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Cyclophosphamide Inhibition of Experimental Allergic Encephalomyelitis in Wistar Rats.* (31888)

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Prevention of auto-immune tissue injury by immunosuppressive drugs carries the promise of additional understanding and better control of harmful immunological responses of animals and man. This paper describes the suppression of experimental allergic encephalomyelitis (EAE)—a prototype auto-immune disease—in rats treated with cyclophosphamide.

Suppression of EAE has been reported by other workers using rabbits and/or guinea pigs treated with nitrogen mustard(1,2), 6-mercaptopurine(3,4), chlorambucil(5), methotrexate(6,7) or cyclophosphamide(7,8). In those animals observed after stopping the immunosuppressive agent in question, EAE usually appeared within a matter of 9 to 20 days, the usual latent period for this disease in rabbits and guinea pigs.

The present report describes inhibition of EAE in rats not only during administration of cyclophosphamide but for at least 3 weeks after discontinuing treatment with this alkylating agent. Cyclophosphamide administration beginning as late as 9 days after nervous tissue sensitization also inhibtis the disease. In addition, this alkylating agent has been found to inhibit production of antibrain antibody in parallel with inhibition of EAE.

Methods. Cyclophosphamide (Cytoxan®).‡ The drug was supplied in vials containing 100 mg dry powder and was stored at 4°-10°C until dissolved in sterile physiological saline and injected intraperitoneally into lightly etherized rats. In most experiments, cyclophosphamide was injected daily or 5 days a week beginning on day of sensitization and continued through the 17th post-sensitization day. Rats were weighed weekly to be sure the same dose of the drug per kilogram was given throughout each course of treatment. *Rats and sensitization procedure.* Female Wistar rats§ (125 to 250 g each) were used and maintained on commercial food pellets and water *ad lib.* Hartley strain guinea pig spinal cord aseptically removed and stored 1 to 17 days at —20°C was homogenized in 0.25% phenol in distilled water to give a 33% suspension and incorporated into an equal volume of Freund's complete adjuvant as previously described(9). Each rat was sensitized by injection of 0.1 ml spinal cord-adjuvant emulsion into each of 6 skin sites over the upper back and 1 skin site over the anterior neck(9). *EAE criteria:* Sensitized rats were observed closely for clinical neurological signs, these usually being ataxic gait and/or paraly-

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‡ Ampules of cyclophosphamide generously supplied as "Cytoxan" through the interest of Dr. Max D. Davis, Mead-Johnson Laboratories, Evansville, Ind.

§ Female Wistar stock (CFN) rats purchased from Carworth, Inc., New City, New York.

TABLE I. Toxicity of Cyclophosphamide (CY) for Normal Wistar Rats.

CY dose/K, per group of rats, mg*	Mean maximum wt change, % of pre-CY weight	Mean fall in hemoglobin, % of pre-CY value	Mean WBC × 10^3/mm^3, days of CY injection			Clinical status thru 17th day CY
			0	9	17	
5	+7.8% (4.5–10.1) †	3.5% (0–6.4)	11.6 (9.6–13.0)	6.3 (5.3–7.7)	6.8 (4.6–9.4)	Clinically well
10	+4.4% (2.4–8.0)	13.0% (10.3–17.6)	10.2 (7.6–12.3)	3.5 (2.4–4.1)	2.2 (1.8–2.7)	*Idem*
15	−10.1% (6.7–13.4)	36.8% (20.0–53.5)	9.7 (7.5–11.8)	1.5 (.8–2.5)	1.3 (1.2–1.4)	2 dead 13th & 15th day; 2 ill on 17th day
25	−7.8%‡ (6.3–8.7)	—	10.9 (9.5–12.0)	1.0 (.3–1.3)	—	All dead 10th thru 17th day

* Daily intraperitoneal injections of CY for 17 days; 4 rats in each group.

† Figures within parentheses represent range of values for each group concerning weight, hemoglobin and leukocyte count.

