Immunology 1987 **61** 167–172

Heterogeneity in the activation requirements of T cells stimulated by phytohaemagglutinin

H. S. WARREN & A. BEZOS *Cancer Research Unit, Woden Valley Hospital, Woden, Australian Capital Territory, Australia*

Accepted for publication 9 February 1987

SUMMARY

Data are presented showing that resting T cells proliferated in response to phytohaemagglutinin (PHA) provided that either sheep erythrocytes (SRC) or interleukin-2 (IL-2) was added. Proliferation requiring PHA and SRC was inhibited by anti-CD2 monoclonal antibody (OKT11) and anti-CD3 monoclonal antibody (OKT3). These monoclonal antibodies only partially inhibited IL-2 production stimulated by PHA and SRC, and added IL-2 did not restore proliferation in these cultures. Low concentrations of cyclosporine inhibited both proliferation and IL-2 production ($ED_{50}=12$ and 9 ng/ml, respectively) and this inhibition was partially relieved in the presence of added IL-2 ($ED_{50}=110$ ng/ml). In contrast, proliferation stimulated by PHA with exogenous IL-2 was not inhibited in the presence of OKT11, and moderate concentrations of cyclosporine were required to inhibit proliferation ($ED_{50}=49$ ng/ml). Evidence was obtained, from analysis of cultures at limiting dilution, to suggest that the different requirements for stimulation of PHA-responsive T cells reflected different T-cell subpopulations. The majority of PHA-responsive cells proliferated in the presence of PHA and SRC, whereas only a minority proliferated in the presence of PHA and exogenous IL-2.

INTRODUCTION

The 50,000 molecular weight (MW) CD2 molecule associated with the sheep erythrocyte (SRC) receptor on human T lymphocytes is important in T-cell activation. The SRC receptor was considered a 'negative signal' receptor (Palacios & Martinez-Maza, 1982) since a number of monoclonal antibodies to the CD2 molecule (OKT11A, 9·6 and 35·1) suppressed T-cell proliferation to antigens and mitogens (Palacios & Martinez-Maza, 1982; Reed *et al.*, 1985; Tadmori *et al.*, 1985), suppressed interleukin-2 (IL-2) receptor expression defined by anti-TAC (Reed *et al.*, 1985), and suppressed interleukin-2 (IL-2) production (Tadmori *et al.*, 1985) at a pretranslational level (Tadmori, Kant & Kamoun, 1986). Several reports, however, documented a role for SRC in augmenting T-cell responses to phytohaemagglutinin (PHA) (Wilkinson & Morris, 1984; Ebert, 1985).

It is now evident that the CD2 molecule is an important site through which T cells can be directly activated. T-cell activation occurs in the presence of certain combinations of monoclonal antibodies recognizing different epitopes on the CD2 molecule (Meuer *et al.*, 1984; Fox *et al.*, 1985; O'Flynn *et al.*, 1986). This activation is independent of accessory cells, involves endogenous IL-2 production and is regulated through the Ti–CD3 complex (Fox, Schlossman & Reinherz, 1986). The identity of the natural ligand for the CD2 molecule is under investigation. This ligand may be a lymphokine involved in amplifying the immune response (Milanese, Richardson & Reinherz, 1986), or may be a 42,000 MW protein isolated from the SRC membrane that regulates T-cell proliferation (Hünig, 1985, 1986).

In this report it is demonstrated that resting T cells proliferated in response to PHA by the addition of either SRC or IL-2. The majority of PHA-responsive T cells were stimulated in the presence of SRC, a process involving the CD2 molecule, and accompanied by IL-2 production. A minority of PHA-responsive T cells proliferated in the presence of exogenous IL-2 by a process apparently independent of the CD2 molecule.

Abbreviations: IL-2; interleukin-2; PBMC, peripheral blood mononuclear cells; PHA, phytohaemagglutinin; SRC, sheep erythrocytes.

Correspondence: Dr H. S. Warren, Cancer Research Unit, Woden Valley Hospital, PO Box 11, Woden, ACT 2606, Australia.

MATERIALS AND METHODS

Tissue culture reagents

Tissue culture medium was Eagle's medium (410-1500; Gibco, Grand Island, NY) containing 100 μg/ml penicillin, 100 μg/ml streptomycin, 80 μg/ml neomycin, 60 μg/ml gentamycin, 24 mM $NaHCO_3$, and 0·1 mM 2-mercaptoethanol. Proliferation assays contained 10% heat-inactivated fetal calf serum. Lymphokine production cultures contained 1% human AB serum (Warren & Pembrey, 1981a). The gas phase for all cultures was 10% CO_2, 7% O_2, and 83% N_2.

