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Evaluation of the immunosuppressive effects of cyclophosphamide in patients with multiple sclerosis

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SUMMARY

In a group of eight patients suffering from clinically definite multiple sclerosis, we studied the effects of treatment with cyclophosphamide on the immune reactivity *in vitro* and *in vivo*. The results are compared with those obtained in a control group consisting of eight patients who received no drug therapy and who were matched with the former group for age, sex and severity of disease. The results indicate that therapy with cyclophosphamide at a mean dose of 100 mg/day induces a profound lymphocytopenia in peripheral blood involving both T and B cells. Serum levels of immunoglobulins as well as primary and secondary antibody responses were depressed. In tests with standardized cell numbers, proliferative responses of lymphocytes *in vitro* and cytotoxic T cell function remained normal, whereas K and NK cell activities were diminished. Secondary cellular immune responses *in vivo* remained intact; however, the primary cellular immune response *in vivo* was markedly depressed. From these data, it is concluded that therapy with cyclophosphamide in man mainly affects humoral immune functions, but also cellular immunity, although to a lesser extent.

INTRODUCTION

The effects of cyclophosphamide on the immune system have been extensively studied in animals, in which experimental conditions, such as the nature of the antigen, the dose of the drug and the time interval between administration of antigen and drug can be readily controlled (Bach, 1975; Hersh, 1974). In contrast, a thorough study of the immunological effects of this drug in a well defined population of human beings, including a matched control group, is still lacking. Previous studies, especially those concerning effects of therapy with cyclophosphamide at doses used for maintenance treatment, have only measured a few selected parameters (for reviews, see Berenbaum, 1975; Gershwin, Goetel & Steinberg, 1974; Hersh, 1974). Moreover, as far as we know, no data are available regarding cytotoxic effector functions of the lymphocytes in such treated patients.

In this study, the immune capacity of 16 patients suffering from multiple sclerosis was

Abbreviations: ADL = antibody-dependent lymphocytotoxicity; ALS = anti-lymphocyte serum; CML = cell-mediated lympholysis; Con A = concanavalin A; DNCB = dinitrochlorobenzene; DTH = delayed type hypersensitivity; DTP = a vaccine containing diphtheria toxoid, tetanus toxoid and inactivated polio virus, types I, II and III; ELISA = enzyme linked immunosorbent assay; FITC = fluorescein isothiocyanate; Ig = immunoglobulin; K cell = killer cell; MLC = mixed lymphocyte culture; NK cell = natural killer cell; PHA = phytohaemagglutinin; PPD = purified protein derivative of tuberculin; PWM = pokeweed mitogen; s.d. = standard deviation; SK/SD = streptokinase/streptodornase.

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investigated. Eight of these were treated with cyclophosphamide in doses as high as possible, depending on the number of leucocytes and thrombocytes in peripheral blood and other possible side effects. Another eight patients, matched for age, sex and severity of disease with the former group, did not receive cyclophosphamide or any other potentially immunosuppressive drug.

Cellular and humoral immune responses *in vivo* (both primary and secondary) were investigated. In addition, the following parameters were measured: cell counts in peripheral blood; levels of serum complement and serum immunoglobulins, and finally proliferative and cytotoxic effector functions of lymphocytes *in vitro*.

PATIENTS

Eight patients (three females, five males) suffering from clinically definite multiple sclerosis who received a daily dose of cyclophosphamide in the range of 75–150 mg during 1–5 months prior to initiation of this study, were investigated. Their age varied from 31 to 53 years. A control group consisted of eight patients, not receiving cyclophosphamide, who were individually matched with the above mentioned patients for age, sex and severity of disease.

Severity of disease was graded according to the Disability Status Scale as described by Kurtzke (1965). From all patients, included in this study, informed consent was obtained.

