Immunoresponsiveness of patients with rheumatoid arthritis receiving cyclophosphamide or gold salts

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Rheumatologists are continually searching for more efficacious modes of therapy for patients with rheumatoid arthritis. Over the past few years, there has been a surge of enthusiasm for the drug cyclophosphamide in the treatment of progressive rheumatoid arthritis unresponsive to conventional therapy. Cyclophosphamide has been reported to be therapeutically beneficial in 50 to 80 per cent. of patients in whom other modes of therapy have failed (Co-operative Clinics, ARA, 1970; Fosdick, Parsons, and Hill, 1968, 1969). The mechanism by which this drug exerts its beneficial effects is under study by many investigators.

Since rheumatoid arthritis is often classified as an autoimmune disease or at least a disease in which the symptomatology is often associated with autoimmune phenomena, we undertook to evaluate the immunological responsiveness of patients receiving cyclophosphamide therapy in conjunction with a clinical trial of drug efficacy. The present study was designed to evaluate humoral and cellular immunity as well as bone marrow reserve in patients receiving cyclophosphamide therapy and a control group receiving maintenance chrysotherapy. Six parameters were evaluated: skin testing, antibody response to influenza vaccines, immunoglobulin assays, circulating leucocyte and lymphocyte counts, lymphocyte transformation, and bone marrow reserve. Of these six parameters only suppression of circulating white blood cells, lymphocyte transformation *in vitro*, and bone marrow reserve were observed to be altered in a statistically significant manner in the cyclophosphamide-treated patients as compared to those given chrysotherapy.

Material and methods

There were 31 patients with definite or classical rheumatoid arthritis under therapy in the Arthritis Research Clinic of the University of Colorado Medical Center. Sixteen received cyclophosphamide and fifteen received chrysotherapy. None was receiving corticosteroid therapy. Unfortunately, because of poor patient cooperation, not all studies were performed on every patient.

Skin testing was done with Parke-Davis commercially available environmental antigens, PPD and histoplasmin, using a 0.1 ml. intradermal injection; 0.5 cm. of induration at 48 hrs was taken to be a positive response. *Candida* antigens were prepared by the Allergy Division, using 1:100 and 1:1000 dilution, and similar criteria were used for a positive response as recorded above for PPD and histoplasmin. Mumps antigen was given intradermally and the erythema response was evaluated as outlined in the product circular. All patients studied were tested with all four intradermal antigens.

Antibody response to bivalent influenza vaccine ((A₂H₁C₆₈-B_{MASS}66) Parke-Davis) was studied in the virology section using haemagglutination inhibition titres. The responses were evaluated at 0, 2, 4, and 6 weeks after subcutaneous injections of 0.5 ml. vaccine. A two-tube rise in the haemagglutination titre was considered to be a positive response.

Immunoglobulin assays for IgG, IgA, and IgM were done by radial diffusion using Hyland plates. The assays were done after 6 months of cytotoxic therapy or Ig. chrysotherapy. No pre-therapy serum samples were available for evaluation.

Peripheral total leucocyte and lymphocyte counts were done before treatment and after 3, 6, and 9 months of therapy.

Lymphocyte transformation was assayed by the incorporation of tritiated thymidine into DNA. Two mitogens were used, pokeweed and phytohaemagglutinin,

which were added at the time of culture. Cultures were maintained for 5 days. One million cells/ml. with 3 ml./tube, in triplicate, were pulsed with tritiated thymidine on the fourth day and harvested on the fifth day. The cells were washed serially with phosphate buffered normal saline (4°C.), 5 per cent. trichloroacetic acid (4°C.), and methanol (-10°C.), and were then digested in hyamine (Newberry, Chandler, Chin, and Kirkpatrick, 1968). An aliquot of the digest was counted in a Packard Tricarb scintillation counter according to the methods recommended by the manufacturer. Results were reported as counts/minute/million lymphocytes.

Finally, bone marrow reserve was evaluated by Piromen stimulation (a polysaccharide endotoxin of pseudomonas furnished by Travenel Laboratories, Morton Grove, Ill.) according to the method of Korbitz, Tonen, Davis, Ramirez, and Ansfield (1969). Patients were admitted to hospital the night before the study. On the day of the study no coffee or tea was permitted and the patients were instructed to remain in bed. Baseline total white counts, differential, and platelet counts were done at 8:00 a.m. At 9:00 a.m. the patients were given Piromen, 0.1 ml./kg. body weight intravenously, and serial total white counts, differential, and platelet counts were done hourly for 5 hours after injection.

