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Autoimmune Diseases and Thalidomide II. Adjuvant Disease, Experimental Allergic Encephalomyelitis and Experimental Allergic Neuritis of the Rat[1, 2]

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There is controversy about the possible immunosuppressive effect of thalidomide ([2, 5, 7, 15]). It seems clear, though, that this drug does not have an all-inclusive effect over immune responses. It is very active on signs and symptoms of reactional lepromatous leprosy ([6]) but does not have any direct effect on *Mycobacterium leprae* ([13, 14]).

Reactional lepromatous leprosy has many of the clinical and histologic features of hypersensitivity reactions ([11, 16]).

We decided to explore the possibility of a suppressive action of thalidomide on experimental autoimmune diseases due to delayed hypersensitivity mechanisms, and where tissue components and mycobacteria had to be administered together (as they occur in lepromatous lesions). In a previous paper ([9]), we studied the effects of thalidomide on experimental autoimmune diseases of the guinea pig. We found the drug to be inactive in experimental allergic encephalomyelitis (EAE) and experimental allergic neuritis (EAN) of this animal. Since drugs may act differently on different species, it was thought interesting to explore the effect of thalidomide in the same diseases in rats. In this animal, the drug has sedative effects and its metabolism has been studied ([1]). We also tested the action of thalidomide on adjuvant disease. This is a condition thought to be peculiar to the rat. It is produced when mycobacteria, or other acid-fast organisms, suspended in an oily vehicle such as Freund's incomplete adjuvant, are injected into the animal. Adjuvant disease is mediated by delayed hypersensitivity mechanisms ([3, 4]) and has features in common with reactional lepromatous leprosy to wit: the existence of a considerable mass of mycobacteria in the tissues; fever, malaise, inflammatory reactions in and around the joints, skin and eyes. Its main features have been described ([3, 4, 12]).

MATERIALS AND METHODS

Thalidomide was supplied in powder form by Chemie Grüenthal GMBH, Stolberg Rheinland, Germany. It was given as a suspension of 40 mg of drug per ml of "vehicle" (0.5% carboxymethylcellulose [CMC Baker & Adams] in phosphate-buffered saline, 0.15 M, pH 7.0). The dose of thalidomide employed was 400 mg per kg daily. The suspension was administered into the stomach by means of a polyethylene catheter passed through the animal's mouth.

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

Albino rats were used throughout the experiments. They were kept in galvanized or stainless steel cages and fed rat pellets and water *ad libitum.* For initial experiments with adjuvant disease, Sprague-Dawley rats from a local colony were used. The bulk of the work was done with Wistar or Lewis/Mai rats kept as random-bred closed colonies. Colonies were begun with animals purchased from Microbiological Associates Inc., Walkersville, Maryland.

Nonspecific experimental granulomas were produced by the intradermal injection

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of 0.1 ml of incomplete Freund's adjuvant into each flank. Their size and microscopic features were evaluated as previously described (8).

Induction and evaluation of autoimmune diseases. *Experimental allergic encephalomyelitis* was induced by the injection of whole guinea pig spinal cord emulsified in fortified Freund's adjuvant (10 mg of dessicated mortar-ground mycobacteria per ml of incomplete Fruend's adjuvant). A clinical classification from 0 (no signs of EAE) to 4 (typical EAE) was established. Details of induction of EAE and features of classification have been previously described (9). At the end of the experiment, or when animals died of the disease, they were autopsied and samples taken from cord, cerebellum and sciatic nerves.

Central nervous system lesions were evaluated miscroscopically as follows:

0 no lesions.
0.5 moderate vascular dilatation.
1 moderate vascular dilatation plus mild perivascular infiltrate.
2 definite vascular dilatation and moderate perivascular infiltrate.
3 marked and diffuse vascular dilatation with marked perivascular infiltrate.
4 similar to 3, but infiltrate becomes granulomatous. There may be necrosis.

Peripheral nerve lesions were also classified. Features will be described in connection with experimental allergic neuritis (see below).

Experimental allergic neuritis was induced by the injection of dog sciatic nerves and brachial plexi, ground and emulsified in fortified Freund's adjuvant. A clinical classification from 0 to 4 and serial *in vivo* determinations of velocity of nerve conduction (VNC) were done as previously described (9). At the end of the experiments, animals were autopsied, samples were taken and, as described for EAE and central nervous system, lesions were classified as mentioned above. Sciatic nerve sections were evaluated as follows:

0 no lesions.
0.5 some vascular dilatation in perineural tissue.
1 moderate vascular dilatation; with blood-filled vessels visible inside nerve tissue.
2 vascular dilatation in and around nerve tissue plus moderate perivascular infiltrate formed mainly by lymphocytes.
3 marked vascular dilatation. Definite infiltrate forming "islands."
4 very marked vascular dilatation. Frank perivascular infiltrate that invades nerve and disrupts nerve architecture.

