Effects of cyclophosphamide on immune arthritis in the guinea-pig

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Certain drugs, originally developed for the chemotherapy of cancer, are now also used in the control of autoimmune diseases in man. Cyclophosphamide, a potent alkylating agent, has an established place in the treatment of the nephrotic syndrome in children (Cameron, 1968) and has been used with some success in systemic lupus erythematosus (Seah, Wong, Chew, Jayaratnam, 1966) and in the lupus-like nephritis of the NZB and NZW F₁ hybrid mouse (Russell and Hicks, 1968). More recently cyclophosphamide (Fosdick, 1968; Smyth, 1968) and also azathioprine (Mason, Currey, Barnes, Dunne, Hazleman, and Strickland, 1969) have been used in the treatment of rheumatoid arthritis, a more chronic and usually less life-threatening disease.

Using an experimental immune arthritis histologically resembling rheumatoid arthritis that can be induced in guinea-pigs (Loewi, 1968), we have attempted to evaluate the effect of cyclophosphamide, both the immunosuppressive effect on the induction of an immune arthritis and also the immunosuppressive and anti-inflammatory effects on established arthritis in guinea-pigs.

Materials and methods

Cyclophosphamide (Endoxana) was kindly supplied by Ward Blenkinsop Ltd.

INDUCTION OF ARTHRITIS

An immune arthritis was induced in Duncin-Hartley guinea-pigs weighing 250 to 500 mg. after the method of Dumonde and Glynn (1962). 1 mg. ovalbumin (Koch-Light Laboratories Ltd., 5X crystallized), emulsified in 1 ml. Freund's complete adjuvant, was injected intradermally. After 10 days each animal was skin-tested, using 10 µg. ovalbumin. Following a positive skin test 24 hours later, 1 mg. ovalbumin in saline was injected into each knee under anaesthesia.

LABORATORY TECHNIQUES

White blood cell counts were performed weekly, using a Coulter counter. At the final count, a blood smear was made for a differential cell count. After death, the knee joints were fixed in formol saline and then decalcified. Full sagittal sections of each knee to include both the suprapatellar and infrapatellar pouches and posterior compartments were stained with haematoxylin and eosin. Each section was read independently in the blind by two of us. Each knee was graded 0 to 4 according to the severity of the degree of synovial inflammation. A maximum score of 16 was therefore possible for any one animal.

Grade 1 Mild: Slight synovial membrane (SM) hypertrophy and hyperplasia, a few inflammatory cells, no increase in vascularity.

Grade 2 Moderate: Definite SM hypertrophy and hyperplasia, moderate mononuclear infiltrate, increased vascularization.

Grade 3 Moderately severe: As 2, with some plasma cells and many lymphocytes.

Grade 4 Severe: Marked SM hypertrophy, large numbers of lymphocytes and plasma cells, often with lymphocytic aggregations. Erosions of marginal cartilage.

EXPERIMENTAL DESIGN (Figure, opposite)

Experiment I was designed to show the effect of increasing dosages of cyclophosphamide on an established arthritis. 2 weeks after the introduction of antigen into the knees, animals were randomly selected into control and treatment groups.

Treated animals received 10 mg., 15 mg., and 20 mg./kg. body weight of cyclophosphamide intraperitoneally daily for 28 days.

Control animals received saline intraperitoneally daily. All were weighed initially and then weekly and the dosage of cyclophosphamide was adjusted accordingly.

Experiment II was designed to show whether cyclophosphamide could influence the development of arthritis, once immunization was complete. One group of animals

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FIGURE 1 Experimental design of study, showing time of joint injection for each group (arrowed) and duration and dosage of cyclophosphamide

started cyclophosphamide at a level of 15 mg./kg. 1 week before knee injection, continuing for 2 weeks after, and were killed 3 weeks after joint injection. Another group started cyclophosphamide (15 mg./kg.) 2 weeks after joint injection, continued treatment for 3 weeks, and were killed 1 week after discontinuing the drug.

Experiment III was designed to explore the effect of cyclophosphamide on the immune response, and animals were divided randomly into groups before immunization. One group received cyclophosphamide (15 mg./kg.) for 22 days, starting on the day of immunization. Another started cyclophosphamide (20 mg./kg.) on the day of immunization but received the drug for 10 days only. A third group was treated with cyclophosphamide (20 mg./kg.) from the 10th to the 22nd day after immunization.

Arthritis was induced by injection of antigen into the knees of all animals on the 11th day. Surviving animals were killed on the 23rd day after initial immunization. A control group of six animals received no treatment.

