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## IL-4 INDUCES LFA-1 AND LFA-3 EXPRESSION ON BURKITT'S LYMPHOMA CELL LINES

### Requirement of Additional Activation by Phorbol Myristate Acetate for Induction of Homotypic Cell Adhesions

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LFA-1 and LFA-3 expression is absent or low on Burkitt's lymphoma cell lines and low on the EBV-transformed B cell line UD61. Incubation of cells of BL2 and of UD61 with various concentrations of IL-4 resulted in induction of LFA-1 and LFA-3 expression in a dose dependent fashion. This effect was already observed after 16 h of incubation whereas maximal expression was obtained after 72 h. Induction of LFA-1 and LFA-3 expression seemed to be specific for IL-4, because IL-1, IL-2, IL-3, IFN- $\alpha$ , IFN- $\gamma$  and a low m.w. B cell growth factor were ineffective. LFA-1 and LFA-3 induction by IL-4 was blocked specifically by an anti-IL-4 antiserum. Induction of LFA-1 expression by IL-4 was furthermore confirmed at the specific LFA-1  $\beta$ -chain mRNA level. IL-4 was unable to induce LFA-1 expression on EBV-transformed lymphoblastoid cell lines of two LFA-1-deficient patients. BL2 grows as single cells, but induction of LFA-1 and LFA-3 expression by IL-4 was insufficient to induce homotypic cell adhesions and required PMA as a second signal. PMA alone did not induce LFA-1 antigen expression and was unable to induce adhesions between BL2 cells in the absence of IL-4 in 22 h assays. Addition of PMA to BL2 cells that expressed LFA-1 Ag upon incubation with IL-4 resulted in aggregate formation within 30 min. Adhesions between BL2 cells induced by IL-4 in combination with PMA were blocked by anti-LFA-1 $\beta$  or anti-LFA-1 $\alpha$ -chains mAb. In addition, these mAbs dispersed preformed aggregates of BL2 cells. Our results indicate that IL-4 can induce the adhesion molecules LFA-1 and LFA-3 on B cell lines, but that an additional activation signal provided by PMA was required for the induction of homotypic cell adhesions.

The LFA-1<sup>4</sup> family consists of the LFA-1 (CD11a),

CR3bi (CD11b), and p150,95 (CD11c) Ag. These heterodimeric molecules share a common  $\beta$ -subunit (CD18) with a  $M_r$  of 95 kDa, but LFA-1, CR3bi, and p150,95 have different  $\alpha$ -subunits with  $M_r$  of 180, 170, and 155 kDa, respectively (1, 2).

Antibodies directed against LFA-1 have been shown to block every immune response requiring heterotypic cell interactions including adhesion reactions between cytotoxic T cells or natural killer cells and target cells (3-6), Th cells and APC (6-8). They also block antibody-dependent killing and mixed leucocyte responses (5, 6, 9). Furthermore, it was demonstrated that anti-LFA-1 mAb prevent or block conjugate formation between cells (9-11) and disrupt existing conjugates (12, 13), indicating that the LFA-1 family Ag act as adhesion molecules.

T or B lymphocytes, EBV-LCL, and monocytoid cell lines can be induced to form aggregates after stimulation with Ag, lectins, or phorbol esters. These cell-cell interactions could be blocked by anti-LFA-1 mAb indicating that this molecule is also associated with homotypic cell interactions (12-15).

One natural ligand for LFA-1 is ICAM-1 (CD54), which is expressed on leukocytes, fibroblasts, epithelial cells, and endothelial cells. mAb against ICAM-1 inhibit conjugate formation between cytotoxic T lymphocytes and target cells and block adhesions between T cells and fibroblasts or endothelial cells (16-18).

The importance of the LFA-1 family molecules in adhesion-dependent processes is illustrated in patients who are deficient for these Ag (19-22). These patients suffer from persistent leukocytosis, impaired wound healing, and severe recurrent bacterial, viral, and fungal infections (23).

Recently, it has been described that fresh lymphoma cells including BL cells lack expression of LFA-1 Ag and it has been suggested that these tumor-cells lacking LFA-1 fail to induce an immune response, and in this way escape from immunosurveillance (24). Furthermore, established BL cell lines have been shown not only to be deficient in LFA-1, but also in ICAM-1 and LFA-3 (CD58) expression (25). Recently, we demonstrated that human rIL-4 induced in a coordinated way the expression of both CD23 and class II MHC antigens on BL cell lines (26). During these investigations, we noticed that also the expression of LFA-1 was modulated by IL-4. In our study, we show that IL-4 specifically induces/enhances the expression of LFA-1 on BL cell lines and on the EBV-LCL

Received for publication August 30, 1988.

