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Autoimmune Diseases and Thalidomide. I. Experimental Allergic Encephalomyelitis and Experimental Allergic Neuritis of the Guinea Pig[1, 2]

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The teratogenic effect of thalidomide (Th) is well-known. Current theories about the mechanism of action of this effect have been well summarized by Woollam ([21]). There is controversy about the possible immunosuppressive action of this compound ([1, 2, 4, 5, 10]) and its influence on chromosome aberrations ([9, 14, 15]). It seems clear, however, that the drug does not have an all-inclusive "blanket" effect over immune responses.

Thalidomide is very active, sometimes dramatically so, on signs and symptoms of reactional lepromatous leprosy ([3]). It does not have, however, any direct effect over *Mycobacterium leprae* and cannot be considered as an anti-leprosy drug ([16]).

Reactional lepromatous leprosy has many clinical and histologic features of hypersensitivity reactions as has been stressed recently ([20]).

We decided to explore the possibility of a suppressive effect of this drug over autoimmune experimental diseases, in which delayed hypersensitivity is all important, and where tissue components and mycobacteria have to be administered together as they occur, for instance, in lepromatous neuritis.

In a first series of experiments, experimental allergic encephalomyelitis (EAE) and experimental allergic neuritis (EAN) of the guinea pig were chosen for study.

MATERIALS AND METHODS

Thalidomide was kindly supplied by courtesy of Dr. H. W. Schrader-Beielstein (Grünenthal Chemie GMBH Stolberg, Rheinland, Germany), in powder form or suspended in oil (200 mg per ml).

Incomplete Freund's adjuvant and dessicated, killed mycobacteria (*M. tuberculosis* H 37-Ra and *M. butyricum*) were purchased from Difco Laboratories, Detroit, Michigan.

Albino guinea pigs were used throughout the experiments. They were kept in galvanized rabbit cages and fed rabbit pellets and water *ad libitum* plus fresh carrots or cabbage twice weekly. For initial experiments with EAE, guinea pigs of anonymous strain, locally purchased, were used. Most of the work was done with Rockefeller-strain guinea pigs, kept as a random bred closed colony and purchased from the Instituto Venezolano de Investigaciones Científicas.

Administration of the drug. Pilot experiments had shown that thalidomide suspension given intraperitoneally produced considerable peritoneal and abdominal wall inflammation. There were also gross unabsorbed deposits of the drug lying in the peritoneal cavity.

For definitive experiments the drug was given either as a suspension of 80 mg of thalidomide per ml of 0.5% carboxymethylcellulose (CMC, Baker Adams) in phosphate buffered saline 0.15 M, pH 7.2, using an intragastric catheter or intramuscularly in paravertebral and scapular areas. Prior experiments using a 0.5% solution of Sudan III in olive oil, showed that this technic was conducive to true intramuscular application. In another series of prelimi-

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nary experiments, we found that thalidomide suspension intragastrically, at the dose of 400 mg/kg/day, did not produce sedation or any detectable untoward effects in guinea pigs. Autopsies showed no detectable gross lesions.

Nonspecific experimental granulomas were produced by injecting 0.1 ml of incomplete Freund's adjuvant intradermally in each flank as previously described ([6,7]). The sizes of the granulomas were estimated by serial determinations of diameters of areas of induration. At the end of the experiment 10 mm punch biopsies were taken, routinely processed and stained by the hematoxylin and eosin (H&E) method.

Induction and evaluation of autoimmune diseases. *Experimental allergic encephalitis.* The disease was induced by injecting into each of the four foot pads and the neck, 0.1 ml of an emulsion of guinea pig spinal cord in fortified Freund's adjuvant. The emulsion was prepared as follows. Spinal cord was obtained from adult guinea pigs, cleaned, washed, weighed and kept frozen at —20°C until use. At the time of injection, thawed cord was ground in a Ten Broeck glass grinder with a volume equal to its weight of 0.5% phenol in water. The resulting suspension was heated to 60°C in a water bath and added drop-wise to an equal volume of heated incomplete Freund's adjuvant to which 10 mg per ml of dessicated, mortar-ground mycobacteria were added. The whole was made into a water/oil emulsion by shaking in a Cyclo-Mixer (Clay Adams).

