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B cell activation by pokeweed mitogen in cultures of normal peripheral blood lymphocytes depleted of T regulator subsets by treatment with OKT4 and OKT8 monoclonal antibodies

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SUMMARY

OKT4$^+$ (T helper/inducer) and OKT8$^+$ (T cytotoxic/suppressor) subsets were depleted from peripheral blood lymphocytes (PBL) by complement-mediated lysis and residual cells examined for responsiveness to pokeweed mitogen (PWM) using a protein A haemolytic plaque assay for immunoglobulin secreting B cells. It was shown that: (1) three cycles of cell killing were required to totally abolish T helper function; (2) OKT4$^-$ PBL did not respond to PWM, but in a co-culture system, an equal number of unfractionated normal PBL could entirely reconstitute responsiveness of the residual B cells; (3) OKT8$^-$ PBL gave enhanced numbers of PWM-induced plaque forming cells (PFC); (4) addition of 4 μg/ml concanavalin A (con A) to PWM stimulated OKT8$^-$ PBL failed to suppress PFC generation, but suppression was induced by 12·5 and 25 μg/ml con A and (5) kinetics of PWM-induced PFC development were similar in the presence or absence of OKT8$^+$ cells.

INTRODUCTION

The possibility exists that newly available reagents such as helper and suppressor factors from T cell lines (Altman & Katz, 1980) and monoclonal antibodies directed against T helper (T_H) and T suppressor (T_S) subpopulations (Reinherz & Schlossman, 1981) could permit therapeutic intervention in hypo- or hyperimmunoglobulinaemic states characterized by defective T regulation. It is therefore essential that clinically applicable methods are developed both for diagnosing the precise nature of cellular defects in such cases and for monitoring attempts to repair the defect.

Functional evaluation of B cells and T regulator subsets is usually performed by co-culturing T depleted patient's peripheral blood lymphocytes (PBL) with normal control PBL-T, or patient's PBL-T with control T depleted PBL, and stimulating with a T-dependent B cell mitogen (Cassidy, Oldham & Platts-Mills, 1979; Chiorazzi *et al.*, 1979; Herrod & Buckley, 1979; Johnsen, Madsen & Kristensen, 1979; Krakauer *et al.*, 1979; De Gast *et al.*, 1980; Delfraissy *et al.*, 1980; Krantman *et al.*, 1980; Levitt, Griffin & Egan, 1980; Nies, Stevens & Louie, 1980, 1981; Reinherz *et al.*, 1981). I have attempted to use a simplified co-culture system to discriminate between (a) intrinsic B cell defects, (b) T_H deficiency and (c) T_S hyperactivity in patients who generate few immunoglobulin (Ig) secreting plaque forming cells (PFC) in response to T-dependent pokeweed mitogen (PWM) (Jones, 1981). The observed number of PFC developing in PWM stimulated cultures of equal numbers of patient's plus normal control unfractionated PBL was compared with the expected value derived from the mean of each individual's PBL cultured separately with PWM. Observed/expected (O/E) values were between 0·81 and 1·25 in normal co-cultures but were severely reduced when normal

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PBL were co-cultured with cord blood lymphocytes (Jones, 1981). Cord blood contains *in vivo* activated T_S (Miyawaki *et al.*, 1979; Moriya *et al.*, 1979), and thus the simple method does appear capable of identifying T_S hyperreactivity. Enhanced O/E values might be predicted in co-cultures of normal plus T_H deficient PBL, since normal T_H would permit patient's B cells to respond and contribute additively to the total number of PFC. In this paper, the validity of this concept has been examined by co-culturing normal PBL with further normal autologous or allogeneic PBL depleted of T_H by prior treatment with monoclonal anti-T_H OKT4 antiserum (Reinherz *et al.*, 1979) and complement.