Accepted for publication June 1, 1989.

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<sup>4</sup> Abbreviations used in this paper: LFA-1, lymphocyte function Ag-1; ICAM-1, intercellular adhesion molecule-1; EBV-LCL, EBV-transformed lymphoblastoid cell line; BL, Burkitt's lymphoma, LAD, leukocyte adhesion deficiency; GM-CSF, granulocyte-macrophage CSF; PKC, protein kinase C; GAPDH, glyceraldehyde phosphate dehydrogenase.



UD61. LFA-1 induction was confirmed at the LFA-1  $\beta$ -chain mRNA level. In addition to LFA-1, also LFA-3 expression was induced by IL-4. Induction of LFA-1 was not sufficient for the formation of adhesions between cells that required a second signal provided by PMA. Aggregate formation was completely prevented by an anti-LFA-1  $\beta$ -chain mAb and to a lesser extent by anti-LFA-1  $\alpha$ -chain mAb. The anti-LFA-1  $\beta$ -chain mAb also dispersed existing aggregates, confirming the notion that adhesions between BL2 cells were mediated by LFA-1 molecules.

#### MATERIALS AND METHODS

**Lymphokines and reagents.** Purified rIL-4 (sp. act.  $10^7$  U/ml), was a kind gift of Dr. S. Nagabhushan (Schering Corporation, Bloomfield, NJ). One unit of IL-4 is defined as the concentration of IL-4 resulting in half maximal proliferation of PHA-activated T lymphoblasts (27). Human rIL-1 $\alpha$  was obtained from Dr. X. Zurawski (DNAX Research Institute, Palo Alto, CA). rIL-2 was purchased from Amgen Biologicals (Amersham, Inc., UK). IL-3 was used in the form of supernatants of Cos 7 cells transfected with the pcD vector containing the cloned human IL-3 cDNA. GM-CSF tested at concentration of 10 ng/ml (700 pM), IFN- $\gamma$  ( $10^7$  U/mg) and IFN- $\alpha$  ( $10^7$  U/mg) used at 250 U/ml were provided by Dr. P. Trotta (Schering Corp.). A commercial preparation of LMW-BCGF was obtained from Cellular Products Inc. (Buffalo, NY) and used at 20% v/v. PMA was obtained from Sigma (Chemical, St. Louis, MO). The FITC-conjugated F(ab')<sub>2</sub> fragments of goat antimouse Ig used in the indirect immunofluorescence assays was purchased from Grub (Vienna, Austria).

**Cells and cultures.** The BL cell lines used in this study were established from Caucasian, North African, and African donors and described previously (28, 29). Both EBV<sup>+</sup> and EBV<sup>-</sup> cell lines were tested for their LFA-1 and LFA-3 expression (Table I). The EBV-LCL UD61 was established from a hyper-IgE patient, whose leucocytes expressed normal levels of LFA-1. EBV-LCL A and B were established from patients suffering from LAD and kindly provided by Drs. F. Miedema (Central Laboratory of the Red Cross, Blood Transfusion Service, Amsterdam, The Netherlands) and R. Callard (Institute for Child Health, London, U.K.) respectively. LCL of the patients A and B expressed approximately 1% and <0.1% of the normal amounts of LFA-1. These LAD patients have been described previously (30-32). Cell cultures were carried out in culture medium consisting of RPMI 1640 supplemented with 10% heat inactivated FCS, 2 mM glutamin, penicillin (100 IU/ml), and streptomycin (100  $\mu$ g/ml), all from Flow Laboratories, (Irvine, Scotland). The cell lines were mycoplasma free. Test cultures in the presence of IL-4 or other lymphokines were carried out as described previously (26).