‡ Weights obtained at 10th, 13th and 15th day of CY treatment.

sis of hindlegs. Animals were sacrificed by cardiac puncture under ether anesthesia at the end of each experiment, their brains and spinal cords formalin fixed and 3 blocks of brain tissue and 4 blocks of spinal cord routinely processed to yield hematoxylin-eosin stained sections for microscopic examination. Presence of one or more focal areas of perivascular cuffing with microglial proliferation indicated that EAE had occurred in individual animals(9). *Blood leucocyte counts.* Total leukocyte counts on whole blood obtained by cut tail vein or cardiac puncture and collected in tubes containing potassium-ammonium oxalate crystals (or rarely 0.1 ml liquid heparin per 9 drops blood), were made in duplicate with standard white cell pipettes and hemocytometer chambers. Total leucocyte counts also were carried out with some samples using a Coulter counter. Differential white cell counts were based on microscopic examination of at least 100 cells in Wright stained blood smears. *Complement-fixation assay for antibrain antibodies.* The procedure used was that described fully elsewhere(10) and employed diluted stock ethanolic extracts of rat brain as antigen and approximately 100-50% hemolytic units of complement ($C'H_{50}$) per reaction mixture. Final results were expressed as number of $C'H_{50}$ specifically fixed, of exactly 100 $C'H_{50}$ available, by 0.25 ml of heat inactivated rat serum and brain antigen.

Results. Cyclophosphamide toxicity in normal Wistar rats. Toxic manifestations of daily injections of cyclophosphamide given over a 17-day period are shown in Table I. At dose levels of 5 mg/k and 10 mg/k, weight loss did not occur and essentially all rats remained active and appeared clinically well throughout the 17 days of drug administration and observation. Despite clinical well being, a decline in hemoglobin and peripheral blood leucocyte count was observed. Dose levels of 15 mg/k and 25 mg/k resulted in obvious weight loss, evidence of systemic illness (ruffled fur, diarrhea, increasing cough and pallor) and death which occurred as soon as 10 days after initiating cyclophosphamide at the higher dose. Severe anemia and marked leucopenia were associated with these toxic doses of the drug. It is to be emphasized that clinical signs suggesting nervous system disease or toxicity were not observed in any rats, even those moribund as a result of drug treatment.

Cyclophosphamide inhibition of EAE. As shown in Table II, cyclophosphamide started on day of sensitization and usually continued through the 17th post-sensitization day inhibited manifestations of EAE in all rats. As anticipated from the toxicity data, those rats sensitized and treated with 15 mg/k exhibited weight loss and pallor, but these signs of cyclophosphamide toxicity were less marked than in normal unsensitized rats. Those rats receiving lower doses appeared clinically well throughout the period of drug

TABLE II. Inhibition of Experimental Allergic Encephalomyelitis (EAE) in Wistar Rats Treated with Cyclophosphamide (CY).

CY dose per K, mg*	No. of rats	No. of rats with Clinical signs of EAE	No. of rats with Lesions of EAE†
15	8	0	0
12	13	0	0
12	6‡	0	0
12	6§	0	0
10	6	0	0
10	7§	0	0
5	7	0	0
None	24	15	23

* CY injected intraperitoneally daily or 5 days per week through 17th day after spinal cord-adjuvant sensitization.

† Rats sacrificed 18th to 20th post-sensitization day.

‡ CY injected 9th through 17th days after sensitization only.

§ Rats observed 18 to 21 days after last injection of CY and not sacrificed until 33rd or 38th day after sensitization.

treatment. The absence of any clinical signs of EAE coupled with the robust appearance of the rats treated with 5 mg/k was especially noteworthy. Clinical signs and characteristic lesions of EAE occurred in each control group of rats, sensitized but not drug treated, carried along with each cyclophosphamide treated group. The cumulative incidence of neurological signs and lesions of EAE in these control rats (Table II) agrees with that observed in previous work with this rat strain in our laboratory(11,12).

Thirteen rats treated with cyclophosphamide 12 mg or 10 mg/k were not sacrificed following the last injection of drug but observed further (Table II). Three of the 6 animals receiving the higher dose (12 mg) developed respiratory distress and appeared moribund 4 and 8 days after stopping drug treatment and were sacrificed at these times. The remaining 3 rats and all 7 animals which received the lower dose (10 mg) of cyclophosphamide gained weight and remained clinically well throughout the 18- to 21-day post-cyclophosphamide observation period. None had demonstrable lesions of EAE.

