EFFECT OF CYCLOPHOSPHAMIDE, HYDROCORTISONE, AND LEVAMISOLE ON COLLAGEN-INDUCED ARTHRITIS IN RATS

JOHN M. STUART, LINDA K. MYERS, ALEXANDER S. TOWNES, and ANDREW H. KANG

The immune response of rats to type II collagen was modified by pretreating the rats with levamisole, hydrocortisone, or cyclophosphamide. Rats were observed for the development of collagen-induced arthritis and were bled serially for the determination of antibody levels to type II collagen. Levamisole had a slight but not significant potentiating effect with arthritis developing earlier, whereas hydrocortisone and cyclophosphamide significantly depressed the incidence of arthritis; cyclophosphamide also delayed its onset. Antibody levels measured by radioimmunoassay paralleled the clinical course of arthritis.

Collagen-induced arthritis is a new animal model of inflammatory polyarthritis (1). It results from the intradermal immunization of rats with type II collagen emulsified with incomplete Freund's adjuvant. The onset and course of the arthritis resemble that of adjuvant arthritis in rats, but the exact relationship between these two animal models of polyarthritis is not well understood. We have previously shown that collagen-induced arthritis is intimately associated with the simultaneous development of intense humoral and cellular immunity to type II collagen (2,3). It has also been reported that arthritis can be passively transferred with spleen and lymph node cells from arthritic rats (4). These observations suggest that immunologic reactivity to collagen is intimately involved with the development of arthritis. In the studies reported here we attempted to modify the development of arthritis and the immune response to type II collagen by pretreating the rats with cyclophosphamide, hydrocortisone, or levamisole. Since adjuvant-induced arthritis has been widely used to test the effect of various drugs on inflammatory arthritis, we have chosen drugs to test collagen-induced arthritis which have been reported to affect adjuvant-induced arthritis when similar dosages were employed (5,6).

From the Veterans Administration Medical Center and the University of Tennessee Center for the Health Sciences, Memphis, Tennessee.

Supported in part by funds from the Veterans Administration and by grant AM-00650 from the National Institutes of Health.

Address reprint requests to Dr. John M. Stuart, Research Service (151), Veterans Administration Medical Center, Memphis, TN 38104.

Submitted for publication September 8, 1980; accepted in revised form January 9, 1981.

MATERIALS AND METHODS

Rats. Outbred female Wistar rats, 100–125 gm, were obtained from Charles Rivers Breeders (Wilmington, MA), housed in metal cages, and fed standard Purina rat chow.

Collagen preparation. Type II collagen was prepared by limited pepsin digestion of pulverized chick sternal cartilage and purified as previously described (3). Lyophilized collagen was stored in a vacuum desicator at 4°C until used.

Immunization procedures. Collagen was dissolved overnight at 4°C in 0.1*M* acetic acid at a concentration at 4 mg/ml. The solution was emulsified in an equal volume of incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI) for 2 minutes at high speed using a Virtis 23 homogenizer (Gardner, NY). Each rat was immunized with 0.1 ml of the cold emulsion by injecting the right hind footpad. Rats were observed daily for the development of arthritis and scored for severity as previously described (7).

Treatment regimens. Rats were randomly allocated to one of three treatment groups or one of two control groups. Hydrocortisone (Merck, Sharp, and Dohme, West Point, PA) was administered intravenously (IV) at a dosage of 125 mg/kg. Cyclophosphamide (Mead Johnson, Evansville, IN) was administered IV at a dosage of 50 mg/kg. Levamisole (Janssen R&D, New Brunswick, NJ) was administered subcutaneously (SC) at a dosage of 5 mg/kg. Control rats were given saline either IV or SC. All IV injections were given after lightly

anesthetizing the rats with ether. Since no difference in incidence, severity of arthritis, or antibody levels were detected between the rats given IV or SC saline, these data have been pooled and were reported as a single control group. All drug and saline control injections were performed 1 day prior to immunization

Antibody assay. Sera were collected at intervals from the internal jugular vein. These were heat inactivated at 56°C for 30 minutes and stored at −80°C until used. Antibodies were quantified by radioimmunoassay.

