50	0	0
	180	0	0	0	25	0	50	0	0

[a] In 48 hours, control cells and cells designated "0" were fully sheeted, confluent and healthy-appearing.

[b] Five tubes tested per dilution.

[c] Percentages indicate approximate degrees of cytopathology on cell monolayers.

TABLE 8

Effect of cycloleucine and hydrocortisone on PMN leukocyte migration[a]

Compound	Expt. No.	Effect of Drug Concentration of:									
		0 mg/ml	0.125 mg/ml	0.5 mg/ml	0.75 mg/ml	1.0 mg/ml	2.5 mg/ml	5 mg/ml	10 mg/ml	20 mg/ml	40 mg/ml
Hydrocortisone[b]	1	21.2	9.2	5.0	4.5	2.4	1.6				
		±1	±0.6	±0.3	±1.3	±0.5	±0.3				
	2	17.4	4.7	1.9	1.0	1.0					
		±0.8	±0.5	±0.4	±0.2	±0.2					
	3	73		28	3.7	0.4	1.0				
		±7		±1.8	±0.4	±0.1	±0.3				
Cycloleucine	1	16.5						13.9	25.4	18.3	7.3
		±0.5						±0.5	±0.7	±0.5	±0.5
	2	27.6					65	51.9	104.6	51.3	21.9
		±0.6					±4.7	±3.3	±3	±1.2	±0.7
	3	22.2					21.8	25.9	28.4	19.8	5.5
		±0.7					±0.7	±1.6	±1	±1	±0.8
	4	20.4	13.9			19.7		21.9	16.7		
		±0.8	±1.0			±2.1		±1.2	±0.7		

[a] Data are expressed as the number (means ± S.E.) of PMN leukocytes migrating through a single high power field of the Millipore filter. Thirty fields were counted per drug dilution (10 fields per filter; 3 filters per dilution). Each experiment was done on a different day.

[b] As the soluble sodium succinate salt.

classified as autoallergic or autoimmune diseases (Paterson, 1966). These two experimental immunopathies are useful models for evaluating and differentiating various anti-inflammatory and immunosuppressive agents (Rosenthale *et al.*, 1969b; Newbould, 1963; Glenn, 1966).

Cycloleucine was found to be an effective therapeutic agent, alleviating the severity and lowering the incidence of both EAE and AA. When administered by various dosage schedules, all beginning on the day of antigen administration, it exhibited similar dose-response relationships against the various paralytic and inflammatory clinical signs of EAE and AA. In rats with EAE, it suppressed the histologic changes in neural tissue, indicating that it does not mask but rather prevents this disease.

At therapeutic doses, cycloleucine was also effective in preventing changes in total body and various stress organ weights and the hematologic changes seen in EAE and AA. Larger, toxic doses decreased body weight and caused involution of the thymus and spleen resulting in a leukopenia with a specific depression of PMN leukocytes. Thus, this agent presents a biphasic picture: low therapeutically effective doses are capable of normalizing the organ, total body and hematologic changes obtained in EAE and AA, whereas larger, more toxic doses can themselves depress these parameters.

The depressant effects of cycloleucine on appetite are well known (Clark, 1966). Antagonism of body weight loss by force-feeding with a basal diet affected neither the therapeutic nor the toxic effects of cycloleucine, indicating a separation of body weight changes from drug activity, or toxicity.