Adjuvant disease. In initial experiments, Sprague-Dawley rats and varying concentrations of *M. butyricum* or *M. tuberculosis* H 37 Ra, up to 3 mg per animal were used.

For all further experiments only Lewis/Mai or Wistar rats were employed. Each animal received 0.1 ml of adjuvant containing 10 mg per ml of *M. butyricum* in the right rear foot pad and in the neck (thus, each animal received a total volume of 0.2 ml containing 2 mg of *M. butyricum).*

The following data were recorded at practically daily intervals: body weight, thickness of injected foot pad, thickness of contralateral noninjected foot pad, and number of inflammatory lesions in feet, tail, eyes and ears (secondary lesions).

Thicknesses were estimated using callipers which give an accuracy of 0.01 mm. Lesions were counted with the aid of a hand lens when needed. Each lesion was given the numerical value of one. Data were recorded using a special form.

RESULTS

Effects of thalidomide on experimental nonspecific granulomas. Wistar rats were distributed into three groups of eight animals each. The first group received thalidomide 400 mg/kg weight daily, beginning three days before adjuvant injection. The second received vehicle alone and the third received no treatment. Incomplete Freund's adjuvant was injected and intensity of response estimated. There were no significant differences in the magnitude of inflammatory responses between groups. Microscopic examination showed typical paraffinomas in all groups. In sum, thalidomide showed no detectable effect on nonspecific granulomas in the rat. This also has been our experience in the case of guinea pigs (9).

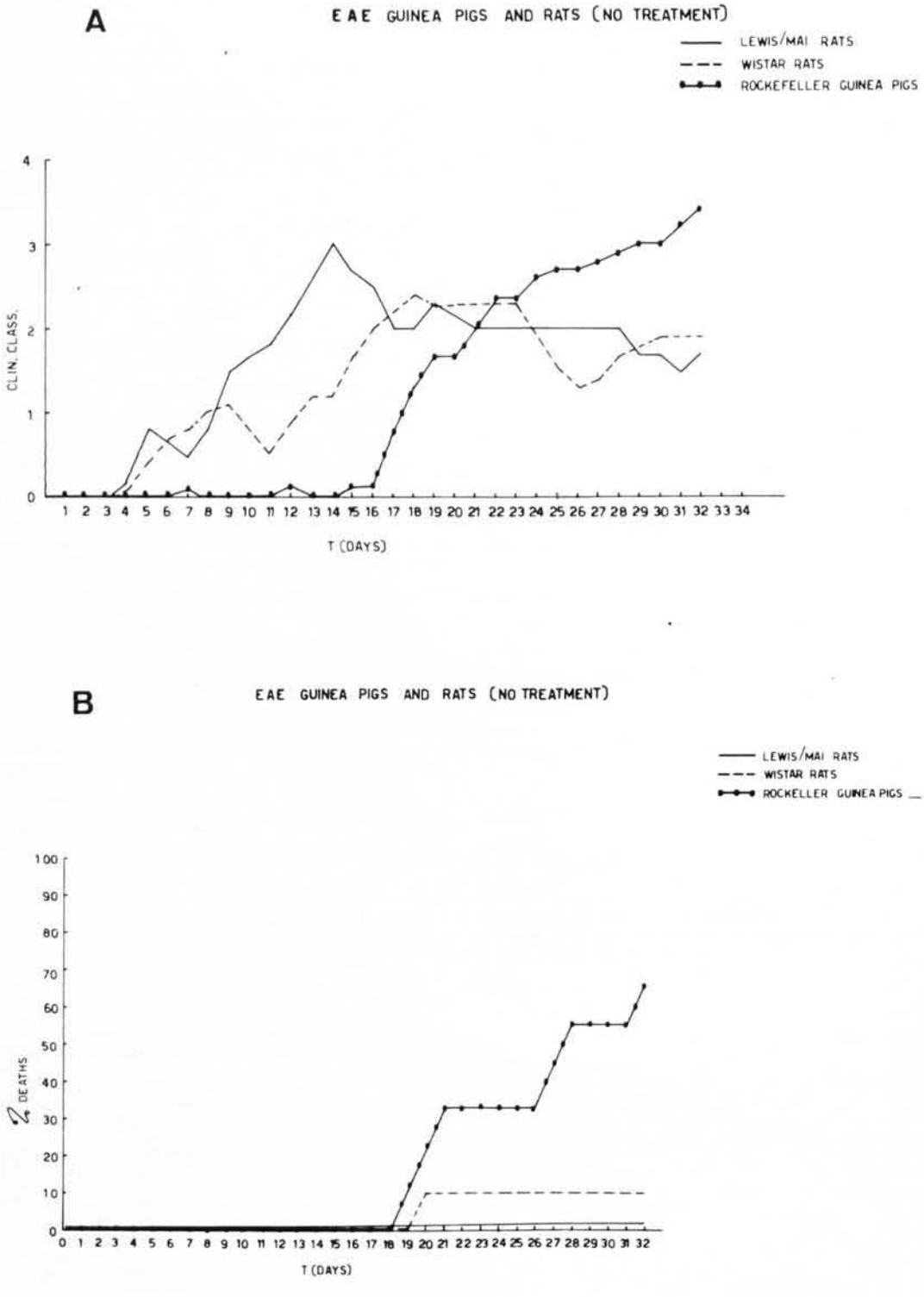
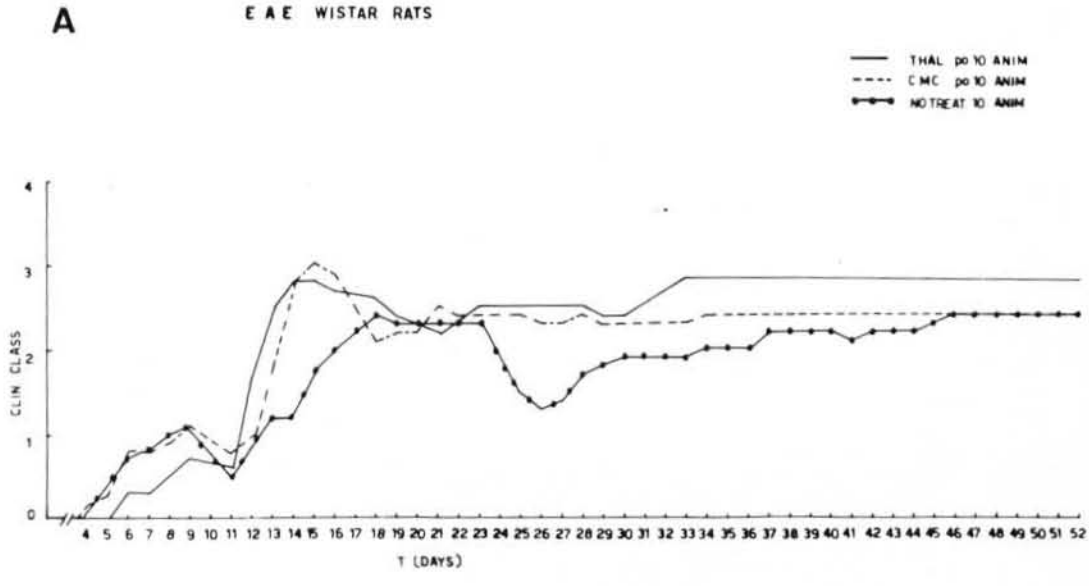