Type II collagen to be used for radioimmunoassay was labeled with ^{125}I by a modification of the technique described by Bolton and Hunter (8,9). This has also been applied to the labeling of collagen by Roll, Madri, and Furthmayr (10). Bolton-Hunter reagent having a specific activity of 2,000 Ci/mmol was obtained from a commercial source (New England Nuclear, Boston, MA). Benzene was evaporated by a gentle stream of N_2, and the vial containing dry reagent was chilled in an ice bath. Collagen was dissolved in phosphate buffer pH 7.6, ionic strength 0.4, at a concentration of 2 mg/ml by stirring overnight at 4°C. Undissolved material was removed by centrifugation, and 10 μl of collagen solution was added to the dry Bolton-Hunter reagent. After 2 hours in an ice bath, the vial was removed and left at 4°C overnight. Unreacted label was separated on a 0.4 × 10 cm column containing Bio-Gel P-10 (Biorad, Richmond, CA) equilibrated with 0.1*M* Tris/0.15*M* NaCl, pH 7.6 (Tris buffered saline). Elution was performed with Tris buffered saline, and fractions of 4 drops each were collected. Collagen eluted in the void volume as a discrete peak in 3–4 fractions which were pooled and diluted in Tris buffered saline containing 1% ovalbumin (Sigma Grade V, St. Louis, MO). Under these conditions, labeled collagen with a specific activity of approximately 2×10^6 counts/minute/μg was obtained. From 85–90% of the ^{125}I in the pooled fraction was precipitable with 5% trichloroacetic acid (TCA). All calculations are based on the number of TCA precipitable counts added.

Radioimmunoassay was performed by diluting test sera in Tris buffered saline containing 1% ovalbumin. To 0.5 ml of diluted serum was added 20,000 cpm of ^{125}I labeled collagen. After incubation for 24 hours at 4°C, sufficient polyvalent rabbit anti-rat immunoglobulin (second antibody) (Cappel Laboratories, Cochranville, PA) was added to precipitate all of the immunoglobulin. The necessary quantity of second antibody to accomplish this was determined experimentally by using serial 2-fold dilutions of sera in a "checkerboard." After an additional incubation at 4°C for 18 hours, precipitates were recovered by centrifugation at 3,000*g* for 30 minutes, washed twice with Tris buffered saline, and counted in a gamma counter. Results are expressed as % binding calculated as follows:

$$\frac{\text{cpm in precipitate}}{\text{TCA precipitable cpm added}} \times 100$$

The standard deviation of replicate samples never exceeded 10%. All of the experiments reported here used a single preparation of labeled collagen and included appropriate positive and negative controls. Variation of duplicate sera within a run and between runs also did not exceed 10%.

RESULTS

Hydrocortisone and cyclophosphamide altered the clinical course of arthritis. Levamisole had a slight but not significant enhancing effect with disease appearing in some animals by 9 days after immunization (Figure 1). None of the control group developed arthritis until day 12. However, in previous studies we have noted that arthritis in immunized but untreated rats may occasionally develop as early as 9 days after immunization. At day 10 the incidence of arthritis in the levamisole group was 20% (8 of 40), compared to none in the control group ($P < 0.005$ by Chi square). The overall incidence of arthritis in the levamisole treated group was also greater than in controls, but this difference was not significant by Chi square analysis. Treatment with either hydrocortisone or cyclophosphamide reduced the incidence of arthritis as compared to the control group ($P < 0.02$ and $P < 0.001$, respectively). With hydrocortisone, this suppression was manifested as a lowering of the incidence of arthritis, but without change in the time of onset. With cyclophosphamide, the time course of development of arthritis after treatment was also altered. Although a few rats receiving cyclophosphamide developed arthritis at 12–16 days similar to controls,

