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Cell number requirements for lymphocyte stimulation *in vitro*: changes during the course of multiple sclerosis and the effects of immunosuppression

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SUMMARY

Peripheral blood lymphocytes from 20 patients with clinically definite, relapsing and remitting multiple sclerosis (MS) were studied during their participation in a double-blind trial of immunosuppressive treatment. Proliferative responses occurring with different numbers of cells in culture and on different days of culture in the presence of phytohaemagglutinin (PHA) or with allogeneic cells from lymphoid cell lines (MLC) were assessed. Cells taken from patients before treatment showed similar responses to cells from laboratory personnel. However, when cells were taken from patients in relapse or from untreated patients as the disease progressed, there was an alteration in the pattern of response; higher numbers of cells were required in culture to produce responses. A change in the responsiveness to PHA or in MLC may therefore accompany the progression of the disease in MS (reflecting clinical relapses and possibly subclinical activity of the disease), perhaps resulting from a simple reduction in the proportion of cells able to respond. After intense immunosuppression followed by long-term maintenance on azathioprine, cells from patients gave similar responses to those found before treatment. Thus long-term immunosuppression prevented the progressive alteration in lymphocyte function. Shifts in the total cell number and time in culture required to allow proliferation with mitogens of cells from untreated MS patients could explain both the 'low' PHA responses reported and the changes of *in vitro* 'suppressor' function of these cells.

INTRODUCTION

There have been many reports demonstrating that lymphocytes from patients with multiple sclerosis (MS) respond poorly to T cell mitogens (e.g. PHA) *in vitro* (for references see Knight, 1977; Ilonen *et al.*, 1981). Recent work has suggested that there is a deficit in 'suppressor' T cells in active multiple sclerosis (Arnason & Antel, 1978; Gonzales, Dan & Spitler, 1979; Huddlestone & Oldstone, 1979; Bach *et al.*, 1980; Reinherz *et al.*, 1980). This work was based on studies using conditions where proliferation was reduced in culture by the addition of cells stimulated with concanavalin A (Con A). The existence of such Con A-induced suppressor cells was not supported by work where a range of culture conditions were studied (Farrant & Newton, 1981). The proliferation of a population of lymphoid cells has been shown to be dependent on the relationship between the total number of cells in culture, the dose of stimulant and the time in culture (Knight *et al.*, 1979; Farrant *et al.*, 1980). Studies mixing monoclonal lymphoid cell line cells suggest that

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helper and suppressor effects on cell proliferation can be demonstrated using the same cells under appropriate conditions (Farrant & Knight, 1979). Similarly, in mixed lymphocyte cultures (MLC) the addition of autologous cells resulted in increases or decreases in responses depending on the total number of cells present and the time of culture as well as on the ratio of responder to stimulator cells (Knight *et al.*, 1979; Knight & Burman, 1980). Thus changes in help or suppression could result from simple changes in the number of responsive cells present. We have investigated the responses to PHA and in MLC of lymphocytes from patients with MS participating in a double-blind trial of intensive immunosuppressive treatment and report here how these responses, measured using a range of conditions, change with the progression of the disease and during relapse.

MATERIALS AND METHODS

Patient material. Patients had clinically definite MS (McAlpine, Lumsden & Acheson, 1972), were between 20 and 45 years of age, had suffered on average at least one relapse per year over the preceding 3 years and were ambulant at the beginning of the trial. They were allocated randomly to receive either immunosuppressive or placebo treatment. During the period of study 20 patients entered the trial and nine were treated and 11 placebo-treated. The treated patients received azathioprine (3 mg per kg per day) for 15 months, prednisolone (initially 150 mg daily, subsequently tapered down to 20 mg and discontinued after 4 weeks) and equine anti-human lymphocyte globulin (ALG, Upjohn), 500–750 mg daily for 15 days starting on day 7 of treatment (Mertin *et al.*, 1980). Peripheral blood lymphocytes were studied before treatment, after 1 week of treatment, at the end of the ALG treatment, 2–6 weeks after the completion of ALG and 4–8 months after the completion of ALG treatment. Cells from five of the untreated patients with clear-cut relapses (defined as the occurrence of one or more new symptoms, or a marked worsening of existing symptoms lasting more than 48 hr) were also studied.

