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Cell cycle arrest rather than apoptosis is associated with measles virus contact-mediated immunosuppression *in vitro*

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Acute measles is associated with pronounced immunosuppression characterized both by leukopenia and impaired lymphocyte functions. In an earlier study, we found that mitogen-dependent proliferation of uninfected human peripheral blood lymphocytes (PBLs) and spontaneous proliferation of human cell lines of lymphocytic or monocytic origin was impaired after contact with UV-inactivated, measles virus (MV)-infected cells, UV-inactivated MV or with cells transfected with MV glycoproteins (gp) F and H. We now show that mitogen-stimulated PBLs and Jurkat cell clones either highly sensitive or resistant to CD95-induced apoptosis have a similar sensitivity to MV-induced inhibition and do not undergo apoptosis. Moreover, unimpaired mitogen-dependent upregulation of important activation markers, including IL-2R, was observed in PBL cultures after contact with MV-infected, UV-irradiated presenter cells. This indicates that the cells were indeed viable and acquire a state of activation. Less IL-2 was released from PBLs after contact with MV-infected presenter cells when compared with that released after contact with uninfected cells. However, mitogen-induced proliferation of PBLs was not restored by addition of IL-2 under these conditions. It appeared that a higher fraction of mitogen-stimulated PBLs accumulated in the G0/G1 phase of the cell cycle after contact with MV-infected cells. Thus, the mitogen-unresponsiveness of PBLs seen after contact with MV-infected cells is due to cell cycle arrest rather than apoptosis.

Introduction

Acute measles is an early childhood disease that is still of great clinical importance due to its high rate of morbidity and mortality (Katz, 1995; Clements & Cutts, 1995). Following infection, an efficient measles virus (MV)-specific immune response is established which leads to clearance of the virus from the peripheral blood and to the generation of lifelong protective immunity (Griffin, 1995). At the same time, a marked lymphopenia, affecting both T and B lymphocytes, and immunological abnormalities are observed including an impairment of delayed type hypersensitivity reactions that lasts for weeks or months and an increased susceptibility towards opportunistic infections. When analysed *ex vivo*, peripheral blood lymphocytes (PBLs) isolated from patients with acute measles reveal a strongly impaired proliferative response to mitogens, allogens and recall antigens (Borrow & Oldstone, 1995; Ward *et al.*, 1995; Tamashiro *et al.*, 1987; Griffin *et al.*, 1987), while the profile of cytokines released from these cells indicates a predominant TH2-type response (Griffin, 1995; Ward & Griffin, 1993; Griffin *et al.*, 1989). In addition, inflammatory cytokines secreted from the monocyte/macrophage fraction of peripheral blood monocytic cells (PBMCs) from measles patients are altered (Ward *et al.*, 1995). The suppression of immune functions by MV is far from being understood. Although it is well documented that both lymphocytes and monocytes are the major targets during the acute infection, the frequency of infected cells is generally low throughout the disease (Nakayama *et al.*, 1995; Hyypiä *et al.*, 1985; Schneider-Schaulies *et al.*, 1991), and virus cannot be detected later than 2 weeks after the onset of the rash. Thus, indirect mechanisms have been proposed to play a central role in this suppressive process. More recently, evidence has been obtained in SCID-mice grafted with human PBLs that the age of the infected host may be a further parameter defining the extent of MV-induced immunosuppression (Tishon *et al.*, 1996).

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As shown *in vitro*, MV infection induces a state of mitogen-unresponsiveness in primary lymphocytes or is associated with growth inhibition of lymphocytic cell lines (McChesney *et al*., 1987, 1988; Yanagi *et al*., 1992). No reduction in the accumulation of transcripts associated with T cell activation such as IL-2, c-Myc, IL-2 receptor or IL-6 has been found in MV-infected T cell cultures (McChesney *et al*., 1988; Yanagi *et al*., 1992). As described in a previous report, neither release of IL-2 nor reactivity towards exogenously added IL-2 was found to be affected after MV infection of IL-2-dependent T cell lines or tonsillar lymphocytes (Borysiewicz *et al*., 1985). Although its molecular basis has not yet been identified, these and other studies indicated that cell cycle arrest might be central to the disruption of immune functions after MV infection of lymphocytes (McChesney *et al*., 1987, 1988; Yanagi *et al*., 1992).

Since only a minor fraction of PBMCs is actually infected *in vivo*, indirect mechanisms based on soluble factors or mediated by contact between uninfected and infected cells have been suggested to be involved in immunosuppression (Borrow & Oldstone, 1995; Yanagi *et al*., 1992). Recently, a strongly impaired synthesis of IL-12 by monocytes/macrophages after MV-induced CD46 ligation has been linked to the suppression of cell-mediated immunity by MV *in vitro* (Karp *et al*., 1996). We have adopted an *in vitro* system to study the interaction between MV-infected, UV-irradiated PBLs (used as 'presenter cells', PCs) and uninfected cells of lymphocytic or monocytic origin (referred to as responder cells, RCs) (Sanchez-Lanier *et al*., 1988). We found that a small number of infected PCs was able to induce a long lasting, profound inhibition of mitogen- and allogen-driven proliferation in uninfected PBLs, and that the induction of this inhibition was completely dependent on the expression of both MV glycoproteins, F and H, by the PCs (Schlender *et al*., 1996). No evidence for a direct effect of soluble factors, cell surface molecules other than F and H, or the MV receptor CD46 could be obtained in this system. Moreover, proliferation of permanent cell lines of lymphocytic and monocytic origin was affected after MV glycoprotein-contact, independent of the expression of the major MV protein receptor CD46. The present study aims to define the mechanisms underlying MV-induced proliferative inhibition *in vitro* at the cellular level by characterizing changes in the RCs after contact with MV-infected PCs. No evidence for the induction of apoptosis, dependent or independent of CD95, could be obtained in the RCs. In contrast, we found an unimpaired upregulation of cellular activation markers on the RCs. Remarkably, IL-2 release was reduced after MV-contact while proliferative inhibition could not be rescued after addition of exogenous IL-2. Our studies indicate that a G0/G1-arrest is induced in RCs after MV-contact.