**Antibodies.** The mAb B1 directed against CD20 was obtained from Coulter (Hiialeah, FL). The mAb L243 anti-HLA-DR was purchased from Becton Dickinson (Monoclonal Antibody Center, Mountain View, CA). mAb SPV-L3 is directed against HLA-DQ (33), W6/32 directed against a monomorphic determinant on HLA-A, B, C was obtained from Sera Lab. (Crawley Down, UK). mAb 25 recognizes the CD23 Ag (34). mAb IOT16 (Immunotech, Marseille, France), SPV-

L7 and SPV-L12 are anti-LFA-1  $\alpha$ -chain antibodies (7). mAb CLB54 is directed against the LFA-1  $\beta$ -chain (7, 35). The anti-CR3bi mAb OKMI was obtained from Ortho (Raritan, NJ). The anti-p150,95 mAb S-HCL3 (36) and anti-LFA-3 mAb TS 2/9 (4) were kindly provided by Dr. R. Schwarting (Freie Universität, Berlin, FRD) and Dr. S. Burakoff (Dana Farber Cancer Institute, Boston, MA) respectively. The polyclonal rabbit anti-IL-4 antiserum was raised in our laboratory. This antiserum blocked specifically IL-4-mediated proliferation of activated T or B cells, IL-4-induced IgE-synthesis, and did not react with rIL-1- $\alpha$ , - $\beta$ , IL-2, IL-3, IL-5, GM-CSF, and IFN- $\gamma$  (37). Preimmune serum from the same rabbit was used as control.

**Immunofluorescence.** A total of  $5 \times 10^5$  cells was incubated with 50  $\mu$ l of the appropriately diluted mAb in 0.2-ml microtiter plate wells (V bottom, Linbro, Titertek, Flow) for 20 min at 4°C. After two washes with PBS containing 1% BSA and 2 mM azide, the cells were incubated with FITC-labeled F(ab')<sub>2</sub> fragments of goat anti-mouse Ig for 20 min at 4°C. After three washes with PBS containing BSA and azide the cells were analyzed with the flow microfluorimetry (FACS 440, Becton Dickinson, Sunnyvale, CA). Fluorescence data were expressed as percentage of fluorescent cells tested with the appropriate mAb compared to nonrelevant mAb of identical isotypes.

In addition fluorescence was measured as mean fluorescence intensity of the positive fluorescent cells expressed as the mean channel number plotted on a logarithmic scale (26).

**mRNA analysis.** RNA was prepared from  $5 \times 10^7$  cells by using the guanidine isothiocyanate/cesium chloride centrifugation method (38). A total of 25  $\mu$ g of each RNA was size fractionated on a 1% agarose gel containing 2.2 M formaldehyde and transferred to gene screen nylon membranes for hybridization. LFA-1  $\beta$ -chain cDNA 3.1.1 kindly provided by Dr. T. Springer (Dana-Farber Institute, Boston, MA) (39) was prepared by random multiprime labeling. The level of LFA-1  $\beta$ -chain mRNA expression in each cell line was compared to the mRNA level of the house keeping gene glyceraldehyde phosphate dehydrogenase using the cDNA probe p RGAPDH.13, generously provided by Dr. M. Piechaczyc (Université des Sciences, Montpellier, France).

**Qualitative determination of aggregate formation.** Aggregation of BL and LCL cells was determined qualitatively as described previously (40). This method is a modification of the method described by Rothlein and Springer (13). Briefly, cells were resuspended in 96-well microtiter plates (no. 3072 Falcon, Meylan, France) at concentrations of  $1 \times 10^5$  cells/well. Cells were incubated at 37°C and 5% CO<sub>2</sub> for the times indicated in the presence or absence of IL-4 (200 U/ml), mAb or PMA. Aggregate formation was counted in coded wells in an inverted microscope by two investigators. Scores ranged from 0 to 5+ in which 0 indicated that essentially no cells were aggregated; 1+, 2+ and 3+ indicate that <10%, <50%, <80% of the cells form loose clusters respectively; 4+ indicated that >80% of the cells formed small dense aggregates and 5+ indicate >90% of the cells formed large compact clusters (40).

**Quantitation of aggregate formation.** Aggregates of BL and LCL cells were measured quantitatively as described previously (40). Briefly, cells were resuspended in 96-well microtiter plates (no. 3072 Falcon) at concentrations of  $1 \times 10^5$  cells/well. Cells were incubated at 37°C and 5% CO<sub>2</sub> for the times indicated in the presence or absence of IL-4 (200 U/ml), mAb (at concentrations of 1 to 10  $\mu$ g/ml) or PMA (1 or 10 ng/ml). The number of nonaggregated cells was counted by using a calibrated ocular in an inverted microscope. The