Results

Table I presents the results of skin testing with the individual antigens. The ratio of patients giving a positive response to one or more skin tests in both the cyclophosphamide-treated group and the gold-treated group is shown. The responses were basically similar, representing 45 per cent. of the cyclophosphamide group and 67 per cent. of the chrysotherapy patients. These results showed no statistical difference by non-parametric analysis between the two groups

with respect to these common environmental antigens as an evaluation of cellular immune response. The columns on the right of Table I shows the number of patients responding over the number of patients challenged with influenza vaccine. Again, no statistical difference appeared between the two study groups, the cyclophosphamide-treated group showing 54 per cent. responsiveness, and the gold-treated 61 per cent. The results show no direct or inverse relationship to therapeutic response or to the other parameters evaluated in this study.

Single determinations of the three immunoglobulins were performed after 6 months of cyclophosphamide therapy or Ig. or more of chrysotherapy. The results are shown in Table II for the two study groups and the normal range for our laboratory. The only statistically significant difference was in the IgA levels, which were significant to a P value of 0.01. From the ranges presented it is obvious that there is a wide range of values from hypo- to hypergamma-globulinaemia. However, at the present time, there is no ready explanation for the relatively high levels of IgA observed in the cyclophosphamide-treated patients as opposed to the gold-treated patients.

Table III outlines the results of the total white cell counts, absolute lymphocyte counts, percentage of lymphocytes, and average dosage levels for the cyclophosphamide-treated group. No variations in these parameters were observed in the gold-treated group. These patients were followed from before treatment through 9 months of cyclophosphamide therapy. A sequential decrease of both lymphocytes and total white cells was noted through the sixth month of therapy. The fall in the absolute lymphocyte

Table 1 *Cellular and humoral response to environmental antigens*

Study group	Response to skin tests*		Response to influenza†	
	(+) Response/No. studied	Per cent. response	(+) Response/No. vaccinated	Per cent. response
Cyclophosphamide	5/11	45	7/13	54
Gold	6/9	67	8/13	61

* Significantly positive response to one or more of the skin tests was considered as positive.

† As discussed above, a two-tube increase in titre was considered a positive response to immunization by the mixed influenza vaccine.

Table II *Immunoglobulin levels*

Immunoglobulins (mg./100 ml. serum)			γ _G	γ _A	γ _M
Group	Cyclophosphamide (16)	($\bar{M}$)	948	322	123
		Range	400—1530	64—972	53—189
	Gold (15)	($\bar{M}$)	912	166	126
		Range	238—2040	24—462	8—384
Normal range*			620—1400	46—418	70—384

* This is the normal range observed in the clinical immunology laboratory of this hospital.

Table III Leucocyte levels during therapy in 16 cyclophosphamide-treated patients

Time of testing	Leucocytes (cells/mm ³ .)	Lymphocytes (cells/mm ³ .) per cent.	Drug dose (mg./24 hrs)
Before treatment	7,528	1,890 (26)	0
At 3 mths	5,722	1,200 (22)	93
At 6 mths	4,521	784 (17)	111
At 9 mths	4,372	729 (17)	111

count was significant to the 0.01 level and to the 0.05 level for the total white count. The averages presented in Table III represent three determinations for each of the fifteen patients included in this portion of the study for each time period illustrated. No statistical relationship to therapeutic response was noted.

Fig. 1 demonstrates the results of lymphocyte transformation induced by pokeweed mitogen and phytohaemagglutinin as compared to control cultures and measured by the incorporation of tritiated thymidine into DNA. The Figure compares a small control series of normal subjects with the gold- and cyclophosphamide-treated rheumatoid arthritis. The bars represent the mean values for the control and stimulated lymphocyte cultures. In order to eliminate undue weighting of the data, the results were subjected to nonparametric rank sum analysis. The results showed statistically significant suppression of tritium incorporation into DNA in the lymphocyte cultures of the cyclophosphamide-treated patients. This was true of the control cultures ($P = 0.01$) and both of the stimulated cultures ($P = 0.01$), as compared to the gold-treated patients and the normal subjects which did not differ statistically.

FIG. 1 Lymphocyte transformation in vitro in normal control subjects and gold- and cyclophosphamide-treated patients with rheumatoid arthritis
C represents control lymphocyte cultures to which no mitogen has been added
PWM, pokeweed mitogen-treated cultures
PHA, phytohaemagglutinin-treated cultures

The bone marrow reserve of these two therapy groups was evaluated according to the Piromen

FIG. 2 Bone marrow reserve expressed as peripheral granulocyte response to intravenous Piromen stimulation. ΔG represents the maximum granulocyte response minus the baseline level. The symbol in the middle of the figure represents the range and the average response in normal subjects

stimulation method of Korbitz and others (1969). The results are presented in Fig. 2 as baseline granulocytic count, peak response to intravenous Piromen, and delta change in total granulocytes. The symbol in the centre of the figure shows the normal variation of response as defined by Korbitz with a minimal normal delta response of 2,850 and an average delta response of 5,767. The control group of rheumatoid arthritis patients treated with chrysotherapy displayed a normal although slightly low response, while six of the eight cyclophosphamide-treated patients available for this study demonstrated a significant suppression of Piromen responsiveness well below the acceptable minimal delta response. The differences between the two groups were statistically significant at the level 0.01 for all three determinations.