Methods

■ **Cell isolation, cell lines and viruses.** PBMCs were isolated by Ficoll/Paque (Pharmacia) density gradient centrifugation of heparinized blood obtained from healthy adult donors with subsequent depletion of monocytes by plastic adherence. PBLs were cultured in RPMI 1640–10% FCS. Human lymphoid Jurkat (J16, JA16.6; $CD4^+$ T cells), BJAB (lymphoblastoid B cells), BJAB cells persistently infected with the Edmonston (ED) B (BJAB-EDp) or WTF (BJAB-WTFp) strain of MV and monocytic cells (HL60) were maintained in RPMI 1640 containing 10% FCS. MV vaccine strain Edmonston-B (ED) was propagated in Vero cells, WTF (wild-type strain) on BJAB cells.

■ **Immunohistochemistry and cell cycle analysis** Cell surface staining was performed using monoclonal antibodies directed against cell surface proteins (CD3, CD4, CD8, CD14, CD19, CD25, CD40L, CD45R0, CD69 Immunotec; CD71 Sigma; CD122, Il2-R_γ Coulter) or virus-specific proteins (MV-F, MV-H) following standard protocols. For annexin V staining, J16 cells (1×10^6 cells) were mixed with UV-irradiated BJAB or BJAB-EDp or BJAB-WTFp at a ratio of 1:4. As a control, J16 or JA16.6 cells were incubated with 250 ng/ml anti-human CD95 IgM-antibody (BIOMOL). Cells were harvested, washed once in PBS and resuspended in 190 µl binding buffer (10 mM HEPES, pH 7·4, 140 mM NaCl, 2·5 mM $CaCl_2$), mixed with 10 µl Annexin–FITC (final concentration 1 µl/ml) and 10 µl of 20 µg/ml propidium iodide stock solution. For TUNEL experiments, $2{\cdot}5 \times 10^6$ PBLs were kept in the presence of 1 µg/ml anti-CD3 (OKT3) monoclonal antibody together with IL-2 (100 U/ml) for 5 days. Cells were washed twice and apoptosis was induced by the addition of anti-CD95 IgM (5 µg/ml) to 1×10^6 cells for 16 h. TUNEL staining was performed following the manufacturer's protocol (Promega). For cell cycle analysis, cells were mixed with detergent solution (0·1% Triton, 0·15 M NaCl, 0·1 M HCl), centrifuged, resuspended in 50 µl RNase solution (RNase A 100 µg/ml, 1% trisodium citrate) and incubated at 37 °C for 15 min. Cells were washed in 0·1 M Tris–HCl pH 7·4, and stained for 10 min (50 µg/ml propidium iodide in 1% trisodium citrate) (Taylor & Milthorpe, 1980). Both immuno-fluorescence and propidium iodide stainings were analysed using FACScan.

■ ***In vitro* proliferation assay.** The assay was performed essentially as described previously (Schlender *et al*., 1996). In brief, presenter cells (PCs) (BJAB-ED, BJAB-WTF, HL-60-ED, BJAB-EDp or BJAB-WTFp) were stained for the expression of MV surface antigens and subsequently UV-irradiated (0·25 J/cm^2 in a biolinker). Proliferative inactivation was controlled after treatment by incorporation of [^{3}H]thymidine. If not described otherwise in the text, responder cells (RCs) (PBLs, J16 or JA16.6 cells) were seeded into a 96-well plate at a density of 5×10^4 (Jurkat cells) and 1×10^5 (PBLs, together with 2·5 µg/ml PHA) in a volume of 100 µl per well. The PCs were added at the concentrations indicated in a volume of 100 µl per well and were incubated for 48 or 72 h. When indicated, PCs were removed from the assay by immunomagnetic sorting using CD19 or CD14 beads. In some experiments, recombinant IL-2 (100 U/ml) was added to the assay. Proliferation rates were determined following a 16 h labelling with [^{3}H]thymidine (0·5 µCi/ml). Assays were performed in triplicate, harvested and the incorporation rates of the label were determined using a β-plate reader. Values of proliferative inhibition were always determined relative to those obtained with RCs cocultured with uninfected PCs.

■ **Cytokines.** For the determination of IL-2 release, 1×10^6 PBLs were cocultivated with 2×10^5 UV-irradiated BJAB or BJAB-EDp cells in a total volume of 500 µl per well. Supernatants were harvested after 24 h and Il-2 concentrations were determined using a commercial ELISA kit (DPC Biermann).

Results

Human PBLs and Jurkat clones defective in CD95-induced apoptosis are sensitive to MV-induced proliferative inhibition

