The Use of a Standardized Adjuvant Arthritis Assay to Differentiate Between Anti-inflammatory and Immunosuppressive Agents (35609)

R. J. Perper, B. Alvarez, C. Colombo and H. Schroder

Geigy Pharmaceuticals, Division of Ciba-Geigy Corporation, Ardsley, New York 10502

The induction of adjuvant arthritis (AA) in rats with mycobacterium bacilli has been described in detail by many authors (1–4). The etiology of the disease is thought to be due to a delayed hypersensitivity reaction (DHS). This assumption is based on the time course of induction, transfer with viable lymphoid cells from sensitized donors, and histological criteria (5). Most authors have attributed the inflammatory event in this disease to the delayed hypersensitivity reaction (5–9). Nonspecific inflammation may make a significant contribution to the development of the lesions, since AA can be induced in thymectomized rats which are incapable of mounting normal DHS reaction to other antigens (10).

Rosenthale and Nagra (11) studied the effects of anti-inflammatory (AI) and immunosuppressive agents (IS) in both AA and in a more "classical" model of autoimmunity, acute allergic encephalomyelitis (EAE) (12). They concluded that both tests were necessary to discriminate between AI and IS compounds. Since two distinct mechanisms, inflammation and delayed hypersensitivity are involved in the mediation of AA, it is possible that an analysis of the time sequence of the development of this disease might indicate methods for discriminating between AI and IS agnts. For this purpose it was necessary to develop a model which was not subject to individual variability and which was characterized by a well-defined time sequence. In this report a method is described for inducing such a model in which it is possible to make an absolute distinction between class specific AI and IS agents.

Materials and Methods. A total of 700 male Spartan rats (Spartan Research, Haslet, Michigan) and Charles River rats (Charles River Farms, Wilmington, Massachusetts), weighing 250–260 g, were used in these studies. Heat killed *M. butyricum* was prepared either as an emulsion or suspension. The suspension was made by thoroughly grinding the bacilli with a mortar and pestle in the presence of a few drops of heavy mineral oil. Additional oil was subsequently added to give a final concentration of 6 mg/ml. For preparation of the emulsion, 100 mg of a suspension of *M. butyricum* prepared as outlined above was added to a 15.6 ml of mineral oil and 1.0 ml of normal saline. The mixture was homogenized at 4° for 10 min in a Virtis "45" at 1400-1600 rpm. The final concentration was 6 mg/ml. The establishment of a stable emulsion was confirmed by microscopic examination. A 0.05-ml dose of the adjuvant preparation (emulsion or suspension) was injected subcutaneously either into the right hind paw (plantar), close to the origin of the tail, or simultaneously into both sites. Plethysmographic measurements of the primary disease (injected paw) and the secondary disease (noninjected paw) were made by mercury displacement. Drugs were administered either during the induction of the disease (nonestablished arthritis) or after the peak of the disease had been reached (established arthritis). The time of drug administraiton and paw measurements were determined by the experimental data reported below (see Table I). The anti-inflammatory drugs used were: indomethacin given at 0.05, 0.25, 1.25, 3.0 mg/kg and phenylbutazone given at 100 mg/kg. The immunosuppressant agents were azathioprine (Imuran) given at 5 and 30 mg/kg and cyclophosphamide used at 10 mg/kg. In addition a corticosteroid, paramethasone, was used at 0.5 mg/kg.

All drugs were prepared in 5% carboxy-

TABLE I.

Established adjuvant arthritis

Day:	0 (adjuvant injection)	← Daily drug therapy → 18	30 (paw measurement)

Nonestablished adjuvant arthritis

Day:	← Daily drug therapy → 0 (adjuvant injection)	12	21 (paw measurement)

methylcellulose (CMC) which contained a final concentration of 5% polyethylene glycol (PEG) 400. A maximum of two drops of Tween 80 was added to each compound prior to adding CMC. All drugs were prepared daily and were administered orally.