FIG. 1. Clinical evolution (A) and lethality (B) of EAE in untreated rats and guinea pigs.



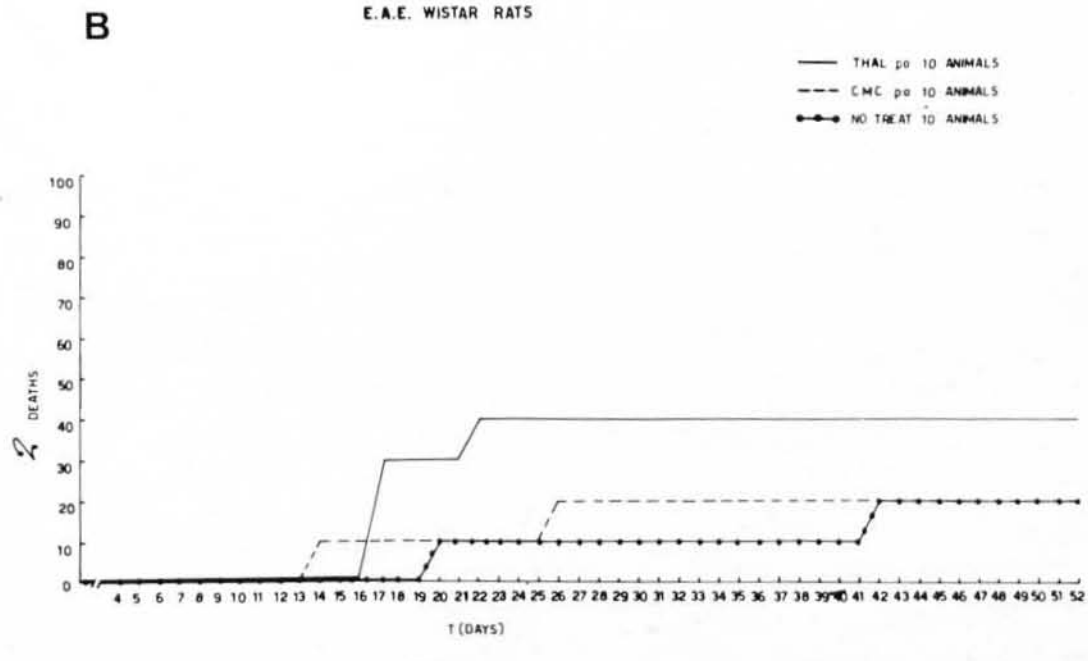


FIG. 2. Clinical evolution (A) and lethality (B) of EAE in Wistar rats. Comparison between thalidomide-treated and control animals.

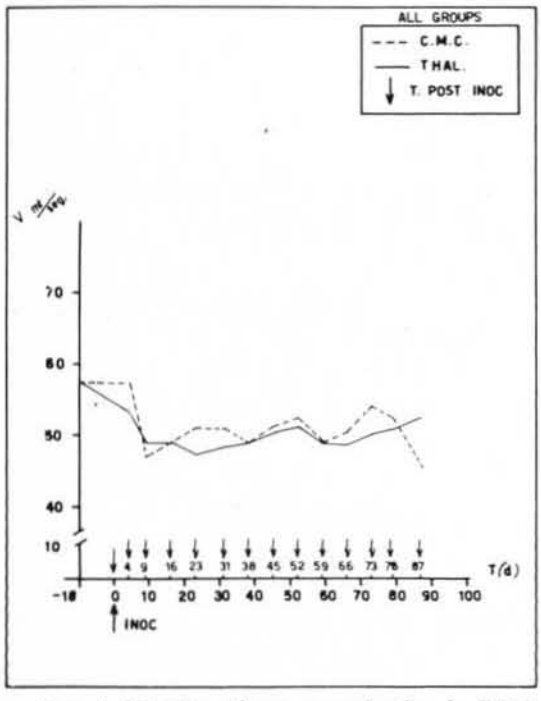


FIG. 3. Velocity of nerve conduction in EAN. Comparison between thalidomide-treated and control animals.

Experimental allergic encephalomyelitis of the rat; effects of thalidomide. We wished to compare evolution of EAE in untreated animals of our two strains of rats. Six Lewis/Mai animals with a mean weight of 233 gm were sensitized with guinea pig cord and evaluated as described. Figure 1 shows evolution of EAE in such rats as compared with Wistar rats and Rockefeller guinea pigs, the latter from previous work (9). It will be noted that Lewis/Mai rats suffered no deaths and the intensity of their disease was not greater than that of Wistar rats. EAE in rats appears even earlier than in guinea pigs and becomes severe, but in time, signs tend to lessen or disappear.

Experiments with Wistar rats. Animals were distributed into three groups as in the nonspecific granulomas experiment, but each group had ten animals. Thalidomide was given daily, beginning five days before sensitization. Disease was evaluated as previously mentioned. The experiment was terminated after 51 days. Surviving animals were autopsied and samples taken from randomly selected animals for histologic study. Figure 2 shows that the disease had similar features to those observed in Lewis/Mai rats with, however, some deaths.

Thalidomide showed no therapeutic effect; if anything, lethality was greater in thalidomide treated animals. Histologically, lesions were milder than those seen in guinea pigs, and there was no significant differences between groups. There were mild but definite lesions in sciatic nerves.

Experimental allergic neuritis. We wished to find out the following: a) evolution of EAN in the rat compared with EAN in the guinea pig, b) magnitude of histologic lesions in sciatic nerves and presence or absence of lesions in central nervous system, and c) therapeutic value of thalidomide.

Thirty-nine Wistar rats were injected with emulsified peripheral dog nerve tissue. For logistic reasons they were injected in two sessions. Twenty animals received thalidomide daily, beginning eight days before sensitization; nineteen received vehicle only. Animals were evaluated at almost daily intervals and VNC determinations were also performed at intervals shown in Figure 3. The experiments lasted 91 or 97 days in the two groups respectively. At the end, or when animals died, autopsies were done and samples were taken for histological analysis (the cerebellum was not studied in all animals and no samples were taken from animals dying early in the experiment). Figure 4 shows that EAN in the rat has an irregular evolution similar to that of guinea pigs, with a tendency toward progression. EAN is not lethal by itself. Determinations of VNC show a definite lowering with progression of the disease (Fig. 3), which appears earlier than in guinea pigs. Microscopic lesions are summarized in Table 1. Lesions were moderate, somewhat milder than in guinea pigs. There was no evidence of central nervous system involvement. Thalidomide had no detectable effect on clinical features, histology or VNC.