Phytohaemagglutinin (PHA-P) was purchased from Commonwealth Serum Laboratories (Melbourne, Australia). Leucoagglutinin was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden).

IL-2-containing lymphokine (IL-2) was prepared by stimulation of tonsil lymphocytes with PHA-P as described previously (Warren & Pembrey, 1981b). This preparation is free of residual mitogen. In some experiments recombinant IL-2 was used (RO 23-6019, Hoffman La Roche, Nutley, NJ). The amount of IL-2 used in all cultures was an amount that allowed maximum proliferation of human T-cell blasts in a 2-day assay (Warren & Pembrey, 1981b). This amount was between 3 and 5 ED_{50} units/ml for crude IL-2 and was 125 ED_{50} units/ml for recombinant IL-2. Sheep red blood cells (SRC) were purchased from Commonwealth Serum Laboratories, Melbourne, Australia. Bovine and rabbit blood was obtained by venepuncture and was stored in Alsever's solution. Erythrocytes were sedimented and washed free of lymphocytes before use.

Monoclonal antibodies were culture supernatants prepared from hybridoma cell lines obtained from the American Type Culture Collection (Rockville, MD). The culture supernatants were OKT11 (CRL 8027), OKT3 (CRL 8001) and OKT8 (CRL 8014) and these had IgG2a concentrations of 4 μg/ml, 3·6 μg/ml and 9·6 μg/ml, respectively. Advice on determination of antibody concentrations was kindly given by Australian Monoclonal Development, Sydney, Australia.

Cyclosporine was a gift from Sandoz Ltd, Basel, Switzerland. It was supplied as a 50 mg/ml concentrate for intravenous infusion containing cremophore EL (65%, w/v) and ethanol (26%, w/v). A 1/1000 dilution was prepared and subsequent dilutions were prepared from this 50 μg/ml stock. Dilutions from the original concentrate were prepared fresh for each experiment.

Lymphocyte isolation

Buffy coats were prepared from normal donors attending the Red Cross Blood Transfusion Service of the Australian Capital Territory. Peripheral blood mononuclear cells (PBMC) were isolated by flotation on Ficoll–Hypaque (Pharmacia). Resting T cells were isolated from PBMC by a previously described procedure (Warren *et al.*, 1983). Briefly, PBMC were cultured with IL-2 for 3 days to allow the activation/growth of IL-2-responsive cells. These cells were separated from IL-2 non-responsive lymphocytes by centrifugation over Percoll (Pharmacia) of density 1·063 g/ml. IL-2-responsive cells were discarded in the lighter fraction, with the IL-2 non-responsive cells collected in the denser fraction., The yield of IL-2 non-responsive lymphocytes was on average 14% of the initial number of PBMC cultured. These cells were greater than 95% E-rosette positive (Warren *et al.*, 1983), and between 85% and 95% were $CD3^+$ (Warren, 1984).

Lymphocyte cultures

Resting T cells (5×10^4) were cultured in triplicate 0·2-ml samples of medium in round-bottomed wells (Linbro trays 76-013-05). Unless stated otherwise, the medium contained PHA-P (5 μg/ml), 0·05% SRC ($2·5 \times 10^6$/well) and/or IL-2. Proliferation was measured on Day 3. For lymphokine production cultures, 25 μg/ml of PHA were used, and supernatants were harvested at 24 hr (Warren & Pembrey, 1981a).

Measurement of lymphocyte proliferation

Proliferation of lymphocytes was measured by the incorporation of [^{3}H]thymidine. One μCi (1 Ci = 37 GBq) of [^{3}H]methyl thymidine (Amersham Corp., Arlington Heights, IL) was added to 0·2-ml cultures, and the incubation was continued for 5 hr (or 18 hr for limit dilution cultures). The cells were harvested onto glass fibre paper with a Dynatech C103 cell culture harvester. The dried paper was counted in liquid scintillation fluid in a Packard Liquid Scintillation Counter.