It is also desirable to develop clinically applicable *in vitro* methods for identifying reduced T_S function, since this may be associated with hyper-Ig secretion in diseases such as systemic lupus erythematosus (Krakauer *et al.*, 1979; Miller & Schwartz, 1979; Gladman *et al.*, 1980), chronic active hepatitis (Tremolada *et al.*, 1980), insulin-dependent diabetes mellitus (Buschard, Madsbad & Rygaard, 1980), myasthenia gravis (Berrih *et al.*, 1981), polyclonal gammopathy (Robinson, Abdou & Abdou, 1981) and atopic hypersensitivity (Geha, 1979; Martinez *et al.*, 1979). Concanavalin A (con A) added directly to PWM stimulated normal PBL at the start of the culture will inhibit subsequent PFC development (Haynes & Fauci, 1977; Pryjma *et al.*, 1980; Jones, 1981), but that this is due to the effects of con A activated T_S has not strictly been proven. Therefore in this study the effect of con A added to PWM stimulated PBL, depleted of T_S by treatment with OKT8 monoclonal antiserum (Reinherz *et al.*, 1980) has been examined. The results confirm that the simple co-culture method using unfractionated PBL can be usefully employed to evaluate T_H function, and that direct addition of low dose con A (but not high doses) to PWM stimulated cultures causes suppression due to activation of $OKT8^+$ T_S.

MATERIALS AND METHODS

Blood samples. Peripheral blood was collected from healthy laboratory staff, aged 25–36 years, into syringes containing 10 units/ml preservative free heparin. PBL were separated over Ficoll-Hypaque and washed three times in RPMI medium (GIBCO, Grand Island, New York, USA) containing 20 mM $NaHCO_3$, 4 mM fresh frozen L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin.

T_H and T_S depletion. PBL at $10–15 \times 10^6$/ml in RPMI were mixed with an equal volume of medium (sham treated) or OKT4 or OKT8 monoclonal antiserum (Orthoclone, Raritan, New Jersey, USA) diluted 1/125 in RPMI, and incubated at room temperature for 45 min. Fresh rabbit serum was used as a source of complement and was added to sham, OKT4 and OKT8 treated PBL at a final concentration of 10% for 60 min at 37°C. The cells were then washed once and subjected to up to two further cycles of cell killing. Finally they were washed three times in RPMI and the viability determined by trypan blue dye exclusion.

Lymphocyte cultures. Treated or untreated PBL were cultured at $0{\cdot}5 \times 10^6$ viable cells/ml in RPMI containing 5% pooled human AB serum, previously heat-inactivated at 56°C for 30 min. One millilitre cultures in round bottomed 12×75 mm plastic tubes (Falcon, Maryland, USA) were stimulated with 20 μg/ml PWM (GIBCO) and in certain cultures con A (Sigma, St Louis, Missouri, USA) was also added at 4–25 μg/ml to activate putative T_S. Co-cultures were performed by mixing equal numbers of untreated or sham treated PBL and OKT4 treated PBL, the latter being either autologous or allogeneic with respect to the control cells. In some experiments, unfractionated PBL, 5×10^6/ml, were treated with mitomycin C (Sigma) at 37·5 μg/ml for 30 min at 37°C, washed three times with RPMI, incubated for 2 hr at 37°C and again washed three times before addition to $OKT4^-$ PBL. Cultures were incubated at 37°C in 5% CO_2, 95% air, 100% humidity, usually for 7 days, though some OKT8 depleted cultures were allowed to continue for up to 14 days to examine the kinetics of PFC development in the absence of T_S. At the end of the incubation period, cells were washed three times in RPMI, the viability determined and the viable cell concentration adjusted as appropriate.