TABLE I  
Major characteristics of BL cell lines used<sup>a</sup>

Cell Line	Origin <sup>b</sup>	EBV	% Positive Cells			Culture Morphology <sup>c</sup>
			LFA-1 $\alpha$ (SPV-L7)	LFA-1 $\beta$ (CLB54)	LFA-3 (TS 2/9)	
BL2	C	-	0	0	0	0
BL18	NA	+	58	68	54	+++
BL29	A	+	0	0	0	+
BL30	C	+	4	4	0	+/-
BL31	C	-	96	81	ND	+++
BL41	C	-	0	0	0	0
BL49	C	-	0	0	ND	+
BL60	NA	+	4	4	0	+
BL70	C	-	6	5	0	0
BL72	C	+	50	55	45	+++
BL74	C	+	22	20	15	+++
Jijoye	A	+	0	0	25	++
Daudi	A	+	24	35	15	+

<sup>a</sup> Presence of EBV in the genome of the cells was determined by immunofluorescence with anti-EBV-nuclear Ag antibodies (28, 29).

<sup>b</sup> C, Caucasian; NA, North African; A, African.

<sup>c</sup> 0, single cells only; +/-, few small loose aggregates; +, clumps of 10 to 20 cells; ++, moderately sized aggregates of 50 cells and considerable numbers of single cells; +++, large aggregates and few single cells.



mean and total number of nonaggregating cells was counted in at least five randomly chosen areas which differed for each well. Counting was carried out independently by two investigators. Percent aggregation was determined according to the formula:

$$\% \text{ aggregation} = 1 - \frac{\text{no. of nonaggregated cells}}{\text{total number of cells}} \times 100\%$$

The experiments were carried out in triplicate. The SD was less than 10%.

## RESULTS

**LFA-1 and LFA-3 expression on BL cell lines and EBV-LCL.** Phenotyping of a series of BL cell lines and LCL revealed that most of the BL cell lines tested (10/13) showed a low or a lack of expression of LFA-1 Ag (Table I), whereas only one LCL (UD61) out of 55 tested had reduced LFA-1 expression (Fig. 1).

The low or lack of expression of LFA-1- $\alpha$  as detected by the mAb SPV-L7, SPV-L12, and IOT16, and LFA-1- $\beta$  as detected by mAb CLB54, was a stable feature of these cell lines. Upon repetitive testing over a prolonged culture period (4 mo), consistent levels of LFA-1- $\alpha$  and LFA-1- $\beta$  expression were observed (Table I). There seems to be an association between the levels of LFA-1 expression and the growth characteristics of the BL cell lines. The BL which have no detectable, or minimal LFA-1 expression grow as single cells (BL2, BL41, BL70), or in small aggregates (BL29, BL30, BL49, BL60) whereas the BL that express LFA-1 (BL18, BL31, BL72, BL74) generally grow in large aggregates. Exceptions are Jijoye, which is LFA-1<sup>-</sup> but grows in medium sized, rather dense aggregates and Daudi, which has relatively high LFA-1 expression but grows in small clumps (Table I). In contrast to EBV-LCL expressing normal levels of LFA-1 and that grow in large dense clumps with hardly any single cells, cultures of UD61 contain small loose aggregates and also single cells (not shown). Finally, it is worth noting that EBV-LCL established from the patients BL2, BL41, BL49, BL70, and BL74 expressed normal levels of LFA-1, excluding that the low LFA-1 expression in these patients is a genetic defect. In addition, leucocytes of donor UD61 expressed normal levels of LFA-1 (not shown).

**Effect of IL-4 on LFA-1 family and LFA-3 Ag expression on BL2 and UD61.** The BL cell line BL2 that failed

to express detectable levels of LFA-1- $\alpha$  and LFA-1- $\beta$  and the EBV-LCL UD61 of which only a proportion of the cells were LFA-1 positive, were selected for further studies. Determination of the expression of the other members of the LFA-1 family on BL2 and UD61 revealed that CR3bi and p150,95 were absent on both cell lines (Table II). In this respect, BL2 does not differ from EBV-LCL, that also have been shown to lack expression of CR3bi and p150,95, but in contrast express high levels of LFA-1 (1, 13).