Animals were examined at practically daily intervals. Motility was observed in a "walking receptacle" which was a wooden square box, 60 cm per side and 5.5 cm in depth, the bottom of which was covered with aluminum foil and rice shavings. Normal animals walked easily on its surface and were able to climb easily over the edge of the receptacle. An arbitrary clinical classification was established as follows:

0 no signs of EAE;
1+ minimal signs, doubtful weakness of hind legs or doubtfully wobbling gait;
2+ fecal impaction or definite weakness of hind legs;
3+ definite weakness of hind legs plus fecal impaction and urine incontinence;
4+ typical features, deteriorated aspect, flaccid paresis, hindquarters dragged by forelegs, fecal impaction and urine incontinence.

When animals died of the disease or at the end of the experiment, they were autopsied. Organs were examined grossly to rule out other causes of death. The central nervous system was examined and samples taken from cerebellum, pons, medulla, and cervical and lumbar spine. Both sciatic nerves were also taken. Samples were fixed in buffered formalin pH 7.2, routinely processed and stained with H&E. Selected samples were also stained by the Luxöl-Fast blue method of Klüver and Barrera ([8]) for myelin.

Experimental allergic neuritis. The disease was induced by injection of dog peripheral nerve tissue emulsified in fortified Freund's adjuvant (FFA). This was processed as described for cord, except that since nerves are much stringier, it was necessary to homogenize the tissue in one and one half volumes of 0.5% phenol in distilled water with an Omni-Mixer (Ivan Sorvall) and to grind the resulting suspension with a Kontes conic glass grinder driven by a motor (Con-Torque Eberbach), before adding the tissue to the adjuvant. The emulsion was injected as described for the cord except that the right rear foot was not injected and instead the right flank was. This permitted use of the right hind leg for determinations of velocity of nerve conduction (VNC) (see below).

The disease was clinically evaluated as follows:

0 no signs of EAN;
1+ doubtful weakness of legs, minimal difficulty in walking;
2+ weakness of right hind leg (not injected), difficulty in climbing the edge of the "walking receptacle";
3+ definite weakness of right hind leg, flaccidity of limbs, marked difficulty in climbing edge of receptacle;
4+ typical, manifest flaccidity of four legs, animal walks with great difficulty and with a wide basis of sustentation, the

legs "hang," climbs with very marked difficulty, or is unable to climb the edge of the receptacle.

Animals did not die of the disease. Some, however, died during general anesthesia (see below). Either then, or at the end of the experiment, animals were autopsied and samples taken and processed as described for EAE.

In vivo serial determinations of peripheral nerve velocity of conduction were done using a modification of the method of Ramerman *et al* ([13]), originally used in the rat. Animals were anesthetized with 25-35 mg/kg of weight of nembutal IP. The fixed hind leg was stimulated with a Grass S 4 stimulator (Grass Medical Instruments, Quincy, Mass.) through a Grass SIU-4 isolation unit, permitting the application of rectangular pulses of 10 to 14 volts with 0.1 msec duration and a frequency of one per second. Action potentials were recorded on a Textronik-502A Oscilloscope (Textronik Inc., Portland, Ore.) and photographed with a Textronik C-27 Oscilloscope camera.

RESULTS

Effect of thalidomide on experimental nonspecific granulomas. Guinea pigs were distributed into three groups. The first received thalidomide intragastrically, 400 mg/kg. The drug was begun three days before injection of incomplete Freund's adjuvant and given daily thereafter. The second group received CMC in the same way. Animals of the third group received no treatment. Incomplete Freund's adjuvant was injected and biopsies taken at the end of the experiment. The diameters of induration were measured at four hours and at 1, 2, 5, 8, 10, 15, 18, and 23 days after injection. As Figure 1 shows, there were no significant differences in the magnitude of inflammatory responses between experimental groups. Histologic features were also identical in all groups, namely, the typical features of paraffinomas.