Estimates of the number of PHA-responsive T cells

The number of PHA-responsive T cells was measured by limiting dilution analysis as described previously (Warren *et al.*, 1983). For each dilution of resting T cells, 24–36 wells were set up and these contained PHA-P (5 μg/ml), IL-2, and SRC (0·05%). Identical cultures were set up containing 8 ng/ml OKT11. Cultures were assayed on Day 7. Wells were considered positive if the amount of isotope incorporated exceeded, by 3 SD, the background level of [^{3}H]thymidine retained on the glass fibre paper from multiple wells containing no cells. Data were plotted as the logarithm of the fraction of negative wells against the number of resting T cells per well, and the number of cells plated giving 37% negative wells was recorded. By Poisson distribution, this is when there is on average one precursor cell per well (Fischer-Lindahl & Wilson, 1977). Results are expressed as the mean percentage of PHA-responsive cells in the resting T-cell population, and the 95% confidence limits are given (Diem & Lentner, 1970).

Measurement of IL-2

IL-2 reactivity in lymphokine preparations was assayed on the murine IL-2-dependent cell line CTLL-2 (kindly provided by Dr R. Ceredig, JCSMR, Australian National University, Canberra). The CTLL-2 cells were maintained by twice weekly subculture in tissue culture medium containing IL-2. The gas phase for these cultures was 10% CO_2 in air. Serial dilutions of lymphokine supernatants were cultured with 10^4 CTLL-2 cells in 0·1 ml medium in flat-bottomed wells (Linbro trays 76-032-05). Cultures were harvested at 24 hr following a 5-hr pulse with 0·5 μCi of [^{3}H]thymidine. IL-2 activity is expressed as the reciprocal of the dilution required to give 50% of maximum growth (ED_{50} units), relative to a standard IL-2 preparation.

Statistical analysis

Data were compared using the Wilcoxon signed rank test.

RESULTS

Resting T cells proliferated when cultured with either PHA and IL-2 or PHA and SRC

The T cells used in these studies were a high-density subpopulation of PBMC which had been selected by their inability to respond to IL-2 during a 3-day culture (Warren *et al.*, 1983). By this criterion, the T cells were defined as a resting population.

The results in Table 1 show that the resting T cells did not proliferate when cultured with IL-2, and proliferated poorly when cultured with PHA alone. The resting T cells proliferated when cultured with PHA and IL-2, confirming earlier results (Warren *et al.*, 1983). Recombinant IL-2 replaced crude IL-2 in these cultures. T-cell proliferation stimulated by PHA alone was higher when measured on Day 2 compared to Day 3 (data not shown), indicating that stimulation with PHA produced IL-2 that was sufficient for a limited proliferative response. At higher PHA concentrations measurable levels of IL-2 were produced

Table 1. Resting T cells proliferate in the presence of either PHA and SRC or PHA and IL-2

				Proliferation (c.p.m. × 10^{-2})		
T cells	PHA	IL-2	SRC	16,090*	15,825	15,493
+	−	+	−	<1	<1	<1
+	+	−	−	2±0	6±3	3±1
+	+	+	−	86±5	111±4 (137±2)	68±11 (112±4)
+	+	−	+	173±38	213±4	82±4
+	+	+	+	195±2	253±5 (196±7)	169±21 (167±28)
+	−	−	+	<1	<1	<1
+	−	+	+	<1	<1	<1

T cells (5×10^4/0·2 ml) were cultured for 3 days with, as indicated, PHA (5 μg/ml), SRC ($2{\cdot}5 \times 10^6$/culture) and crude IL-2 or recombinant IL-2 (values in parentheses).

* Donor number.

(Table 4), and this was sufficient to sustain proliferation until Day 3 without the need for additional IL-2 (data not shown).

The results in Table 1 also show that the resting T cells proliferated when stimulated with PHA and SRC. Significant proliferation required as few as one SRC to one T cell (Fig. 1), although SRC to T cell ratios of 50:1 were used in most of the studies. Specific interaction of SRC with T cells appeared necessary since the effect of SRC could not be duplicated by bovine or rabbit erythrocytes, which were unable to bind to human T cells (data not shown). T-cell proliferation still occurred, although to a lesser extent, if T cells were treated with PHA and washed prior to culture with SRC (data not shown). The results demonstrated that PHA together with SRC provided adequate signalling for T cells to initiate and maintain a proliferative response.

When resting T cells were cultured with PHA and both SRC and IL-2, proliferation was always greater than proliferation measured in cultures containing PHA and exogenous IL-2, and was usually greater than proliferation stimulated by PHA and SRC (Table 1). No proliferation occurred when the resting T cells were cultured with SRC or SRC and IL-2 in the absence of PHA.

