Immunology 1989 **68** 108–113

Properties of lymph-borne (veiled) dendritic cells in culture

II. EXPRESSION OF THE IL-2 RECEPTOR: ROLE OF GM-CSF

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Accepted for publication 27 May 1989

SUMMARY

Fresh lymph-borne (veiled) dendritic cells (L-DC) in the rat are almost totally negative for the interleukin-2 (IL-2) receptor detected by the monoclonal antibody (mAb) MRC OX39. After 16 hr culture more than 90% of L-DC are OX39 positive, and increased levels of expression can be seen within 5 hr culture. In cultures of L-DC and allogeneic lymphocytes, L-DC appear to express the IL-2 receptor more rapidly than lymphocytes. The intensity of labelling of L-DC is variable but maximal levels are similar to those seen on lymphoblasts. Culture in the presence of concanavalin A (Con A)-stimulated spleen cell supernatants or recombinant granulocyte–macrophage colony-stimulating factor (GM-CSF) results in a more rapid and intense expression of the IL-2 receptor by L-DC. L-DC cultured following rigorous T-cell depletion, or derived from athymic rats also express the IL-2 receptor after culture with GM-CSF. Cultured, but not fresh, L-DC bind iodinated recombinant IL-2 in a dose-dependent manner and binding is inhibited by excess unlabelled ligand. The amount of IL-2 bound varies but maximal amounts are similar to those bound by lymphoblasts. Following intravenous endotoxin injection, a large proportion of freshly collected L-DC express the IL-2 receptor and the number of L-DC released into the lymph is increased. An antibody to the IL-2 receptor which blocks an allogeneic MLR has no effect on a xenogeneic MLR using rat L-DC as stimulators and mouse lymphocytes as responders.

INTRODUCTION

The interleukin-2 (IL-2) receptor was first identified on activated T cells but has subsequently been described on several different cell types, including B cells (Lowenthal *et al.*, 1985), macrophages and monocytes (Herrmann *et al.*, 1985; Holter *et al.*, 1986; Hancock, Muller & Cotran, 1987; Malkovsky *et al.*, 1987; Wahl *et al.*, 1987), glial cells (Beneviste & Merrill, 1986) and Langerhans' cells (Steiner *et al.*, 1986). On T, B and glial cells IL-2 has a mitogenic effect and had been claimed to activate macrophages (Malkovsky *et al.*, 1987; Wahl *et al.*, 1987). Human natural killer (NK) cells respond to IL-2 but do not express the IL-2 receptor detectable by antibody (Tsudo *et al.*, 1987). No functional effects of IL-2 on Langerhans' cells have been described.

In the previous paper we described the properties of cultured lymph-borne dendritic cells (L-DC). Amongst the changes observed was the expression of an antigen subsequently shown to be the rat IL-2 receptor (Paterson *et al.*, 1987). In this paper we characterize the expression of the IL-2 receptor by L-DC and examine some aspects of the regulation of its expression. We also examine the role of the L-DC IL-2 receptor in T-cell activation.

Abbreviations: BSA, bovine serum albumin; CAS, supernatant from spleen cells cultured with Concanavalin A; FCS, fetal calf serum; GM-CSF, granulocyte-macrophage colony stimulating factor; [^{3}H]TdR, tritiated thymidine; IFN, interferon; IL-2, interleukin 2; L-DC, lymph-borne (veiled) dendritic cell; mAb, monoclonal antibody; MHC, major histocompatibility complex; MLR, mixed leucocyte reaction; PBA, phosphate-buffered saline with 10 mM sodium azide; PBS, phosphate-buffered saline; PLL, poly-L-lysine; TDL, thoracic duct lymph cells; XTDL, thoracic duct lymph cells from mesenteric-lymphadenectomized rats.

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MATERIALS AND METHODS

Animals and cells

Animals used and methods of cell collection, culture and immunocytochemistry are described in the accompanying paper (MacPherson, 1989). Congenitally athymic nude rats backcrossed to the PVG (RT1^c) strain were bred in the Dept. of Anatomy, University of Oslo (Fossum, 1984).