Lymphocyte cultures. Triplicate cultures (0·2 ml in Cooke microtitre plates, flat wells) in Dulbecco's medium with 10% fetal calf serum (GIBCO) with added L-glutamine 2 mM, penicillin (100 units/ml) and streptomycin (100 μg/ml), contained 10, 5, 2·5 or $1{\cdot}25 \times 10^5$ lymphocytes per ml which were separated from defribinated peripheral blood by sedimentation with gelatin, giving a population of leucocytes which usually contained over 90% lymphocytes. Cultures received 1 μl PHA (Burroughs Wellcome, HA 15, final dilution 1/120 v/v). Twenty-four hours before harvesting cultures on days 3, 4, 5 or 6, ^{3}H-thymidine (Amersham, 0·6 μg per culture, 150 mCi/mmol) was added to each culture. The mean uptake into the acid-precipitable fraction of the cells was assessed by scintillation counting (Knight *et al.*, 1975). Alternatively, duplicate cultures (20 μl) contained different numbers of lymphocytes and different concentrations of mitomycin-treated cell line cells (50 μg/ml mitomycin C, Sigma, at 37°C for 30 min, washed twice in saline and once in medium). Seven human lymphoid cell lines each believed to be homozygous for antigens of the HLA-D locus (HLA-Dw1–Dw7) were used which were kindly supplied by Dr Bodmer. Cultures in Terasaki plates (Falcon, 60 well) were inverted above saline in a plastic box in a well-humidified incubator gassed with 5% CO_2. Tritiated thymidine (1 μl to give 1 μg/ml of thymidine of specific activity 2 Ci/mmol) was added 2 hr before harvesting by blotting the hanging drops onto filter discs cut in a harvester plate as previously described (O'Brien *et al.*, 1979).

Analysis of the results. The PHA responses were analysed by comparing the mean $\log_{10}$ uptakes from triplicate cultures using analysis of variance with the following factors: (*A*) four cell concentrations, (*B*) 4 days of culture, (*C*) number of individuals within each treatment group, and (*D*) treated or placebo groups. Using this method of analysis changes which were significantly above the variations seen between individuals within each group were assessed. The program used for analysis was intended for factorial designs and to analyse this nested design (i.e. individual 1 in group 1 was not the same as individual 1 in group 2), terms were combined to make the appropriate comparisons, e.g. to see whether an effect of cell concentration (*A*) in two groups of patients (*D*) containing a number of individuals (*C*) was significantly different from the variations between individuals in the group, a mean square ratio (*F*) between the variance of DA and $AC+ACD$ was used (Winer, 1971). Comparisons between placebo-treated and treated patients in MLC used a

similar technique for analysis of the data and the factors used are indicated for individual experiments in the text.

RESULTS

Before treatment

The mean PHA responses of lymphocytes from 12 laboratory controls and from 12 patients (six treated and six placebo) before treatment are shown in Fig. 1. There were no significant differences between these two groups of responses. In each case, over the early days of culture an almost linear increase in uptake of ^{3}H-thymidine occurred with increasing concentrations of cells in culture; a plateau of response was not reached over the range of cell concentrations used. On the 5th day of culture the response showed a plateau with 2·5 and 5×10^5 cells per ml and responses decreased at both higher and lower cell concentrations. The two groups of patients, one of which was to receive immunosuppressive treatment and the other placebo materials, were not different in the way their lymphocytes responded to PHA before treatment. This is demonstrated in Table 1 which shows that there was no indication of any difference between the two groups of patients (factor *D*) before treatment. In addition there was no difference between the two groups in the responsiveness at different cell concentrations (*DA*) or in the way in which responses developed with time in culture (*DB*).

After early intensive treatment

Similar PHA responses were found when cells from treated and placebo-treated patients were compared after 1 week of treatment with azathioprine and prednisolone (Table 2). After a further 2 weeks of treatment which included ALG, the time course of the response was different in the two groups of patients. This change in responsiveness was also seen by comparing the cells taken from the same patients before and after treatment. The development of the response with time in culture was the same before and after placebo treatment but the response had altered significantly in those patients studied at the end of ALG treatment (Fig. 2).