Plaque assay. The protein A haemolytic plaque assay (PrA-HPA) was performed as described previously (Jones, 1981). Briefly, partially purified PrA (Sigma) was coupled to sheep erythrocytes

(ShE) by the method of Gronowicz, Coutinho & Melchers (1976). One hundred microlitres of PWM activated PBL, $1–5 \times 10^6$ viable cells/ml (the concentration chosen depending on the expected number of PFC), were mixed with 80 μl PrA-ShE, 10^9/ml, 6 μl ShE absorbed guinea-pig complement and 14 μl rabbit anti-human IgG, IgA or IgM (IgG fraction, Dako, Copenhagen, Denmark) diluted 1/17·5 in RPMI. The mixtures were introduced into triplicate 40 μl Cunningham chambers (Cunningham & Szenberg, 1968) which were then sealed with paraffin wax and incubated in the horizontal position at 37°C for 4 hr. The number of PFC/chamber was determined by low magnification microscopy and converted to PFC/10^6 cells plated or PFC/culture.

Percentage suppression by con A was calculated from the following formula:

$$\% \text{ suppression} = \frac{\text{PFC in the presence of PWM} - \text{PFC in the presence of PWM} + \text{con A}}{\text{PFC in the presence of PWM}} \times 100.$$

O/E co-culture values were calculated as follows:

$$\text{O/E} = \frac{\text{PFC in co-cultures of equal numbers of control plus treated PBL stimulated with PWM.}}{\text{Mean of PFC in control and treated PBL stimulated separately with PWM.}}$$

Each experiment was repeated at least once and results were reproducible. All data are from representative experiments and results are expressed as the mean number of PFC in triplicates tested. Standard errors did not exceed 15% of the mean values shown.

RESULTS

T_H depletion

In initial experiments, the number of cycles of treatment with OKT4 antiserum required to abolish helper function was determined, and representative results from one of three experiments are shown in Table 1. Sham treated PBL were >98% viable after one, two and three cycles of incubation with medium followed by complement and the number of IgG-, IgA- and IgM-PFC/10^6 were not changed by this treatment. OKT4 antiserum produced 17, 27 and 31% cell killing after one, two and three cycles of treatment. These values may be falsely low since there was some clumping of dead cells and trypan blue stained preparations could not be counted accurately. A single killing cycle had virtually no effect on PWM-induced PFC, even though at least half of the OKT4$^+$ cells had been inactivated, and O/E values in sham plus OKT4 treated autologous co-cultures were close to

Table 1. PWM-induced PFC/10^6 in cultures depleted of OKT4$^+$ PBL by one, two or three cycles of cell killing

Treatment	Viability (%)	PFC/10^6 IgG	IgA	IgM
None	99	32,500	31,000	27,500
Sham ×1	98	28,000	30,000	26,500
OKT4 ×1	83	24,000	27,000	27,500
OKT4+sham ×1		27,500	28,250	28,000
O/E		1·06	0·99	1·04
Sham ×2	99	38,000	32,500	26,000
OKT4 ×2	73	16,000	7,750	7,500
OKT4+sham ×2		35,500	29,500	23,000
O/E		1·31	1·48	1·37
Sham ×3	98	30,000	36,000	23,500
OKT4 ×3	69	1,000	1,250	0
OKT4+sham ×3		31,000	34,000	27,000
O/E		2·00	1·82	2·30

1·0. Two cycles reduced PFC/10^6 by 58–76% and O/E values were slightly above the normal limit of 1·25. However, three cycles of treatment with OKT4 and complement abolished subsequent PWM-induced PFC generation and O/E values were close to 2·0, which is the value expected if OKT4 treated PBL were totally lacking T_H function, and if sham treated PBL could provide sufficient help to enable both sets of B cells in the co-culture to fully express PWM responsiveness.

To confirm that thrice treated OKT4$^-$ PBL contained PWM responsive B cells, mitomycin C treated unfractionated PBL were used as a source of T_H for autologous OKT4$^-$ PBL. In one typical experiment, negligable PFC/10^6 were produced when mitomycin C or OKT4 treated PBL were cultured separately with PWM, whereas mixtures of equal numbers of these cell preparations gave 13,738 IgG-, 15,444 IgA- and 12,574 IgM-PFC/10^6. Control untreated PBL from the same donor gave 31,500 IgG-, 42,333 IgA- and 31,667 IgM-PFC/10^6.