^{125}I-IL-2 binding assay

Fresh L-DC or cells cultured for 16 hr in Hb102 (NEN, Boston, MA) supplemented with a 10% concentration of supernatant

from Con A-stimulated spleen cell cultures (CAS) were washed and resuspended in phosphate-buffered saline (PBS) containing 10 mM sodium azide (PBA) at 4°. Ten microlitres of a suspension of cells at 5×10^5/ml were allowed to adhere to poly-L-lysine-coated 12-spot slides (Hendley, Loughton, Essex) for 30 min at 4°. Ten microlitres of cold fetal calf serum (FCS) were added for 5 min to block poly-L-lysine (PLL) binding sites; this was sucked off and replaced with 10 μl ^{125}I-IL-2 in PBA, containing 0–4×10^4 c.p.m. ^{125}I, with or without a 200–400-fold excess of unlabelled IL-2. After incubating at 4° for 40 min, slides were washed for 5–10 seconds with ice-cold PBA from a wash bottle, dipped in 5% BSA, dried, and autoradiographed in Ilford K5 emulsion. To assess labelling quantitatively, grains were counted over an artibrary area of a cell, defined by an eyepiece graticule, including the majority of the cell body. Background counts were estimated by counting an equal area of adjacent blank slide.

^{125}I-IL-2 was recombinant human IL-2, purchased from NEN, Chicago, IL. The specific activity was 20–50 μCi/μg.

Xenogeneic MLR

MLR's were set up as described earlier (MacPherson, 1989). Responder cells were allogeneic (rat) TDL or spleen cells (RT1^a), or mouse spleen cells (C57Bl or BALB/c). Stimulator cells were RT1^c L-DC enriched to 60–80% by metrizamide separation, or mouse (C57Bl or BALB/c) spleen cells, all irradiated with 10 Gy. In some experiments mouse responder spleen cells were depleted of cells expressing Ia by treatment with an anti-Ia monoclonal antibody (a kind gift from Dr J. M. Austyn, John Radcliffe Hospital, Oxford) and complement.

Endotoxin treatment

Normal or irradiated lymphadenectomized rats with thoracic duct cannulae, after one overnight collection of lymph, were injected, via a tail vein cannula, with 50–200 μg of endotoxin (*Salmonella typhimurium*; Difco, East Moseley, Surrey) over a period of 10 min. Lymph samples were collected for up to 3 days after treatment, quantified and processed for immunocytochemistry.

RESULTS

Cultured L-DC up-regulate IL-2 receptor expression

Between 0% and 5% of fresh L-DC were stained weakly by MRC OX39, specific for the rat IL-2 receptor. After 24 hr culture *in vitro*, the majority of L-DC and some lymphocytes expressed OX39. Cultured L-DC were also recognized by another mAb (C5a) specific for the rat IL-2 receptor (Dr M. Dallman, Nuffield Department of Surgery, John Radcliffe Hospital, Oxford). This mAb and MRC OX39 do not cross-react in competitive binding assays (M. Dallman, personal communication). When mixtures of the two mAbs were used to stain cultured L-DC, the proportion of positive cells was the same as when either antibody was used alone, but the intensity of staining increased (data not shown).

Cultured L-DC bind IL-2 specifically

To ascertain if the antigen identified immunocytochemically as the IL-2 receptor on L-DC was associated with IL-2 binding, we used ^{125}I-IL-2 in single cell binding assays. Fresh or cultured L-DC on PLL-coated microscope slides were incubated with different concentrations of ^{125}I-IL-2 for 40 min at 4° and autoradiographed. Preparations incubated with a 200–400-fold excess of unlabelled IL-2 were used as specificity controls. Positive controls were lymphocytes present in the L-DC preparation and spleen cells stimulated with Con A for 2–3 days.

The results (Table 1 and Fig. 1) show that most cultured, but few fresh, L-DC bound significant amounts of ^{125}I-IL-2 and that binding was completely inhibited by the addition of excess unlabelled IL-2. Comparison with IL-2 binding on activated T cells suggests that L-DC and T cells bind similar amounts of IL-2.