In an experiment investigating the effect of this treatment on the responses of lymphocytes in MLC, the following factors were studied: five concentrations of responder cells (20, 10, 5, 2·5 and $1{\cdot}25 \times 10^5$/ml), four concentrations of stimulator cells (10, 5, 2·5 and $1{\cdot}25 \times 10^5$/ml), two types of stimulator cells (Dw1 and Dw2), three responder individuals (one laboratory control and two treated patients), 4 days of culture (days 2, 3, 4 and 5) and three occasions (pretreatment, immediately post ALG treatment and 2 weeks after the end of ALG treatment). Cells from both the MS patients gave lower responses to the Dw2 cells than to the Dw1 cells whereas the laboratory

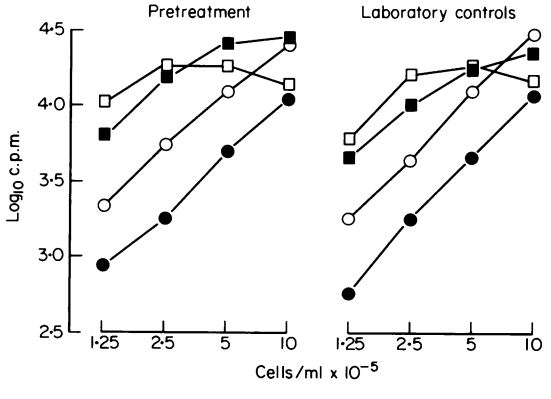

Fig. 1. Mean uptake of ^{3}H-thymidine in lymphocytes from 12 multiple sclerosis patients before treatment and from 12 laboratory personnel. Different concentrations of cells were stimulated with PHA and the uptake starting on days 2 (●), 3 (○), 4 (■) or 5 (□) was measured.

Table 1. Comparisons, before treatment, of PHA responses of cells from patients allocated to the treatment and placebo treatment groups

	Source of variation	Sum of squares of deviation	Degrees of freedom	Mean square	*F*	*P*
D	Group	0·000525	1	0·000525	0·0007	0·98
C+*CD*	Individual within group	7·68	10	0·768		
DA	Group × cell concentration	0·211	3	0·0704	0·683	0·57
AC+*ACD*	Cell concentration × individual within group	3·09	30	0·103		
DB	Group × period of culture	0·0483	3	0·0161	0·265	0·84
BC+*BCD*	Period of culture × individual within group	1·81	30	0·604		
DAB	Group × cell concentration × period of culture	0·112	9	0·0125	0·72	0·69
ABC+*ABCD*	Cell concentration × period of culture × individual within group	1·54	90	0·017		

Data expressed as $\log_{10}$ c.p.m., means from triplicate cultures. *A* = four cell concentrations (1·25, 2·5, 5 and 10×10^5/ml), *B* = four periods of culture (days 3, 4, 5 and 6), *C* = six individuals in each group, *D* = two groups (patients to receive immunosuppression and patients to have placebo).

Table 2. Comparison between treatment and placebo treatment groups

	Treatment stage				
Source of variation	Pre	1 week (pre-ALG)	3 weeks (post-ALG)	5–9 weeks	4–8 months
Group	0·98	0·66	0·21	0·26	0·41
Group × cell concentration	0·57	0·54	0·25	0·025	0·024
Group × time	0·84	0·48	0·044	0·27	0·37
Group × cell concentration × time	0·69	0·43	0·56	0·056	0·08

Significance levels obtained as shown in Table 1.

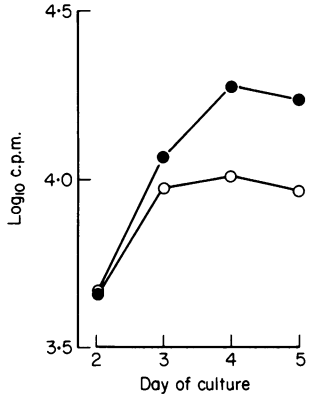

Fig. 2. Mean responses to PHA averaged over four cell concentrations (1·25, 2·5, 5 and 10 × 10^5/ml) are given for cultures pulsed with ^{3}H-thymidine on the days shown. Cells were from five patients studied before treatment (●) and from the same five patients at the end of ALG treatment (○).

control gave similar responses to each cell line. This effect was due to the requirement for higher numbers of responder cells from the patients (Fig. 3a) to produce responses to the Dw2 line than to the Dw1 line. These relative responses to the two cell lines were similar on all occasions of study and were not changed by the treatment (Fig. 3b). However, immediately after the end of ALG treatment, the responses to both cell lines were significantly reduced compared with the control (Fig. 3). The responses of cells taken from patients immediately after the end of ALG treatment were also significantly altered in their time course and responsiveness with different concentrations of stimulator cells.