In sum, thalidomide had no detectable effect on the nature or magnitude of the

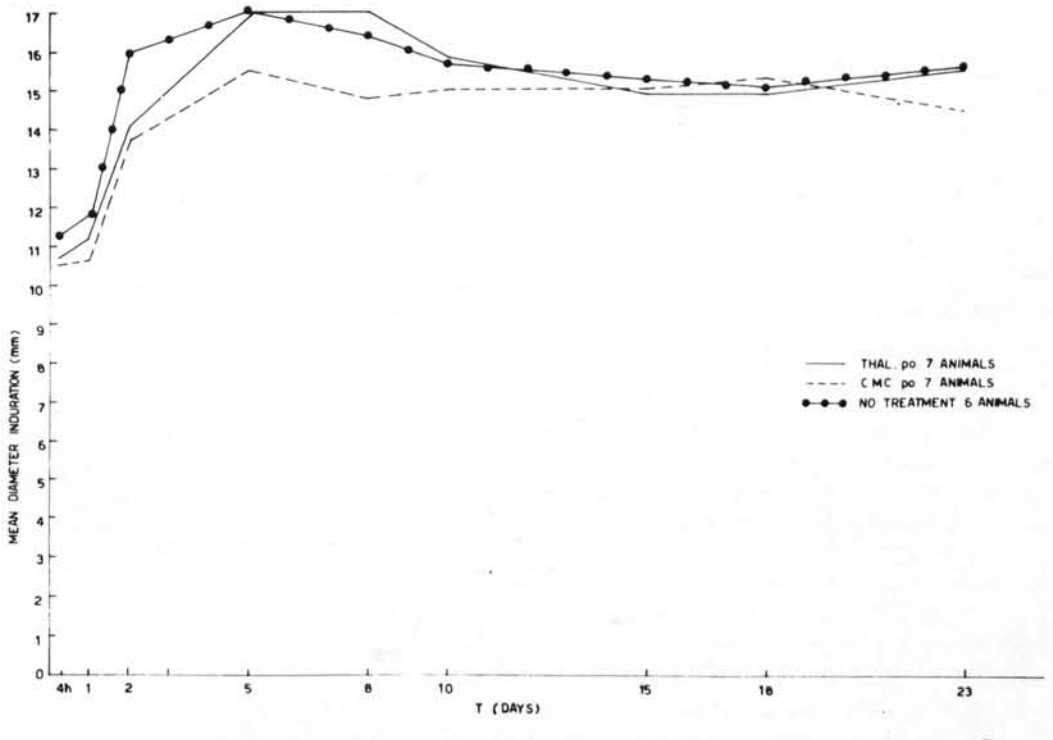

FIG. 1. Mean readings (mm induration) after intradermal injection of incomplete Freund's adjuvant.