Table 1. ^{125}I-IL-2 binding to L-DC and Con A-stimulated spleen cells

Cell type	C.p.m. added	Cold IL-2	Mean GC (+1 SD)	Ratio cell/BG	% +ve cells (>2×BG)
Cultured L-DC	3×10^4	—	7·20±4·5	1·53	24
	6×10^4	—	16·30±8·7	3·01	74
	$1{\cdot}25 \times 10^5$	—	19·20+10·8	3·21	70
	$1{\cdot}25 \times 10^5$	400×	6·20+4·1	0·88	4
Fresh L-DC	3×10^4	—	9·60±5·9	1·18	6
	6×10^4	—	5·40±4·9	0·63	4
	$1{\cdot}25 \times 10^5$	—	5·80±4·3	1·0	8
Con A Spleen cells	3×10^4	—	8·06±5·6	2·40	48
	6×10^4	—	12·80±6·8	1·73	30
	$1{\cdot}25 \times 10^5$	—	8·70+5·6	1·81	40

L-DC were enriched from XTDL by centrifugation over Metrizamide and used fresh or after 24 hr culture in Hb102 supplemented with 10% CAS. Spleen cells were cultured for 72 hr in medium containing 1 μg/ml ConA and washed with α-methyl mannoside before use. Cells were adhered to PLL-coated slides and incubated with different concentrations of ^{125}I-IL-2 for 40 min at 4°. After a brief wash they were air dried and autoradiographs made. Grain counts were made over standard areas, defined by an eye-piece graticule, of 50 cells for each preparation. Background counts were made over blank areas of the same size and subtracted from the cell counts. Cells were counted as positive if they had more than twice the number of background grains per unit area.

GC, grain count; BG, background.

Up-regulation of IL-2 receptor expression by cultured L-DC is rapid and partially dependent on GM-CSF

Metrizamide-separated L-DC were cultured with or without CAS or rGM-CSF and cytospin preparations stained for the IL-2 receptor at different intervals. Preparations were coded and L-DC classified as negative, weakly or strongly stained, using OX6 staining (αIa) to indicate maximal staining intensity and to estimate the overall frequency of L-DC in cytospin preparations.

In the presence of CAS, increased IL-2 receptor expression was detected within 5 hr, and by 24 hr over 90% of L-DC were stained (Fig. 2b). Without CAS, IL-2 receptor expression was slower and weaker (Fig. 2a).