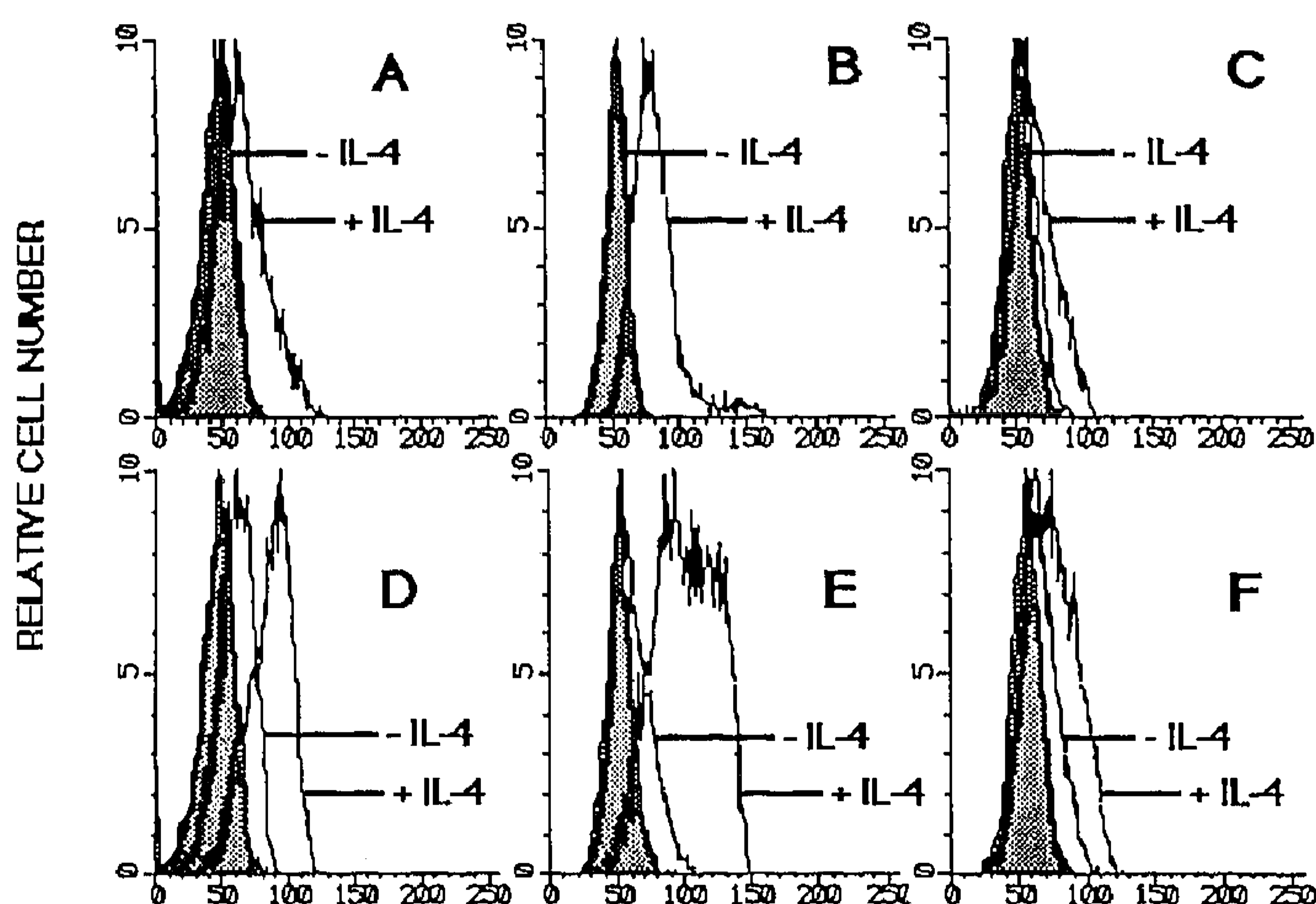
Incubation of BL2 and UD61 for 48 h in the presence of 200 U/ml of IL-4 induced a significant expression of LFA-1- $\alpha$  and LFA-1- $\beta$  on a proportion of the cells (Fig. 1, Table II). In contrast, IL-4 failed to induce the expression of CR3bi and p150,95. (Table II). In addition, IL-4 did not affect the expression of class I MHC Ag and the specific B cell marker CD20 as judged by both the percentage of positive cells (Table II) and mean fluorescence intensity (not shown), but enhanced, as was shown previously the expression of low affinity receptor (Fc $\epsilon$ RII/CD23) and the expression of class II MHC Ag (26). IL-4 which has been shown to have growth promoting activity for activated B cells (41) did not affect the growth of BL2 or UD61 cells because no differences in [<sup>3</sup>H]TdR incorporation were observed in the presence or absence of IL-4 (Table II).

BL2 also failed to express LFA-3, whereas LFA-3 expression on UD61 was very low (Fig. 1). Culturing of BL2 and UD61 in presence of IL-4 resulted in the induction of LFA-3 expression on a significant proportion of both cell lines (Fig. 1).