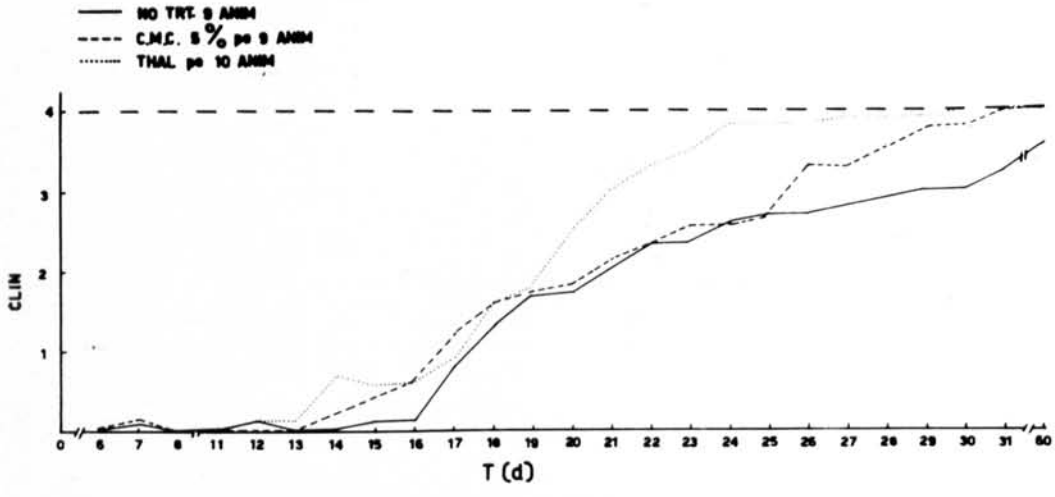

FIG. 2. Comparison of intensity of experimental allergic encephalomyelitis between guinea pigs treated with oral thalidomide and controls.

inflammatory response induced by intradermal incomplete Freund's adjuvant.

Effect of thalidomide on EAE. *Initial experiments.* If guinea pigs of the anonymous strain and H37-Ra were used, the intensity of EAE varied enormously. This went from an absence of obvious signs, to the usual, rather acute, form described in the literature ([11, 12]). There were also relapsing "sinusoid" forms. If previously sensitized animals were reinjected with the cord emulsion in both flanks ten days after initial sensitization, some developed a hyperacute disease, dying in two to nine days after this second stimulus. Others developed a very chronic disease, which at first had exacerbations and remissions but later stabilized, showing a typical spastic paresis and even pressure sores. We were able to follow one such guinea pig for ten months before death occurred. On the other hand, if Rockefeller guinea pigs and *M. butyricum* were used, the typical, rather acute, and highly lethal form of EAE in the guinea pig appeared. All further experiments were done with these animals and this mycobacterium.

Effect of intragastric thalidomide on EAE. Guinea pigs were distributed in three groups as in the incomplete Freund's adjuvant experiments. Thalidomide was given daily, 400 mg/kg beginning six days before sensitization and continuing until death or the end of the experiment. Observation of animals was prolonged for 60 days, although by the end of 30 days many animals had already died. Dates of death were recorded. For analysis of data, an animal that died of EAE was given a value of 4+ for further calculations (see Materials and Methods). Figures 2 and 3 give further data about the composition of groups and show that there was no appreciable benefit derived from the administration of thalidomide on the intensity of the disease, the incubation time or survival.

Specimens from animals of all groups showed typical histologic features as described in the literature ([11, 12]). These were more marked in sections from lumbar cord. No differences could be detected between groups. Sciatic nerves were not affected except for a mild perineural and intraneural vascular dilatation seen in a few cases.

Effect of intramuscular "depot" thalidomide on EAE. Guinea pigs received 800 mg/kg of thalidomide intramuscularly five days before sensitization. One day after sensitization they were given thalidomide 400 mg/kg. This dose was repeated weekly thereafter. Control animals received comparable volumes of sterile olive oil intramuscularly or no treatment. Clinical features were recorded and histologic analysis carried out as described previously.