For the experiments described in this paper, identical results were obtained using leucoagglutinin, the highly purified form of PHA. Therefore, it was unlikely that the PHA preparation used contained different mitogenic components (O'Flynn *et al.*, 1986) which then stimulated T-cell proliferation in the presence of either SRC or added IL-2.

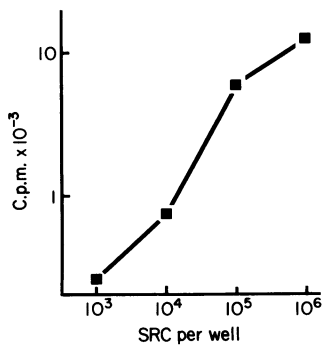

Figure 1. Resting T cells (5×10^4) were cultured for 3 days with PHA (5 μg/ml) and SRC at the numbers indicated.

The effect of OKT11 and OKT3 on T-cell proliferation stimulated by either PHA and SRC or PHA and IL-2

The anti-CD2 monoclonal antibody, OKT11, inhibited T-cell proliferation stimulated by PHA and SRC, but was without effect on proliferation stimulated by PHA with exogenous IL-2 (Table 2). OKT11 did not stimulate T-cell proliferation in the presence of IL-2. OKT11 inhibited proliferation stimulated by PHA and SRC at SRC:T cell ratios of 2:1 and 50:1. If OKT11 was added to cultures containing PHA, SRC and IL-2, then proliferation was reduced to the levels measured in cultures containing only PHA and IL-2. Therefore, addition of IL-2 did not relieve the inhibition caused by OKT11. For six different donors, proliferation stimulated by PHA and SRC was abrogated by OKT11 in the concentration range 16 ng/ml. T-cell proliferation stimulated by PHA and SRC was not inhibited in the presence of a control monoclonal antibody, OKT8, at concentrations as high as 600 ng/ml. The anti-CD2 monoclonal antibody used in the studies described in Table 2 did not inhibit T-cell binding to neuraminidase-treated SRC. Rosette formation was inhibited when the OKT11-treated T cells were incubated at 37° with rabbit anti-mouse immunoglobulin before incubation with SRC (data not shown). This treatment would induce capping of the CD2 molecule, and this has been reported to give optimum inhibition of rosetting with some anti-CD2 monoclonal antibodies (Verbi *et al.*, 1982). Therefore, OKT11 must be interacting with the CD2 molecule at a site distant from the SRC binding site and must be inhibiting T-cell proliferation at a step following binding of SRC to T cells.

The anti-CD3 monoclonal antibody, OKT3, inhibited proliferation of T cells stimulated by PHA and SRC (Table 3). This result was obtained with three different donors, and in all cases proliferation stimulated by PHA and SRC was abrogated at 225 ng/ml of OKT3. The addition of IL-2 to these cultures did not

Table 2. OKT11 inhibits T-cell proliferation stimulated by PHA and SRC but not T-cell proliferation stimulated by PHA and IL-2

	Proliferation (c.p.m. × 10^{-2}/culture)		
OKT11 (ng/ml)	PHA+SRC	PHA+IL-2	PHA+SRC+IL-2
0	134±3	67±5	132±9
0·5	128±2	72±9	135±8
1	123±10	69±2	135±8
2	115±6	74±3	115±10
4	69±3	66±6	117±10
8	21±1	70±6	91±8
16	7±1	71±10	74±4
32	6±1	70±5	70±6

T cells (5×10^4/0·2 ml) were cultured for 3 days with, as indicated, PHA (5 μg/ml), SRC (10^5/culture) and IL-2.

Table 3. The effect of OKT3 on T-cell proliferation stimulated by PHA and SRC compared to T-cell proliferation stimulated by PHA and IL-2

OKT3 (ng/ml)	Proliferation (c.p.m. × 10^{-2}/culture) PHA+SRC	PHA+IL-2	PHA+SRC+IL-2
0	156 (<1)	43 (<1)	137 (<1)
7	123 (<1)	39 (17)	118 (9)
14	112 (<1)	40 (20)	109 (13)
28	77 (<1)	41 (27)	100 (21)
56	52 (<1)	40 (29)	95 (28)
112	30 (<1)	38 (29)	73 (30)
225	6 (<1)	35 (30)	57 (28)
450	2 (<1)	32 (28)	44 (33)

T cells (5×10^4/0·2 ml) were cultured for 3 days with, as indicated, PHA (5 μg/ml), SRC (10^5/culture) and IL-2. Results are the mean of triplicate cultures. Values in parentheses show the proliferation stimulated by OKT3 in the absence of PHA.

significantly increase proliferation over that measured in cultures containing PHA, IL-2 and OKT3. The magnitude of proliferation in cultures containing PHA with exogenous IL-2 was not reduced in the presence of OKT3. OKT3 with IL-2, in the absence of PHA, stimulated T-cell proliferation. Therefore it cannot be determined whether OKT3 inhibited T-cell proliferation stimulated by PHA with exogenous IL-2.