Adjuvant disease. The disease was induced and data recorded as previously described.

At the end of the experiments, random samples were taken for microscopic examination. These were processed for routine hematoxylin and eosin staining. Bone-containing samples were decalcified.

In initial experiments using Sprague/Dawley rats, we were able to obtain a few lesions in only 2 of 16 animals. Such rats

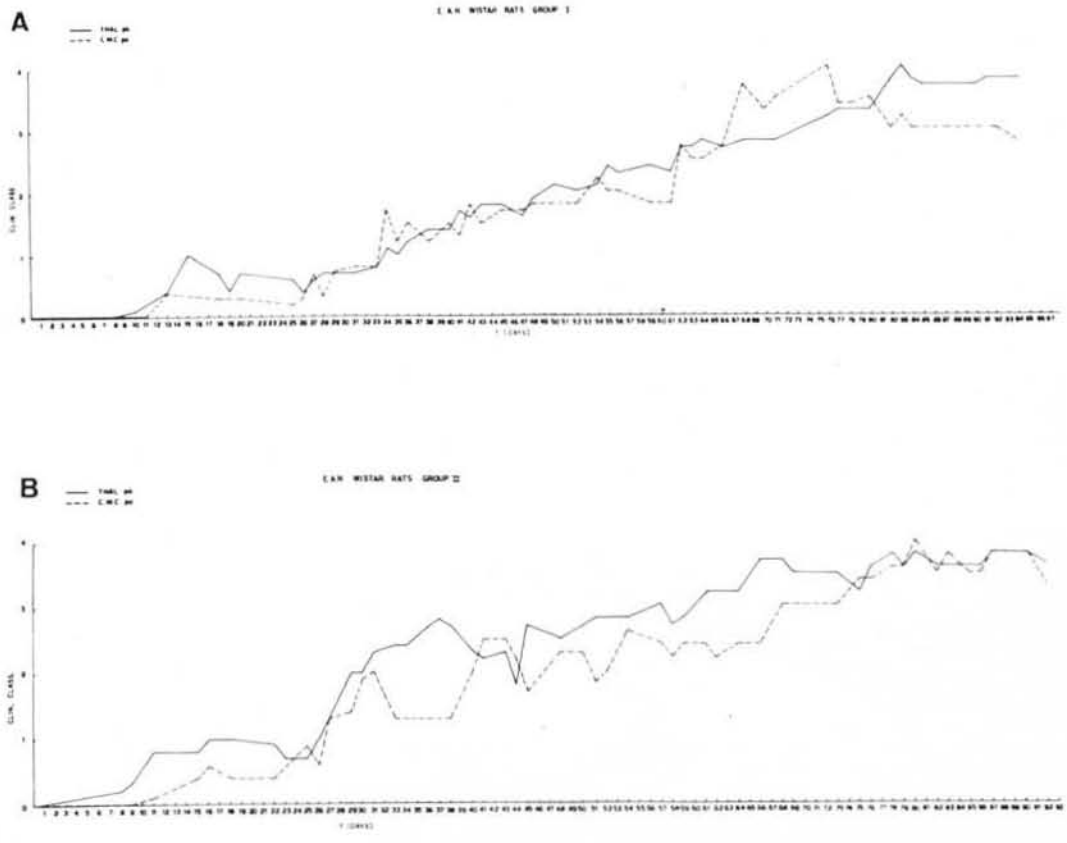


FIG. 4. Evolution of EAN in Wistar rats (A & B). Comparison between thalidomide-treated and control animals.

were deemed unsuitable for further work.

Definitive experiments were done with Wistar and Lewis/Mai rats. The former were divided into three groups of ten animals each, receiving respectively, thalidomide, vehicle only or no treatment. Drug treatment began 14 days before sensitization. Lewis/Mai rats were distributed in the same way except that the group which received no treatment was composed of only five rats.

Rats gained no weight during the period of the experiment. There was no increase in thickness of the noninjected foot pad. Injected pads showed great inerease in thickness. The above data provided no noteworthy differences between strains or experimental groups.

Of much greater interest was the number of lesions seen far from the injected areas (secondary lesions). Figure 5 shows the evolution of disease, expressed as number of secondary lesions, in untreated Wistar and Lewis/Mai rats. Lesions appeared in the former at a later date and they had fewer

TABLE 1. *Microscopic lesions in experimental allergic neuritis of the rat.*

Group	Cerebellum and pons	Dorsal cord	Lumbar cord	Right sciatic nerve	Left sciatic nerve
Vehicle alone (Control)	0.40[a] (5)[b]	0.32 (14)	0.38 (13)	1.36 (11)	1.68 (16)
Thalidomide	0.25[a] (4)	0.10 (14)	0.20 (15)	1.58 (12)	1.38 (18)

[a] Mean of numerical value of lesions.
[b] Figures in brackets indicate the number of animals examined.