As found in our previous studies, human peripheral blood lymphocytes or monocytes (presenter cells, PCs), lytically infected with MV and then UV-irradiated, efficiently prevent mitogen-dependent proliferation of uninfected PBLs or spontaneous proliferation of lympho- or monocytic cell lines (responder cells, RCs) in cocultivation assays (Schlender *et al.*, 1996). To further extend our studies and to exclude variables in infection efficiencies, a comparative analysis was carried out of the ability of BJAB cells lytically infected (BJAB-ED) or persistently infected (BJAB-EDp) with MV-ED to induce proliferative arrest of mitogen-stimulated PBLs. We found that BJAB-EDp cells were highly potent PCs in our system, as were BJAB-ED cells (Table 1). Moreover, BJAB cells persistently infected with the MV wild-type strain WTF (BJAB-WTFp) were as effective as BJAB-EDp (Table 1) or BJAB cells lytically infected with WTF (not shown). To test whether the same PCs would also interfere with spontaneous proliferation of T cells and whether proliferative inhibition could possibly be due to the induction of apoptosis, we used two Jurkat cell clones as RCs: J16, which is selected for high sensitivity to both PHA- and CD95-induced apoptosis, and JA16.6, a CD-95-resistant subclone (J. Borst, unpublished). The sensitive or resistant phenotype of the clones to CD95-induced apoptosis was tested prior to the cocultivation experiments (not shown). The proliferative activity of both cell lines was highly sensitive to cocultivation with BJAB-ED, BJAB-EDp and BJAB-WTFp cells over a wide range of PC/RC ratios (two PC/RC ratios are shown in Table 1), and no differences in the extent of inhibition were observed between J16 and JA16.6 cells. Similarly, treatment of both cell lines with cell-free, UV-inactivated MV at two different doses (corresponding to an m.o.i. of 1 and 5 of infectious MV) led to a dose-dependent proliferative arrest (Table 1). Although the inhibition was less pronounced than that observed with PCs, no significant differences were observed between J16 and JA16.6 cells. These findings indicate that a variety of presenter systems can be used to induce a state of unresponsiveness in PBLs or proliferative arrest in Jurkat cells. Moreover, as the response of J16 and JA16.6 was similar, CD95-mediated signalling was not likely to be involved.

Jurkat clones and PBLs do not undergo apoptosis after MV contact

J16 cells were cocultivated with UV-irradiated PCs (BJAB-EDp or BJAB-WTFp) at a PC/RC ratio of 1/4 or treated with anti-CD95 to induce apoptosis, which was subsequently detected by the binding of FITC-labelled annexin V to translocated phosphatidylserine. As expected, J16 revealed a significant increase in annexin V binding after treatment with anti-CD95 (representatively shown for a 3 h incubation period in Fig. 1*b* as compared to untreated cells, Fig. 1*a*). In contrast, binding of annexin V was not significantly enhanced when BJAB-WTFp or BJAB-EDp were used as PCs at any time during the cocultivation (representatively shown for a cocultivation time of 6·5 h in Fig. 1*c*, *d*). Thus, the signal provided by MV-infected PCs led to a proliferative arrest of J16 cells which was independent of apoptosis. To determine whether apoptosis might be involved in the proliferative inhibition of mitogen-stimulated PBLs after MV contact, we used the TUNEL detection system as PBLs spontaneously bound high amounts of annexin V. As a positive control, apoptosis was induced by anti-CD95 treatment of PBLs that had been prestimulated by anti-CD3 in the presence of 100 U/ml IL-2

Table 1. MV contact-mediated proliferative inhibition of Jurkat cell clones sensitive or insensitive to CD95-triggered apoptosis

Responder cells (RCs), either PHA-stimulated PBLs (*a*) or Jurkat cell lines J16 and JA16.6 (*b*) were cocultured for 72 h with infected or uninfected BJAB cells that had been UV-irradiated (presenter cells, PCs) at a PC/RC ratio of 1:1 or 1:16 or were cocultured with UV-irradiated MV-ED (corresponding to an m.o.i. of 1 or 5) followed by a 16 h labelling period. The different PC cells were BJAB-ED (BJAB cells lytically infected with MV-ED), BJAB-EDp (BJAB cells persistently infected with MV-ED) or BJAB-WTF (BJAB cells persistently infected with MV-WTF). Proliferative inhibition of RCs after coculture with MV-infected PCs or UV-MV was determined in comparison to uninfected BJAB cells and is indicated as a percentage. Values represent means of at least three independent experiments, each of which was carried out in triplicate. N.D. Not determined.

	BJAB-ED		BJAB-EDp		BJAB-WTFp		UV-MV	
RCs	1/1	1/16	1/1	1/16	1/1	1/16	m.o.i.	m.o.i.
PBLs[a]	97 ± 2	58 ± 9	96 ± 1·2	60 ± 0·5	96 ± 3	66 ± 11	ND	ND
J16[b]	89 ± 3	41 ± 4	99 ± 1	80 ± 5	98 ± 2	79 ± 12	44 ± 13	20 ± 1·4
JA16.6[b]	96 ± 4	38 ± 5	99 ± 1	95 ± 2	98·6 ± 2	85 ± 5	50 ± 11	29·5 ± 1

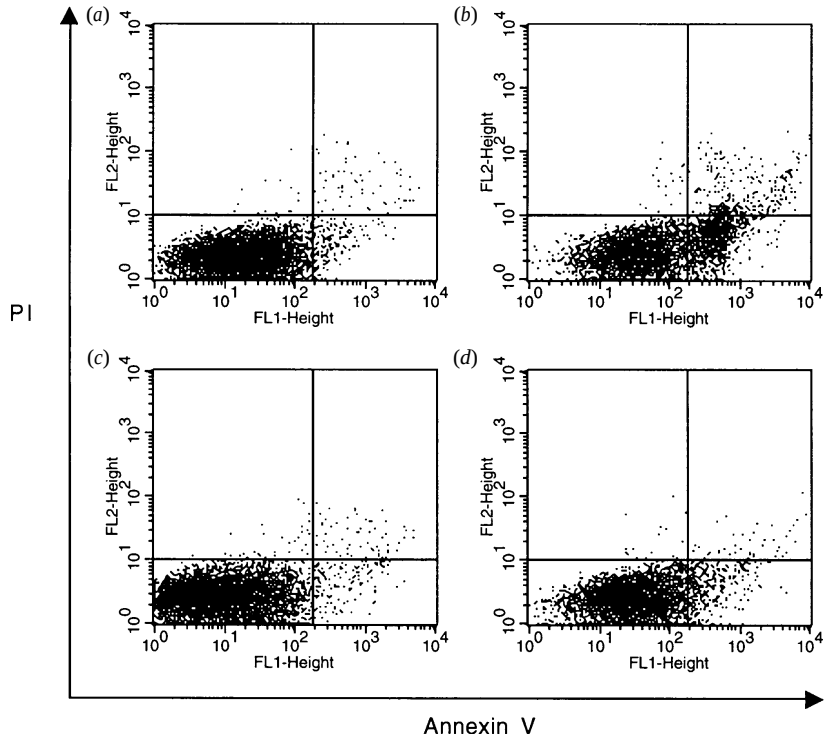

Fig. 1. CD95 crosslinking but not cocultivation with BJAB-EDp or BJAB-WTFp induces apoptosis in J16 cells. J16 cells (RCs) were left untreated (*a*), treated with anti-CD95 for 3 h (*b*) or cocultivated with BJAB-WTFp (*c*) or BJAB-EDp (*d*) cells (PCs) for 6·5 h at a PC/RC ratio of 1 : 4 and subsequently analysed for binding of annexin V–FITC and incorporation of propidium iodide.