IL-2 is produced by PHA and SRC stimulation of T cells

The results in Table 4 show that resting T cells produced some IL-2 when stimulated with PHA. This was anticipated since some T-cell proliferation occurred in the presence of PHA alone (see above). The amount of IL-2 produced was up to 10 times greater when the resting T cells were stimulated with PHA and SRC. OKT11 and OKT3 only partially inhibited IL-2 production when tested at concentrations sufficient to inhibit totally proliferation stimulated by PHA and SRC (Tables 2 and 3). The same result was obtained using lower PHA concentrations of 5 μg/ml, which stimulated less IL-2 production.

Table 4. OKT11 and OKT3 partially inhibit, but do not abrogate, IL-2 production from T cells stimulated by PHA and SRC

	Units of IL-2 produced			% inhibition of IL-2 production			
				OKT11 (ng/ml)		OKT3 (ng/ml)	
Donor	PHA	SRC	PHA+SRC	32	16	450	225
18,732	1·5	<0·02	13·0	47%	32%	19%	0%
19,494	0·7	<0·02	7·1	53%	45%	43%	29%
16,985	1·7	<0·02	10·5	53%	34%	25%	9%

IL-2 produced from resting T cells (10^5/0·2 ml) during 24 hr of culture with PHA (25 μg/ml) and SRC ($2·5 \times 10^6$/culture). IL-2 activity expressed as ED_{50} units was compared to a standard IL-2 preparation, measured using the IL-2-dependent murine CTLL-2 cell line.

The effect of cyclosporine on the stimulation of resting T-cells with either PHA and SRC or PHA and IL-2

Cyclosporine is an immunosuppressive agent which at pharmacological concentrations inhibits T-cell activation following interaction with antigen or mitogen (Hess, 1985). T-cell proliferation stimulated either by PHA and SRC or by PHA with exogenous IL-2 was inhibited by cyclosporine, but the concentration required for 50% inhibition (ED_{50}) differed by four-fold (Table 5). T-cell proliferation stimulated by PHA and SRC was sensitive to low concentrations of cyclosporine ($ED_{50} = 12$ ng/ml). IL-2 production stimulated by PHA and SRC was sensitive to equally low concentrations of cyclosporine ($ED_{50} = 9$ ng/ml), indicating that cyclosporine inhibited T-cell proliferation at the level of IL-2 production. The cyclosporine sensitivity of these cultures decreased in the presence of added IL-2 ($ED_{50} = 110$ ng/ml). This concentration of cyclosporine was significantly different ($P < 0·05$) to that giving the same degree of inhibition of proliferation in cultures containing PHA with exogenous IL-2 ($ED_{50} = 49$ ng/ml).

Different T cells proliferate when stimulated with either PHA and SRC or PHA and IL-2

Cultures were analysed at limiting dilution to examine whether different T-cell subpopulations were stimulated by PHA and SRC compared to PHA with exogenous IL-2. For this analysis, limiting numbers of T cells were cultured with PHA, SRC and IL-2 to allow proliferation of all PHA-responsive cells. Identical cultures were analysed in which OKT11 was included to inhibit the PHA- and SRC-stimulated component of the response. Cultures were scored as either responding or not responding, and from these data the percentage of PHA-responsive cells was estimated (Fischer-Lindahl & Wilson, 1977). If the same T cells

Table 5. The effect of cyclosporine on T-cell proliferation and IL-2 production stimulated by PHA and SRC and on T-cell proliferation stimulated by PHA and IL-2

	Concentration of cyclosporine (ng/ml) for 50% inhibition				
	T-cell proliferation			IL-2 production	
Donor	PHA + SRC	PHA + IL-2	PHA + SRC+IL-2	Donor	PHA + SRC
1559	10	50	100	49,103	2
43,426	14	28	100	35,118	18
16,090	8	40	100	6791	6
15,825	22	45	125		
15,493	8	80	125		
Mean±SD	12±6	49±19	110±14		9±8