When murine rGM-CSF, at 50 U/m, was added to cultures

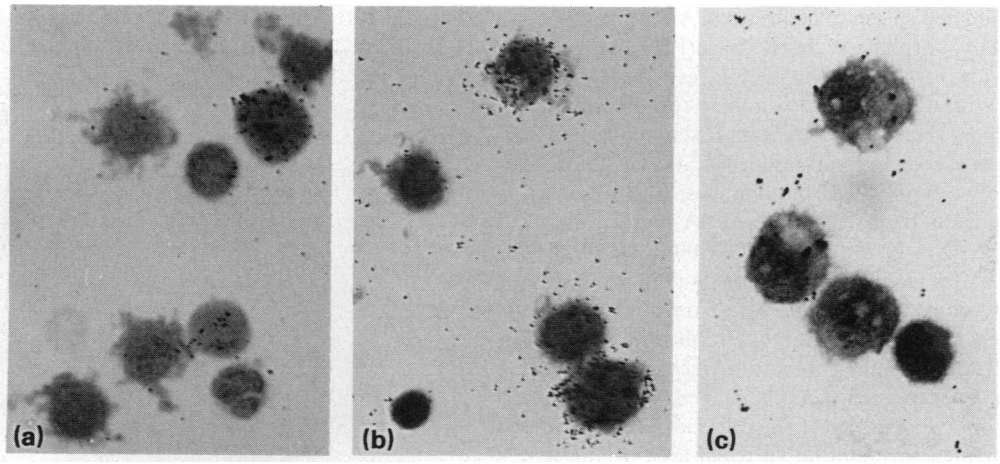

Figure 1. Fresh or cultured L-DC or spleen cells cultured with Con A for 72 hr were adhered to PLL-coated microscope slides and incubated at 4° for 40 min with ^{125}I-IL-2 at differing concentrations with or without a 400-fold excess of unlabelled IL-2. After rapid washing they were air-dried and autoradiographs prepared. (a) Fresh L-DC. L-DC are unlabelled. A lymphoblast shows moderate labelling. (b) Cultured L-DC. Three L-DC show heavy labelling. One L-DC and a small lymphocyte are unlabelled. (c) Cultured L-DC with excess unlabelled IL-2. No cells show significant labelling.

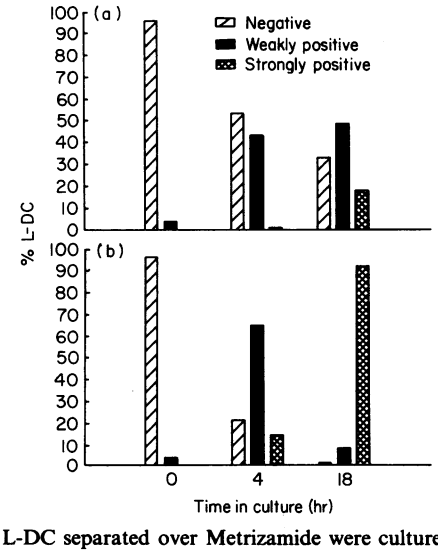

Figure 2. L-DC separated over Metrizamide were cultured in Hb102 either without (a) or with (b) the addition of 10% CAS. After 4 or 18 hr cultures were harvested and cytopsin preparations stained for the presence of the IL-2 receptor using MRC OX39. Preparations were coded and L-DC scored as negative, weakly or strongly positive.

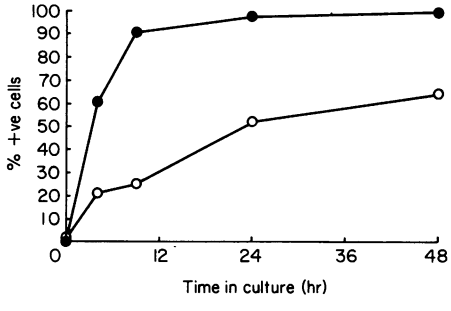

Figure 3. L-DC separated over Metrizamide were cultured with a 10-fold excess of allogeneic lymphocytes in the presence of 50 U/m GM-CSF for different intervals and cytospin preparations stained for the IL-2 receptor using MRC OX39. L-DC were scored as negative or positive (●). Lymphocytes were scored when they were in contact with a L-DC (clustered; ○).

of L-DC, IL-2 receptor expression was induced as rapidly and intensely as with CAS (Figs 2a and 3).

The kinetics of IL-2 receptor expression in L-DC were compared with those of lymphocytes by culturing L-DC with a 10-fold excess of allogeneic lymphocytes in the presence of GM-CSF. At intervals, cells were gently resuspended and cytospin preparations stained for IL-2 receptor expression. Figures 3 and 4 show that the expression of the IL-2 receptor was up-regulated at least as rapidly in L-DC as in lymphocytes. The maximal intensity of staining was similar for L-DC and lymphoblasts, suggesting a similar level of expression of IL-2 receptor.

L-DC from nude rats express the IL-2 receptor after culture

To rule out the possibility that the L-DC passively acquired the IL-2 receptor or functional and antigenic fragments from it, we collected L-DC from nude rats. L-DC were enriched on Percoll gradients (Fossum, 1988) yielding a purity of 70–90%, the majority of contaminating cells being B lymphoblasts. Fresh nude L-DC were uniformly negative for the IL-2 receptor but after 24 hr culture with CAS 92·5% were positive, and with GM-CSF (50 U/ml) 89·8% were positive (Fig. 5). As can be seen from the figure, most of the contaminating cells were negative for the IL-2 receptor even after culture with CAS or GM-CSF.