Allogeneic co-cultures

Three experiments were performed in which untreated PBL were co-cultured with an equal number of allogeneic sham or OKT4 treated (three cycles of killing) PBL and stimulated with PWM. Untreated plus sham treated PBL gave O/E values close to 1·0, whereas untreated plus OKT4 depleted PBL produced significantly enhanced O/E values. Addition of untreated allogeneic PBL to T_H depleted PBL (which produced almost no PFC when cultured alone with PWM) provided sufficient help for total expression of B cell activation by the latter, since untreated plus OKT4 depleted PFC/10^6 were approximately equal to untreated plus sham treated PFC/10^6 (Table 2).

T_S depletion

OKT8 antiserum produced 12, 20 and 23% cell killing after one, two and three cycles of treatment, and this resulted in increased numbers of IgG, IgA and IgM PWM-induced PFC as suppressor influences were removed. Concurrently, reduced suppression induced by con A at 4 μg/ml was observed and was abolished after three cycles of cell killing. However, suppression remained consistently high when the concentration of con A was increased to 12·5 or 25 μg/ml (Table 3).

Kinetics of cell growth and PFC development in OKT8 depleted cultures

Cultures of sham treated and OKT8 treated (three cycles of killing) PBL were stimulated with PWM and harvested at intervals between 4 and 14 days later. Representative results from one of two

Table 2. PFC/10^6 and O/E values in co-cultures of untreated plus allogeneic sham or OKT4 treated (three cycles of cell killing) PBL

Treatment	Cells	PFC/10^6 (O/E) IgG	IgA	IgM
—	A	15,000	27,000	30,000
Sham	B	16,500	24,000	25,000
OKT4	B	2,500	0	500
Untreated + sham	A + B	17,000 (1·08)	25,500 (1·00)	27,000 (0·98)
Untreated + OKT4$^-$	A + B	13,500 (1·54)	22,000 (1·63)	26,500 (1·74)
—	C	17,000	11,000	20,500
Sham	D	44,500	60,000	26,500
OKT4	D	500	500	0
Untreated + sham	C + D	30,000 (0·98)	37,500 (1·06)	22,500 (0·96)
Untreated + OKT4$^-$	C + D	30,500 (3·49)	28,500 (4·96)	20,000 (1·95)
—	E	20,000	19,000	13,500
Sham	F	20,000	9,000	12,000
OKT4	F	0	0	0
Untreated + sham	E + F	22,000 (1·10)	13,000 (0·93)	12,000 (0·94)
Untreated + OKT4$^-$	E + F	18,000 (1·80)	15,500 (1·63)	10,500 (1·56)

Table 3. PWM-induced PFC/10^6 and percentage con A-induced suppression in cultures depleted of OKT8$^+$ PBL by one, two or three cycles of cell killing

Treatment	Viability (%)	Con A (μg/ml)	PFC/10^6 (% con A-induced suppression) IgG	IgA	IgM
Sham ×3	98	0	25,500	14,000	31,500
		4	1,050 (96)	750 (95)	475 (98)
		12·5	550 (98)	475 (97)	0 (100)
		25	475 (98)	325 (98)	0 (100)
OKT8 ×1	88	0	31,000	18,000	33,750
		4	1,550 (95)	500 (97)	575 (98)
		12·5	1,750 (94)	450 (98)	750 (98)
		25	500 (98)	350 (98)	0 (100)
OKT8 ×2	80	0	52,500	24,000	41,500
		4	19,500 (63)	8,750 (64)	14,500 (65)
		12·5	1,250 (98)	2,250 (91)	750 (98)
		25	825 (98)	1,500 (94)	1,250 (97)
OKT8 ×3	77	0	70,000	44,000	63,500
		4	71,500 (−2)	42,750 (3)	64,000 (−1)
		12·5	2,250 (97)	500 (99)	1,500 (98)
		25	1,125 (98)	750 (98)	750 (99)

experiments are shown in Fig. 1. The yield of cells (expressed as a percentage of the number of cells initially put into culture) in sham treated PWM stimulated cultures fell to 31·5% after 4 days, rose to 59% on day 7 and thereafter gradually decreased to 21·5% on day 14. OKT8 depleted PWM stimulated PBL followed a similar time course of expansion but peaked at a much higher cell yield (approximately 160% on days 6 and 7).