EXPERIMENTAL PROTOCOL

The design of the study was as follows. After collection of blood, each patient (except patient 8) was immunized subcutaneously with 1·0 mg haemocyanin from α-*Helix pomatia* to elicit a primary humoral immune response, and with a patch containing 1 mg DNCB to elicit a primary cellular immune response. Secondary humoral immune responses were evoked by immunization with 1 ml of alum precipitated DTP vaccine, containing diphtheria toxoid (2·5 Lf), tetanus toxoid (5 Lf) and inactivated polio virus, types I, II and III. After 14 days, blood was again drawn and skin tests were performed with DNCB and, to test secondary cellular immune responses, with a variety of recall antigens (PPD, varidase, mumps, trichophyton and candida). Skin tests were read 48 hr after challenging.

MATERIALS AND METHODS

Blood samples. Serum obtained from each individual was frozen and kept at −20°C. Mononuclear cells were isolated from defibrinated blood by means of Ficoll-Isopaque density gradient centrifugation and preserved in liquid nitrogen (Du Bois *et al.*, 1976). Lymphocyte cultures of one patient and the matched control patients were performed in one experiment on the same day, thus avoiding day-to-day variations in the lymphocyte cultures.

Cell numbers in peripheral blood. The absolute numbers of lymphocytes and monocytes in peripheral blood were determined by electronic cell counting (Coulter counter) and May-Grün-wald-Giemsa stained blood smears. T lymphocytes were determined by rosette formation with sheep erythrocytes (Jondal, Holm & Wigzell, 1972). B lymphocytes were determined by a direct immunofluorescence test using $F(ab')_2$ fragments of sheep anti-human Ig serum conjugated to FITC (Pernis, Forni & Amante, 1971).

Serum proteins. The total serum protein concentration and serum protein spectrum were determined by standard methods. The levels of IgG, IgM and IgA were determined by a nephelometric technique, and those of IgD and IgE by a radioimmunoassay. The levels of complement components were determined by radial immunodiffusion.

Specific humoral immune responses. Specific antibodies of the IgM, IgG and IgA classes to the antigen haemocyanin, as well as total antibody titre against diphtheria toxoid were measured by means of an ELISA technique. Total antibodies and those of the IgG class against tetanus toxoid

were determined by a radioimmunoassay. The IgM and IgA anti-tetanus antibodies were estimated by a semi-quantitative Ouchterlony technique combined with autoradiography (Thorbecke, Hochwald & Williams, 1971). Anti-polio virus antibodies were measured by virus neutralization.

Cellular immune reactivity in vitro. After thawing, the viability of the cell suspensions used for the experiments was determined by trypan blue exclusion and found to be always higher than 95%. The proliferative capacity of the lymphocytes was assessed as described previously (ten Berge *et al.*, 1981). It should be noted that under the culture conditions chosen, the ^{3}H-thymidine incorporation was linearly dependent on the number of lymphocytes. On each occasion when the assays were performed, cryopreserved lymphocytes from healthy donors were tested in parallel. The results obtained from these control lymphocyte cultures were consistently within the same, normal range, i.e. from 80 to 115% of the mean value from 10 experiments.

The effector functions of lymphocytes were assessed by different cytotoxicity assays, namely the cell-mediated lympholysis (CML) (Zeijlemaker, Van Oers & Eijsvoogel, 1976), the antibody-dependent lympholysis (ADL) (Zeijlemaker *et al.*, 1975), and the NK cell function using K-562 cells as target cells (Ortaldo *et al.*, 1977).

Cellular immune reactivity in vivo. Sensitization to DNCB was tested after 14 days with two concentrations of DNCB (3 and 10 μg per patch). Forty-eight hours later, the test was evaluated as follows:

	Score
Erythema	1
Erythema and induration	2
Erythema, induration and blistering	3
Erythema, induration, blistering and ulcer	4

The final DNCB score was calculated as the sum of the scores of each patch. Delayed type hypersensitivity to recall antigens was determined by skin tests using five different antigens, viz PPD (10 TU/ml), varidase (50 units SK/SD/ml), mumps (20 CFU/ml), trichophyton (0·1%) and candida (1:300). Each antigen solution (0·1 ml) was injected intradermally on the volar surface of the forearm. An induration of 5 mm or more at two perpendicular diameters after 48 hr was considered positive.

Statistical methods. Standard statistical methods were used: cytotoxicity assays were tested by regression analysis; analysis of variance and covariance and some distribution free tests were used for analysis of the other parameters. All tests were two tailed.