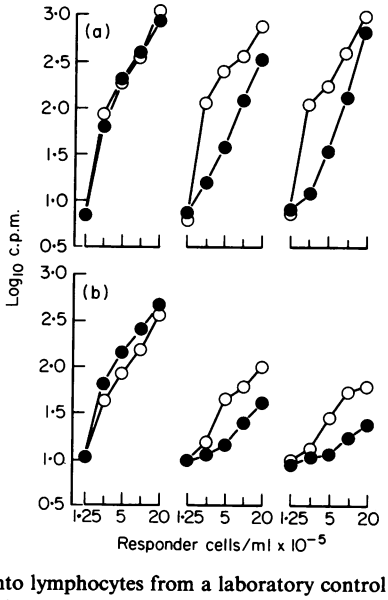

Fig. 3. Uptake of ^{3}H-thymidine into lymphocytes from a laboratory control (1st graph of each set) and into lymphocytes from two patients with multiple sclerosis. Mean responses of cells averaged over 4 days of culture (2–5) and four different concentrations of stimulator cells (10, 5, 2·5 and 1·25 × 10^5/ml) from the cell lines HOM 1 (Dw2) (●——●) or HOM 2 (Dw1) (○——○) are shown. The responses of cells from all three individuals were studied in a single experiment on each of two occasions (a & b). Cells from the two patients were taken immediately before treatment (a) and at the end of ALG treatment (b).

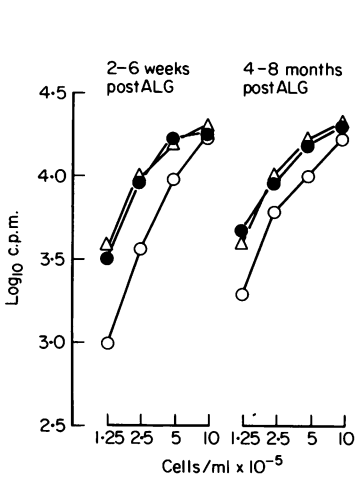

Fig. 4

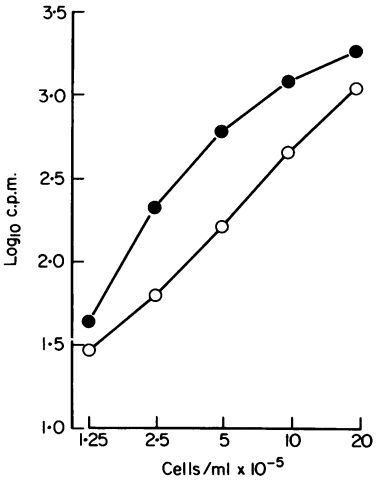

Fig. 5

Fig. 4. Mean ^{3}H-thymidine uptake from cells stimulated with PHA averaged over 4 days of culture. Responses at each of four cell concentrations are shown. (▵——▵) Mean of cells from 12 patients before treatment, (●——●) mean of cells from four treated patients and (○——○) from four placebo-treated patients.

Fig. 5. Mean ^{3}H-thymidine uptake into lymphocytes from three placebo-treated patients (○——○) or three treated patients (●——●). Cells at different concentrations were stimulated with 20,000 mitomycin-treated cells from each of seven lymphoid cell lines (Dw1–Dw7) and the response on day 5 of culture was measured. The graph pools the responses to demonstrate the major difference observed between the two groups of patients.