In these experiments, numbers of Ig secreting B cells were expressed as PFC/culture rather than PFC/10^6 cells plated to take into account the variation in cell yields at different times of culture. Kinetics of IgG-, IgA- and IgM-PFC development are shown in Fig. 1 b–d. Numbers of PFC of each isotype were very low on day 4 of culture, peaked on day 7 and fell gradually over the next 7 days in both sham and OKT8 treated PBL, but at the height of the response PFC were approximately twice as numerous in T_S depleted cultures.

To determine whether the peak PFC response could be extended beyond day 7, one further experiment was performed in which sham or OKT8 treated PBL were fed with fresh culture medium + PWM on alternate days throughout the culture period. Essentially similar results to those given in Fig. 1 were obtained.

DISCUSSION

The purpose of this study was two-fold: first to determine whether a simple co-culture system using unfractionated PBL could be used to identify T_H defects in one of the co-cultivants, and second to determine whether the observed suppression of PFC development in cultures of normal PBL stimulated simultaneously with PWM and con A was due to T_S activation by the latter.

Normal PBL were depleted of specific T subsets using OKT4 and OKT8 monoclonal antisera and complement-mediated cytotoxicity, and subsequently assessed for helper function and con A-induced suppressor function in the PWM driven PrA-HPA for activated Ig secreting B cells. It was shown that three cycles of treatment were required to totally deplete functional activity of T_H or T_S from whole PBL, whereas other workers have used a single cell killing cycle to obtain functionally pure T_H or T_S after first isolating T cells by ShE rosetting (Miyawaki *et al.*, 1982). These