A delayed appearance of clinical signs of either AA or EAE in rats after cessation of treatment with various anti-inflammatory or immunosuppressive agents has been described, thus suggesting a relatively short duration of action (Rosenthale *et al.*, 1969b; Newbould, 1963). As indicated by single-dose inhibition of EAE or AA (table 1), cycloleucine has a long duration of action in rats. It is nonmetabolizable (Sterling *et al.*, 1962) and its half-life varies over a wide range, depending on the species in which it is utilized. Its half-life in the male rat is reportedly 22 days; in the mouse, 42 days; and in the rabbit, 39 days (Christensen and Clifford, 1962). We have observed a half life of 23 to 27 days in dogs, 8 to 9 days in squirrel monkeys, 3 to 4 days in African Green monkeys and 15 to 20 days in miniature swine (Owen *et al.*, 1969). In man, on the other hand, cycloleucine has a

relatively short half-life of only 3 to 4 days (Christensen and Clifford, 1962). The persistence in animals probably is due to a combination of slow metabolism and good renal tubular reabsorption, in a manner similar to that of various neutral amino acids (Sterling *et al.*, 1962). It most likely explains the long duration of action of the drug in the studies described.

In its pharmacologic spectrum against EAE and AA, cycloleucine resembles the various immunosuppressive rather than the so-called "anti-inflammatory" steroidal or nonsteroidal agents. Nonsteroidal anti-inflammatory drugs (NAID) are ineffective in EAE, whereas cycloleucine, glucocorticoids and immunosuppressive drugs are quite active. In AA, the initial phase, consisting of an acute inflammation of the injected paw which appears on days 3 to 6, is highly susceptible to various NAID and steroidal agents but is not affected by cycloleucine or various immunosuppressive agents. Similarly, an established AA is susceptible to both NAID and glucocorticoids but is resistant to cycloleucine and various anti-immune agents. In rats with either AA or EAE, cycloleucine loses its effectiveness if administered later than nine days after antigen, again resembling other immunosuppressive agents. EAE and AA thus resemble mature antibody synthesizing cells in that they are resistant to drug therapy after the initial critical period of cellular proliferation (post log growth base) (Makinodan *et al.*, 1970).

Further evidence concerning the dissimilarity between cycloleucine and the steroidal agents and NAID is obtained from studies (unpublished observations) in which it was shown to be completely inactive in preventing carrageenin paw edema in the rat, bradykinin-induced bronchoconstriction in the guinea pig and urate-induced synovitis in the dog, all tests which respond to NAID and, in certain cases, to glucocorticoids. The ability of cycloleucine to depress both humoral and cellular immune responses in various test systems in several species (Rosenthale and Gluckman, 1968) suggests that its primary pharmacologic effect is inhibition of the proliferation of immunologically stimulated lymphoid cellular elements and that its anti-inflammatory activity is secondary to, and a result of, this effect.

The acute body weight loss, decreased therapeutic activity of drugs and initial appearance of inflammatory neurologic lesions which occur as early as eight or nine days after administration of encephalitogenic antigen indicate that this is a critical period in the development of EAE. Similarly in AA, Newbould (1964) has shown that removal of draining lymph nodes within five, but not seven, days after injection of adjuvant prevents development of the secondary lesions which appear on day 10 and probably represent a delayed hypersensitivity response. In the present studies, when L-valine was used to terminate the effects of cycloleucine, adjuvant-afflicted rats were found to be responsive to single doses of cycloleucine only during the period from day 5 to day 9 after antigen, a period of maximal lymphoid cell activity. Thus, a critical time period appears to exist for the selective destruction by this drug of lymphoid elements responding to adjuvant stimulation.

At the present time, the mechanism of action of cycloleucine can only be speculated upon. Recent studies in our laboratories indicate that cycloleucine can normalize the decrease in lysosomal membrane permeability seen in AA (Grant *et al.*, 1971). Cycloleucine does not inhibit enzymatic oxidation or amino acid transamination and is not incorporated into protein but is an inhibitor of the methionine adenosyltransferase reaction (Lombardini *et al.*, 1970). Aminoaciduria has been reported after oral administration in man, but this is probably not responsible for the antitumor action (Sterling and Henderson, 1963; Brown, 1967). The observed concentration of cycloleucine in rapidly proliferating cells may be due to the ability of tumor (and possibly immunologically stimulated) cells to concentrate amino acids to a greater extent than normal tissue (Sterling and Henderson, 1963).