**Dose response and kinetics of IL-4 induced LFA-1 and LFA-3 expression on BL2 and UD61.** Culturing BL2 at various concentrations of IL-4 for 48 h indicated that 5 U/ml of IL-4 already induced a significant expression of LFA-1, that increased in a dose-dependent fashion. Maximal expression was obtained at IL-4 concentrations between 200-1000 U/ml (Fig. 2). Even at saturating IL-4 concentration, only a proportion of the cells ( $37 \pm 6\%$  for BL2) could be induced to express detectable levels of LFA-1. Kinetic studies indicated that IL-4 added at saturating concentrations of 200 U/ml induced significant LFA-1 expression on both BL2 and UD61 after 12 to 24 h (Fig. 3), whereas maximal LFA-1 expression was obtained after 72 h of incubation. Prolonged incubation with IL-4 resulted in a decrease in LFA-1 expression (Fig. 3) which may be due in part to exhaustion of the medium and unfavorable culture conditions, because the elevated levels of LFA-1 expression were maintained by splitting the cultures in fresh medium and IL-4 (not shown). The induction of LFA-1 expression by IL-4 was completely inhibited by anti-IL-4 antiserum, whereas control rabbit sera were ineffective (Fig. 3). These results indicate that the effect is indeed specific for IL-4, IL-1, IL-2, IL-3, IFN- $\alpha$ , IFN- $\gamma$ , GM-CSF and LMW-BCGF, tested at various concentrations, were ineffective in inducing LFA-1 on UD61 and BL2 cells (Table III).

Induction of LFA-3 followed similar kinetics as shown for LFA-1. Also the IL-4 induced LFA-3 expression was inhibited by the anti-IL-4 antiserum at a dilution of 1/250, whereas the control preimmune rabbit-serum was ineffective (Fig. 4). These results indicate that IL-4 induces the expression of LFA-1 and LFA-3 in a coordinated way.

**IL-4 induces transcription of LFA-1  $\beta$ -chain-specific mRNA.** The notion that IL-4 induces LFA-1 expression



**Figure 1.** Induction of LFA-1 and LFA-3 expression on BL2 and UD61 by IL-4. Flow microfluorimetry profiles of BL2 and UD61 cells incubated without or with IL-4 (200 U/ml) for 48 h. A, BL2, LFA-1 $\alpha$ ; B, BL2, LFA-1 $\beta$ ; C, BL2, LFA-3; D, UD61, LFA-1 $\alpha$ ; E, UD61, LFA-1 $\beta$ ; F, UD61, LFA-3 mAb SPV-L7 (anti-LFA-1 $\alpha$ ), CLB54 (anti-LFA-1 $\beta$ ) and TS 2/9 (anti-LFA-3) were used. Conjugate control: (shaded diagrams). Abscissa, log fluorescence intensity; ordinate, relative cell number.



TABLE II  
Induction of LFA-1 Ag on BL2 and UD61 by IL-4<sup>a</sup>

Cell Line	Lymphokine Added	<sup>3</sup> H]TdR Inc. (cpm × 10 <sup>-3</sup> ± SD)	% Positive Cells (Mean ± SD)						
			LFA-1α	LFA-1β	CR3bi	p150,95	CD23	HLA-A,B,C	CD20
BL2	0	92 ± 1	1 ± 1	0 ± 0	0 ± 0	0 ± 0	0 ± 0	96 ± 2	93 ± 3
	IL-4	94 ± 2	31 ± 8	30 ± 11	3 ± 1	0 ± 0	47 ± 5	95 ± 4	96 ± 5
UD61	0	83 ± 1	17 ± 7	14 ± 3	0 ± 0	0 ± 0	25 ± 7	95 ± 3	79 ± 7
	IL-4	86 ± 4	50 ± 7	51 ± 6	1 ± 1	0 ± 0	63 ± 9	95 ± 3	82 ± 9

<sup>a</sup> BL2 and UD61 cells were incubated with 200 U/ml IL-4 for 48 h and analyzed with the FACS. BL2 (mean ± SD of seven experiments); UD61 (mean ± SD of five experiments). LFA-1α, LFA-1β, CR3bi, p150,95, CD23, HLA-A,B,C and CD20 were determined by using mAb SPV-L7, CLB54, OKM-1, S-HCL3, 25, W6/32, and Leu 16, respectively. Proliferation as measured by [<sup>3</sup>H]TdR incorporation is expressed as mean cpm × 10<sup>-3</sup> ± SD of two different experiments.