An additional group of 6 rats was treated with cyclophosphamide 12 mg/k beginning on the 9th day after sensitization (Table II). All 6 rats remained well and none had lesions of EAE when sacrificed on the 18th day. This finding indicates that cyclophosphamide need not be administered continuously to inhibit EAE in rats and can exert a profound suppressive effect even when started at a time when lesions of EAE are just beginning to appear in the neuraxis, *i.e.*, 9-10 days post-sensitization.

Not shown in Table II were 2 groups of 5 rats each given a single injection of cyclophosphamide of 15 mg/k and 10 mg/k on day of nervous tissue-adjuvant sensitization, respectively. Eight of the 10 developed typical hindleg paresis by the 17th post-sensitization day. Nine had severe disseminated lesions of EAE. Current work in our laboratory indicates that it takes much larger single doses of cyclophosphamide, of the order of 100 mg/k and preferably given on the 2nd post-sensitization day, in order to inhibit the disease.

Leucocyte counts in cyclophosphamide treated-sensitized rats. Total peripheral blood leucocyte counts fell progressively during cyclophosphamide treatment but not to the same degree as seen in normal rats. Perhaps this is best explained by the brisk proliferative cell response elicited by spinal cord-adjuvant sensitization and which might well blunt the leucopenic effect of cyclophosphamide. Leucopenia resulted in large part from a decrease in circulating lymphocytes. While detailed analysis of the relationships between leucocyte count, cyclophosphamide dose and inhibition of EAE will be reported elsewhere, one aspect should be emphasized here. Severe leucopenia less than 3000 cells/mm^3 was associated regularly only with the 12 and 15 mg/k dose levels of cyclophosphamide. Depression of circulating leucocytes of this degree was uncommon with 10 mg/k and not observed at all with the 5 mg/k doses. Furthermore, the marked leucopenia induced by the larger doses gave way promptly to a striking leucocytosis after stopping the drug, resulting in peak circulating leucocyte levels approximating those of sensitized control rats.

Representative data relevant to this point, and fully confirmed by current work, are shown in Fig. 1. Sensitized rats (K 381 and K 382) not treated with cyclophosphamide developed peak leucocyte counts 9 to 17 days

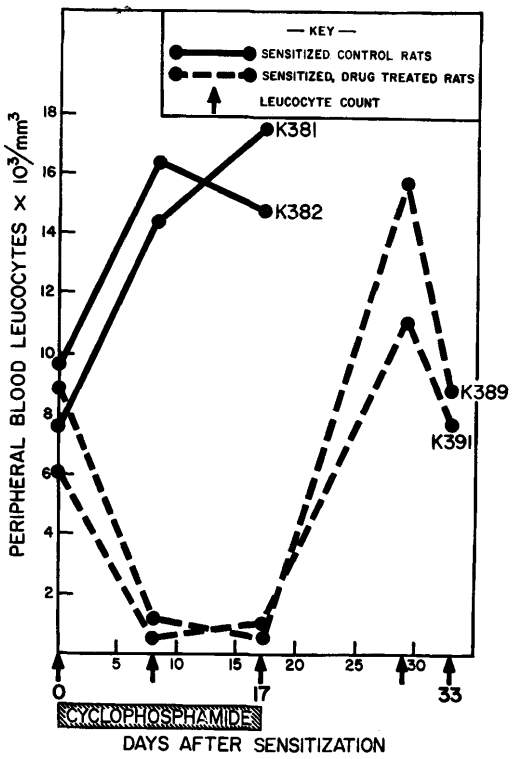

Fig. 1. Serial total peripheral blood white cell counts in rats sensitized to spinal cord-adjuvant and treated with cyclophosphamide or not treated.

post-sensitization during which time paralysis occurred and EAE lesions were appearing in the neuraxis. Sensitized rats (K 389 and K 391) treated with 12 mg/k cyclophosphamide developed profound leucopenia of less than 1000 cells/mm^3 by 9 to 17 days post-sensitization. After stopping cyclophosphamide, the leucocyte count rose to elevated levels of 11 and 16 $\times$ 10^3 cells/mm^3 within 9 days and then began to fall. Both rats remained free of any clinical signs of EAE until sacrificed on the 33rd post-sensitization day; none had demonstrable EAE lesions.