RESULTS

Cell numbers in peripheral blood

The numbers of both T and B lymphocytes in peripheral blood were severely depressed (Table 1). The absolute number of monocytes was not affected, whereas the absolute number of granulocytes was moderately depressed.

Serum proteins

Whereas treatment with cyclophosphamide did not influence the levels of total protein and serum albumin, it did reduce levels of immunoglobulins of all classes, except for IgE (Table 1). These data, obtained by comparison between the two groups of patients, are confirmed by our findings in three patients studied both before and 6 weeks after initiation of cyclophosphamide treatment: in each of these patients, the levels of immunoglobulins showed a decrease. Complement levels were not affected.

Humoral immune responses

The primary antibody response to haemocyanin *in vivo*, 14 days after immunization, was clearly depressed (Fig. 1).

Table 1. Cell counts in peripheral blood and serum proteins

	Patients	Control patients	*P* value
Total number of lymphocytes $\times 10^9$/l	0·63 ± 0·24	1·95 ± 0·51	0·005
Number of T lymphocytes $\times 10^9$/l	0·47 ± 0·17	1·46 ± 0·44	< 0·00001
Number of B lymphocytes $\times 10^9$/l	0·03 ± 0·01	0·21 ± 0·08	< 0·00001
Number of monocytes $\times 10^9$/l	0·20 ± 0·05	0·26 ± 0·11	n.s.
Number of granulocytes $\times 10^9$/l	2·5 ± 0·7	4·8 ± 1·6	0·025
Albumin (g/l)	41 ± 7	44 ± 5	n.s.
Immunoglobulins			
IgM (IU/ml)	150 ± 16	222 ± 64	0·038
IgG (IU/ml)	125 ± 24	168 ± 14	0·008
IgA (IU/ml)	91 ± 24	212 ± 61	< 0·0004
IgD (IU/ml)	11 ± 8	28 ± 20	0·006
IgE (IU/ml)	34 ± 29	52 ± 25	n.s.
C3 (%)	147 ± 36	180 ± 39	n.s.
C4 (%)	183 ± 73	177 ± 78	n.s.

All values represent the mean ± s.d.
n.s. = not significant.

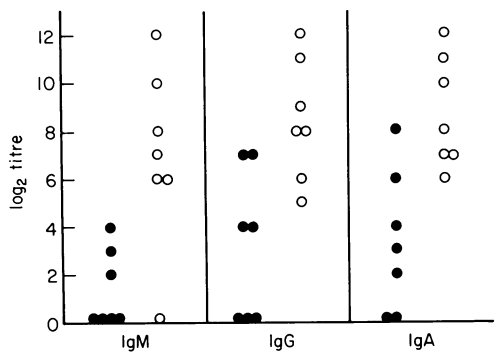

Fig. 1. Primary antibody response 14 days after immunization with haemocyanin. ● = patients receiving cyclophosphamide; ○ = control patients. Responses are expressed as $\log_2$ titre, starting at a dilution of 1:100. Statistical analysis = IgM–anti-haemocyanin, $P < 0{\cdot}005$; IgG–anti-haemocyanin, $P = 0{\cdot}01$; IgA–anti-haemocyanin, $P < 0{\cdot}005$.

With regard to secondary antibody responses, Table 2 shows that both the absolute levels and the rise in antibody titre after immunization with diphtheria and tetanus toxoid in the patients receiving cyclophosphamide was decreased compared to the group of patients not receiving cyclophosphamide. The same holds for the response against polio virus, types I and II. Only the response to polio virus type III was quantitatively in the same order of magnitude for both groups of patients.

The difference in responses to tetanus toxoid between both groups was not significant, which is probably due to the large variation in responses observed in healthy individuals, and the relatively small number of patients included in this study. In all patients, the antibodies produced against tetanus toxoid occurred in the IgM, IgG and IgA classes.