Anti-IL-2 receptor antibody does not block a xenogeneic MLR

To investigate the role of the L-DC IL-2 receptor in lymphocyte activation, we used a xenogeneic MLR, with rat L-DC as stimulator and mouse spleen cells as responder cells. An anti-

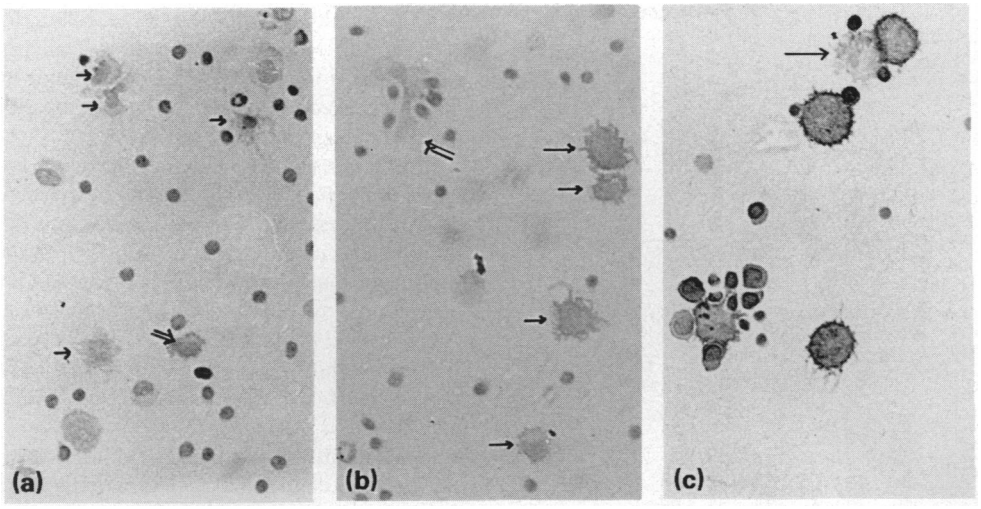

Figure 4. Photomicrographs of cells from the experiment shown in Fig. 3 stained with MRC OX39. (a) Fresh ×200. Most L-DC are negative (single arrows) but one is weakly positive (double arrow). (b) 9 hr culture, ×320. Most L-DC are weakly or moderately positive (single arrows but one is negative (double arrow). (c) 24 hr culture, ×320. Four L-DC are moderately or strongly positive but one (arrow) remains negative. Lymphoblasts and lymphocytes adherent to one L-DC are positive. The apparent intensity of staining of L-DC and lymphocytes is similar.

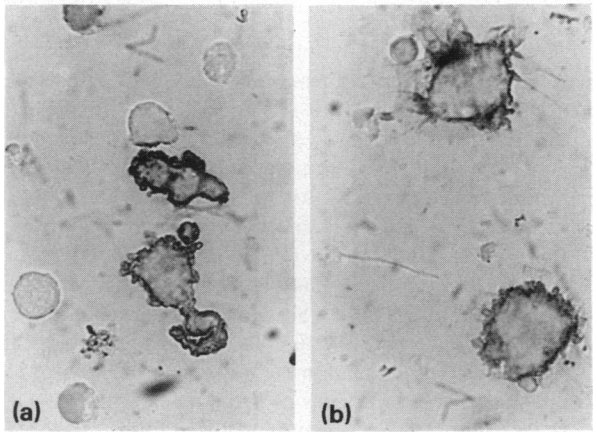

Figure 5. L-DC were separated from XTDL collected from athymic (nude) rats and cultured in the presence of 10% CAS (a) or 50 μ/ml rGM-CSF (b) for 24 hr. Cytospin preparations were stained for expression of the IL-2 receptor using MRC OX39. In these experiments 92·5% (a) and 89·8% (b) of L-DC were positive. Note that in (a) four lymphocytes are negative for OX39.