Results

Effect of increasing dosages of cyclophosphamide on established arthritis (Table I)

Little difference could be found in the severity of the arthritis between animals which had received cyclophosphamide at dosage levels of 10, 15, and 20 mg./kg. and between these animals and the first two control groups. However, the control animals of the 20 mg./kg. group had a significantly more severe arthritis ($t = 2.63$; $P = 0.027$) than the other control animals, and therefore the arthritis of the simultaneously infected treated group is significantly less severe ($t = 3.23$; $P = 0.01$). If we compare the 20 mg./kg. treated animals with all sixteen control animals, the difference is not significant ($t = 861$; $P = 0.399$). It is known that there is considerable variation in the severity of arthritis from animal to animal, and sometimes between the two knees of one animal.

Effect of cyclophosphamide started before and after joint challenge (Table II, overleaf)

There was no difference between those that started cyclophosphamide at 15 mg./kg. 1 week before joint challenge and those that started at the same dose 2 weeks after challenge. Both groups were treated for 3 weeks.

Effect of cyclophosphamide started at time of immunization and of joint challenge (Table III, overleaf)

The arthritis was considerably less severe in all the treated animals than in the control animals, although there was little difference between the three treatment groups. The arthritis is known to be more severe in its initial stages, and the scores represent a significant improvement. The arthritis was also less marked than in the animals of the previous experiment which received 15 mg./kg. starting 1 week before joint challenge.

Table I To show the effect of varying dosages of cyclophosphamide

Series	Dosage (mg./kg.)	Number of animals	Survivors	Weight gain (per cent.)	Mean WBC/ c.mm. during treatment	Arthritis		
						Mean score	±	SD
Cyclophosphamide	10	7	7	17	6,000	8.64	±	0.90
Saline		7	7	17	12,000	7.57	±	2.62†
Cyclophosphamide	15	5	5	25	4,600	6.50	±	1.69
Saline		5	5	38	14,000	7.00	±	1.37†
Cyclophosphamide	20	7	2 (7)†	-5	2,200	7.36	±	2.19*
Saline		4	4	13	13,600	11.37	±	1.49*†

* Treated v. controls: $t = 3.23$; $P = 0.01$.

† 20 mg./kg. animals v. all 16 controls: $t = 861$; $P = 0.399$.

† ( ) Number examined histologically.

In this experiment we also tested the effect of cyclophosphamide on delayed hypersensitivity skin reactions to old tuberculin (10 units) induced by the killed tubercle bacilli in Freund's complete adjuvant. Those animals receiving cyclophosphamide for the whole 22 days remained tuberculin-negative. By the 10th day after stopping cyclophosphamide, the three survivors in the second group were all tuberculin-positive, all five having been negative up to the 6th day. In the third group, all seven were tuberculin-positive on starting cyclophosphamide on the 11th day after immunization. In one animal this was suppressed by the 4th day of cyclophosphamide treatment, and in all five survivors it was suppressed by the 12th day of treatment.

Mortality and morbidity of cyclophosphamide

Our experience was similar to that of Berenbaum (1965). No animals died at a dosage of 10 mg./kg., while three of our 21 animals died at a dosage of 15 mg./kg. for 3 weeks—two in the 2nd week, one in the 4th week. At 20 mg./kg., eight out of seventeen animals died; five out of seven in a 4-week course, three out of ten in a 10 to 13-day course. Four died in the 2nd week of treatment. Profound leucocyte depression was found, with total absence of lymphocytes in some and polymorph leucopenia in others. Nucleated red cells were not infrequently seen. No consistent effect on the differential white count was noted.

Discussion

Immune arthritis, produced by immunization with a protein followed by introduction of the immunogen into joints, provides a reasonable experimental model by which to test the function of immunosuppressive drugs. The arthritis resembles rheumatoid arthritis histologically, especially in rabbits, in which plasma cells are more frequently seen than in guinea-pigs. Loewi (1968) has shown by transfer experiments that the arthritis can be produced by the transfer of cells or of serum, but to produce a long-lasting arthritis by transfer, both cellular and humoral mechanisms are necessary.

Maguire and Maibach (1961) showed that cyclophosphamide was a potent inhibitor both of delayed hypersensitivity and of antibody production and anaphylactic sensitization (Maibach and Maguire, 1963). It has been shown to produce significant prolongation of homograft survival at a dosage level of 20 mg./kg. (Berenbaum, 1965), although as a chronic regime this was almost universally lethal. Turk (1964) has suggested that cyclophosphamide may have a non-specific anti-inflammatory effect as well, and that some of the depression of peripheral markers of delayed hypersensitivity such as skin tests may be due to this effect, while the central sensitization continues (Turk, 1967).

This latter point may well explain the failure of cyclophosphamide to prevent the development of immune arthritis. Cyclophosphamide at the maximum dosage modified the arthritis when started

Table II *Effect of cyclophosphamide started before and after induction of arthritis*

<i>Start of Cyclophosphamide</i> 15mg./kg.	<i>No. of animals</i>	<i>Survivors</i>	<i>Mean WBC/c.mm.</i> <i>during treatment</i>	<i>Arthritis</i>		
				<i>Mean score</i>	$\pm$	<i>SD</i>
1 week before	5	5	2,800	9.70	$\pm$	1.99
2 weeks after	6	4 (5)*	3,000	10.33	$\pm$	1.37

* ( ) Number examined histologically.

