Cyclophosphamide: Effect on Experimental Allergic Encephalomyelitis in Lewis Rats

Abstract. *Lewis rats sensitized to spinal cord in adjuvant and exhibiting advanced clinical paralytic signs of experimental allergic encephalomyelitis were treated with cyclophosphamide (5 milligrams per kilogram) daily. Of 30 treated animals, 21 recovered rapidly and appeared clinically well within 7 to 12 days. This immunosuppressive agent may prove therapeutically useful in the treatment of autoimmune diseases.*

The capacity of cytotoxic drugs to suppress antibody synthesis, delayed-type hypersensitivity, and allergic tissue injury elicited by a variety of antigenic stimuli is well established (*1*). Optimum immunosuppression usually has required administration of the cytotoxic agent at the time of or soon after antigenic stimulation; that is, well in advance of the time when the immune response in question would have otherwise occurred (*1*). This prerequisite for immunosuppression has severely restricted the use of most cytotoxic drugs in treatment of immunologic diseases.

Experimental allergic encephalomyelitis (EAE) is a promising model system for the study of immunologic factors implicated in central nervous system disease of animals and man (*2, 3*). Complete inhibition of EAE has been reported in rats sensitized to spinal cord and treated daily with cyclophosphamide for 17 days after sensitization (*4, 5*).

In our study (*5*), with albino rats, equivalent results were obtained when injections of this drug were delayed until the 9th day after sensitization, that is, several days before the appearance of paralysis in control animals. Furthermore, rats sensitized and then treated daily or 5 days per week for 17 days after sensitization did not develop EAE for as many as 112 days (*5, 6*). Thus treatment with cyclophosphamide initiated after onset of EAE might exert a beneficial effect on the course of the disease. In that Lewis rats regularly develop a severe and often fatal form of EAE, they can be used for critical testing of any therapeutic action of a drug (*7, 8*). We now report striking improvement and the return to clinical well-being of Lewis rats with EAE treated with cyclophosphamide (CY) for 7 to 12 days after onset of paralytic signs.

Under light ether anesthesia male rats 7 to 10 weeks of age were sensitized to guinea pig (Hartley) spinal cord in adjuvant (H37Rv or BCG) by injection into the skin of the back (*9*). The animals then were placed in different treatment groups at random, housed in groups of eight in stainless steel cages and given free access to food pellets and water. Sensitized rats were observed daily for clinical neurologic signs of EAE—ataxic gait, weakness or frank paralysis of hind legs, "cord" bladder, incontinence of urine, and fecal impaction (*8*). Paralytic signs appeared in all animals 12 to 18 days after sensitization. On the day when first paralyzed, each rat was transferred to an individual cage for the duration of each experiment. Lewis rats become extremely hyperirritable after onset of paralytic signs (*8*), and separation of animals eliminates trauma inflicted by fighting among cage mates. The treatment group was started on daily injections 1 to 5 days after onset of paralytic signs. The drug was supplied (*10*) in ampules containing 100 mg of dry powder and was hydrated and diluted with distilled water to provide a dose of 5 mg/kg in a volume of 1.0 to 1.5 ml. This volume was injected intraperitoneally into etherized rats each day.

The control rats treated with salt solution were handled in the same way and received a corresponding volume of physiological saline intraperitoneally each day, beginning 1 to 5 days after onset of paralysis. Other control rats were not treated; they were left in individual cages and simply observed. All rats were killed 22 to 24 days after they were sensitized. Brains and spinal cords from these animals, as well as those that died before this time, were fixed in Formalin and prepared for histological examination (*9*). A minimum of three blocks of brain and four blocks of spinal cord from each rat was embedded in paraffin and sectioned; the sections were stained with hematoxylin and eosin. Microscopic lesions were graded according to an arbitrary scale of 0 to +++ (*11*).

Table 1. Clinical status of Lewis rats with EAE after cyclophosphamide treatment.

Treatment	Rats in group (No.)	Rats (No.)		
		Dead	Paralytic signs*	Clinically well†
Cyclophosphamide	30	6	3	21
Saline	28	13	12	3
Not treated	22	11	11	0

* Unsteady gait, hindleg weakness or paraplegia, and "cord" bladder. † No detectable clinical neurological abnormalities; appearance indistinguishable from normal rats. Probabilities from chi-square calculations with Yates correction for small numbers: CY versus saline <.001; CY versus not-treated <.001; CY versus saline plus not-treated <.001.

The mean number of days after sensitization when cyclophosphamide and saline injections began was 14.5 (range 12 to 18) and 14.4 (range 12 to 18), respectively. Mean number of days after onset of paralytic signs when either type of treatment was started was 2.1 (range 1 to 5) for both groups (*12*).

Of 30 rats with EAE treated with the drug (Table 1), only three of the survivors still had slight ataxia or questionable hind-leg weakness 22 to 24 days after sensitization. The 21 rats that recovered from paralysis appeared clinically well, active, and alert by this time. Striking improvement usually began within 2 or 3 days in most treated rats, including return of full strength to hind legs which had previously shown flaccid paralysis, disappearance of "cord" bladder and urinary incontinence, disappearance of fecal impaction, increasing ambulation and return of interest in grooming of body hair.