Table 2. Apoptosis is not induced in PBLs after cocultivation with MV-infected PCs

PBLs (RCs) were cocultured with UV-irradiated mock-infected or MV-infected HL-60 cells (*c*) at a ratio of 1 : 4 (PC/RC) in the presence of PHA for the intervals indicated (*a*). To induce apoptosis, PBLs were activated for 5 days by CD3-crosslinking (1 μg/ml) in the presence of 100 U/ml IL-2, and subsequently treated with anti-CD95 (5 μg/ml) or left untreated. Cells were stained for apoptosis using the TUNEL technique. By FACScan, the PCs (HL-60) could be separated from the RCs based on their size and morphology. The number of cells within the RC gate stained by TUNEL is indicated as a percentage (*b*). Values represent a mean of at least three independent experiments. ND, Not determined.

	Conditions applied to the RC-population			
	HL-60[c]	HL-60-MV[c]	α-CD3/IL-2[d]	α-CD3/IL-2/ α-CD95[d]
12 h[a]	2·3 ± 0·6[b]	2·3 ± 1·5	ND	ND
16 h	ND	ND	5 ± 2·5	57 ± 2·5
24 h	2·5 ± 2	5 ± 2	ND	ND
48 h	3 ± 1·5	4·5 ± 1·4	ND	ND

for 5 days (Table 2). In this particular experiment, MV-ED-infected HL-60 cells were used as PCs (PC/RC ratio of 1 : 4), as after TUNEL staining these cells could easily be distinguished morphologically from the RC population by FACScan analysis. No significant increase in the proportion of apoptotic cells was detected over a 2 day observation period in the RC population after cocultivation with UV-irradiated HL-60-MV cells (Table 2), although inhibition of mitogen-dependent proliferation of the RCs was 84 ± 4% after 72 h, as determined by the incorporation of [^{3}H]thymidine. These findings indicate that inhibition of both mitogen-dependent and spontaneous proliferation of RCs after contact with MV-infected PCs is not associated with the induction of apoptosis.

Mitogen-dependent T cell proliferation is not restored by an exogenous supply of IL-2

As apoptosis does not account for the unresponsiveness of RCs to mitogen stimulation after cocultivation with MV-

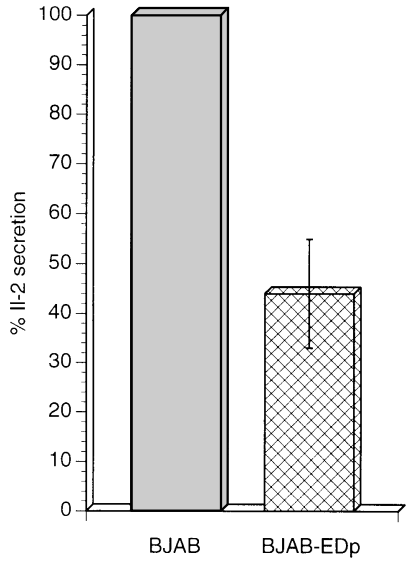

Fig. 2. IL-2 release from mitogen-stimulated PBLs is reduced after cocultivation with BJAB-EDp cells. PBLs isolated from four donors were cocultivated with UV-irradiated BJAB or BJAB-EDp cells at a PC/RC ratio of 1:4 in the presence of PHA (2·5 μg/ml). The amount of IL-2 released into the supernatant was determined by ELISA. IL-2 release after cocultivation with BJAB-EDp is indicated as a mean value compared to the controls.

infected PCs we investigated whether these cells show altered IL-2 release or altered responsiveness to IL-2. PBLs from four different donors were cultivated with PCs (BJAB cells or BJAB-EDp cells at PC/RC ratios of 1/4) in the presence of PHA, and the levels of cytokines released into the supernatants were determined after 24 h. Whereas high levels of IL-10 and IFN-γ were detected in the supernatants of the RCs cocultivated with both infected and uninfected PCs (not shown), levels of IL-2 were found to be reduced after contact with MV-infected PCs compared to those released after cocultivation with uninfected PCs (Fig. 2). Using lower PC/RC ratios, this reduction in IL-2 release was less pronounced (not shown). If reduced IL-2 levels accounted for the proliferative arrest of RCs after MV contact, then this block should be released after exogenous addition of IL-2. Therefore, RCs were cocultured with PCs (BJAB cells or BJAB-EDp at a PC/RC ratio of 1:4) for 48 h in the presence of IL-2 (100 U/ml) at the time of PHA stimulation. Exogenous addition of IL-2 did not restore the proliferative activity of the RCs towards mitogen stimulation. Similarly, mitogen-dependent proliferation of the RCs was not restored by addition of IL-2 when the PCs were depleted from the reaction by anti-CD19 selection after a 6 h contact period (not shown).

