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In vitro mixed skin cell lymphocyte culture reaction (MSLR) in man: analysis of the epidermal cell and T cell subpopulations

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SUMMARY

The nature of normal human epidermal cells (EC) and peripheral blood (PB) cells that react *in vitro* in allogeneic mixed skin cell lymphocyte culture reaction (MSLR) was investigated using monoclonal antibodies (MCAB) specific for cell subpopulations. T cells and helper–inducer and suppressor–cytotoxic T cell subsets were defined by OKT3, OKT4, OKT8 MCAB, respectively, whereas, among EC, Langerhans cells were characterized by reactivity with OKT6 or anti-HLA-DR MCAB. MSLR were conducted with untreated cell suspensions as controls and cells suspensions depleted of various functionally active cell subset(s). Two approaches were used for cell depletion: (1) complement (C′)-mediated lysis by MCAB of T cells, T cell subsets, HLA-DR or OKT6 positive cells; (2) panning of PB cells or EC after pre-incubation with the appropriate MCAB to deplete or enrich (OKT6) cell suspensions with the respective cell subset. Responses in MSLR were abolished after treatment of PB cells with OKT3+C′ or OKT4+C′, significantly reduced with OKT8+C′; they were abolished after incubation of EC with anti-HLA-DR+C′ and significantly reduced with OKT6+C′. After panning, OKT3 and OKT4 depleted populations did not proliferate in MSLR while OKT8 depleted populations respond as controls. OKT6 depleted EC were not able to stimulate PB cells, yet proliferation rates were increased after stimulation by OKT6 enriched EC. Data show that helper-inducer T cells ($OKT3^+$; $OKT4^+$) play the major role in MSLR and that the presence of Langerhans cells is necessary for the stimulation of PB cells. They also suggest that co-operation between helper and suppressor cells is necessary for an optimal response. Differences in results using either OKT6 or anti-HLA-Dr-C′-mediated treatment of EC may be related to differences in the cellular expression of these markers by EC.

INTRODUCTION

In the *in vitro* mixed skin cell lymphocyte culture reaction (MSLR), epidermal cells (EC) are recognized by peripheral blood (PB) lymphocytes (L) which are stimulated to proliferate in culture (Lane, Jackson & Ling, 1975; Hirschberg & Thorsby, 1975; Beucher & Saurat, 1979). We (Faure *et al.*, 1982) and others (Morhenn, Engleman & Farber, 1978; Braathen & Thorsby, 1980) have shown that human PBL may proliferate upon stimulation with allogeneic and autologous EC. This MSLR exhibits characteristics of an immune response, memory and specificity and is in many points similar to the classical one way mixed lymphocyte reaction (MLR) (Tanaka & Sakai, 1979).

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Although it is generally considered that the responder cells are T cells, the nature of the T cell subpopulation proliferating in MSLR has not been clearly established. The stimulating ability of EC is usually related to the expression by human EC of HLA antigens, blood group substances and specific epidermal differentiation antigens (Bystryn, 1977) and/or to the presence among EC of Langerhans cells which express the HLA-DR-Ia like antigens (Klaresgog *et al.*, 1977; Rowden, Levis & Sullivan, 1977; Stingl *et al.*, 1978a, 1978b).

The aim of the study was to characterize the various cell types involved in the MSLR in man. We have examined the ability of human T cell subpopulations recognized by OKT3, OKT4 or OKT8 MCAB to respond in allogeneic MSLR, and that of OKT6 reactive (LC) EC to stimulate the proliferation of PB cells. The depletion or enrichment of cell populations in the various subsets considered was obtained either through complement-mediated lysis by the appropriate antibody or after separation on immunoglobulin (Ig) coated plates of the specifically reactive cells.