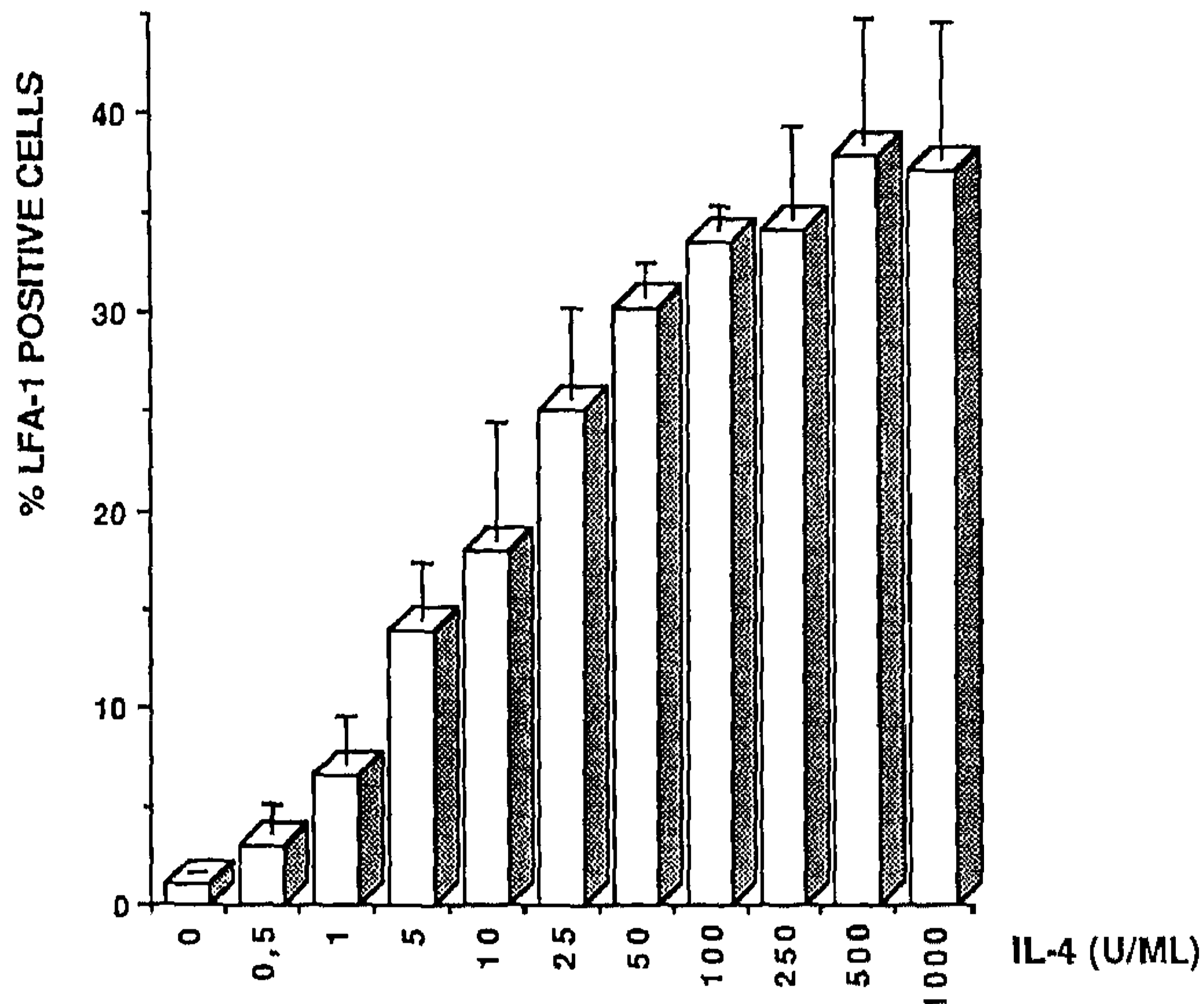


Figure 2. Dose response of IL-4-induced LFA-1 expression on BL2 cells. BL2 cells were incubated for 72 h with different concentrations of IL-4. Mean ± SD of three experiments.