To establish whether the inability of the RCs to respond to exogenous IL-2 was linked to an impaired expression of the IL-2 receptor, we characterized the induction of the IL-2 receptor α- (CD25) β- (CD122) and the common γ-chain (CD132) on the surface of the RCs (derived from two different donors, 1 and 2) after contact with BJAB or BJAB-EDp cells (Fig. 3*a–c*). The expression frequencies for CD25 and CD132 were not found to be markedly different on RCs cocultured with UV-irradiated MV-infected PCs when compared to those cultured in the presence of the control-PCs for up to 72 h (Fig. 3*a*, *c*). Although the expression levels of CD122 were generally low, again no alterations occurred after coculture with MV-infected PCs (Fig. 3*b*). Similarly, expression of population markers such as CD3 (Fig. 4*d*), CD4 and CD8 were unaffected (not shown). No major, reproducible differences were detected in the expression of cellular and activation markers such as CD40L, CD45RO, CD49d, CD69 and CD71 (Fig. 3*e, f*) on the surface of RCs after a 24 h (and later, not shown) coculture with UV-irradiated MV-infected or uninfected PCs. These findings indicate that contact with MV-infected PCs does not impair the viability or the mitogen-dependent activation of RCs as indicated by the induction of activation markers. Although the release of IL-2 was significantly impaired after MV contact, exogenous supply of IL-2 did not restore the proliferative activity of the RCs.

MV-induced proliferative arrest occurs independently of preactivation of the RCs

To determine whether preactivation of the RCs would influence this non-responsiveness and whether the effect can be sustained, we separated the positive (PHA-stimulation of PBLs) and the negative signal (cocultivation with MV-infected PCs). In a first set of experiments, PBLs were cocultured with UV-irradiated BJAB-EDp or mock-infected BJAB cells at a PC/RC ratio of 1:4 for 6 h before depletion of the PCs by immunomagnetic sorting. The RCs were then either stimulated with PHA immediately (Fig. 4*a*, lane 1) or after an incubation period in medium for 24 (Fig. 4*a*, lane 2), 48 (Fig. 4*a*, lane 3) and 96 h (Fig. 4*a*, lane 4). In all cases the cells were cultured for further 48 h in the presence of PHA prior to labelling with [^{3}H]thymidine. We found that the negative signal imposed on the RCs by MV-infected PCs is sustained for the entire observation period, as even after 96 h the RCs were still refractory to mitogen-induced proliferation (Fig. 4*a*, lane 4). In a second set of experiments, we analysed the impact of MV-infected PCs on RCs activated prior to cocultivation. We prestimulated PBLs with PHA for 48 or 96 h before cocultivation with PCs or alternatively cocultured PBLs and PCs in the presence of PHA over the same time-period. The PCs used were BJAB-EDp (Fig. 4*b*) and BJAB-WTFp (Fig. 4*c*). After a further 48 h culture period in the presence of PHA, preactivation of the RCs did not prevent MV-contact-mediated inhibition of mitogen-dependent proliferation by the PCs, irrespective whether BJAB-EDp or BJAB-WTFp were used.

Accumulation of RCs in the G0/G1 phase of the cell cycle is observed after MV contact

PBLs were cocultured with UV-irradiated BJAB-EDp or BJAB cells at a PC/RC ratio of 1:5 in the presence of PHA and