MATERIALS AND METHODS

Mixed skin cell lymphocyte culture reaction (MSLR). Normal human EC were obtained from fresh surgical skin specimens (healthy volunteers) through skin trypsinization as already described (Eisinger *et al.*, 1979; Faure, Eisinger & Bystryn, 1981). Peripheral blood lymphocytes were separated by the Ficoll-Hypaque method (Böyum, 1976). MSLR were performed in U bottomed microtitre plates (Cook Lab. Alexandria, Virginia, USA) as already described (Czernielewski *et al.*, 1982; Faure *et al.*, 1982). Briefly, in each well, 1×10^5 PB cells were co-cultivated with 1×10^5 allogeneic EC (responder:stimulator (R:S) ratio 1:1) in 0·2 ml RPMI 1640 (Eurobio, Paris, France) + 10% heat-inactivated AB human serum, 2 mM L-glutamine and antibiotics, at 37°C in a humidified atmosphere of 5% CO_2. Non-treated cells or cells pre-treated with monoclonal antibodies were studied. Cultures were established in triplicate. As controls EC alone and PB cells alone were cultured under culture conditions otherwise identical to those of MSLR. After 5 days of culture, ^{3}H-thymidine (Amersham, UK) was added (1 μCi/well) and the cells were harvested 18 hr later. Thymidine uptake was evaluated by means of liquid scintillation counting (SL 3000 Intertechnique France) and expressed as mean c.p.m. of triplicates.

Monoclonal antibodies. For isolation or lysis of T cells and T cell subsets, three MCAB were used: OKT3, OKT4, OKT8 (Ortho Pharmaceutical Corp., Raritan, New jersey, USA) (Reinherz *et al.*, 1979, 1980a, 1980b; Haynes, 1981).

OKT6 (Fithian *et al.*, 1981; Murphy *et al.*, 1981) was used to obtain EC suspensions depleted or enriched in functionally active OKT6$^+$ cells. Results in further MSLR were then compared to those obtained in parallel experiments using an anti-HLA-DR MCAB, named BL2 (J. Brochier, INSERM U 80, Lyon, France). Its specificity was shown to be identical to that monomorphic anti-HLA-DR; it precipitates with material from B cell membranes showing two bands (mol. wt 28 K and 33 K), and belongs to the IgG2b isotype and is able to fix complement.

Complement-mediated lysis of cell suspensions with MCAB. (a) PB cells: to obtain T cell populations depleted in functionally active OKT3, OKT4 or OKT8 reactive cells, PB cells were suspended in culture medium (5–10^6 cells/ml), to which was added OKT3, OKT4 or OKT8 MCAB (final dilution of 1:200). Cells were incubated for 45 min at 4°C, then washed three times. Fresh rabbit complement (C′) (Mérieux, France) was then added (final dilution 1:10) and incubation was carried out, 1 hr at 37°C. This procedure was repeated twice and cells washed twice before MSLR. As controls, only complement treated cells were used in MSLR.

(b) EC; 2×10^6 EC in medium were incubated with 0·2 ml of OKT6 MCAB (diluted 1:200) at 37°C for 45 min. Cells were then washed twice. C′ was then added (diluted 1:10) and incubation was then carried out in a fashion identical to that used with PB cells.

As controls, EC were incubated with C′ only, and with anti-HLA-DR MCAB (final dilution 1:20) and C′ in conditions otherwise identical to those used for C′-mediated lysis by OKT6.

Separation of cell subpopulations by immunoglobulin (Ig) coated plates. A modification of the plate technique described by Wysocki & Sato (1978) and Reinherz *et al.* (1981) was used. Briefly, plastic plates (Corning 14830, New York, USA) were coated with affinity purified goat anti-mouse

Ig (Tago, Inc, Burlingame California, USA). Fifteen to twenty million unfractionated PB cells, or 4–5 × 10^6 EC in culture medium were incubated with the appropriate antibody (OKT3, OKT4, OKT8 or OKT6; diluted 1:200) for 45 min at 4°C (PB cells) or 1 hr at room temperature (EC). Cells were next washed in PBS with 2% FCS, and 3 ml of suspension at a concentration of 5 × 10^6/ml (PB cells) 3–4 × 10^6/ml (EC) was applied to a single goat anti-mouse Ig coated plate. After 60 min, the supernatant was removed (non-adherent (NA) fraction). The NA fraction only of PB cells was used for MSLR. For study of EC suspensions, the adherent (A) cells were removed by scraping with a rubber policeman. The two fractions of EC suspensions, NA and A were used in MSLR. As controls, the NA fraction of PB cells and both A and NA fractions of EC were analysed in indirect immunofluorescence (IF) for the percentage of OKT3, OKT4, OKT8 or OKT6 positive cells.