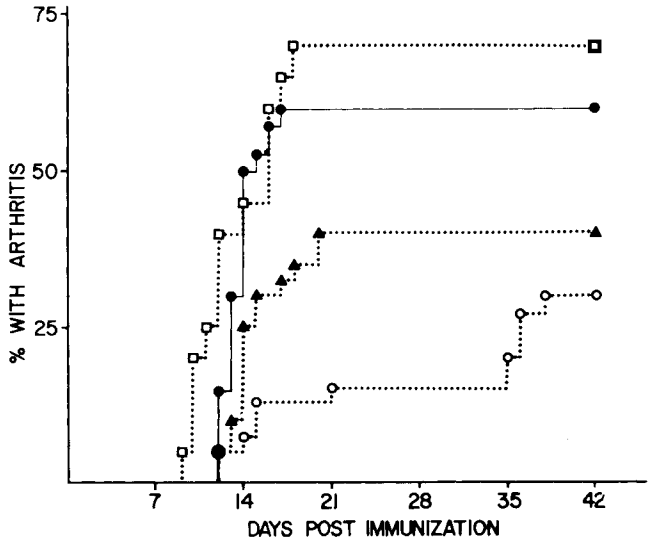

Figure 1. Cumulative incidence of arthritis. Forty rats were included in each group. Levamisole (□ . . . . . □), hydrocortisone (▲ . . . . . ▲), cyclophosphamide (○ . . . . . ○), or saline (●——●) was administered at day 1 and all rats were immunized with 200 μg of type II collagen on day 0. Rats were observed daily for the development of arthritis. All rats with definite arthritis were included from the day disease began even if the arthritis was transient.

some had a delayed onset and developed arthritis 5–6 weeks after immunization.

All rats were bled at 14, 21, and 42 days after immunization. Sera from two arthritic and two non-arthritic control rats collected at day 21 were selected to determine representative antibody binding curves (Figure 2). Various dilutions of sera were employed, and all of the immunoglobulin was precipitated by polyvalent rabbit anti-rat immunoglobulin. At low dilutions of serum, a high degree of binding of labeled collagen occurred with all sera; however, the binding curves of the nonarthritic sera were shifted to the left, compared to the arthritic sera. It was evident that a serum dilution of 1:100 was in the area of the inflection of the curves, and consequently a 1:100 dilution was chosen for use in comparison studies.

When sera from rats in each treatment group were individually tested for collagen binding, it was found that the mean antibody level in all treatment groups was different from control levels at 14 days after

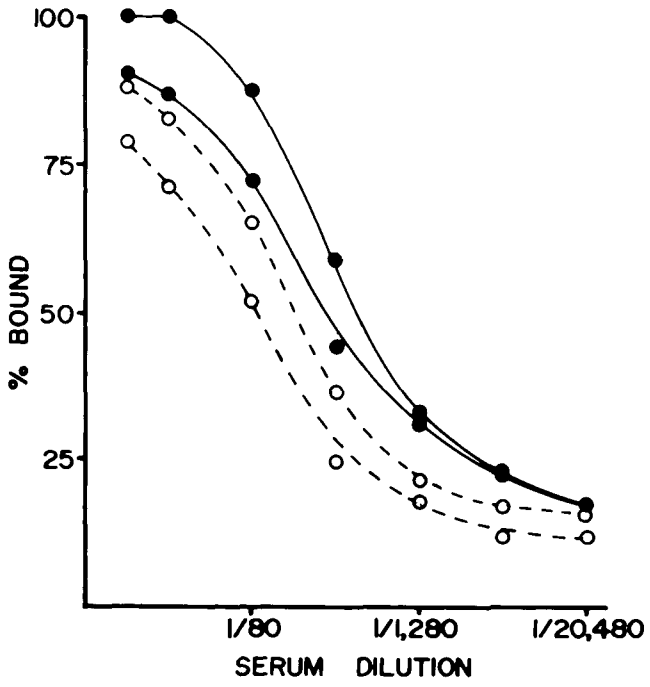

Figure 2. Representative antigen binding curves of 2 arthritic (●——●) and 2 nonarthritic (○- - - - -○) rats. All of the rats were immunized with 200 μg of type II collagen in incomplete Freund's adjuvant. Serum was obtained 3 weeks after immunization and assayed for anti-collagen antibodies using ^{125}I labeled type II collagen in a radioimmunoassay (see Materials and Methods). Normal serum at various dilutions and a buffer control gave approximately 5% binding. All points represent the mean of triplicate samples.