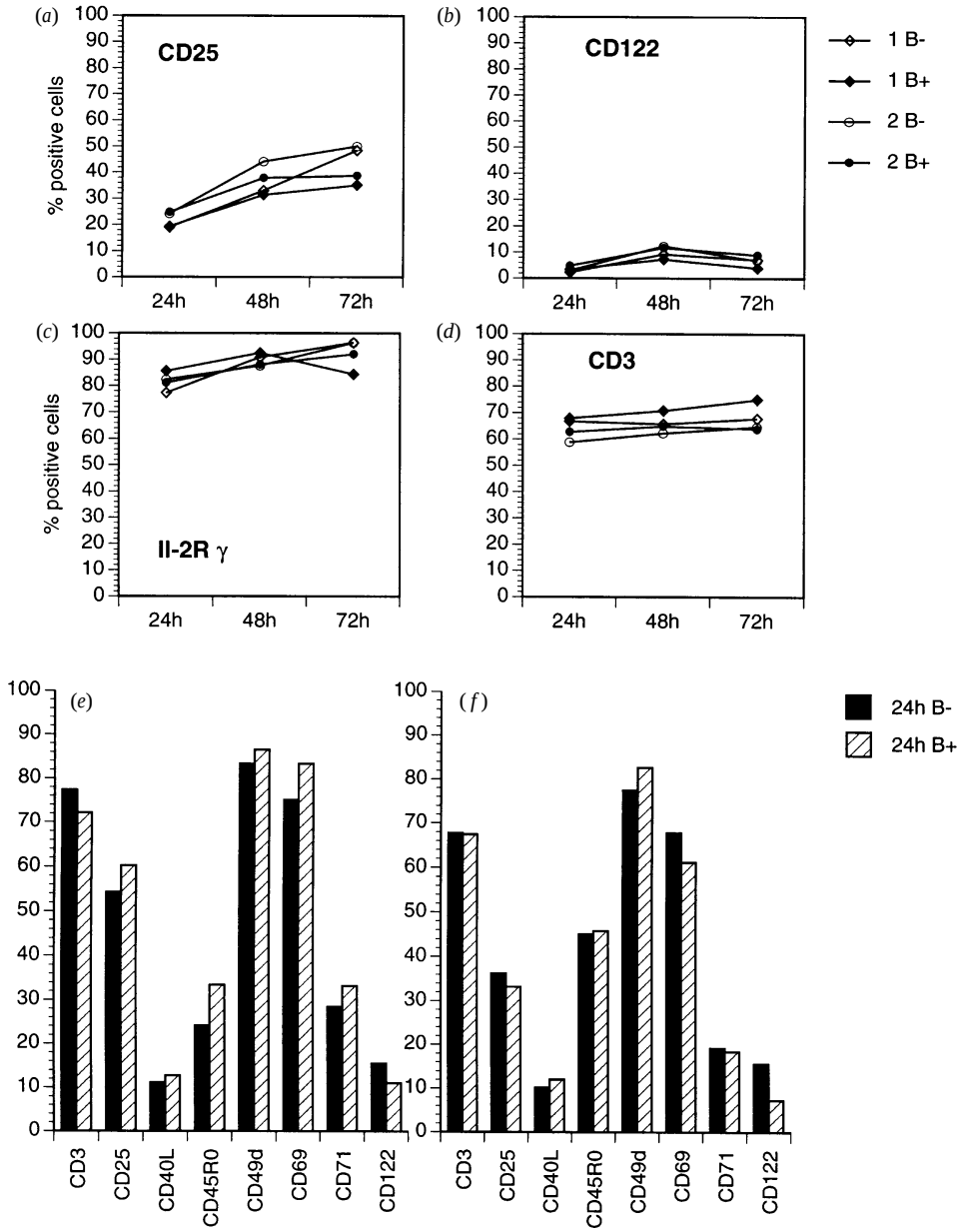

Fig. 3. Expression of population and activation cell surface markers on mitogen-stimulated PBLs is not affected after cocultivation with BJAB-EDp cells. PBLs from two different donors (1 and 2) were cocultured with UV-irradiated BJAB cells (B−, open symbols) or BJAB-EDp cells (B+, filled symbols) in the presence of PHA at a PC/RC ratio of 1:5. The expression levels of CD25 (*a*), CD122 (*b*), IL-2Rγ chain (*c*) and CD3 (*d*) were determined after 24, 48 and 72 h. PBLs isolated from two other donors (*e* and *f*) were cocultivated with uninfected (B−, black bars) or MV-infected (B+, hatched bars) PCs as described above and the expression of population (CD3) and activation markers was determined 24 h after PHA stimulation.

analysed for their cell cycle distribution after 48 and 72 h by propidium iodide staining. Whereas 72·6% (Fig. 5*a*, Table 3) or 69·2% (Fig. 5*b*, Table 3) of the RCs cocultured with mock-infected PCs accumulate in the G0/G1 phase of the cell cycle 48 and 72 h after mitogen-stimulation, 82·8% and 82·9% of the RCs are in the G0/G1 phase after cocultivation with MV-infected PCs (Fig. 5*c*, *d*, Table 3). Similarly, RCs isolated from two further donors were found to accumulate in the G0/G1 phase after a 72 h contact with MV-infected PCs (Table 3).

Discussion

A strongly impaired proliferative response to a variety of stimuli and an impairment of effector functions such as cytokine release are characteristic for MV-induced immunosuppression, both *in vivo* and *in vitro* (Borrow & Oldstone, 1995). As the marked immunosuppression *in vivo* is established in the presence of a very limited number of infected PBMCs, indirect mechanisms including virus-induced apoptosis (Esolen *et al.*,

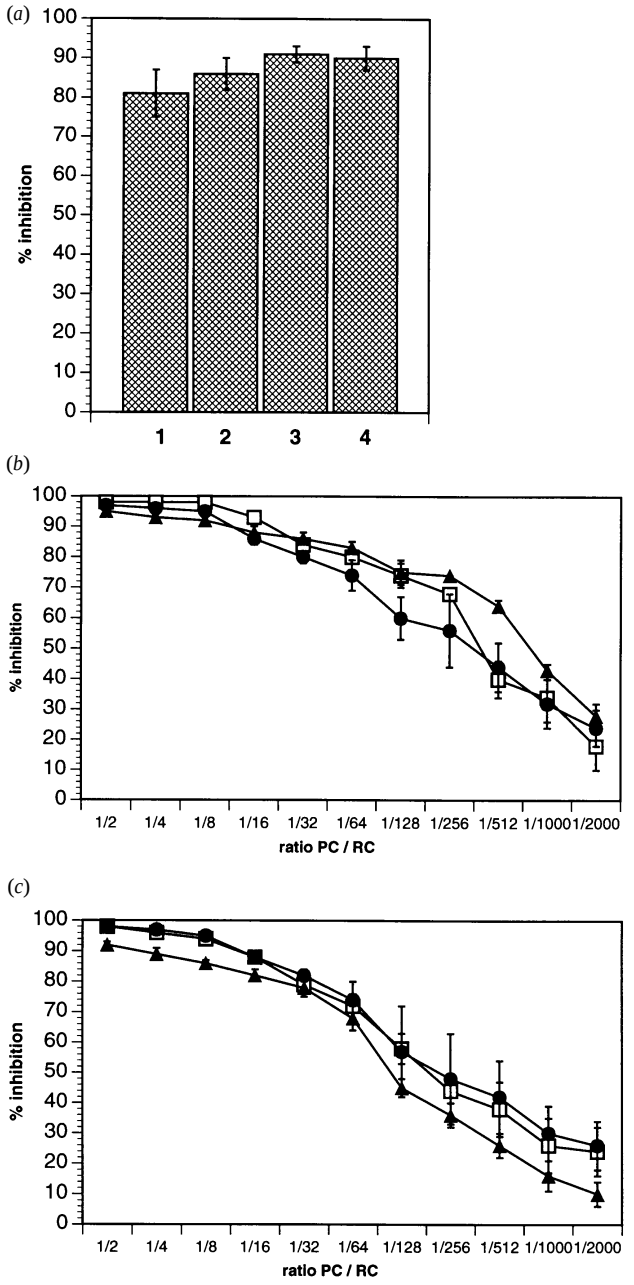

Fig. 4. Inhibition of mitogen-dependent proliferation of PBLs is sustained and preactivation does not prevent its induction. (*a*) PBLs were cocultivated with UV-irradiated BJAB or BJAB-EDp cells at a PC/RC ratio of 1:4 for 6 h after which the PCs were depleted. PBLs were stimulated with PHA (2·5 μg/ml) immediately (lane 1) or after 24 (lane 2), 48 (lane 3) or 96 h (lane 4). Proliferative inhibition (as compared to PBLs cocultivated with uninfected BJAB cells) was determined after a further 48 h incubation by labelling for 16 h with [^{3}H]thymidine. Counts obtained ranged from 40000 to 70000 c.p.m. (*b*, *c*) PBLs were PHA-stimulated for 96 (▲) or 48 h (●) prior to addition of UV-irradiated BJAB, BJAB-WTFp (*b*) or BJAB-EDp (*c*) at the PC/RC ratios indicated or left untreated (□). PBLs were restimulated with PHA for a further 48 h and proliferation rates were determined after a 16 h labelling period. Proliferative inhibition was determined in relation to the values obtained with uninfected BJABs. Values indicated represent means of three independent experiments which were performed in triplicate assays. Absolute counts ranged from 50000 to 70000 c.p.m.