Results. The arthritis which developed in the injected paw was defined as a primary lesion; whereas the swelling in the contralateral paw was designed as a secondary lesion. Animals injected only in the tail developed secondary lesions which were quantitated by using the mean value of the swelling for both hind paws. The results illustrated in Figs. 1 and 2 indicated that, regardless of the strain of rat or the method of preparing the adjuvant, the tail injection produced a less severe secondary disease than either the primary or secondary disease induced by subplantar injections. In addition, there was a lower incidence of disease in that only 30–65% of rats became arthritic when they were injected in the tail, compared with 67–100% when the adjuvant was injected into the paw (Figs. 1 and 2). The tail injection, therefore, represented an unsatisfactory method for producing arthritis. This criterion is especially important for the nonestablished disease, since it must be assumed that all animals injected with the adjuvant would have become arthritic in a situation in which a test compound was shown to prevent its induction. The simultaneous injection of adjuvant into both the tail and paw induced primary and secondary lesions which were as statistically acceptable as the plantar injections.

The suspension versus emulsion method of adjuvant preparation was compared. Figures 1 and 2 show that the emulsion induced both primary and secondary lesions in a greater percentage of the animals with less variability than did the suspension. The best results were obtained following the injection of

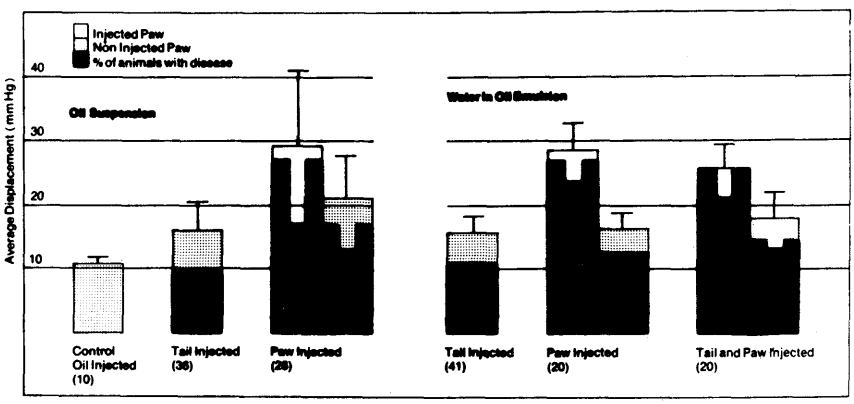

FIG. 1. Groups of male Spartan rats were injected in various sites with an emulsion or suspension of *M. butyricum* in oil. Measurements of the injected and noninjected rear paws were made 21 days later. (vertical bar) standard deviation; number of animals per group is given in parentheses.

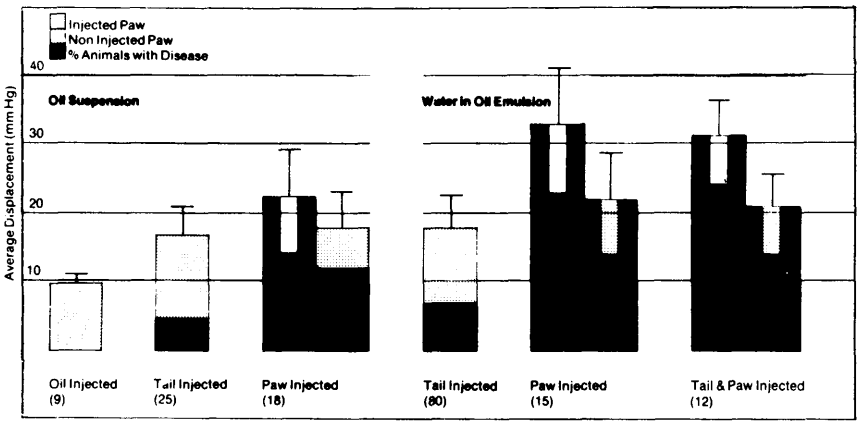

FIG. 2. The same experiments as outlined in Fig. 1 were performed on Charles River rats.

emulsion directly in the paw. When comparing all groups, 7/10 or 70% of the groups injected with emulsion had 90–100% incidence of disease, whereas only 2/6 or 33% of the groups injected with suspension had 90–100% incidence of disease.