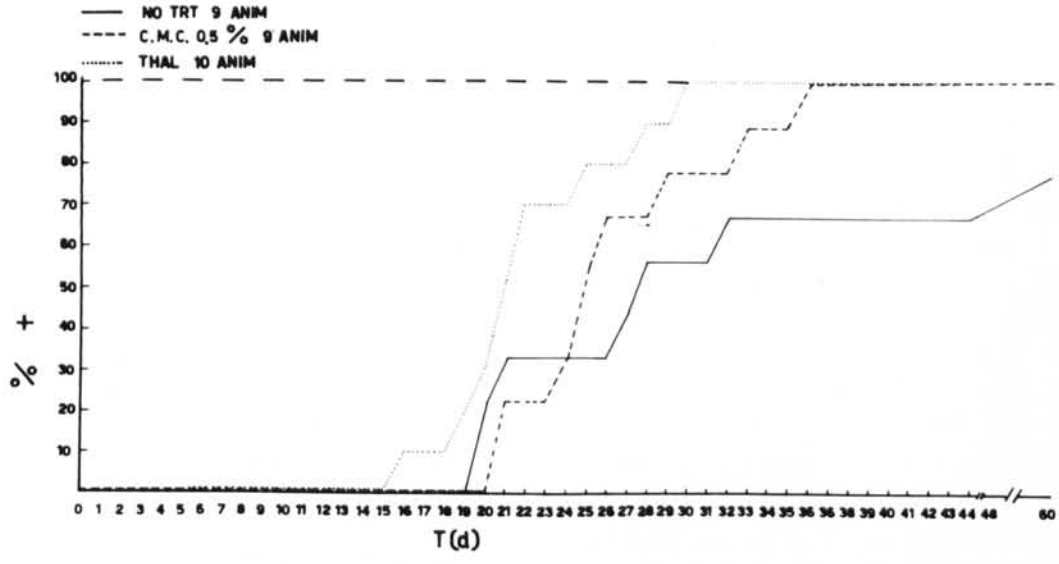

FIG. 3. Comparison of mortality produced by experimental allergic encephalomyelitis between guinea pigs treated with oral thalidomide and controls.

Figures 4 and 5 show that animals receiving thalidomide developed EAE later and survived longer than untreated controls. However, olive oil recipients had an even longer delay and survival. No benefit can then be attributed to thalidomide but rather to the oily vehicle.

Histologic features were identical to those mentioned in the preceding experiment, with no difference between experimental groups.

Effect of thalidomide on EAN. *Pilot experiments* showed that mycobacteria in Freund's adjuvant (with no tissue added) did not induce clinical features of EAN, nor altered velocity of nerve conduction.

Repeated determinations of VNC did not appreciably alter the values obtained. However, if performed too frequently, they produced considerable mortality. There was wide variation in values obtained from individual animals. Thus, it was better to

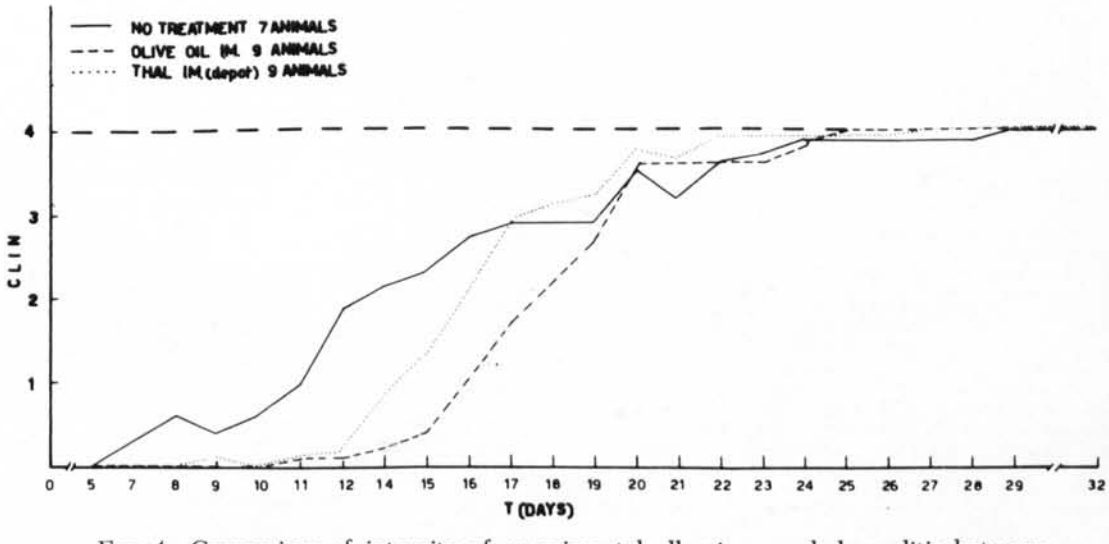

FIG. 4. Comparison of intensity of experimental allergic encephalomyelitis between guinea pigs treated with intramuscular "depot" thalidomide and controls.