Conditions for T-cell proliferation and IL-2 production are those described in the footnotes to Tables 1 and 4.

were stimulated by either PHA and SRC or by PHA and exogenous IL-2, then estimates of the number of PHA-responsive cells would be the same in both cultures. A summary of the data for seven donors is presented in Table 6. In all cases, the number of PHA-responsive cells was substantially reduced in cultures containing OKT11. For the different donors, between 5% and 45% (mean 16·7%) of resting T cells responded to PHA in the presence of both SRC and IL-2. When the PHA- and SRC-stimulated component of the response was inhibited by the presence of OKT11, the number of T cells responding to PHA was reduced by a factor of 3–18, with a mean of only 3% of T cells responding.

DISCUSSION

The results presented in this paper showed that the proliferation of resting T cells in response to PHA required either costimulation with SRC or exogenous IL-2. The data obtained support the notion that these two pathways for stimulation of T-cell proliferation were fundamentally different, and involved different T-cell subpopulations. Only T-cell proliferation requiring PHA and SRC was inhibited by anti-CD2 monoclonal antibody, demonstrating the requirement for the CD2 molecule in this activation process. Analysis of cultures at limiting dilution showed that the total number of PHA-responsive T cells estimated in cultures containing PHA with SRC and IL-2 was on average five times greater than the number of PHA-responsive T cells stimulated in identical cultures but where the SRC-stimulated component was inhibited by anti-CD2 monoclonal antibody. These data are most simply interpreted by there being two subpopulations of PHA-responsive T cells, one subpopulation stimulated by PHA and SRC and involving the CD2 molecule, and one subpopulation stimulated by PHA in the presence of exogenous IL-2. The possibility cannot be excluded that cells responding to PHA with exogenous IL-2 *also* responded to PHA and SRC. The possibility is unlikely, since in the presence of anti-CD2 and anti-CD3 monoclonal antibodies T cells stimulated by PHA and SRC produced IL-2, but proliferation in these cultures was totally inhibited.

Table 6. Estimates of the number of PHA-responsive T cells stimulated by either PHA, SRC and IL-2 or PHA and IL-2

	%-PHA-responsive cells*			
Donor	PHA+SRC +IL-2		PHA+SRC +IL-2 +OKT11†	
34,858	5·3	(4·3–6·3)	1·6	(1·2–2·5)
1559	11·0	(7·8–18·9)	2·0	(1·5–3·1)
43,426	4·8	(3·1–11·1)	1·5	(1·0–2·6)
15,825	16·7	(12·8–23·8)	4·0	(2·9–6·7)
14,274	23·0	(15·6–43·5)	8·9	(6·8–12·8)
16,090	45·0	(35·7–62·5)	2·5	(1·9–3·6)
6791	11·1	(8·5–17·9)	1·3	(1·0–1·9)
Mean ± 1 SD	16·7 ± 14·0		3·1 ± 2·7	

* Mean percentage responders, with 95% confidence limits in parentheses.

† OKT11 was added at a concentration of 8 ng/ml.

It has been reported previously that the CD2 molecule is the activation site through which PHA stimulates T-cell proliferation (O'Flynn *et al.*, 1985, 1986; Reed *et al.*, 1985). T-cell activation through the CD2 molecule can be stimulated directly using combinations of anti-CD2 monoclonal antibodies (Meuer *et al.*, 1984; O'Flynn *et al.*, 1986; Fox *et al.*, 1985). This activation site is regulated by, and is regarded as acting 'in-series' with, the Ti–CD3 activation site (Fox *et al.*, 1986). As with T-cell activation stimulated by the combinations of mitogenic anti-CD2 monoclonal antibodies, T-cell proliferation stimulated by PHA and SRC involved endogenous IL-2 production, did not require the addition of accessory cells, and was inhibited by anti-CD3 monoclonal antibody. Thus, T-cell proliferation stimulated by PHA and SRC may be an alternative method for stimulating T cells directly through the CD2 molecule. The T cells used in these studies were a resting population, therefore indicating that SRC were involved in initiating T-cell proliferation in response to PHA. The studies reported here extend earlier reports that documented a role for SRC in augmenting T-cell responses, but where the T cells used were not a selected resting population (Wilkinson & Morris, 1984; Ebert, 1985).