Table 2. Secondary antibody responses *in vivo*

	Patients		Control patients		
	Before Immunization	After Immunization	Before Immunization	After Immunization	*P* value*
Diphtheria toxoid	0·85† ± 1·32	1·74 ± 1·73	2·30 ± 1·58	7·26 ± 3·45	< 0·01
Tetanus toxoid					
Total	0·82‡ ± 1·18	4·3 ± 5·0	0·43 ± 1·13	16·8 ± 22·6	n.s.
IgG	8·1§ ± 19·1	25·8 ± 25·9	0·17 ± 0·41	45·0 ± 65·3	n.s.
Polio virus					
type I	2·39¶ ± 4·33	4·93 ± 4·89	1·94 ± 2·01	53·85 ± 41·21	0·01
type II	0·94 ± 1·92	5·89 ± 2·66	2·37 ± 3·67	46·70 ± 35·83	0·01
type III	2·56 ± 2·55	58·88 ± 61·23	2·79 ± 2·69	47·80 ± 56·23	n.s.

All values represent the mean ± s.d.

n.s. = not significant.

**P* values indicate significant differences in increase of antibody titre between both groups.

† Expressed as extinction in ELISA test.

‡ Expressed as IU/ml.

§ Expressed as a percentage of a standard reference serum.

¶ Expressed as IU/ml.

Cellular immune reactivity in vitro

The proliferative capacity of lymphocytes *in vitro* after stimulation with non-specific mitogens was only slightly affected, not reaching significance (not shown). Also the proliferative capacity of lymphocytes to alloantigens as well as to anamnestic antigens remained normal.

The generation of cytotoxic T lymphocytes in CML was not affected by treatment with cyclophosphamide (results not shown), indicating that apparently a normal proportion of cytotoxic T lymphocyte precursors was present. However, cytotoxic activities of K and NK cells were depressed (see Fig. 2).

Cellular immune reactivity in vivo

The primary cellular immune response was depressed in patients receiving cyclophosphamide: three of them did not react ($P < 0{\cdot}0001$), whereas all control patients displayed a positive skin test. However, the secondary cellular immune responses *in vivo* were not affected (Fig. 3).

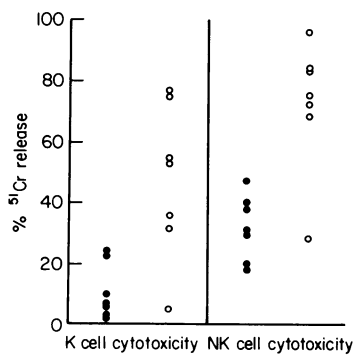

Fig. 2. K and NK cell cytotoxicity. ● = patients receiving cyclophosphamide; ○ = control patients. Percentage ^{51}Cr release is shown at an effector : target ratio of 2·5 : 1 for K cell activity, and at an effector : target ratio of 20 : 1 for NK cell activity. Statistical analysis: K cell cytotoxicity, $P < 0{\cdot}0001$; NK cell cytotoxicity, $P < 0{\cdot}0001$.

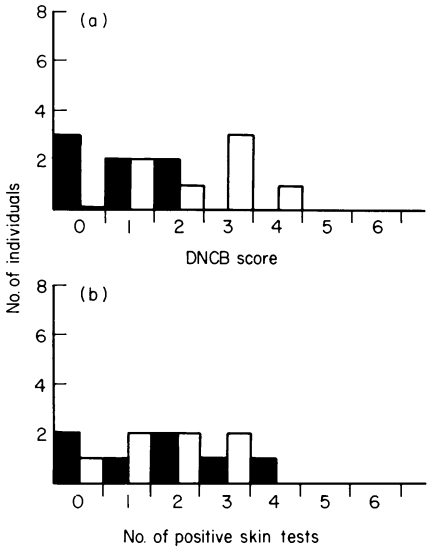

Fig. 3. Primary and secondary cellular responses *in vivo*. ■ = patients receiving cyclophosphamide; □ = control patients. Statistical analysis = DNCB, $P < 0{\cdot}0001$; secondary responses = not significant. (a) Primary cellular response *in vivo* after sensitization with DNCB; (b) secondary DTH response to soluble antigens.

DISCUSSION

This study leads us to the following conclusions upon administration of cyclophosphamide: the number of T and B lymphocytes as well as granulocytes in peripheral blood is diminished; immunoglobulin levels and antibody production *in vivo* after both primary and secondary immunization are decreased, and the primary cellular immune response *in vivo* is depressed. Secondary cellular immune reactivity *in vivo* remains intact; in addition, K and NK cell activity are depressed.