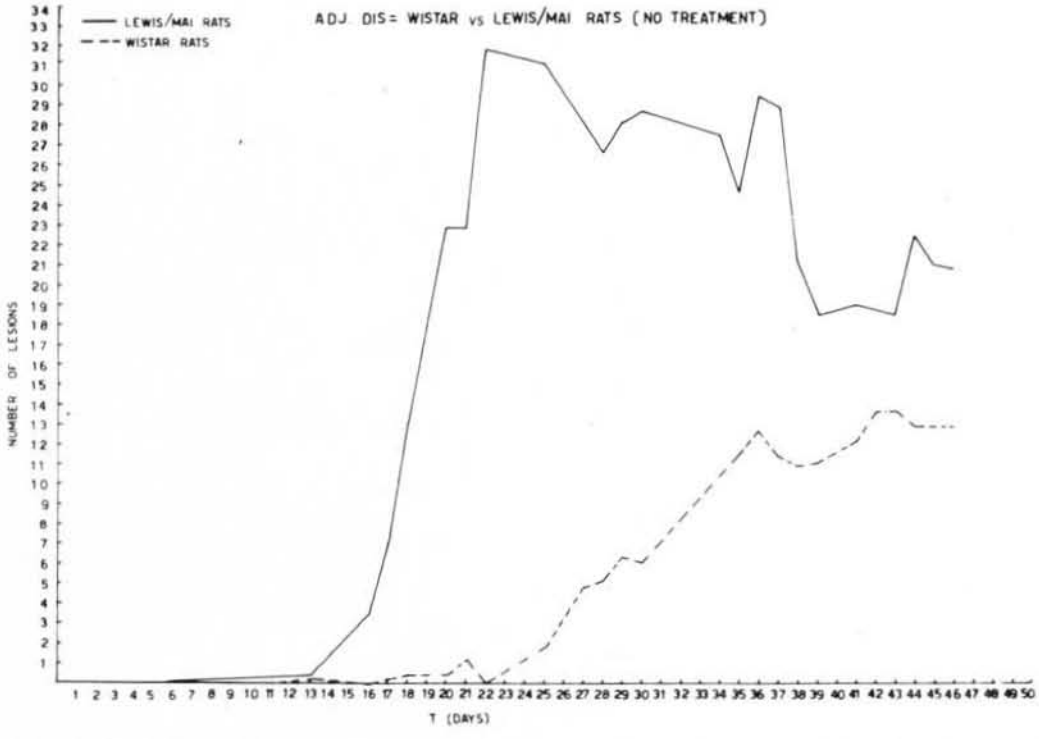


FIG. 5. Evolution of adjuvant disease (number of secondary lesions as a function of time) in untreated rats.

lesions than Lewis/Mai animals. We have already mentioned the lack of response on the part of Sprague/Dawley rats.

Thalidomide-treated Lewis/Mai rats had fewer lesions than untreated controls (Fig. 6). Differences, however, did not reach statistical significance.

Thalidomide-treated Wistar animals had fewer lesions than vehicle treated or untreated controls (Fig. 7). The difference between treated and untreated animals was significant ($p \leq 0.05$, t-test) at 29, 30, 35, 36, 37, 38, 39, 41, 44, 45, 46, 49, 50, 51, 52, 53, 55, 57–62 days; and highly significant ($p \leq 0.01$) at 35, 36, 37, 38, 39, 41, 51, 52, 53, 55 and 57 days. Vehicle-treated animals also had fewer lesions than untreated ones; the difference was significant, at 35, 36, 38, 42, 43, 52, 53, and 55 days. The effect of vehicle was much less than that of thalidomide. Animals treated with this drug had significantly fewer lesions than those receiving vehicle only, on days 29, 30, 36, 38, 46, 50, 51 and approaching statistical significance ($0.1 > p > 0.05$) on days 35, 37, 39, 41, 48 and 62. Furthermore, there was no instance in which animals receiving vehicle alone had significantly fewer lesions than those treated with the drug.

Histologic features were determined at the end of the experiment. There was no intention of quantitating the intensity of response. In general, there was vascular dilatation with hyperemia, and an infiltrate formed almost exclusively by mononuclear histiocytes; there were a few lymphocytes and practically no polymorphonuclear leukocytes. In the case of the eyes, lesions occurred on the iris and ciliary processes. In the ears, the infiltrate was located around dermal blood vessels with no involvement of cartilage. In joints there was not only involvement of synovium but also of periarticular soft parts. There was not only arthritis but also periarthritis.