T-cell proliferation stimulated by PHA and SRC was inhibited by anti-CD2 and anti-CD3 monoclonal antibodies, and by cyclosporine, but apparently involving different mechanisms. IL-2 production was only partially inhibited by the monoclonal antibodies, and exogenous IL-2 did not restore proliferation in these cultures. In contrast, inhibition of proliferation by cyclosporine was by inhibition of IL-2 production, and some relief of inhibition occurred in the presence of exogenous IL-2. Although not tested, it is most likely that IL-2 receptor expression was inhibited by the anti-CD2 and anti-CD3 monoclonal antibodies, and was inhibited only at high concentrations of cyclosporine.

T cells stimulated by PHA with exogenous IL-2 were quiescent, since they did not proliferate when cultured with IL-2 alone, but were probably not naive, since stimulation by PHA alone initiated proliferation of these cells. T cells stimulated by PHA alone produced inadequate amounts of IL-2 to maintain proliferation, indicating that these T cells had a limited capacity for IL-2 production, or required additional stimulation to produce IL-2. Katzen *et al.* (1985) reported that IL-2 was not produced when resting T cells were stimulated with PHA and IL-2. Cells stimulated by PHA with IL-2 did express the CD2 molecule (data not shown), but proliferation could not be inhibited by anti-CD2 monoclonal antibody. Thus, the CD2 molecule is either not involved in activation of these cells, or is already predisposed in some way for activation such that proliferation is now refractory to inhibition by anti-CD2 monoclonal antibody. T-cell proliferation stimulated by PHA with exogenous IL-2 was inhibited by cyclosporine, but at different concentrations to that inhibiting T-cell proliferation stimulated by PHA and SRC. The identity of the T-cell subpopulation proliferating in the presence of PHA and exogenous IL-2 has yet to be determined.

The results presented here are consistent with earlier studies indicating that there is heterogeneity in the activation requirements of PHA-responsive T cells (Katzen *et al.*, 1985), but also provide evidence to suggest that this heterogeneity reflects

different subpopulations of PHA-responsive T cells. This conclusion may necessitate a re-evaluation of previous reports where the addition of IL-2 to PHA-stimulated T-cell cultures 'overcame' the inhibitory effect of anti-CD2 monoclonal antibodies (Reed *et al.*, 1985) and was used to assess immune response capability by T cells from immunodeficient patients (Warren *et al.*, 1983; Kruger *et al.*, 1984).

ACKNOWLEDGMENTS

This work was supported by a grant from the National Health and Medical Research Council of Australia. The support of Dr R. G. Pembrey, the excellent assistance of Ms Bronwyn Dachs, and the generous donations of Buffy Coats by the ACT Red Cross Blood Transfusion Service are gratefully acknowledged.

REFERENCES

DIEM K. & LENTNER C. (1970) *Scientific Tables*, 7th edn. Ciba-Geigy Ltd, Basel, Switzerland.

EBERT E.C. (1985) Sheep red blood cells enhance T-lymphocyte proliferation. *Clin. Immunol. Immunopathol.* **37,** 203.

FISCHER-LINDAHL K. & WILSON D.B. (1977) Histocompatibility antigen-activated cytotoxic T lymphocytes. I. Estimates of the absolute precursor frequency of killer cells generated *in vitro*. *J. exp. Med.* **145,** 508.

FOX D.A., HUSSEY R.E., FITZGERALD K.A., BENSUSSAN A., DALEY J.F., SCHLOSSMAN S.F. & REINHERZ E.L. (1985) Activation of human thymocytes via the 50KD T11 sheep erythrocyte binding protein induces the expression of interleukin 2 receptors on both $T3^+$ and $T3^-$ populations. *J. Immunol.* **134,** 330.

FOX D.A., SCHLOSSMAN S.F. & REINHERZ E.L. (1986) Regulation of the alternate pathway of T cell activation by anti-T3 monoclonal antibody. *J. Immunol.* **136,** 1945.

HESS A.D. (1985) Effect of interleukin 2 on the immunosuppressive action of cyclosporine *Transplantation*, **39,** 62.

HÜNIG T. (1985) The cell surface molecule recognized by the erythrocyte receptor of T lymphocytes. *J. exp. Med.* **162,** 890.

HÜNIG T.R. (1986) The ligand of the erythrocyte receptor of T lymphocytes: expression on white blood cells and possible involvement in T cell activation. *J. Immunol.* **136,** 2103.