Discussion

Gabrielsen, Cooper, Peterson, and Good (1969) have proposed a two-component system as the basis for the immunological defence mechanism. This system has been divided into humoral immunity mediated through bone marrow dependent lymphocytes (B cells) and plasma cells, and cellular immunity

mediated through thymic dependent lymphocytes (T cells). In the present study no significant degree of suppression of humoral immunity was demonstrated. The evaluation of immunoglobulin levels after 6 months of cyclophosphamide therapy or 1g. gold revealed no difference between the two groups studied. In fact, a higher average IgA level was noted in the cyclophosphamide group. These findings correlate with those of total gamma globulin levels reported by Levin, Landy, and Frei (1964) after 28 days of therapy with 6-mercaptopurine. However, in contrast, Alepa, Zvaifler, and Sliwinski (1970), and Denman, Denman, Greenwood, Gall, and Heath (1970) demonstrated significant suppression of individual immunoglobulins upon serial evaluation during cytotoxic therapy. Unfortunately, in the present study, we were not able to evaluate these patients serially, but one would have expected a significant difference between the two groups if the alteration of immunoglobulin levels was strictly on the basis of antimetabolite therapy as has been postulated.

With respect to cellular immunity, the degree of immune suppression achieved in the present study shows conflicting results. There is little doubt that our data demonstrates a quantitative reduction in circulating lymphocytes as demonstrated by the serial white blood cell and lymphocyte counts performed. This is in agreement with other investigators' observations (Cooperative Clinics, ARA, 1970; Alepa and others 1970; Alepa, 1971). Whether this represents a true parameter of immunosuppression is questionable, but is supported in part by the results observed in the NZB/NZW mice receiving cyclophosphamide as studied by Lemmel, Hurd, and Ziff (1971).

Another parameter of cellular immunity evaluated was the response to skin testing with the common environmental antigens discussed above. No significant difference in responsiveness was demonstrated between cyclophosphamide and gold-treated groups of rheumatoid arthritis patients.

The most convincing evidence of suppression of cellular immunity was that afforded by the studies of lymphocyte transformation *in vitro* carried out with two mitogens, pokeweed and phytohaemagglutinin. The results show uniform suppression of lymphocyte responsiveness in the cyclophosphamide-treated patients. This data and that published by Alepa and others (1970) and Alepa (1971) support the contention of immune suppression. The data were also examined

from the standpoint of a delta relationship between control values and the responses to pokeweed and phytohaemagglutinin for each of the three groups studied and presented in Fig. 1. It was noted that, with pokeweed mitogen stimulation, the delta change (maximum response-control) in incorporation of H³-thymidine lacks statistical significance. On the other hand, if the same relationship is examined with respect to phytohaemagglutinin, the cyclophosphamide-treated patients demonstrated a more significant difference when compared to the gold-treated rheumatoid patients. This latter relationship was examined by the Wilcoxon rank sum test for unpaired samples using a one-tailed analysis. In this manner, while just barely missing a significance of 0.05 (probably because of our sample size), it does suggest greater suppression of phytohaemagglutinin stimulation.

These results, while expressing uniform suppression of lymphocyte transformation overall, are further suggestive of additional selective suppression of phytohaemagglutinin responsive cells. If the studies reported by Stockman, Gallagher, Heim, South, and Trentin (1971), Meuwissen, Van Alten, Cooper, and Good (1969), and Davies, Festenstein, Leuchars, Wallis, and Doenhoff (1968) of variability in transformation of T and B lymphocytes to the stimulation of phytohaemagglutinin and pokeweed mitogen respectively are applicable, the application of these hypotheses to the present study would suggest a partially selective suppression of T cells. Further studies concerning this observation are in progress.

Summary

This study compares two groups of patients suffering from rheumatoid arthritis, one treated with chrysotherapy and the other with cyclophosphamide. The cyclophosphamide-treated group demonstrated suppression of circulating leucocytes and a depressed bone marrow granulocyte reserve. The evidence for immunosuppression demonstrated by this study lies only in the cellular immunity component (the thymus-dependent lymphocytes) of the immunoresponsive system of these patients. This suppression was demonstrated by studies of lymphocyte transformation *in vitro*.

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