Statistical analysis. Statistical analysis was performed using the Student's *t*-test, with $P < 0.05$ indicating significant differences.

RESULTS

MSLR with untreated cells

Table 1 shows the results obtained in 10 different experiments, using PB cells and EC from different donors in allogeneic MSLR. The ^{3}H-thymidine uptake, expressed as the mean ± s.d. of the 10 experiments was 4,506 ± 1,950 c.p.m. when PB cells were cultured alone and 41,536 ± 20,049 c.p.m. in MSLR. Results in MSLR were expressed by the stimulation indexes (SI), i.e. in each case, the ratio of the c.p.m. counts in MSLR to those obtained when PB cells were cultured alone, as insignificant c.p.m. counts (50 ± 20) were noted when EC were cultured alone and previous studies have shown that EC do not proliferate in MSLR (Czernielewski *et al.*, 1982). SI ranged from 4·5 to 19·9 (mean SI = 10·2 ± 5·1).

MSLR using PB cells after C′-mediated lysis with MCAB OKT3, OKT4, OKT8

After treatment of the PB cell suspensions by MCAB and complement, OKT3 treated suspensions contained 30 ± 10% viable cells, OKT4 treated suspensions 55 ± 10% and OKT8 treated 70 ± 15% viable cells. Viability of untreated or only C′ treated cells was higher than 95% (trypan blue exclusion test). Fig. 1 shows results obtained in 10 different allogeneic MSLR using 10 different PB cell suspensions and different EC in each case. In each case, results were expressed as the percentage of the stimulation obtained in allogeneic MSLR with the same untreated PB cells as controls. As

Table 1. Proliferative responses of PB lymphocytes in allogeneic MSLR

Exp†	Lymphocytes alone	Allogeneic L*/EC†	SI‡
A	3,753 ± 834§	29,748 ± 3,186	7·9
B	1,848 ± 327	10,046 ± 3,001	5·4
C	1,909 ± 401	38,442 ± 827	19·9
D	6,176 ± 1,271	38,442 ± 1,086	6·2
E	6,005 ± 1,301	74,037 ± 3,596	12·3
F	1,702 ± 804	25,850 ± 5,126	15·2
G	4,588 ± 627	66,933 ± 6,186	14·6
H	6,857 ± 381	68,245 ± 3,276	9·9
I	6,327 ± 1,003	28,445 ± 1,888	4·5
J	6,897 ± 821	35,611 ± 3,901	6·0
		Mean SI ± s.d. = 10·2 ± 5·1	

* L = lymphocytes.

† EC = epidermal cells.

‡ Stimulation index expressed as ratio L/EC c.p.m./L c.p.m. (L alone).

§ Mean c.p.m. ± s.d. (each experiment performed in triplicate).

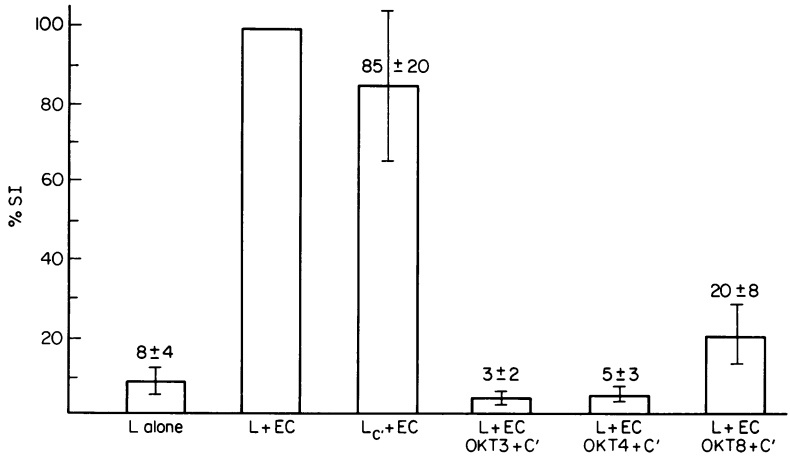

Fig. 1. Proliferative responses of PB cells in allogeneic MSLR using untreated cells (L+EC), complement treated (LC'+EC), monoclonal antibody OKT3, OKT4 or OKT8 and complement treated cells (respectively LOKT3+C'+EC; LOKT4+C'+EC; LOKT8+C'+EC). Results are expressed as means ±s.d. of percentages of results in control MSLR (L+EC). (L alone=cultures in the absence of EC).