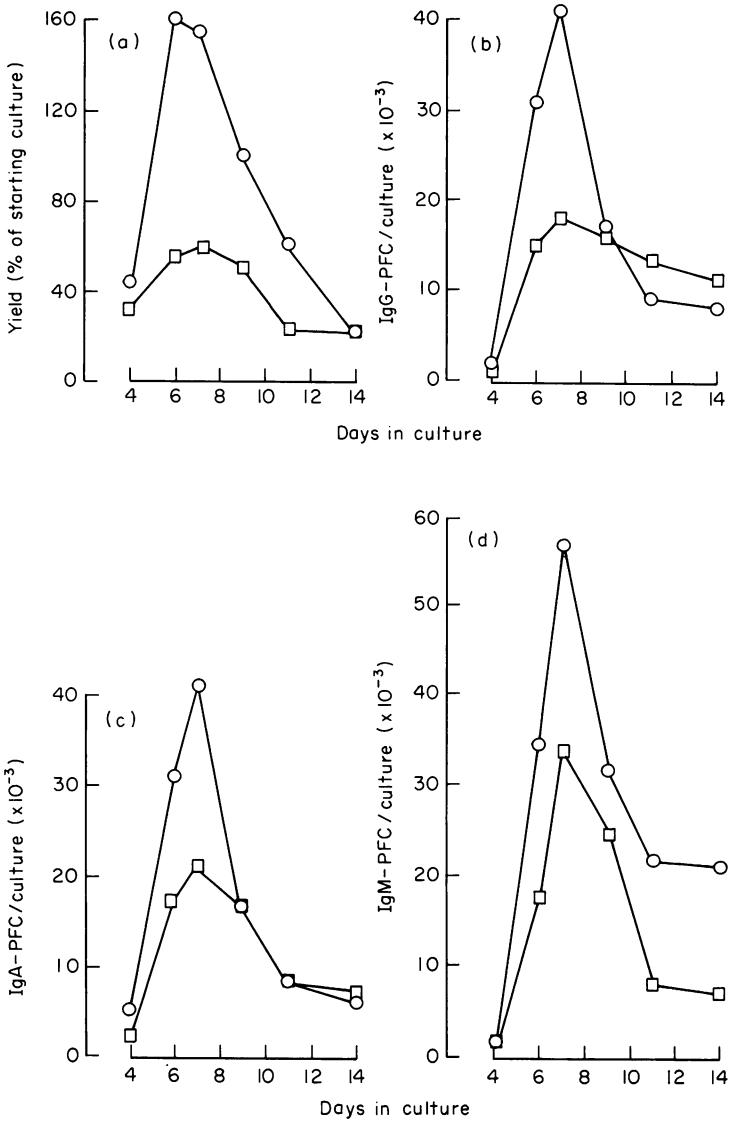

Fig. 1. Kinetics of cell growth and PFC development in PWM stimulated cultures. (a) Cell yields, expressed as percentages of the starting cell number; (b) IgG PFC/culture; (c) IgA PFC/culture; (d) IgM PFC/culture. □——□ = sham treated PWM stimulated PBL; ○——○ = OKT8 treated PWM stimulated PBL.

different findings could be related to the source of rabbit complement used or the sensitivity of the assay system for evaluating residual helper or suppressor activity; alternatively purified T cells may be more susceptible to complement lysis after OKT treatment than a population of cells also containing B cells and monocytes.

The results obtained in this study have confirmed that a co-culture system employing unfractionated PBL can be used to identify the precise nature of cellular defects in cases of humoral immunodeficiency, ie. when T-dependent PWM fails to induce significant PFC generation, discrimination can be made between B cell deficiency, T_H deficiency and T_S hyperactivity. The validity of the co-culture method hinges on there being minimal allogeneic effects when PBL from unrelated individuals are mixed and stimulated with PWM. Other workers have found that Ig

secreting B cells (Garovoy, Reddish & Abbas, 1979) and both helper (Chiorazzi, Fu & Kunkel, 1979) and suppressor (Hirano & Nordin, 1976) influences may be generated in mixed lymphocyte cultures, but in a previous study (Jones, 1981) we found no PFC generation in allogeneic co-cultures lacking PWM and neither enhancement nor suppression in PWM stimulated normal allogeneic co-cultures (O/E 0·81–1·25). Differences in culture conditions for the one-way mixed lymphocyte reaction and the two-way PWM stimulated co-culture, notably a much higher cell concentration in the latter, may account for these conflicting findings.

When lymphocytes from one of the co-cultivants are rich in *in vivo* activated T_S (e.g. cord blood lymphocytes) O/E values are severely reduced, due to suppression of PFC generation in the control population (Jones, 1981). Conversely, as has been shown here, PBL entirely depleted of T_H by three cycles of treatment with OKT4 antiserum and complement can generate PFC when co-cultured with unfractionated normal PBL, giving enhanced O/E values. Normal PBL could in fact provide sufficient help to entirely reconstitute PWM-induced PFC generation in an equal number of T_H depleted PBL, since (a) sham treated PBL (xPFC/10^6) plus autologous OKT4 depleted PBL (0 PFC/10^6) produced as many PFC/10^6 as sham treated PBL alone, ie. $O/E = x/\frac{1}{2}(x+0) = 2{\cdot}0$ (Table 1), and (b) untreated plus allogeneic sham treated and untreated plus allogeneic OKT4 treated PBL gave similar numbers of PWM-induced PFC (Table 2).

Of particular interest was the finding that a single treatment cycle with OKT4 antiserum failed to reduce subsequent PFC responses even though at least one half of the $OKT4^+$ cells had been killed. This would suggest either that $OKT4^+$ cells are heterogeneous with respect to T_H function, those cells most susceptible to complement-mediated killing providing relatively little helper activity, or more likely, that in normal PBL there is a relative excess of T_H which can be reduced by at least one half without affecting the response to PWM.