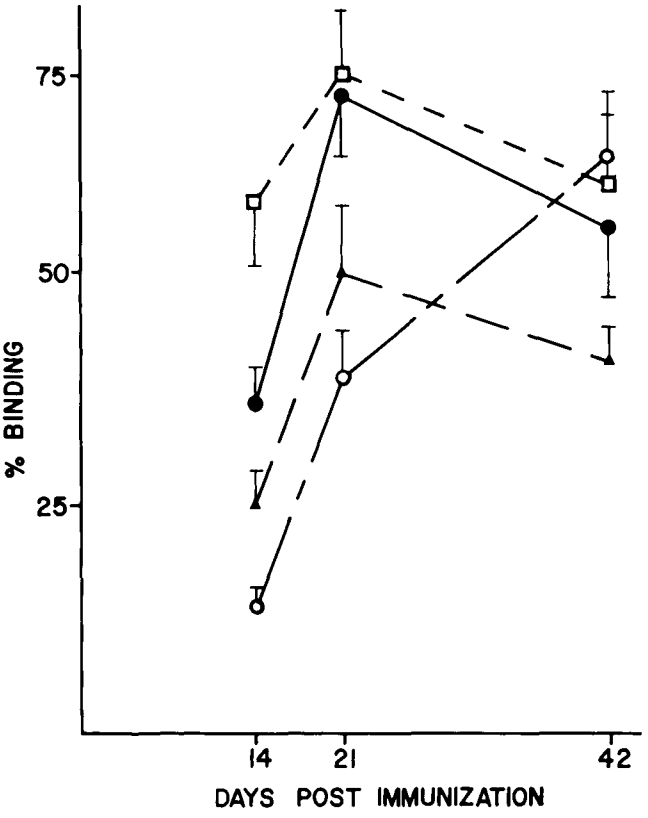

Figure 3. Time course of antigen binding. Rats were treated with levamisole (□- - - - -□), hydrocortisone (▲— —▲), cyclophosphamide (○- - - - -○), or saline (●——●) at day 1 and immunized with 200 μg of type II collagen on day 0. Sera were obtained serially and stored at −80°C. All samples were assayed for antibodies to collagen by radioimmunoassay at 1:100 dilution in duplicate. Each point represents the mean of all the rats in a treatment or the control group with the standard error of the mean given by the vertical bar and crosshatch.

immunization ($P < 0.01$ for each group by Student's t-test). Mean level of binding by sera from levamisole treated rats was higher than from controls, and binding by hydrocortisone and cyclophosphamide groups was lower (Figure 3). By 21 days, control levels had risen and were similar to the levamisole group, although cyclophosphamide and hydrocortisone levels remained low ($P < 0.05$). By 42 days, however, the cyclophosphamide group had risen to control levels and only hydrocortisone remained low ($P < 0.05$). This rise in antibody levels in the cyclophosphamide treated rats paralleled an increase in the incidence of arthritis (Figure 1).

Rats receiving each treatment regimen and the controls were subdivided into arthritic and nonarthritic subgroups. Maximum levels of collagen binding for