seen in Fig. 1, after treatment by OKT3 and C', the proliferation was totally abolished (%3±2, $P<0{\cdot}001$). Similar results were obtained with OKT4 and C' treated cells (%5±3, $P<0{\cdot}01$). After treatment by OKT8 and C', only 20±8% of the maximal stimulation was observed ($P<0{\cdot}02$). No significant difference was noted between the stimulation observed with untreated and only C' treated cells.

MSLR with PB cell subsets separated with Ig coated plates and MCAB

After panning, the NA fraction was composed of less than 5% OKT3, OKT4 or OKT8 reactive cells. Viability was 95–99% in each case. The NA cell suspensions used in the MSLR were thus considered as OKT3, OKT4 or OKT8 reactive cell depleted populations (designated $OKT3^-$, $OKT4^-$ or $OKT8^-$ respectively). As seen in Fig. 2 no proliferation in allogeneic MSLR was

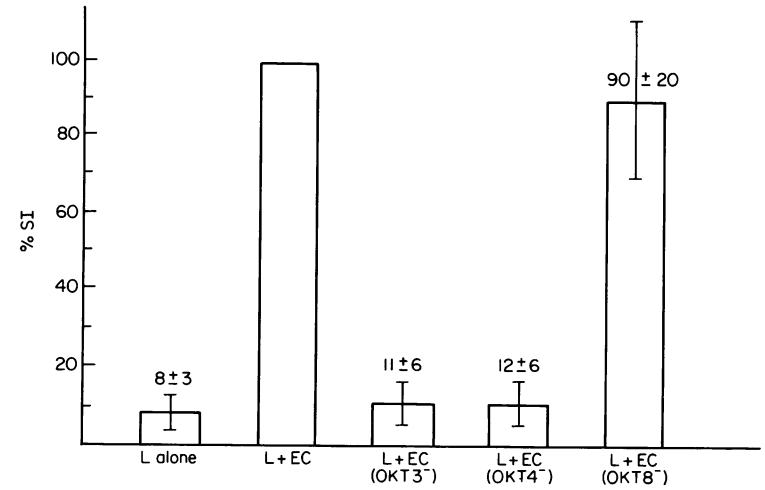

Fig. 2. Proliferative responses in allogeneic MSLR using PB cell suspensions depleted in T cells and T cell subsets through incubation in Ig coated dishes. Results using PB cells without EC (L alone) and in MSLR PB cell suspension depleted in T cells (L ($OKT3^-$)+EC), in helper T cells [L ($OKT4^-$)+EC) and in suppressor T cells (L $OKT8^-$)+EC) are expressed as means ±s.d. of percentages of responses in control MSLR (L+EC).