DISCUSSION

The present work is a continuation study of different autoimmune diseases in diverse animals; it includes the effect of drugs such as thalidomide on autoimmune diseases.

EAN was similar in rats and in guinea pigs. EAE was a self-healing condition in

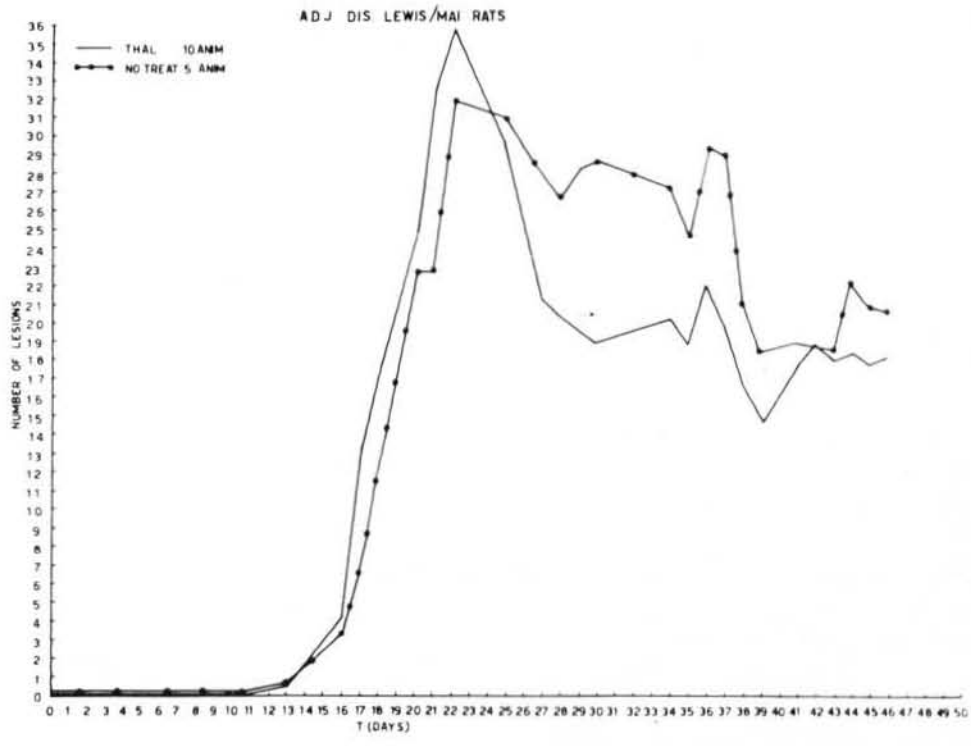


FIG. 6. Adjuvant disease (Lewis/Mai rats). Number of secondary lesions in thalidomide-treated and untreated animals.