Table III *Effect of cyclophosphamide at various periods of immunization*

<i>Cyclophosphamide</i>	<i>Dosage</i> (mg./kg.)	<i>No. of animals</i>	<i>Survivors</i>	<i>Mean WBC/c.mm.</i>	<i>Arthritis</i>		
					<i>Mean score</i>	$\pm$	<i>SD</i>
Throughout	15	5	4 (4)	2,300	5.625	$\pm$	1.49 ¹
Preliminary immunization	20	5	3 (4)	2,000	6.50	$\pm$	1.68 ²
At joint challenge	20	5	4 (5)	2,200	5.50	$\pm$	1.225 ³
Controls	—	6	5 (6)	—	12.50	$\pm$	4.669

Treated v. controls:

(1) $t = 3.23$; $P = 0.01$. (2) $t = 2.68$; $P = 0.028$. (3) $t = 2.80$; $P = 0.023$.

before or at the induction of arthritis, but when the dose was reduced to a less lethal level, this effect disappeared.

Our results may be compared with those of Arinovic and Loewi (1970) who used bovine gamma globulin as their immunogen. Cyclophosphamide at a dosage of 20 mg./kg. started before joint challenge suppressed the inflammatory reaction 48 hrs after joint challenge, but 4 or 10 days after challenge no difference between treated and control animals was found. Cyclophosphamide given for 8 days at the time of joint injection produced some improvement, but when started 3 days after joint challenge it produced no improvement. Cyclophosphamide did produce suppression of skin testing with purified protein derivative and bovine gamma globulin.

Summary

Cyclophosphamide failed to prevent the induction of immune arthritis in guinea-pigs, but considerably modified it when given in lethal doses before or at the time of joint challenge. Peripheral markers of delayed hypersensitivity were completely suppressed. Cyclophosphamide did not modify the arthritis, compared with control animals, when it was started 2 weeks after joint challenge.

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References

- ARINOVICH, R., AND LOEWI, G. (1970) *Ann. rheum. Dis.*, **29**, 32 (Comparison of the effects of two cytotoxic drugs and of antilymphocytic serum).
- BERENBAUM, M. C. (1965) *Transplantation*, **3**, 671 (Effect of cyclophosphamide on the homograft response in the guinea-pig).
- CAMERON, J. S. (1968) British Medical Abstracts 312. Treatment of the Nephrotic syndrome.
- DUMONDE, D. C., AND GLYNN, L. E. (1962) *Brit. J. exp. Path.*, **43**, 373 (The production of arthritis in rabbits by an immunological reaction to fibrin).
- FOSDICK, W. M. (1968) *Med. Clin. N. Amer.*, **52**, 747 (Cytotoxic therapy in rheumatoid arthritis).
- LOEWI, G. (1968) *Immunology*, **15**, 417 (Experimental immune inflammation in the synovial membrane. I. The immunological mechanism).
- MAGUIRE, H. C., AND MAIBACH, H. I. (1961) *J. invest. Derm.*, **37**, 427 (Effect of cyclophosphamide and other drugs on the acquisition of delayed hypersensitivity (DNCB contact dermatitis) in the guinea-pig).
- MAIBACH, H. L., AND MAGUIRE, H. E. (1963) *Nature (Lond.)*, **197**, 82 (Role of species specificity in failure of inhibition of guinea-pig anaphylactic sensitization with cancer chemotherapy drugs).
- MASON, M., CURREY, H. L. F., BARNES, C. G., DUNNE, J. F., HAZLEMAN, B. L., AND STRICKLAND, I. D. (1969) *Brit. med. J.*, **1**, 420 (Azathioprine in rheumatoid arthritis).
- RUSSELL, P. J., AND HICKS, J. D. (1968) *Lancet*, **1**, 440 (Cyclophosphamide treatment of renal disease in (NZB × NZW) F₁ hybrid mice).
- SEAH, C. S., WONG, K. H., CHEW, A. G. K., AND JAYARATNAM, F. J. (1966) *Brit. med. J.*, **1**, 333 (Cyclophosphamide in the treatment of S.L.E.).
- SMYTH, C. J. (1968) *J. Amer. med. Ass.*, **2-4**, No. 11, Medical News, p. 42. (Report on cyclophosphamide in rheumatoid arthritis).
- TURK, J. L. (1964) *Int. Arch. Allergy*, **24**, 191 (Studies on the mechanism of action of methotrexate and cyclophosphamide on contact sensitivity in the guinea-pig).
- (1967) "The effect of immunosuppressive drugs on cellular changes after antigenic stimulation", in "Immunity, Cancer, and Chemotherapy", ed. E. Mihich. Academic Press, New York and London.