The course of EAE in the 28 saline-treated rats and the 22 rats which were not treated was as expected from previous studies of this disease in this rat strain in our laboratory (*8*). Many of the 23 control rats that survived until 22 to 24 days after sensitization still exhibited paraplegia or severe weakness of the hind legs and "cord" bladder.

Four of the six deaths in the CY-treated group occurred after only 1 to 4 days of treatment, perhaps before the drug could exert adequate effects. An additional death occurred after 9 days of treatment in a rat that had recovered after 4 days of treatment and had remained clinically well for 4 days. He then developed an unsteady gait on day 23 and was found dead the next day. The sixth rat was killed on day 20 when he suddenly appeared ill and moribund after dramatic improvement. Urine and blood cultures yielded *Proteus* sp. Severe urinary tract infection and bacteremia would appear to account for his clinical deterioration.

An experiment with seven rats sensitized and treated with the drug after onset of paralysis is not included in

Table 1, because no control group of simultaneously sensitized animals was included. Rapid recovery from paraplegia occurred in all seven animals. They appeared clinically well from day 18 to day 23 after sensitization, when they were killed.

Histological studies on animals in three experiments confirmed the clinical observations. Of 19 rats treated with cyclophosphamide, eight had no demonstrable microscopic lesions of EAE and nine were scored 1+. Only two were scored as 2+. None of the 24 saline-treated or nontreated rats were free of EAE lesions. Two were scored 1+, and 8 as 2+, and 14 as 3+.

Our findings provide additional evidence of the potent immunosuppressive actions exerted by cyclophosphamide in averting tissue damage that occurs in EAE (*4, 5, 13, 14*), allergic thyroiditis (*15*), and allograft rejection (*16*). We believe this is the first report of complete reversal of an experimental autoimmune disease initially treated with an immunosuppressive drug several days after "full-blown" clinical manifestations of the disease have appeared. Brandriss (*17, 18*) reported clinical improvement of guinea pigs with moderately severe EAE (more than 50 percent being paralyzed by day 13) treated with methotrexate, starting on the 1st day of EAE signs. In other experiments with animals developing more severe EAE (more than 50 percent paralyzed by day 10), methotrexate had little, if any, influence on the course of disease. While 6-mercaptopurine, chlorambucil, nitrogen mustard, and x-irradiation have been reported to inhibit development of EAE, none has been shown capable of interrupting the course of this disease if administered after onset of disease (*3, 19*).

Whether the preventative and therapeutic effects of the drug reside in a common mechanism or different mechanisms of action is not known. The preventative effects have a firm immunologic basis (*5, 14*). There are insufficient data to permit any statement now about the therapeutic action of the drug.

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High-Resolution Autoradiography of Intracellular Plutonium

Abstract. *Intracellular incorporation of polymeric plutonium injected into mice was demonstrated in liver and spleen by electron-microscopic autoradiography. The observations are not inconsistent with other evidence indicating the association of plutonium with lysosomal components. Early results with a quantitative electron-microscopic technique may lead to microdosimetry of cells and cellular components.*

The projected use of increasing amounts of plutonium and transplutonium elements in reactors, mobile power sources, and so forth, may be expected to increase the incidence of human contamination by these highly toxic radionuclides. Nuclides of the transition and actinide series tend to hydrolyze and polymerize under physiological conditions. For this reason their deposition in tissues, in addition to being a function of their chemical properties, is complicated by the biological process of phagocytosis. Although there are some differences in the physiological behavior of these elements, plutonium-239 may be considered to be a prototype of this class of radionuclides. Metabolic and therapeutic studies have shown that the hazard of this radionuclide is, in part, related to the long-term retention of particulate plutonium in liver, spleen, and bone marrow (*1*). A large fraction of this plutonium is inaccessible to the action of chelating agents such as diethylenetriaminepentaacetic acid (DTPA) (*2*). To devise effective therapeutic procedures, therefore, it is important to determine, as exactly as possible, the mode of plutonium deposition in these organs.

The usefulness of autoradiography, combined with electron and light microscopy, has been investigated (*3, 4*), and in the latter a quantitative technique for the assay of plutonium deposited in specific areas of animal tissues has been demonstrated. Particulate plutonium administered in the form of PuO_2, as demonstrated by electron microscopy (EM), is incorporated in pulmonary macrophages after administration as an aerosol (*5*) and in peritoneal macrophages removed after intraperitoneal injection of a saline suspension (*6*). Quantitative autoradiography with the electron microscope would permit radionuclide microdosimetry in tissue regions occupied by intracellular components.

Most EM autoradiographic studies have employed tritium, which emits beta particles of low energy (0.018 Mev maximum). In such preparations the reduced silver grains are formed nearly exactly above the deposited radionuclide. However, with nuclides that emit alpha particles of relatively high energy (about 5.15 Mev in the case of plutonium-239), the relationship between grain and source extends over a longer distance, and the grains (seen either singly or arranged as "tracks" made up of five or more aligned grains) may be formed as far as 25 to 30 μm from the source. Two or more tracks with a common origin form a "star." A grain or track in the photographic emulsion represents only a small fraction of the total alpha radiation delivered to the surrounding tissue by the embedded nuclide.

Although it is nearly impossible, in cells or cellular organelles, to locate deposits containing plutonium by the appearance of isolated grains, the convergence area of two or more tracks should