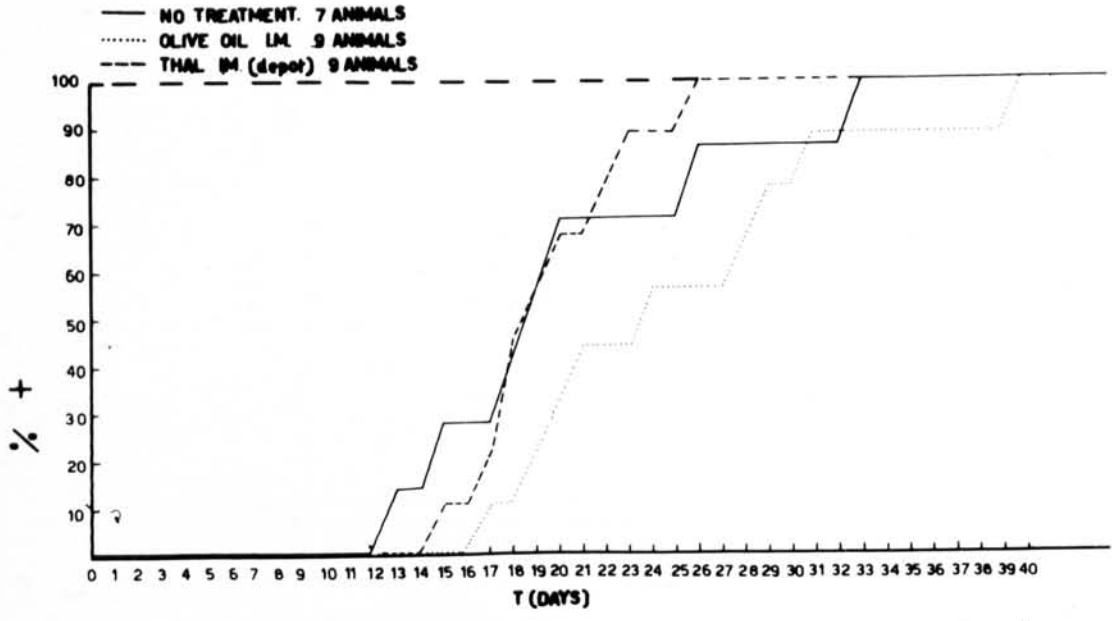

FIG. 5. Comparison of mortality produced by experimental allergic encephalomyelitis between guinea pigs treated with intramuscular "depot" thalidomide and controls.

perform serial determinations in a large group where each animal would be tested repeatedly serving as his own control instead of dividing the animals into smaller groups where individuals would be tested once only.

Definitive experiments. Forty guinea pigs were injected with emulsified peripheral nervous tissue. For logistic reasons, twenty animals were injected in a given session, thus, two groups were formed which differed only in the day of sensitization.

Half of the animals received thalidomide intragastrically, 400 mg/kg/day beginning eight days before sensitization and continuing daily thereafter. The remainder received CMC. Animals dying during anesthesia were excluded from further calculations. Figure 6 presents the clinical evolution and indicates that the thalidomide had no appreciable effect on signs of disease.

Successive determinations of VNC showed a progressive lowering. This became statistically significant ($P<0.05$, t~test) after the sixth determination, i.e. 36 days after sensitization. As indicated in Figure 7, there was no significant difference between animals treated with thalidomide and the controls.

Biopsies showed very moderate lesions in the CNS, consisting mainly of vascular dilatation in meninges and inside nervous tissue. The lumbar cord tended to be more affected than other organs. Sciatic nerves showed great variation in intensity of lesions. Some showed moderate intraneural and perineural vascular dilatation with mild lymphocytic perivascular infiltrate. In others, the vascular dilatation was marked and there was edema of tissue and a marked inflammatory infiltrate which disrupted nerve architectures. There was a rough correlation between intensity of sciatic nerve involvement and the clinical features. No consistent difference could be appreciated between left and right sciatic nerves. There was no correlation between magnitude of peripheral nerve lesions and of CNS involvement.

We could not detect any noteworthy difference in histologic changes between groups treated with thalidomide and the control animals.