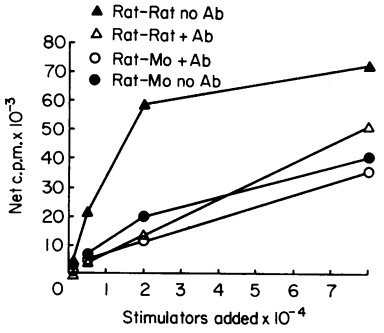

Figure 6. Mixed lymphocyte reactions were set up using graded numbers of rat spleen cells as stimulators and 5×10^5 rat TDL or mouse (MO) spleen cells as responders. Cells were cultured for 4 days with or without the addition of a saturating amount of anti-IL-2 receptor antibody (purified IgG) or an equivalent amount of a non-binding antibody. Results are expressed as net c.p.m. after subtraction of background counts.

IL-2 receptor antibody effectively blocked a rat–rat MLR but had no effect on the rat–mouse MLR (a typical experiment is shown in Fig. 6). To exclude the possibility of rat MHC antigens being processed and presented by mouse cells, the responder population was treated with a cytotoxic anti-mouse Ia monoclonal antibody and complement. The stimulation of mouse responder cells by rat L-DC was not affected by this treatment.

Endotoxin stimulates IL-2 receptor expression by L-DC *in vivo*

Endotoxin is a powerful stimulant for GM-CSF release by macrophages. Data presented above shows that GM-CSF is a major stimulant of IL-2 receptor expression *in vitro*. To see whether stimulation of expression could be achieved *in vivo*, normal or irradiated lymphadenectomized rats were cannulated and injected with 50–200 μg of bacterial endotoxin. Lymph samples were collected and cytospin preparations stained with OX39 to display the IL-2 receptor. Increased IL-2 receptor expression was detected within 4 hr of of endotoxin administration and at the time of peak IL-2 receptor expression by L-DC there was no significant increase in IL-2 receptor expression by lymphocytes (Fig. 7a). Endotoxin added directly to cultures of L-DC had no effect on IL-2 receptor expression or on L-DC survival (data not shown).

Endotoxin stimulates L-DC release into peripheral lymph

Total cells and L-DC were quantified in the lymph samples described above. The numbers of L-DC collected between 6 hr

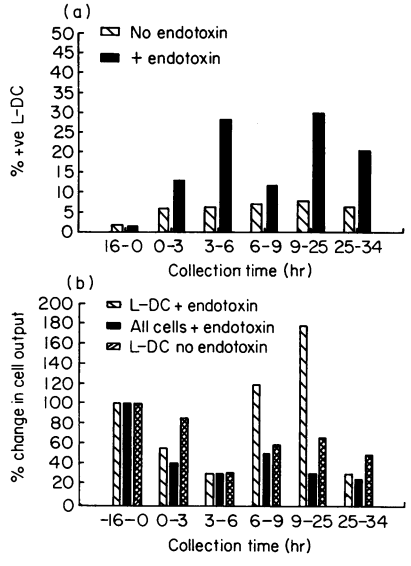

Figure 7. Five irradiated MLNX rats were cannulated and, 24 hr later, three injected i.v. with 200 μg endotoxin. The remaining rats were given an equivalent volume of PBS. Lymph was collected for 34 hr after injection and total cell numbers and the numbers of L-DC quantified. (a) Cytospin preparations were stained for the IL-2 receptor using MRC OX39, coded and the numbers of positive L-DC counted. (b) The graph shows the changes in L-DC output with or without endotoxin and in total cell output after endotoxin.

and 25 hr after giving endotoxin increased by more than three-fold (Fig. 7b). In control rats L-DC made up about 5% of all cells during this period but in the treated animals the proportion rose to almost 50%.