KATZEN D., CHU E., TERHORST C., LEUNG D.Y., GESNER M., MILLER R.A. & GEHA R.S. (1985) Mechanisms of human T cell response to mitogens: IL 2 induces IL 2 receptor expression and proliferation but not IL 2 synthesis in PHA-stimulated T cells. *J. Immunol.* **135,** 1840.

KRUGER G., WELTE K., CIOBANU N., CUNNINGHAM-RUNDLES C., RALPH P., VENUTA S., FELDMAN S., KOZINER B., WANG C.Y., MOORE M.A. & MERTELSMANN R. (1984) Interleukin-2 correction of defective *in vitro* T-cell mitogenesis in patients with common varied immunodeficiency. *J. clin. Immunol.* **4,** 295.

MEUER S.C., HUSSEY R.E., FABBI M., FOX D., ACUTO O., FITZGERALD K.A., HODGDON J.C., PROTENTIS J.F., SCHLOSSMAN S.F. & REINHERZ E.L. (1984) An alternate pathway of T-cell activation: a functional role for the 50KD T11 sheep erythrocyte receptor protein. *Cell*, **36,** 897.

MILANESE C., RICHARDSON N.E. & REINHERZ E.L. (1986) Identification of a T helper cell-derived lymphokine that activates resting T lymphocytes. *Science*, **234,** 1056.

O'FLYNN K., KRENSKY A.M., BEVERLEY P.C.L., BURAKOFF S.J. & LINCH D.C. (1985) Phytohaemagglutinin activation of T cells through the sheep red blood cell receptor. *Nature (Lond.)*, **313,** 686.

O'FLYNN K., RUSSUL-SAIB M., ANDO I., WALLACE D.L., BEVERLEY P.C.L., BOYLSTON A.W. & LINCH D.C. (1986) Different pathways of human T-cell activation revealed by PHA-P and PHA-M. *Immunology*, **57,** 55.

PALACIOS R. & MARTINEZ-MAZA O. (1982) Is the E receptor on human T lymphocytes a 'negative signal receptor'? *J. Immunol.* **129,** 2479.

REED J.C., TADMORI W., KAMOUN M., KORETZKY G. & NOWELL P.C. (1985) Suppression of Interleukin 2 receptor acquisition by monoclonal antibodies recognizing the 50 KD protein associated with the sheep erythrocyte receptor of human T lymphocytes. *J. Immunol.* **134,** 1631.

TADMORI W., KANT J.A. & KAMOUN M. (1986) Down regulation of IL 2 mRNA by antibody to the 50-kd protein associated with E receptors on human T lymphocytes. *J. Immunol.* **136,** 1155.

TADMORI W., REED J.C,. NOWELL P.C. & KAMOUN M. (1985) Functional properties of the 50 kd protein associated with the E-receptor on human T lymphocytes: suppression of IL2 production by anti-p50 monoclonal antibodies. *J. Immunol.* **134,** 1709.

VERBI W., GREAVES M.F., SCHNEIDER C., KOUBEK K., JANOSSY G., STEIN H., KUNG P. & GOLDSTEIN G. (1982) Monoclonal antibodies OKT11 and OKT11A have pan-T reactivity and block sheep erythrocyte 'receptors'. *Eur. J. Immunol.* **12,** 81.

WARREN H.S. (1984) Differentation of NK-like cells from $OKT3^-$, $OKT11^+$, and $OKM1^+$ small resting lymphocytes by culture with autologous T cell blasts and lymphokine. *J. Immunol.* **132,** 2888.

WARREN H.S., ATKINSON K., PEMBREY R.G. & BIGGS J.C. (1983) Human bone marrow allograft recipients: production of, and responsiveness to, Interleukin 2. *J. Immunol.* **131,** 1771.

WARREN H.S. & PEMBREY R.G. (1981a) Lymphokine production by peripheral blood leukocytes: quantitation of T-cell growth facor activity for assessment of immune response capability. *Aust. N.Z. J. Med.* **11,** 475.

WARREN H.S. & PEMBREY R.G. (1981b) A method for the production and quantitative assay of human lymphokine preparations *J. immunol. Meth.* **41,** 9.

WILKINSON M. & MORRIS A.G. (1984) Role of the E receptor in interferon-gamma expression: sheep erythrocytes augment interferon-gamma production by human lymphocytes. *Cell. Immunol.* **86,** 109.