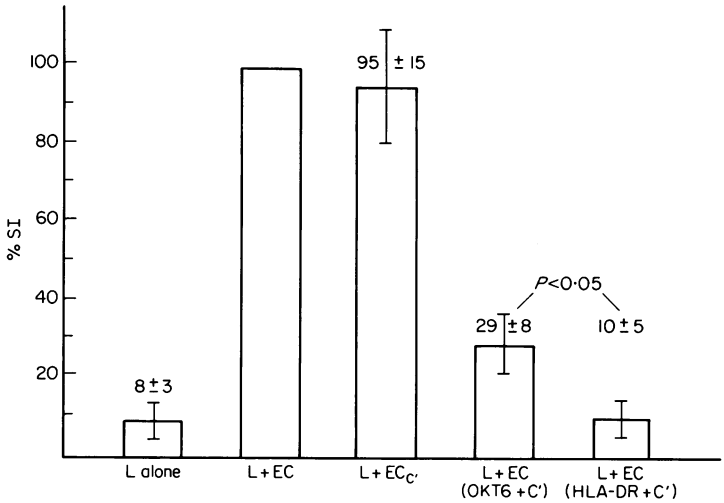

Fig. 3. Proliferative responses in allogeneic MSLR using untreated PB cells and either untreated EC (L+EC as controls) or EC suspensions preincubated with either OKT6 and complement (L+EC (OKT6+C')) or HLA-DR monoclonal antibody and complement (L+EC (HLA-DR+C')). Responses are expressed as means ±s.d. of percentages of responses in control MSLR (L+EC); L indicates proliferation rate of PB cells when cultured in the absence of EC; L+EC$_{C'}$, proliferation in MSLR using only complement incubated EC.

obtained using OKT3$^-$ or OKT4$^-$ populations (10 separate experiments in each case). The percentages of the maximal proliferation obtained were 11 ± 6 and 12 ± 6 respectively ($P < 0{\cdot}001$ as compared to results in control MSLR). No significant difference was noted between MSLR conducted with OKT3$^-$ cells and with OKT4$^-$ cells. Furthermore no significant reduction in the stimulation in MSLR was noted with OKT8$^-$ populations.

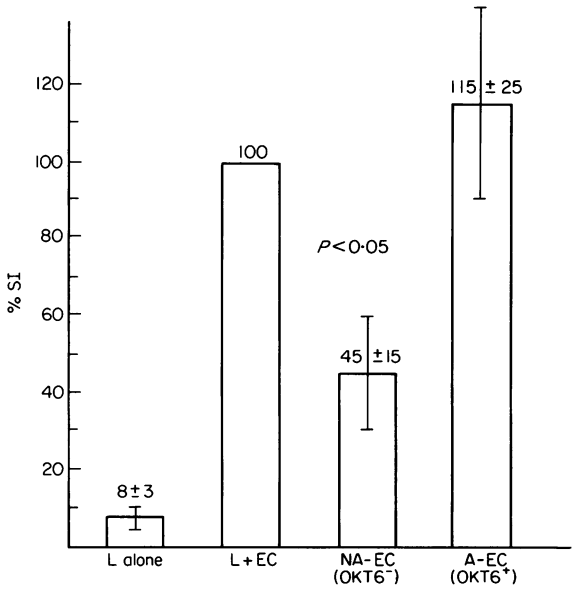

Fig. 4. Proliferative responses in allogeneic MSLR using as stimulator cells either Langerhans cells (OKT6$^+$) depleted suspensions (non-adherent: NA (OKT6$^-$) EC) or Langerhans cells enriched suspensions (adherent: A (OKT6$^+$) EC). Results are expressed as mean ±s.d. of percentages of control MSLR (L+EC) performed with original EC suspensions without incubation with OKT6 and further incubation in Ig coated dishes. (L alone = proliferation rate of PB cells in the absence of EC).

Allogeneic MSLR with EC pre-treated with OKT6+C′ or HLA-DR+C′

As seen in Fig. 3 (10 different experiments), stimulation with either OKT6+C′ or anti-HLA-DR+C′ treated cells was significantly reduced as compared to the stimulation using untreated EC($29 \pm 8\%$ $P < 0.05$ and $10 \pm 5\%$ $P < 0.001$ respectively). No significant difference was noted between MSLR performed with untreated or only C′ treated EC. The degree of reduction was significantly higher with anti-HLA-DR+C′ treated EC than with OKT6+C′ treated cells ($P < 0.02$). With anti-HLA-DR+C′ treated cells, the stimulation in MSLR did not differ from the spontaneous proliferation of PB cells cultured alone ($8 \pm 3\%$).

MSLR with EC populations depleted or enriched in OKT6 reactive cells

After panning, the unfractionated EC suspensions contained 2–4% of OKT6 positive cells, the A population 10–15%, the NA population 1–1·5% only (IF). As seen in Fig. 4, a significant reduction of the proliferation was obtained using the NA population ($45 \pm 15\%$) as compared to results in MSLR with unfractionated cells (100%; $P < 0.05$) (six experiments). Stimulation obtained with the A population was increased, but was not significantly higher ($115 \pm 25\%$) than that obtained with the unfractionated EC.