After long-term treatment

The change of responsiveness immediately following ALG treatment was transient. After longer periods of maintenance treatment on azathioprine this effect was no longer seen and the cells from the treated patients showed no significant alterations from the responses obtained before treatment (Fig. 4). However, higher numbers of cells in culture were required to obtain responses in the placebo-treated patients (Fig. 4). This effect was seen with cells taken between 2 and 6 weeks after

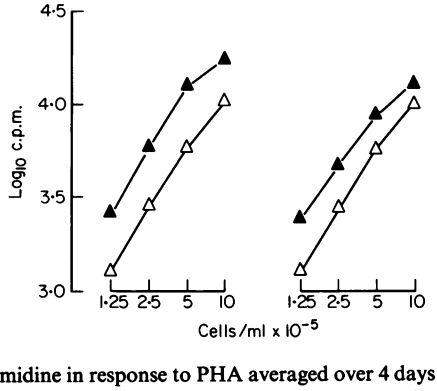

Fig. 6. Mean uptake of ^{3}H-thymidine in response to PHA averaged over 4 days of culture is shown for each of four cell concentrations. (▵——▵) Mean responses of cells from five placebo-treated patients taken during relapse, (▲——▲) mean response of cells from the same five patients taken during remission at the beginning of the trial (*left*), and mean responses of five placebo-treated patients studied on the same occasions as the relapse patients (*right*).

the end of ALG treatment and with cells from patients treated with azathioprine for more than 4 months (Table 2). It was also confirmed by comparing directly in a single experiment the PHA responses of cells from another three treated and three placebo-treated patients who had been in the trial for more than 3 months. Cells from these individuals were also stimulated with allogeneic cells from seven B cell lines and changes similar to those in the PHA responses were found. There were many variations in the numbers of cells required to produce responses to the various cell lines, probably reflecting the HLA-D locus specificities of the cells. However, in addition to these specific effects with individual cell lines the responses on average were significantly lower in the placebo-treated patients ($P=0{\cdot}029$). This was a reflection of the requirement for roughly twice as many cells in culture in order to see the responses in placebo-treated patients (Fig. 5), the changing response with different cell concentrations being significantly different in the two groups of patients ($P=0{\cdot}0027$).

The effect of relapse

As shown in Fig. 6, when patients went into relapse the responses of their cells were lower than the responses of cells from the same patients in remission at the beginning of the trial. This was due to the requirement for approximately twice as many cells to produce equivalent responses. The same trend was seen when these responses were compared with the responses of cells from patients not in relapse but studied in the same experiment performed on the same day (Fig. 6). All patients were from the placebo-treated group.

DISCUSSION

Evidence presented here shows that the low responses to PHA which have been reported with cells from MS patients may be due to a requirement for higher numbers of cells in culture to allow responses. Similar changes occurred in mixed lymphocyte reactivity. This effect was not seen in the cells from early ambulatory patients in remission entering this trial but became evident in untreated patients with time or when patients were in clinical relapse. The treatment with immunosuppressive drugs prevented this change in responsiveness. The treated patients also had fewer relapses than the untreated patients (Mertin *et al.*, 1980). This suggests that progression of disease is associated with a change in responsiveness to PHA or in MLC.

Our results show the importance of studying the responsiveness using a range of culture conditions in order to assess the capacity of cells to proliferate in response to stimulation. However, the handling of data where several factors are studied simultaneously has presented problems. Comparisons of responses to PHA at different cell concentrations and at different times in culture in the treated and untreated patients took into account the variation between individuals within these groups of patients. Since the studies span an 18-month period this dealt with both innate variations in responses between individuals as well as any possible variations in the assay system over that time period. The dose of stimulant also interacts with the time in culture and the concentration of cells used (Knight *et al.*, 1979; Farrant *et al.*, 1980). A single dose of PHA which could be varied approximately five-fold with little alteration in the responses of cells from normal individuals was chosen for this study. With the MLC in the smaller cultures (20 μl) it was possible to include varying doses of stimulator cells and this caused differences in the kinetics of the response. However, at all stimulator cell doses, higher cell numbers from untreated than treated MS patients were required to produce responses.