Reports from other investigators, on the effect of treatment with cyclophosphamide on the primary humoral response *in vivo* are difficult to compare with our results, because the effects of either high doses, administered for only 7 days, (Santos, Owens & Sensenbrenner, 1964) or rather low doses (Curtis *et al.*, 1973) were studied; or a control group was lacking (Fauci, Wolff & Johnson, 1971). A well controlled study by Townes, Sowa & Shulman (1976) shows a normal antibody response to Vi antigen from *E. coli*, which is in contrast with our finding of a diminished antibody response to the antigen haemocyanin from *α-Helix pomatia*.

Regarding the effect of treatment with maintenance doses of cyclophosphamide on secondary humoral responses *in vivo*, only one study has been performed (Winkelstein *et al.*, 1974), using only one antigen (bivalent influenza vaccine), which induced a normal response. We studied secondary antibody responses to both bacterial and viral antigens, and found both to be depressed.

The differences between our results and those of others regarding both primary and secondary antibody responses can possibly be explained by the different nature and strength of the test antigens and differences in the dose of cyclophosphamide.

At variance with other investigators (Alepa, Zvaifler & Sliwinski, 1970; Levy, 1975; Strong, Bartholomew & Smyth, 1973; Winkelstein *et al.*, 1974) who observed a diminished proliferative capacity of lymphocytes *in vitro*, we did not detect any difference between responses of cyclophosphamide treated and non-treated patients. This discrepancy may be due to different doses and/or duration of cyclophosphamide treatment, but probably also to differences in culture techniques. Nevertheless, data on three of our patients studied before onset of therapy as well as about 6 weeks thereafter also did not reveal any alteration in proliferative capacity of lymphocytes *in vitro*. However, when the depressed number of lymphocytes in the peripheral blood is taken into

account, it is obvious that the mitogenic capacity, calculated per ml peripheral blood, is depressed.

As far as we know, cytotoxic functions of peripheral blood lymphocytes in patients receiving maintenance therapy with cyclophosphamide, have not been investigated in the past. The differential effect of cyclophosphamide on pre-cytotoxic T cells versus K/NK cells to cyclophosphamide, as shown in our patients, may reflect differences in turnover rate and/or ability to repair damaged DNA. Moreover, the data of Astaldi *et al.* (1982) indicate that at least part of the K/NK cells does not belong to the T cell lineage, which may account for their different susceptibility to the effect of cyclophosphamide.

Our findings of depressed primary as opposed to normal secondary cellular immune responses *in vivo* confirm earlier studies regarding the influence of cyclophosphamide on delayed type hypersensitivity reactions in man (Fauci *et al.*, 1971; Townes *et al.*, 1976; Strong *et al.*, 1973; Santos *et al.*, 1964). These results indicate that during treatment with cyclophosphamide, at least in the doses used in this study, not only the reactivity of T lymphocytes to anamnestic antigens remains normal, but that there are also no disturbances in recruitment and/or function of accessory cells needed for the expression of cellular immunity *in vivo*. The latter is supported by the finding of a normal Rebuck-skin-window test (Townes *et al.*, 1976; Dale, Fauci & Wolff, 1973).

In conclusion, therapy with cyclophosphamide in a dose of $\pm$ 100 mg/day in man appears to have its main influence on primary responses *in vivo*, for which the severe depression in numbers of circulating T and B lymphocytes may at least partially be responsible. In addition, secondary antibody responses are diminished. In view of the normal skin tests to anamnestic antigens, the inflammatory response occurring in type IV DTH reactions (classification by Coombs & Gell, 1975) remains intact. However, because the number of granulocytes and K and NK cell activities are depressed, it may be expected that type II and type III reactions are diminished. Thus, from a therapeutic point of view, cyclophosphamide may be effective at at least two levels: first, it can lower the production of (auto)antibodies, and second, the effector stages of type II and type III hypersensitivity reactions may be affected, resulting in less tissue destruction.

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