The second aspect of T regulation examined here was activation of T_S by con A. This is generally assessed by culturing PBL for 24–48 hr with mitogenic doses of con A and subsequently assessing the ability of activated T_S to suppress reactivity in a second culture of fresh PBL (Hubert, Delespesse & Govaerts, 1976; Shou, Schwartz & Good, 1976). The method has been simplified by incubating PBL with both PWM and con A, the number of PFC generated from normal PBL being reduced compared with PWM stimulated cultures without con A (Haynes & Fauci, 1976; Pryjma *et al.*, 1980; Jones, 1981). In this study it was shown that removal of $OKT8^+$ T_S prior to incubation with PWM and 4 μg/ml con A abrogated suppression, but when con A was added at 12·5 or 25 μg/ml, suppression was seen in $OKT8^-$ cultures (Table 3). Damle & Gupta (1982) have shown that precursers of con A inducible T_S reside in both $OKT4^+$ and $OKT8^+$ populations, and it would appear from our results that $OKT8^+$ T_S are induced by 4 μg/ml con A, but that higher concentrations may activate $OKT8^-$ T_S.

PWM-induced PFC development in untreated PBL peaked on day 7 and thereafter declined rapidly. This was not due to exhaustion of culture nutrients nor induction of $OKT8^+$ T_S by PWM, since feeding sham or OKT8 treated cultures with fresh medium on alternate days did not extend the time course of PFC development. Instead it would appear that Ig secreting B cells or T_H have an intrinsically limited life span *in vitro*.

Approximately twice as many PFC developed in $OKT8^-$ cultures compared with sham treated cultures when both initially contained $0{\cdot}5 \times 10^6$ cells (Fig. 1), but enhancement was no longer evident when the initial cell concentration was increased to $1{\cdot}0 \times 10^6$/ml (results not shown). In fact optimal PFC responses were obtained in cultures initially containing only $0{\cdot}1$–$0{\cdot}2 \times 10^6$ $OKT8^-$ PBL, and in these cases the final yield of cells on day 7 were equivalent to those obtained in cultures of sham treated PWM stimulated PBL at $0{\cdot}5$–$1{\cdot}0 \times 10^6$/ml, and the number of PFC/10^6 cells cultured was 4–6 times higher in OKT8 depleted cultures. It is likely that unrestricted growth in $OKT8^-$ cultures leads to overcrowding and non-specific suppression of the PFC response when the initial cell concentration is greater than $0{\cdot}2 \times 10^6$/ml, whereas sham treated cultures become overcrowded only when initial cell concentrations are higher than $1{\cdot}5 \times 10^6$/ml.

The observed enhancement of the PFC response following OKT8 depletion could form the basis of new methods for assessing T_S function. The presence of high numbers of PWM-induced PFC in untreated PBL and failure to produce significantly increased numbers of PFC after OKT8 treatment would be indicative of T_S deficiency, while recovery of PWM responsiveness in previously

unresponsive PBL after OKT8 depletion would indicate hyperactivity of T_S. This approach could also be used to assess the functional activity of T_S involved in antigen specific reactions by using antigen coated ShE as target cells in the plaque assay (McLachlan *et al.*, 1981). For example, normal subjects might show increased numbers of PWM-induced autoantigen specific PFC after T_S depletion, while patients lacking specific T_S would show no such increase. This possibility is currently being investigated in patients with autoimmune thyroiditis, using thyroid extract coated ShE as target cells in the plaque assay.

While the simple co-culture technique employing unfractionated patient's and control PWM stimulated PBL can yield preliminary information as to the nature of cellular defects responsible for lack of reactivity in the PrA-HPA, definitive information can only be obtained by admixing defined, highly purified lymphocyte subpopulations from the patient and the normal control. Monoclonal antisera are likely to prove invaluable in the isolation of lymphoid cell subsets for such assays; for example the presence or absence of PWM-induced PFC in mixtures of control OKT4 depleted PBL and patient's $OKT4^+$ cells would clearly define T_H status, while T_S hyperactivity could be investigated by adding patient's $OKT8^+$ cells to normal unfractionated PBL. The clinical applicability of such procedures awaits confirmation.

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