When comparisons were made between the two rat strains under optimum conditions (emulsified adjuvant injected into the paw), as shown in Figs. 1 and 2, 95–100% of both strains were affected with a disease of sufficient magnitude to discriminate for drug effectiveness. The standard deviations given in Figs. 1 and 2 show that the variability of response in the Charles River rats was greater than that in the Spartan rats (24% of the mean versus 14%). In addition, a significant proportion of Charles River rats had an unacceptable severe disease; many of the animals had ulcerated and

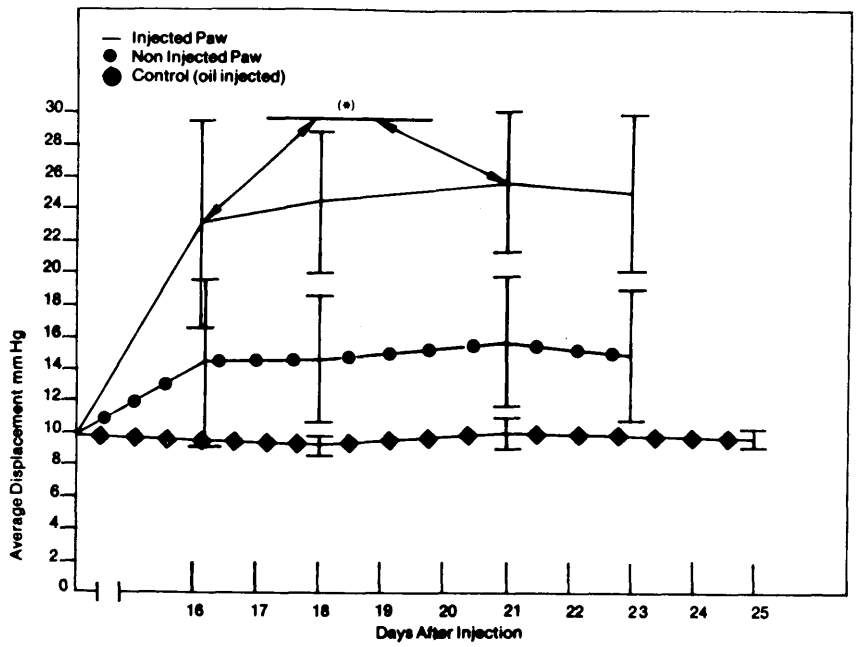

FIG. 3. Development of adjuvant arthritis in 51 male Spartan rats injected with a water in oil emulsion of *M. butyricum*: (vertical bar) standard deviation; (*) difference is significant ($p \leqq 0.05$).

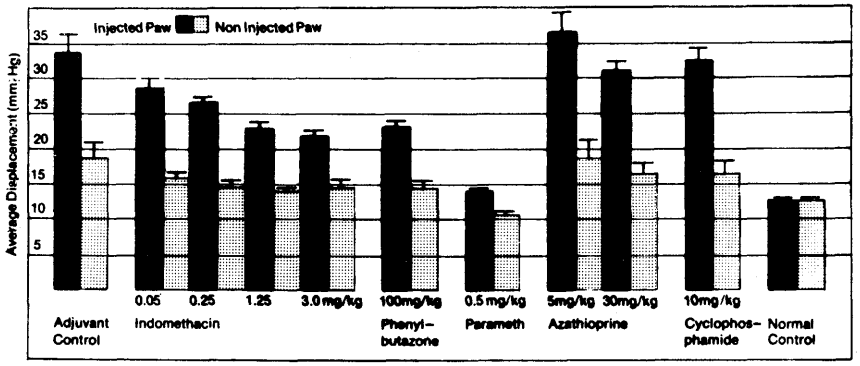

FIG. 4. Effect of various drugs on established adjuvant arthritis induced in groups of 10–20 male Spartan rats by the subplantar injection of emulsified *M. butyricum*. Treatment was given po daily for 12 days starting 18 days after adjuvant injection. The average swelling and the standard error (vertical bar) was determined on the last day of treatment (day 30).

severely edematous lesions in the paws which became secondarily infected.

In order to determine the peak of the disease, paw measurements were made in Spartan rats at different time intervals following emulsified adjuvant injection. The results of this study (Fig. 3) indicate that there was a significant increase ($p<0.05$) in swelling between days 16 and 21. There was no statistical difference between days 18 and 21. It is apparent that the peak of AA, as determined by paw volume, was between days 18 and 21.