DISCUSSION

These studies show the importance of guinea pig strain and schedule of sensitiza-

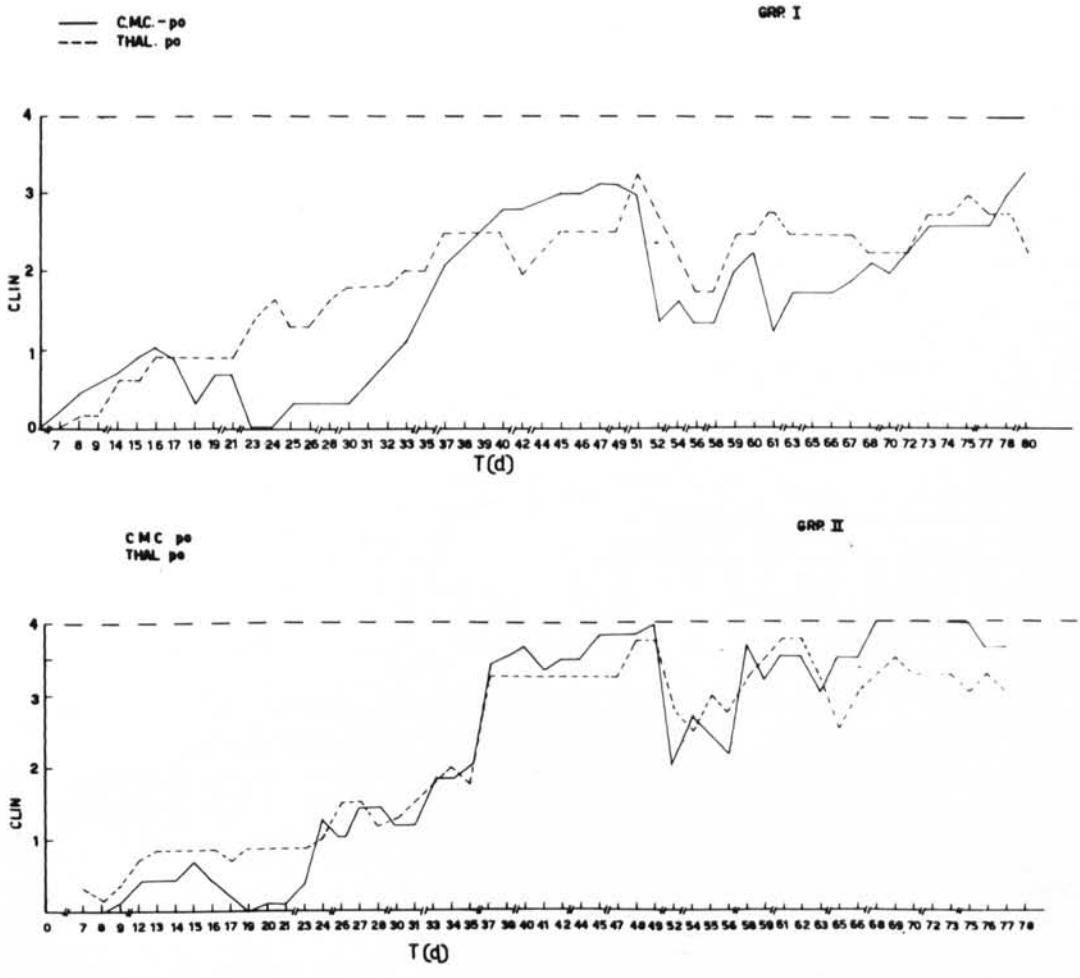

FIG. 6. Comparison of intensity of experimental allergic neuritis between guinea pigs treated with oral thalidomide and controls (Groups I and II).

tion in the development of the clinical features of EAE. Strain, age and sex factors were also shown to be of interest by Stone *et al* ([17]).

Determinations of VNC were useful as an additional method for evaluating EAN. Under our experimental conditions, however, this method was not more accurate or sensitive than gross examination of animals.

The working hypothesis was that if thalidomide were active, this activity should take place in a discrete link of the immune chain. This link need not be present in all animal species nor in all entities explored since we knew that the drug was not active in immediate hypersensitivity of the guinea pig ([18]).