DISCUSSION

Originally described on T lymphocytes, the IL-2 receptor has now been shown to be expressed on B cells (Lowenthal *et al.*, 1985), monocytes (Herrmann *et al.*, 1985) and Langerhans' cells (Steiner *et al.*, 1986). The IL-2 receptor exists in high and low affinity forms and the high-affinity form includes a novel chain (Sharon *et al.*, 1986; Dukovich *et al.*, 1987). On T lymphocytes, only the high affinity receptor has been shown to mediate biological function (Robb *et al.*, 1984) and to internalize IL-2 (Fujii *et al.*, 1986; Weissman *et al.*, 1986). IL-2 has a functional effect in augmenting human monocyte cytotoxicity (Malkovsky *et al.*, 1987) and microbicidal activity (Wahl *et al.*, 1987) but it is not known if these cells express a high affinity receptor.

That L-DC can express the IL-2 receptor is shown by the staining of cultured L-DC with two antibodies recognizing discrete epitopes on the receptor (Paterson *et al.*, 1987; M. Dallman, personal communication) and by the binding of ^{125}I-IL-2 to cultured L-DC, inhibitable by excess unlabelled IL-2. The need to use single cell assays has so far precluded the characterization of the IL-2 receptor as low or high affinity. We also do not know if L-DC IL-2 receptors are able to mediate IL-2 internalization.

It seems unlikely that L-DC are cytophilically binding soluble IL-2 receptors (Rutin, Kirman & Fritz, 1985; Osawa, Josimovic-Alasevic & Diamanstein, 1986; Jaques *et al.*, 1987). IL-2 receptor expression by L-DC is blocked by cycloheximide but this could be affecting other cells. However, L-DC in cultures depleted of lymphocytes or from athymic rats rapidly expressed the IL-2 receptor when cultured with rGM-CSF and under these conditions there is little possibility of free IL-2 receptors being present.

The expression of the IL-2 receptor by L-DC increases remarkably quickly in culture, a marked increase being detectable within less than 5 hr. This rapidity contrasts with cultured Langerhans' cells (Steiner *et al.*, 1986) where only a small proportion was positive after 24–48 hr and it took 4–5 days for the majority of cells to express the receptor. Similarly, Herrmann *et al.* (1985) showed that a human macrophage-like line, U937, and normal human monocytes could be induced to express the IL-2 receptor, but that expression was only detected after 24–48 hr culture.

Our results show that rapid and strong IL-2 receptor expression is dependent on culture in the presence of GM-CSF. This observation contrasts with results obtained with monocytes, where interferon-gamma (IFN-γ) appeared to be the major inducer (Herrmann *et al.*, 1985). In our system, IFN-γ had no effect on L-DC survival or IL-2 receptor expression.

It remains to be shown if IL-2 mediates any functional effects on L-DC. IL-2 has been shown to increase monocyte cytotoxicity (Malkovsky *et al.*, 1987) and microbicidal activity (Wahl *et al.*, 1987), and to have a small effect on the proliferation of U937 cells (Herrmann *et al.*, 1985), but no effects on cells of the DC lineage have been reported. In lymphocytes functional effects depend on the expression of the high-affinity IL-2 receptor but at present we do not know if L-DC express this form. A potent anti-IL-2 receptor antibody which effectively blocks T-cell proliferation in an allogeneic MLR had no significant effects when added to a xenogeneic MLR, suggesting that the L-DC IL-2 receptor was not functionally important in this reaction, but it is difficult to relate this to *in vivo* activation of T cells by conventional antigens where the numbers of antigen-specific cells are much smaller. Should the IL-2 receptor have no functional effects on L-DC it might be that the presence of a low affinity receptor could increase the local concentration of IL-2 and thus increase the efficiency of T-cell activation.

We conclude that L-DC can be induced to express an IL-2 receptor rapidly both *in vitro* and *in vivo* by potentially physiological stimuli and that the expression may have a role in the primary activation of T cells.

ACKNOWLEDGMENTS

We are extremely grateful for the skilled technical assistance of Chris Jenkins. Pam Woodward typed the manuscript quickly and accurately. Dr A. F. Williams, Sir William Dunn School of Pathology, Oxford and members of the Cellular Immunology Unit generously supplied advice and antibodies. Stan Buckingham prepared the photographs. we are grateful to Dr R. M. Steinman, Rockefeller University, NY for his critical reading of the manuscript.

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