It has been suggested that cycloleucine functions as an amino acid antagonist and that it acts by preventing the attachment of valine to transfer ribonucleic acid (Berlinguet *et al.*, 1962). Studies in ascites tumor cells indicate that it inhibits the transport of leucine and valine into these cells in a competitive fashion. Previous demonstrations of the antagonistic effect of L-valine against the antitumor and toxic effects of cycloleucine (Abshire and Pineau, 1967; Macklin *et al.*, 1963) were substantiated in the rats with EAE, which we found exhibited some separation of these effects. The antidotal effect of L-valine in these studies, however, probably is due to competition for reabsorptive transport sites in the renal tubules, resulting in an enhanced

excretion of cycloleucine in its presence (Owen *et al.*, 1969). Competition between L-valine and cycloleucine for attachment to transfer ribonucleic acid may be an additional factor (Berlinguet *et al.*, 1962). As Sokoloff and Newbould (1970) have shown, the therapeutic action of methotrexate in EAE can be "rescued" by suppressing the toxic effects with folinic acid. A similar relationship may exist between cycloleucine and L-valine and the mechanism of this antagonism remains to be defined in greater detail.

An entirely different approach to a possible mechanism of anti-immune activity of cycloleucine has been postulated by Frisch and Wilson (1969) who indicated that this drug may interfere with the macrophage processing of antigen in the immediate (humoral) antibody response. The decreased effectiveness of cycloleucine when administered after antigen may indicate that it acts primarily at a preinduction phase, at least as regards humoral antibody synthesis. Its effectiveness in EAE and AA as late as nine days postantigen suggests possible differences in responsiveness of a humoral, as contrasted with a cellular inflammatory response such as that obtained in EAE or AA.

The cytotoxicity of antitumor agents in cell cultures was demonstrated by Foley and Eagle (1958) and has been proposed as a screen for the detection of these agents. In the present cell culture study, cycloleucine showed minimal cytotoxic effects compared to 6-MP, MTX and hydrocortisone. The dose-response curves in both EAE and AA indicate that as an immunosuppressive agent, cycloleucine lies between cortisone and 6-MP in potency; thus, compared with these agents, it is considerably less toxic to cells *in vitro*, while having a similar *in vivo* therapeutic dose level. *In vivo*, cycloleucine acts in a nonmetabolizable form (Carter, 1970); hence its lack of effect in these studies appears truly reflective of a lack of toxicity on the tissue culture cells used.

Cycloleucine also proved incapable of preventing PMN leukocyte migration when administered at concentrations as high as 40 mg/ml (3×10^{-1} M). Hydrocortisone was quite active in this test and virtually complete inhibition was obtained at 0.5 mg/ml (1×10^{-3} M). Similar inhibition occurs with a variety of steroidal and nonsteroidal anti-inflammatory drugs, antimalarials, colchicine and several inhibitors of protein synthesis such as puromycin, actinomycin D and mitomycin (Ward, 1966, 1968). The inability of cycloleucine to affect PMN leukocyte migration substantiates the low level of toxicity shown in the tissue culture studies and differentiates its mechanism of action from that of the NAID, the inhibitors of protein synthesis and the steroidal, cytotoxic, and immunosuppressive agents.

Conclusions

Immunosuppressive agents have demonstrated anti-inflammatory activity both in humans (Swanson and Schwartz, 1967) and animals (Denman, 1969). Conversely, NAID have shown anti-immune activity in a variety of *in vivo* and *in vitro* test systems (Whitehouse, 1967; Brown and Mackey, 1968). Thus, the relationship between anti-immune and anti-inflammatory activity of the classes of agents currently useful in the inflammatory response is presently obscure and is the subject of intensive investigation. Cycloleucine appears to be a unique type of immunosuppressive agent with a different mechanism of action; its effectiveness in experimental immunopathologic models of autoimmune disease indicates a potential usefulness as an anti-inflammatory agent.

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