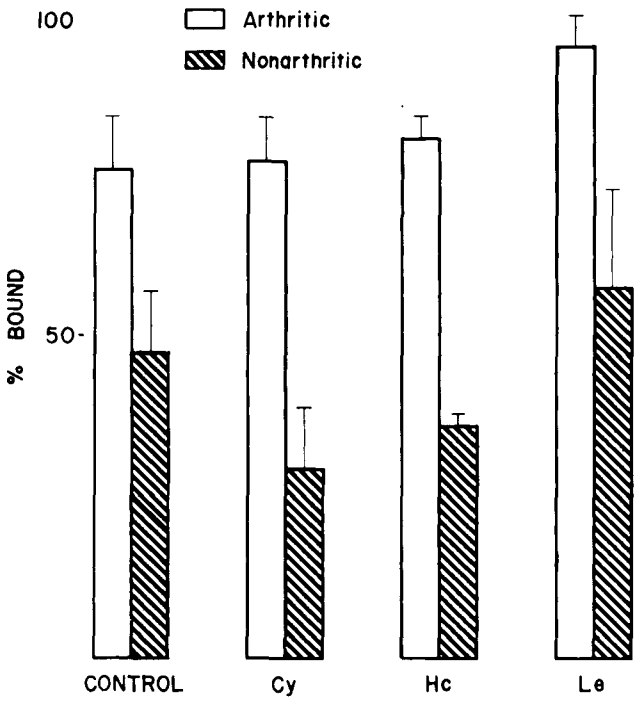

Figure 4. Peak antibody levels. Rats were treated with saline (**control**), levamisole (**Le**), hydrocortisone (**Hc**), or cyclophosphamide (**Cy**) on day 1 and immunized with 200 μg of type II collagen on day 0. They were bled serially and the sera (1 : 100 dilution) were assayed for antibodies to collagen by radioimmunoassay. Maximum levels of antigen binding by arthritic and nonarthritic rats in each group regardless of the time interval at which they occurred were compared.

each subgroup were compared regardless of the time interval at which this binding occurred (Figure 4). In each group, significantly greater binding of collagen occurred in those rats developing arthritis as compared to animals in the same treatment group which did not develop arthritis. Furthermore, the level of binding by arthritic rats was not different regardless of the treatment regimen.

DISCUSSION

These studies were undertaken to determine whether pretreatment of collagen immunized rats could: 1) alter the clinical course of collagen-induced arthritis, 2) alter the immunologic response to the inciting antigen (type II collagen), and 3) determine if the alteration in clinical course was related to the alteration of immune response. All of the drugs were given as a single dose one day prior to immunization with collagen, and although preliminary studies used other doses of the drugs, the most reliable effects were obtained with the dosages indicated.

All of the drugs studied altered the immune response, but only hydrocortisone and cyclophosphamide significantly altered the clinical course of disease. Levamisole had a slight enhancing effect with disease appearing in some animals by day 9, although the overall incidence was not significantly increased. At day 14, however, the antibody levels in the levamisole treated group were significantly higher than in controls. Both hydrocortisone and cyclophosphamide suppressed collagen-induced arthritis and also suppressed the immune response to collagen as measured by antibody levels. In the case of cyclophosphamide, there was a delayed onset of arthritis. A few rats developed disease at the usual time of 2–3 weeks after immunization, but additional rats had the onset of disease 5–6 weeks after immunization. The antibody response curve in the cyclophosphamide group was distorted compared to controls. The highest antibody levels in the cyclophosphamide group were detected at a time when control levels were declining, whereas with hydrocortisone the peak antibody level was lowered without affecting the shape of the time-course curve. In all instances, however, alteration of arthritis and antibody levels were of comparable magnitude and direction.

Previous studies of the drugs used have shown diverse effects on immunity. Cyclophosphamide given prior to immunization of rats with CFA resulted in decreased plaque forming cells and increased delayed hypersensitivity to purified protein derivative at 21 days after immunization. Skin allograft rejection time was unchanged. This data suggested that cyclophosphamide had a selective effect on B cells which resulted in B cell depletion (11). Studies of hydrocortisone (100 mg/kg) given in single doses to rats with established immunity to ovalbumin had no effect on skin test reactivity or antibody production although multiple doses resulted in marked suppression of skin test reactivity (12). However, glucocorticoids in general have been found to exert more profound effects when administered prior to immunization (13), and increased glucocorticoid receptor levels have been found in antigen stimulated lymphocytes (14). Both B and T lymphocytes are probably affected, and consistent inhibition of circulating antibody was detected after treatment with both cyclophosphamide and hydrocortisone. Our data are consistent with these findings.

It is apparent that collagen-induced arthritis can be altered with immune modulating drugs. Other agents known to affect human arthritis may not have a detectable effect, however. McCune et al administered subcutaneous gold sodium thiomalate to rats and immunized

them with type II collagen (15). They were unable to detect any differences in severity of disease, antibody levels, or cell-mediated immunity to collagen in gold treated rats as compared to uninjected or placebo-injected control rats.

We have found that alteration of the clinical course of arthritis was accompanied by alteration of the antibody response to type II collagen. Morgan et al and Clague et al have also studied the relationship of the level of antibodies to type II collagen with the development of arthritis (16,17). They reported that arthritic rats had higher levels of both IgM and IgG antibodies to type II collagen, and among three different strains of rats, the highest incidence of arthritis was in the strain with the highest antibody levels. Neither they nor we found any rats which had low antibody levels and arthritis. These data suggest the possibility that a threshold level may exist which must be exceeded before arthritis develops. Other factors are also involved since some rats without arthritis have higher antibody levels than do some arthritic rats. We did not measure cell-mediated immunity in these trials, and the role of cell-mediated immunity in development of collagen-induced arthritis remains unclear.