DISCUSSION

In the present study, two independant experimental approaches were used to analyse which cell populations participate in the MSLR. First, complement-mediated lysis using appropriate monoclonal antibodies was used to deplete functionally active cell subsets from either PB cells or EC suspensions. Second, these MCAB were utilized to isolate distinct immunoregulatory cell populations after panning as described recently (Reinherz *et al.*, 1981).

As shown here and also previously reported (Braathen & Thorsby, 1980) pre-incubation of EC in the presence of anti-HLA-DR serum plus C′ results in an inhibition of lymphocyte stimulation. Pre-incubation with anti-HLA-DR alone, without C′ (results in four experiments, not reported in the present study) does not inhibit the responses, which would suggest that HLA-DR positive cells are necessary and are the stimulting cells in MSLR. HLA-DR antigens have been demonstrated on epidermal langerhans Cells, the role of which as antigen presenting and allo-reactivating cells is now well established (Braathen & Thorsby, 1980, Stingl *et al.*, 1978c). In fact recent data suggest that HLA-DR antigens may well not be restricted to LC among EC (Tjernlund, 1981; Lampert, Suitters & Chisholm, 1981; Mason, Dallman & Barclay, 1981). Recently, human LC have been shown to express a membrane associated antigen which distinguishes them from classical monocytes (Fithian *et al.*, 1981). This distinctive surface differentiation antigen is identified by OKT6 MCAB, and represents a specific marker of LC (Murphy *et al.*, 1981). Using OKT6 plus C′, we found that such a treatment of EC suspensions leads to a significant reduction of the proliferate response in MSLR. The inhibition was less marked than that noted with anti-HLA-DR antibody plus C′. This might be related to a difference in the proportion of the cells that have been inactivated using either OKT6 or anti-HLA-DR and/or to the fact that not only LC but other EC express the HLA-DR antigens, as has been recently suggested (Tjernlund, 1981).

The Ig coated plate technique has already been utilized to isolate PB cell subsets after incubation of unfractionated T cells with the appropriate antibody specific for T cell differentiation antigen (Reinherz *et al.*, 1981). We have adapted this method in the present study to obtain OKT6 (LC) depleted or OKT6 enriched EC suspensions. The proliferation of PB cells in MSLR was significantly reduced when OKT6 depleted populations were used as stimulator cells. Moreover, OKT6 enriched populations stimulate more vigorously than the unfractionated EC, although this increase in the proliferation rate was not significant. This shows that the OKT6 MCAB itself does not inhibit the functional ability of LC *in vitro*, and that the Ig coated plate technique may usefully represent a new mode of enrichment of human EC in LC. These data are further evidence for the role of Langerhans cells (OKT6 reactive) in the stimulation of PB cells in MSLR.

Treatment of PB cell suspensions with OKT3 or OKT4 and C′ significantly inhibited the proliferation of PB cells in MSLR. These results correlate well with those obtained after panning.

OKT3 depleted and OKT4 depleted populations failed to proliferate in MSLR. This does not necessarily indicate that the helper/inducer T cells are the responding cell types but rather that these cells are necessary for a response to occur. Using a similar Ig coated technique, Engleman *et al.* (1981) recently demonstrated that helper/inducer cells are necessary in MLR.

Further evidence for the role of helper/inducer T cells in MSLR is that no difference was observed between results with NA (OKT8 reactive depleted) cells and the unfractionated populations. However, this differs from the 20% of the maximal response observed using PB cells after incubation with OKT8 and C′. This reduction of the response cannot be related to the effect of C′ as C′ alone had no significant effect. The reason for this divergence remains somewhat obscure. However, it was recently shown that similar lysis of suppressor/cytotoxic T cells may lead to a decrease in the proliferative response of T cells in allogeneic MLR (Damle *et al.*, 1981), although the rate of proliferation is still higher than that noted with helper/inducer depleted cells, as was also noted here in MSLR. The possibility that cooperation between $OKT4^+$ and $OKT8^+$ T cell subsets is necessary for optimal responses in MSLR cannot be excluded, but data presented here strongly suggest that helper/inducer T cells play a major role in allogeneic MSLR.

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