Complement-fixing (CF) antibrain antibody in cyclophosphamide treated rats. Sera from 11 of the 21 rats treated with 15 mg/k and 10 mg/k (Table II) collected at time of sacrifice were assayed for antibodies specifically reactive with ethanol-soluble rat brain antigen. None of the sera fixed more than 9 C'H$_{50}$, of exactly 100 C'H$_{50}$ available, in the presence of the brain antigen. Based on prior observations made in this laboratory(11,12), at least 70% of sensitized Wistar rats bled 2-4 weeks after sensitization have appreciable levels of CF antibrain antibody, their sera fixing from 12 to 80 C'H$_{50}$, of 100 C'H$_{50}$ available, with rat brain extracts. Thus, cyclophosphamide exerts a clear cut inhibitory effect on CF antibrain production, a characteristic response to nervous tissue sensitization in the Wistar rat.

Discussion. These data (Tables I and II) indicate that cyclophosphamide, an alkylating agent chemically similar to nitrogen mustard but requiring activation by host tissues, can inhibit all manifestations of EAE in rats and at dose levels carrying little serious toxicity for this host. Our findings, thus, amplify and strengthen work from other laboratories showing that a number of immunosuppressive drugs are capable of inhibiting EAE in other species of laboratory animals (guinea pigs and rabbits).

The present report extends the subject of immunosuppression of EAE on two scores. First, direct evidence has been obtained that cyclophosphamide inhibits antibrain antibody production in parallel with prevention of the entire disease. Although it is clear that the antibrain antibodies in question, *viz.*, those avidly fixing complement with ethanol soluble rat brain antigen, probably are not of etiological significance in the development of EAE, they are on important biological tag for evaluating responses to nervous tissue sensitization. The fact that cyclophosphamide inhibits both EAE and antibrain antibody production supports the idea that the disease-inhibiting action of the drug is mediated *via* immunological channels, rather than through some other mechanism, *e.g.*, anti-inflammatory effects. The inhibition of EAE with doses of cyclophosphamide which do not result in severe leucopenia and the extended inhibition of EAE after stopping the drug despite a precipitous increase in circulating leucocytes (Fig. 1) are two additional observations which support this view.

Second, evidence has been obtained with the EAE-rat system that the immunosuppressive effects of cyclophosphamide may extend for appreciable periods of time after stopping the drug. Work now in progress and to be

reported separately indicates that rats sensitized and treated for 17 days with cyclophosphamide do not develop evidence of EAE at least for 6 weeks after discontinuing drug treatment and probably longer. Furthermore, such rats if reinjected with spinal cord-adjuvant still do not develop typical EAE. This extended immunosuppressive action of cyclophosphamide in rats is in direct contrast to the short-lived action of nitrogen mustard and 6-mercaptopurine reported in work with guinea pigs and rabbits(1-4). In the cyclophosphamide-EAE study in guinea pigs reported by Calne and Liebowitz(7,8), animals were followed only 8 days after an immunosuppressive 16-day course of treatment, an observation period too short to determine how lasting the immunosuppressive effect may be in this species.

Summary. Experimental allergic encephalomyelitis can be inhibited in Wistar rats treated with doses of cyclophosphamide which carry little or no toxicity for this species of laboratory animal. Inhibition of the disease lasts for at least 3 weeks after discontinuing cyclophosphamide treatment. The suppressive effect of this alkylating agent can be achieved starting treatment as late as 9 days after nervous tissue-adjuvant sensitization. Cyclophosphamide inhibits production of complement-fixing antibrain antibody in parallel with its EAE-inhibitory effect.

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Human Cytomegalovirus. Studies on the Mechanism of Viral Cytopathology and Inclusion Body Formation.* (31889)

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Several investigators have attempted to define the biochemical events leading to viral cytopathic effects (CPE) by the use of metabolic inhibitors(1-3). Cells infected with human cytomegalovirus (CMV) are especially suitable for certain aspects of such study because they slowly develop, during a 72-hour period, CPE and intranuclear and intracytoplasmic inclusion bodies. By means of cytochemical(4), immunofluorescent(4,5), and autoradiographic techniques(6), previous studies have correlated the morphological changes with the synthesis of RNA, DNA and protein as well as the synthesis and release of infectious virus during one cycle of virus infection.

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