Thalidomide had no appreciable anti-inflammatory effect on experimental granulomas. It did not show any beneficial effects, at the doses and schedules employed, over EAE and EAN in the guinea pig. It should be remembered, that the doses were, in terms of mg/kg, about 100 times greater than the usual human dose.

We have not tested all possible schedules of administration, but it is not likely that changes in these might radically alter our results. The drug is inactivated at an alkaline pH, but the guinea pig stomach has a pH of about one and it has been shown that the drug in aqueous suspension is absorbed and can be detected in the blood ([18]).

There are routes still open for exploration. Thus, the possibility of thalidomide

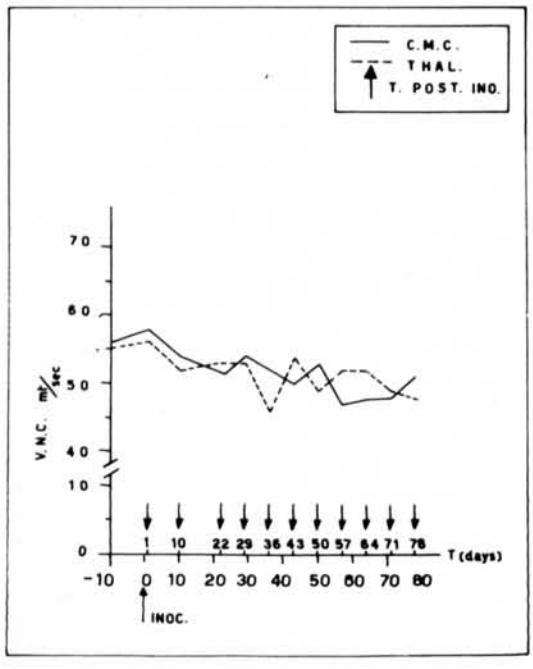

FIG. 7. Comparison of diminution of peripheral nerve conduction velocity induced by experimental allergic neuritis, between guinea pigs treated with thalidomide and controls.

exerting a potentiating effect over other drugs may be worth exploration. This has been shown to occur with Duazomycin A and 6-mercapto purine ([19]). The study of the same experimental diseases in other animals and the study of other experimental autoimmune diseases may be useful. These three alternatives are not mutually exclusive. We have begun experiments on autoimmune diseases in the rat. Absorption and metabolism of thalidomide have been better studied in this animal than in the guinea pig and the rat is sedated by thalidomide given intragastrically.

SUMMARY

Work reported here is part of a more extensive study on exploring the eventual effect of thalidomide on experimental autoimmune diseases and related topics.

In the guinea pig, the drug does not inhibit the formation of nonspecific granulomas and it does not show sedative effects. This compound had no detectable value in treatment or prevention of experimental allergic encephalitis or neuritis, at least in the doses and schedules employed. In the course of experiments we found that in the guinea pig, strain and sensitization schedules could radically alter features of EAE.

RESUMEN

Los experimentos descritos representan parte de un estudio más extenso sobre la exploración de posibles efectos de la thalidomida sobre ciertas afecciones experimentales de origen immune y aspectos relacionados con esto último. En el cobayo, la droga no inhibe la formación de granulomas inespecíficos, no posee efectos sedativos notorios y no tiene acción evidente sobre la evolución de la EAE y la NAE, al menos en las dosis y esquemas de administración empleadas.

En el transcurso de los experimentos encontramos que la cepa de cobayo empleada y los esquemas de inmunización podían hacer variar de modo radical el cuadro clínico de la EAE.

RÉSUMÉ

On décrit des expériences qui font part d'un étude sur les actions éventuelles de la thalidomide sur certaines maladies experimentelles immunes.

Dans le cobaye, la thalidomide n'empeche pas la formation des granulomes expérimentaux non-spécifiques. Elle n'a pas des effects sédatifs et ne montre pas aucune action évidente sur l'evolution de l'EAE ou la NAE.

Au cours des expériences nons avons trouvé que la souche de cobaye employée et les schémas de sénsibilization peuvent faire varier radicalement les charactères et l'evolution de l'EAE.

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