In order to discriminate between AI and IS activities, standard compounds were tested in Spartan rats injected in the paw with emulsified adjuvant. In the established disease, drug treatment was started 18 days after the adjuvant injection and continued daily for 12 days. Measurements were made on day 30. In previous experiments, it was observed that in order to obtain maximum effect with active agents, it was necessary to treat for 12 or 14 days, whereas 8 day treatment was suboptimal. Indomethacin (0.05, 0.025, 1.25, and 3.0 mg/kg) gave a dose related reduction in swelling in the injected paw (Fig. 4). Less protection was produced in the noninjected paw, and the inhibition was not dose related. Phenylbutazone (100 mg/kg) gave significant protection against the primary and secondary disease. Azathioprine (5 mg/kg) showed no reduction in swelling in either the injected or noninjected paw. Azathioprine (30 mg/kg) and cyclophosphamide (10 mg/kg) gave a slight reduction in the swelling of both paws, but this effect had little or no statistical significance. Paramethasone (0.5 mg/kg) was effective against both lesions (Fig. 4).

The nonestablished model was produced by giving 12 daily doses of drug from days 0 to 12. Azathioprine (5 and 30 mg/kg) and indomethacin (0.5 mg/kg) were each given to groups of 10 rats. Measurements of the injected paw were made on day 0, 10, 14, 21. The results (Fig. 5) show that only at day 21 was there a clear distinction between the two agents. Indomethacin gave no protection, whereas azathioprine, at both dose levels, showed significant effects. When measurements were made on day 10 or 14, both compounds were effective. On the basis of these results, other class specific compounds were tested in the nonestablished model and measurements were made on day 21 (Fig. 6). Azathioprine (5 and 30 mg/kg) and cyclophosphamide (10 mg/kg) were effective in totally inhibiting the secondary lesion and in significantly preventing primary arthritis. Indomethacin (0.5 mg/kg) and phenylbutazone (100 mg/kg) were ineffective in preventing the disease in the noninjected paw. However, they did give small, significant effects in the injected extremity. On the other hand, paramethasone, a compound with both anti-inflammatory and immunosuppressive activity, was active in inhibiting both primary and secondary lesions (Fig. 6).

Discussion. Potential therapeutic agents can be tested in AA in two ways: *i.e.*, prevention of the disease (nonestablished arthritis)

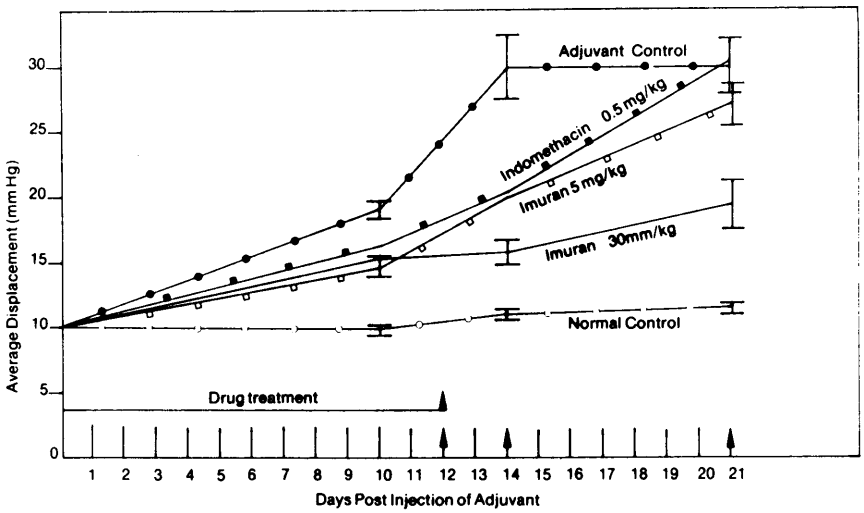

FIG. 5. Effect of 12 daily oral doses of standard compounds on the development of arthritis in groups of 10–20 male Spartan rats injected subplantarly with an emulsion of *M. butyricum*.

and treatment of an already established disease (established arthritis). To properly evaluate the efficacy of agents in the nonestablished arthritis, there are two essential requirements: most, if not all, the animals must become arthritic, and the variability of response must be low enough to attain statistical validity in comparison of treated versus nontreated groups. To properly evaluate potential therapeutic agents in the established disease, other variables must be controlled: treatment should commence at the peak of the disease, and, as before, variability of the individual response must be reduced to a minimum. In this latter regard, too severe a disease which is characterized by excessive swelling and trauma-induced ulcerations, must be eliminated, since these extra variables would tend to mask the underlying disease process. The purpose of the present work was to find methods for inducing AA which would meet the above criteria and which could be used to analyze the biological activity of class specific compounds.