1995; Auwaerter *et al.*, 1996), the release of inhibitory factors or the depletion of cytokines essentially involved in stimulating primary T cell activation (Karp *et al.*, 1996) have been postulated. To define the mechanisms underlying MV-induced immunosuppression, we have established an *in vitro* system based on the interaction between MV-infected, UV-irradiated presenter cells (PCs) and uninfected PBLs (responder cells, RCs). We found that after cocultivation with UV-irradiated PCs (either PBLs or primary monocytes/macrophages) infected with either the MV-ED or the MV-WTF strain, the proliferative response of PBLs to mitogen or allogen is reduced in a dose-dependent manner. The spontaneous proliferation of human cell lines of lymphocytic and monocytic origin was similarly affected (Schlender *et al.*, 1996). We also demonstrated that the negative signal was generated by surface contact between PCs expressing the MV glycoprotein complex and a so far unknown receptor molecule on the RCs which was not identical to CD46, the major protein receptor for the MV-ED strain (Naniche *et al.*, 1993; Dörig *et al.*, 1993).

We now show that the proliferative arrest of both mitogen-stimulated PBLs and a human CD4$^+$ T cell line after contact with MV-infected PCs was not associated with the induction of apoptosis (Fig. 1, Table 2). Apoptosis found after MV-infection of tissue culture cells (Esolen *et al.*, 1995) may be a direct consequence of infection and therefore cannot be directly compared to our system, which is based on surface-contact alone. In a second system, MV-infection of SCID mice grafted with human thymus material resulted in apoptosis of the human thymocytes, although direct infection of the thymocytes appeared unlikely (Auwaerter *et al.*, 1996). However, the study did not address whether apoptosis resulted from the interaction of virus-infected cells of the thymic epithelium with thymocytes or by other mechanisms, such as the induction of inflammatory cytokines (e.g. TNF). Also the different abilities of a wild-type and a vaccine strain of MV to induce apoptosis shown in this study are clearly different from our studies where both ED and WTF induce a similar proliferative inhibition and both do not directly induce apoptosis (Fig. 1, Table 2). In contrast, mitogen-stimulated RCs acquired an activated phenotype in spite of their proliferative arrest, and could therefore be sensitive to activation-induced cell death (AICD). We have not, however, experimentally addressed whether an enhanced sensitivity to AICD could be observed in the RCs after cocultivation with MV-infected PCs when compared to uninfected PCs. In this context it is also interesting to note that enhanced programmed cell death (PCD) of T cells isolated from measles patients was observed after triggering of the TCR–CD3 complex (Addae *et al.*, 1995).

Our data clearly indicate that the RCs, although the majority are arrested in the G1 phase of the cell cycle, are in fact viable after contact with MV-infected PCs as no alterations in the expression of either population or activation markers, including the IL-2R chains, have been observed (Fig. 3). With the exception of a lowered IL-2 secretion in proliferation