When comparing the responses of cells from MS patients to cell lines homozygous either for HLA-Dw1 or Dw2, a high proportion of patients gave lower responses to the Dw2 cells. This was due to a requirement for higher cell numbers in order to produce the response to the Dw2 cells (Knight *et al.*, 1979; Knight & Burman, 1981). This is believed to reflect the presence of Dw2 antigens in a high proportion of MS patients (Batchelor, 1977). Similar 'typing' effects were seen in this study on stimulation using the range of cell lines. Although both the course of the disease and the effects of early intensive immunosuppressive treatment changed the response to the cell line cells,

the relative responses to the Dw1 and Dw2 cell lines were maintained. Changes in the total cell number required to produce proliferation during the course of MS could result in difficulties in typing antigens of the HLA-D locus by MLC using a single set of culture conditions.

Although this multifactor monitoring of immune response is more complex in the culturing and analysis of data than that normally used, the results present a simple message: in untreated MS higher numbers of cells are required to produce responses to PHA or MLC *in vitro*. A simple interpretation could be that there are therefore fewer responsive T cells in the blood of untreated patients. This raises the question as to whether cells are removed or are blocked in some way. There are suggestions of increases in the proportions of B lymphocytes present in MS (for references see Knight, 1977) as well as changes in the populations of T cells with certain cell surface markers (Bach *et al.*, 1980; Reinherz *et al.*, 1980). However, the prevention of the change in responsiveness by long-term treatment with the antimetabolite azathioprine may indicate that dilution of the peripheral pool of lymphocytes by the production of an abnormal population of cells not responsive to mitogen is being blocked. In other trials of immunosuppression in MS it has been suggested that it is patients with early disease who benefit from immunosuppressive therapy (Hommes, Lamers & Reekers, 1980; Patzold & Pocklington, 1980). It is not clear whether an abnormal pattern of responsiveness can be converted to a normal one by immunosuppressive treatment. Since the patients entering this trial did not show changes in their lymphocyte stimulation values, this cannot be ascertained in this trial. In patients already showing a requirement for higher cell numbers to see T cell stimulation, long-term immunosuppression might at least prevent further major alterations in lymphocyte populations. However, the picture in these patients, if confirmed in further studies, could also provide a theoretical basis for a treatment which would amplify the numbers of reactive T cells (Basten *et al.*, 1980).

From the pattern of PHA response studied (e.g. in Fig. 1) it can be seen that a shift of this whole pattern of response to higher cell concentrations can lead to apparently complex changes—increases or decreases in response—when single times in culture or concentrations of cells are used. This would also be true on adding extra cells to cultures in the way used for assessing 'helper' and 'suppressor' cells. The fact that responses to PHA increase, reach a plateau and then decrease as higher concentrations of cells are put into culture, taken together with the requirements for more cells to see this effect when the cells are from MS patients, would perhaps account for the changes in suppressor activity observed with MS cells. Since similar growth patterns are seen when different numbers of monoclonal cells from lymphoid cell lines are put into culture, it seems that such effects do not necessitate the presence of cells with innate helper or suppressor function. We therefore suggest the possibility that changes in helper and suppressor activity *in vitro* when adding cells into culture could be consequent upon a simple change in the numbers of responder cells present in MS. Clearly this may be an over-simplification since other factors besides the basic proportion of specific responder cells present can influence responses, e.g. the addition of mitomycin-treated cells (Farrant & Knight, 1979) or the presence of macrophages (Seeger & Oppenheim, 1970). However, two sources of evidence support the more simple view. Firstly, it was not possible to demonstrate suppression occurring in putative Con A suppressor systems when time and cell concentrations were taken into account (Farrant & Newton, 1981). Secondly, experiments where the proportion of specific cells responding to stimulation with an alloantigen were increased by priming showed that many fewer cells (either responder or stimulator cells) were then required in culture to produce specific responses which also occurred at earlier culture times (Corley, 1977; Knight & Burman, unpublished observations). This demonstrates the extensive changes in culture conditions required when simple changes in the proportion of responder cells occur.

We are grateful to Dr Julia Bodmer for her gift of the human B cell lines. We are also indebted to Mrs Caroline Doré, Dr John Farrant and Professor Michael Healy for their help and discussion on the analysis of the data. The double-blind trial of immunosuppression was approved by the Ethical Committee of Northwick Park Hospital. Dr Peter Rudge selected patients entering the trial.

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