These studies suggest that the humoral response to type II collagen may be more intimately involved in the development of collagen-induced arthritis than previously realized. Additional studies of the role of both humoral and cell-mediated immunity in the development of disease are needed. Furthermore, collagen-induced arthritis may prove useful in delineating the mechanism of action of some antiarthritic drugs since various parameters of the immune response to the specific inciting antigen can be measured and correlated with disease.

ACKNOWLEDGMENT

We would like to thank Ms Susie Muirhead for her excellent technical assistance.

REFERENCES

1. Trentham DE, Townes AS, Kang AH: Autoimmunity to type II collagen: an experimental model of arthritis. J Exp Med 146:857–868, 1977
2. Trentham DE, Townes AS, Kang AH, David JR: Humoral and cellular sensitivity to collagen in type II collagen-induced arthritis in rats. J Clin Invest 61:89–96, 1978
3. Stuart JM, Cremer MA, Kang AH, Townes AS: Collagen-induced arthritis in rats: evaluation of early immunologic events. Arthritis Rheum 22:1344–1351, 1979
4. Trentham DE, Dynesius RA, David JR: Passive transfer by cells of type II collagen-induced arthritis in rats. J Clin Invest 62:359–366, 1976
5. Trabert U, Rosenthal M, Muller W: The effect of levamisole on adjuvant arthritis in the rat. J Rheumatol 3:166–174, 1976
6. Kayashima K, Koga T, Onoue K: Role of T lymphocytes in adjuvant arthritis. II. Different subpopulations of T lymphocytes functioning in the development of the disease. J Immunol 120:1127–1131, 1978
7. Wood FO, Pearson CM, Tanaka A: Capacity of mycobacterial wax D and its subfractions to induce adjuvant arthritis in rats. Int Arch Allergy Appl Immunol 35:456–467, 1969
8. Bolton AE, Hunter WM: A new method for labelling protein hormones with radioiodine for use in the radioimmunoassay. J Endocrinol 55:30–31, 1972
9. Hunter WM: Radioimmunoassay, Handbook of Experimental Immunology. Edited by DM Weir. Philadelphia, J.B. Lippincott Co., 1978, pp 14.1–14.40
10. Roll FJ, Madri JA, Furthmayr H: A new method of iodinating collagens for use in radioimmunoassay. Anal Biochem 96:489–499, 1979
11. Mackenzie AR, Pick CR, Sibley PR, White BP: Suppression of rat adjuvant disease by cyclophosphamide pretreatment: evidence for antibody mediated component in the pathogenesis of the disease. Clin Exp Immunol 32:86–96, 1978
12. Mansour A, Nelson DS: Effect of hydrocortisone, cyclophosphamide, azathioprine and methotrexate on cutaneous delayed and arthus hypersensitivity in the rat. Int Archs Allergy Appl Immunol 60:50–59, 1979
13. Claman HN: Corticosteroids and lymphoid cells. N Engl J Med 287:388–397, 1972
14. Crabtree GR, Munck A, Smith KA: Glucocorticoids and lymphocytes: increased glucocorticoid receptor levels in antigen-stimulated lymphocytes, J Immunol 124:2430–2435, 1980
15. McCune WJ, Trentham DE, David JR: Gold does not alter the arthritic, humoral, or cellular responses in rats with type II collagen-induced arthritis. Arthritis Rheum 23:932–935, 1980
16. Morgan K, Clague RB, Shaw MJ, Holt PJL: Native type II collagen-induced arthritis in the rat. I. Incidence and humoral response to collagen. Ann Rheum Dis 39:285–290, 1980
17. Clague RB, Morgan K, Shaw MJ, Holt PJL: Native type II collagen-induced arthritis in the rat. 2. Relationship between the humoral immune response to native type II collagen and arthritis. J Rheumatol (in press)