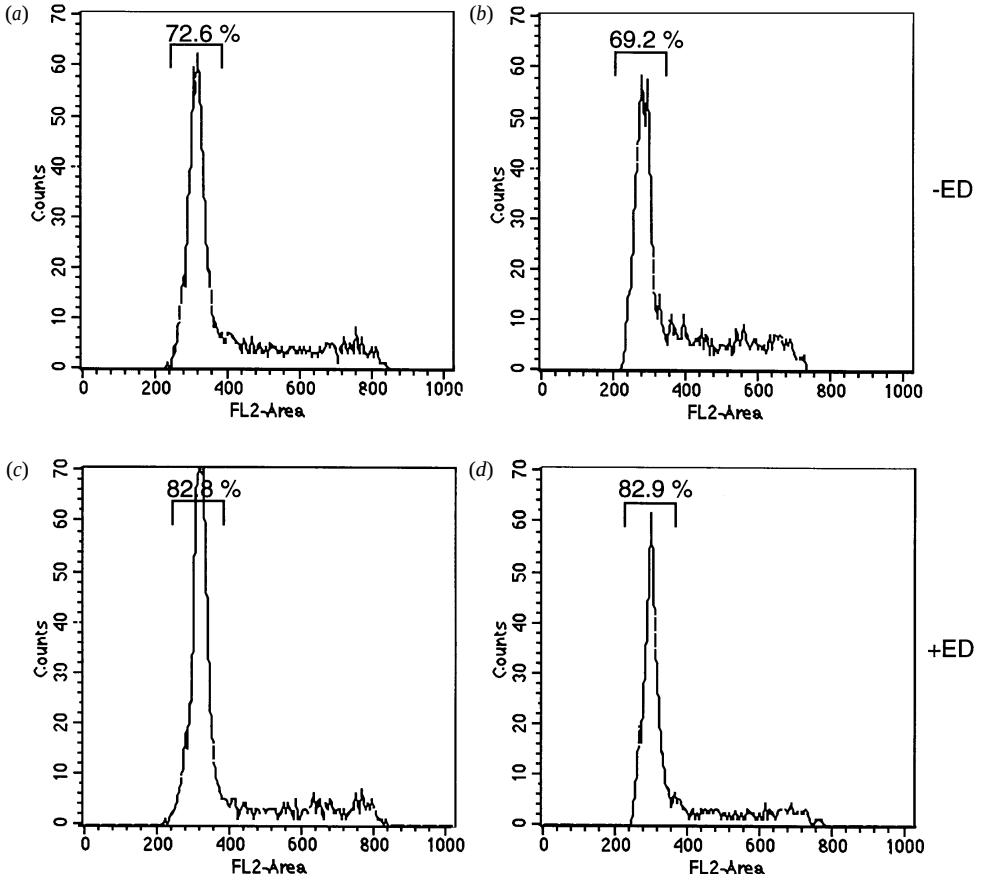

Fig. 5. Accumulation of mitogen-stimulated PBLs in the G0/G1 phase of the cell cycle after cocultivation with MV-infected PCs. PBLs were cocultivated with BJAB (*a*, *b*) or BJAB-EDp cells (*c*, *d*) at a PC/RC ratio of 1:5 in the presence of PHA and the cell cycle distribution was determined after 48 (*a*, *c*) or 72 (*b*, *d*) h by propidium iodide staining followed by FACScan. Values indicate the percentage of cells in the G0/G1 phase of the cell cycle.

Table 3. Increased frequency of PHA-stimulated PBLs in the G0/G1 phase after cocultivation with MV-infected PCs

PBLs from three individual donors were cocultivated with UV-irradiated uninfected BJAB cells or BJAB-EDp cells (*a*) at a PC/RC ratio of 1:5 in the presence of PHA (5 μg/ml) for 72 h. Cell cycle distribution of the RCs (*c*) was determined by propidium iodide staining and subsequent FACS analysis (*b*).

Donor	PC[a]	G0/G1[b]	S	G2
A	BJAB	69·2[c]	26·7	4·1
A	BJAB-EDp	82·9	14·6	2·4
B	BJAB	67·1	31·7	1·2
B	BJAB-EDp	83·6	14·5	1·9
C	BJAB	76·8	19·9	3·3
C	BJAB-EDp	88·5	9·8	1·8

arrested cultures, other cytokines such as IL-10 and IFN-γ were produced in high amounts (not shown). Similarly, suppression of mitogen-induced proliferation *in vitro* has been linked to a G1 arrest after MV-infection of B and T cells (McChesney *et al.*, 1987, 1988; Yanagi, *et al.*, 1992), and arrested cells revealed an increased cell size, normal expression of cell surface markers such as CD98, CD71, HLA-DR (McChesney *et al.*, 1988) and unimpaired PMA/ionomycin-induced IL-2 and PHA-induced IFN-γ secretion (McChesney *et al.*, 1988). Moreover, no alterations in the mRNA levels for IL-2 or IL-2R were detected after PHA-stimulation of MV-infected T cells when compared to uninfected cells (Yanagi *et al.* 1992), and IL-2 levels released from human tonsillar lymphocytes after PHA stimulation were found to be unaffected by MV infection (Borysiewicz *et al.*, 1985). As these studies were performed using an m.o.i. of 3, effects resulting from viral surface contact cannot be distinguished from those which are mediated by the infection.

The activation state of PBMCs isolated from measles patients has also been characterized after stimulation *in vitro*. Whereas one study states that the fraction of PBMCs expressing IL-2R during acute measles is unchanged or even elevated (Griffin *et al.*, 1986), other investigators found a strongly reduced amount of CD25 on $CD4^+$ and $CD8^+$ T cells which reverted to normal levels after PMA treatment (Addae *et al.*, 1995). While our experiments do not allow a rec-

onciliation of these findings, they do clearly indicate that potential alterations in the expression of the IL-2R complex on uninfected PBLs do not result from signalling induced by the MV glycoprotein complex.

The state of the RCs after cocultivation with MV-infected PCs apparently does not correspond to a state of anergy which can normally be overcome by exogenous addition of IL-2. After cocultivation with MV-infected PCs, levels of IL-2 released from mitogen-stimulated PBLs were found to be reduced by approximately 50% when compared to the controls (Fig. 2). This reduction is, however, dose-dependent and IL-2 levels return to normal when lower PC/RC ratios are analysed, although a marked inhibition of RC proliferation is still observed (not shown). Moreover, proliferation is not resumed after exogenous addition of IL-2 (not shown). As revealed by the analysis of activation markers, defective or reduced expression of the IL-2R complex is not detectable and does not explain the inability to respond to exogenous IL-2. In agreement with these findings, PBMCs isolated from measles patients produced only low levels of IL-2 after CD3-triggering (Griffin *et al.*, 1993). In contrast to our findings, however, inhibition of mitogen-dependent proliferation of PBLs isolated from measles patients could partly be rescued by the addition of IL-2 (Griffin *et al.*, 1987; Ward & Griffin, 1993). This discrepancy can possibly be explained as our experiments do not rule out that IL-2 treatment may in fact counteract the proliferative arrest to a certain extent once the negative signal provided by the infected PCs is removed from the reaction. That the negative signal provided by MV-infected PCs is largely independent of alterations in IL-2 release or responsiveness is, however, supported by our previous findings that spontaneous proliferation of cell lines that grow independently of IL-2 and do not express IL-2R was also affected (Schlender *et al.*, 1996). The sensitivity of these cell lines to MV-contact-mediated inhibition agrees with our observation that proliferation of preactivated PBLs is effectively inhibited (Fig. 4*b*, *c*). In summary, our findings indicate that a state of general unresponsiveness is established in PBLs after contact with MV-infected PCs that does not interfere with cellular activation and is based on a G1 arrest. As the inhibitory effect extends to cells whose proliferation is clearly independent of IL-2, it is not likely that classical anergy, as defined for T cells, is responsible. However, it is quite possible that signalling pathways are used that are similar to those involved in downregulation of IL-2 during the induction of the anergic state in T cells.

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