The foot pad injection of a water in oil emulsion of *M. butyricum* in Spartan rats induced a predictable and statistically acceptable primary and secondary disease in almost all animals. These studies indicated that the rat strain used, the preparation of the

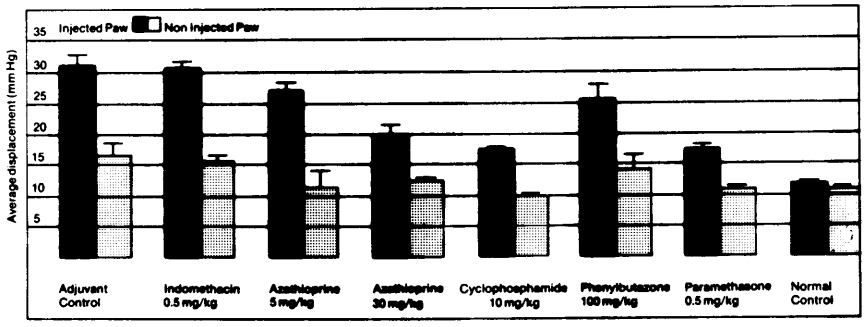

FIG. 6. Twelve daily doses of standard compaunds were administered po to groups of 10–20 male Spartan rats given subplantar injections of an emulsion of *M. butyricum*. Treatment was from days 0 to 12 and the average paw size of each group and the standard error (vertical bar) was determined on day 21.

adjuvant, the site of injection, and the time of measurement were all critical to the analysis of selective drug action.

The fact that AA is an immunologically mediated disease is well established (6, 13). The immunological event, however, is measured by an inflammatory sequela. Therefore, for a compound to be considered immunosuppressive in the assay, it should be capable of totally preventing the induction of the immunological disease state. Arthritic lesions in the noninjected paw represent the immunological component of the disease minus local inflammatory effects. On the other hand, the disease in the *injected* paw, when measured 21 days after injection, is due to a local inflammatory response which may or may not be directly related to the immunological event. Therefore, a true immunosuppressive agent should produce a profound, if not absolute, reduction in the disease in the noninjected paw with less effect on the injected extremity. As was demonstrated in this study, azathioprine, cyclophosphamide and paramethasone were effective in this regard.

The AI agents, indomethacin and phenylbutazone, given at doses active in the established disease, had no significant activity in non-established arthritis. It was therefore possible to demonstrate an absolute separation of AI from IS agents. To demonstrate this separation it was necessary to treat for a finite period of time (first 12 days) and measure at the peak of the disease (21 days). If measurements were made too close to the termination of drug therapy, the residual effects of AI compounds were still evident and protection was observed.

When the same compounds were given for the same length of time after the peak of the disease had been reached, diametrically opposite results were attained. Azathioprine and cyclophosphamide were relatively ineffective in protecting against the primary and secondary disease, whereas indomethacin and phenylbutazone were highly active against both lesions. In this case, measurements of the injected extremity was the most sensitive indicator of drug effect since a good dose–response relationship was obtained only in the primary disease. Paramethasone, a compound with both AI and IS activity, was effective in both established and the nonestablished disease.

All the agents used in this study have been reported to be active in rheumatoid arthritis. They all were capable of inhibiting adjuvant arthritis when the optimum testing protocol was used. What is of importance in this study is that class specific compounds with potential clinical utility can be inactive in AA if they are tested at arbitrary times during the induction or after the establishment of this disease.

Summary. Various methods for inducing AA in rats were examined using different rat strains, adjuvant preparations, and sites of injection. The time course of the development of arthritis in the injected and noninjected extremity was determined and methods for inducing a disease with limited variability and a high incidence was demonstrated. Standard compounds were tested in two models of AA with special reference to defining a method for discriminating between inhibition due to immunosuppression (IS) and that due to anti-inflammatory (AI) effects. 12 daily doses of IS agents, started on the day of adjuvant injection, totally prevented the disease in the noninjected extremity when measurements were made on day 21. Standard AI agents were ineffective. This test (nonestablished arthritis) was therefore capable of detecting compounds with immunosuppressive potential. The same standard compounds were administered daily for 12 days after the peak of the disease had been reached (established arthritis). In this test the IS compounds were ineffective in reducing the disease in the injected extremity, whereas the anti-inflammatory agents inhibited the disease in a dose related manner. The results indicate that with the use of both models of AA it was possible to detect compounds which were selectively either AI or IS.

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Received Jan. 28, 1971. P.S.E.B.M., 1971, Vol. 137.