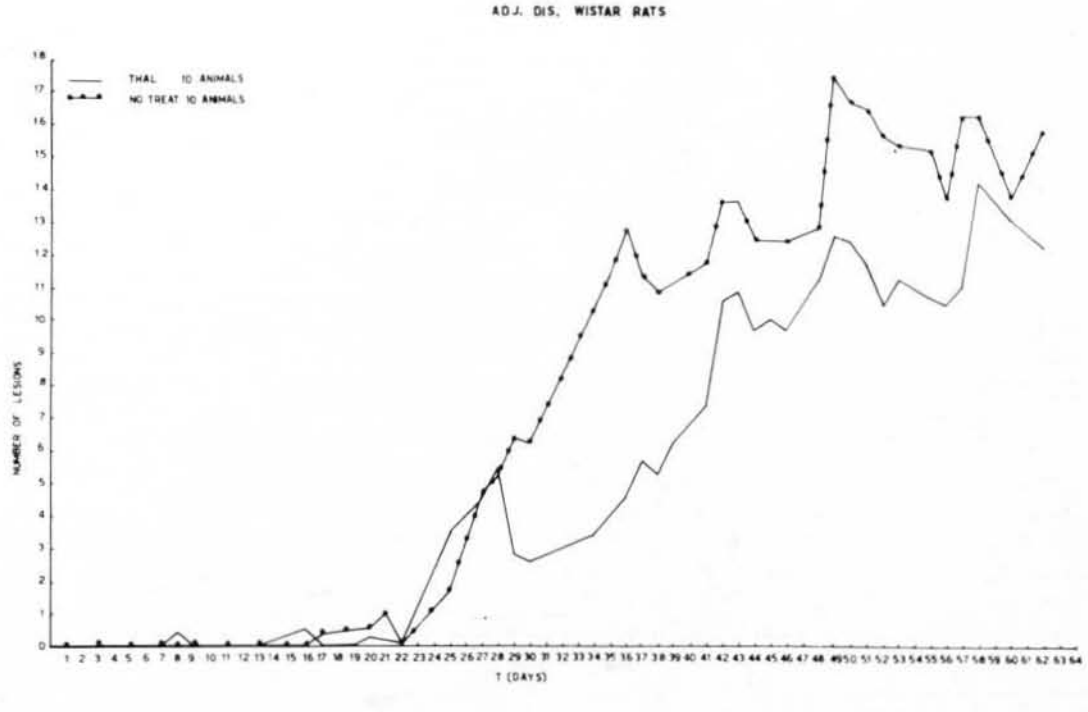


FIG. 7. Adjuvant disease (Wistar rats). Number of secondary lesions in thalidomide-treated and untreated animals.

rats. Thalidomide has no therapeutic effect on any of these diseases. Neither did it show nonspecific anti-inflammatory action.

The results obtained with adjuvant disease are of interest. There was a definite influence of strain factors on the intensity of this disease. Thalidomide significantly diminished the intensity of adjuvant disease of Wistar rats. The effect on this condition in Lewis/Mai rats was not statistically significant. The milder but noticeable effect of vehicle may be explained by the stress involved in restraining the animals daily and passing a catheter through the mouth into the stomach. Adrenocortical hormones are effective in adjuvant disease ([10]).

There are similarities between this experimental malady and reactional lepromatous leprosy. In both there is fever, malaise, inflammatory lesions on skin, joints and eyes and an important mass of mycobacteria in tissues. Inflammatory lesions are not fixed, but wax and wane, and the disease may follow a sinusoidal curve. While adjuvant disease is supposed to be due to delayed hypersensitivity mechanisms, the antigens, mycobacterial or otherwise, which lymphocytes react to are not known; moreover, there is a strong admixture of polymorphonuclear leukocytes in early lesions ([3]).

While our experiments do not clarify the mechanism of action of thalidomide, they suggest that there are intermediate steps in adjuvant disease that are sensitive to thalidomide; these may be common to those occurring in reactional lepromatous leprosy.

The intensity of adjuvant disease in different strains of animals is also of interest. It may be pointed out that genetic factors are thought to be important in susceptibility to lepromatous leprosy and to reactional episodes.

SUMMARY

Nonspecific granulomas, experimental allergic encephalomyelitis, experimental allergic neuritis, and adjuvant disease were induced in rats. Different strains of animals were tested and the effect of thalidomide explored. There were no detectable effects of the drug on experimental allergic encephalomyelitis, neuritis or granuloma formation. Intensity of adjuvant disease was very much dependent on the strain employed. It was almost impossible to induce it on Sprague/Dawley rats, it was moderate in Wistar animals and very marked in Lewis/Mai rats.

Thalidomide was active in diminishing intensity of adjuvant disease. This effect was statistically significant in Wistar rats. The interesting similarities between adjuvant disease and reactional lepromatous leprosy are pointed out.

RESUMEN

Se injudo la EAE, la NAE, granulomas inespecificos y enfermedad por adyuvante en ratas de diferentes cepas. Se exploró asimismo el eventual efecto de la thalidomida. Esta droga no mostró ninguna acción terapéutica en la EAE, la NAE ni los granulomas.

La intensidad de la enfermedad por adyuvante era función de la cepa empleada. Fué muy difícil producirla en ratas Sprague Dawley, era de intensidad moderada en ratas Wistar y era de gran intensidad en ratas Lewis/Mai. La thalidomida tuvo acción terapéutica en esta entidad. La acción fué estadísticamente significativa en el caso de la enfermedad por adyuvante de las ratas Wistar.

Se menciona la interesante similitud que existe entre la enfermedad por adyuvante y la lepra lepromatosa reaccional.

RÉSUMÉ

Des granulomes non-spécifiques, de la EAE, de la NAE et la maladie par adjuvant, on été produits dans des rats de souches différentes. L'éffet de la thalidomide avait été exploré. La thalidomide n'avait pas aucune action évidente sur les granulomes, la EAE ou la NAE. L'intensité de la maladie par adjuvant dépendait de la souche employée. Elle était plus marquée dans les rats Lewis/Mai que dans les animaux Wistar. Il était presqu'impossible de produire la maladie dans les rats Sprague/Dawley. La thalidomide avait une action thérapeutique dans cette maladie. Cette action était statistiquement significative dans les rats Wistar. On fait compte des points de similarité entre la maladie par adjuvant et la lèpre lépromateuse reactionnel.

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