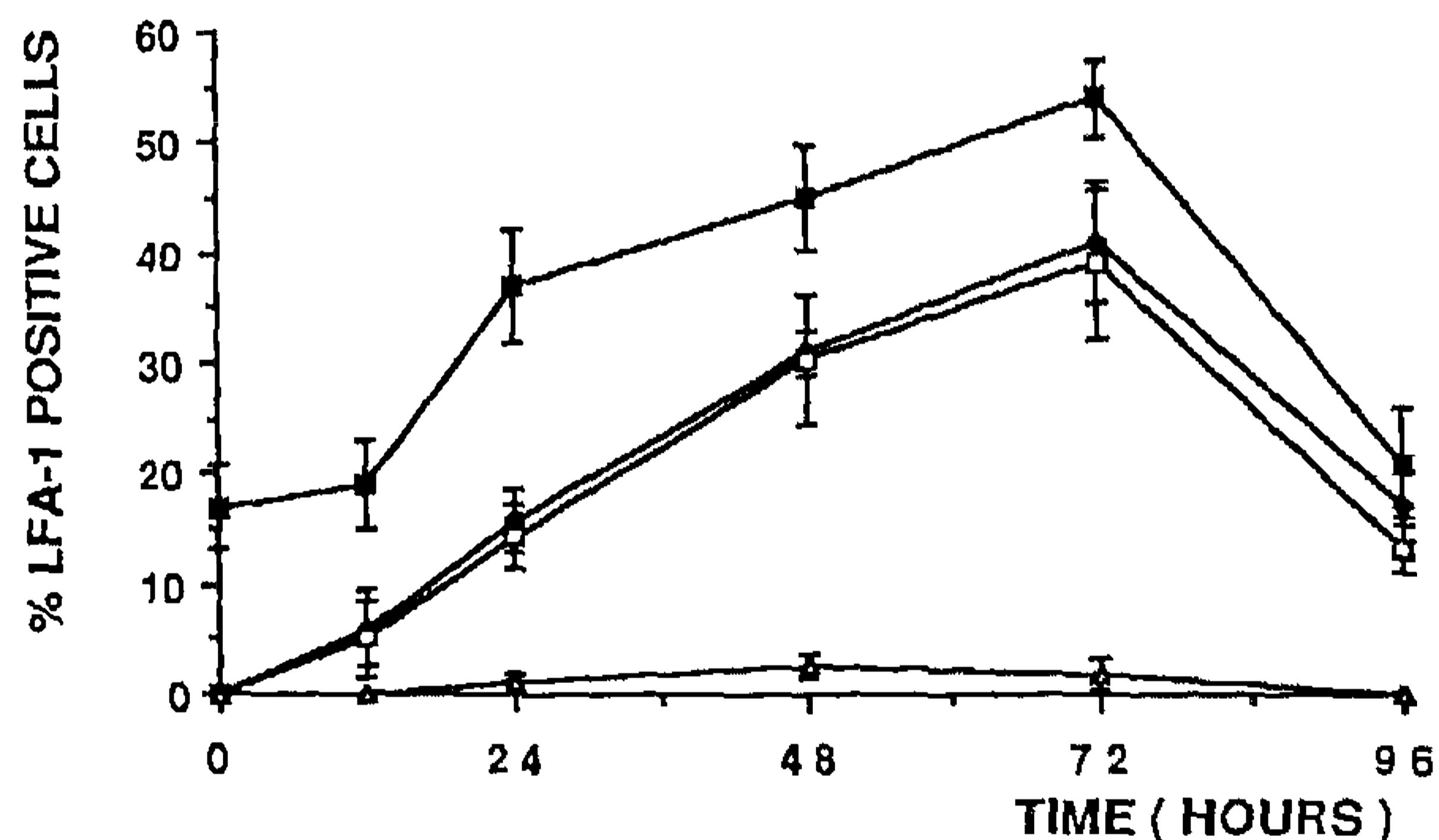


Figure 3. Kinetics of IL-4-induced LFA-1 expression on BL2 and UD61 cells and its inhibition by anti-IL-4 antiserum. IL-4 was added at a concentration of 200 U/ml. □, BL2 + IL-4; ◇, BL2 + IL-4 + preimmune rabbit serum (1/250); △, BL2 + IL-4 + anti-IL-4 antiserum (1/250). ■, UD61 + IL-4. Mean ± SD of four experiments. IL-4-induced LFA-1 expression was also not inhibited by a rabbit anti-human erythrocyte antiserum (1/250, not shown).

was supported by the finding that IL-4 induced the expression of LFA-1 β-chain specific mRNA. BL2 cultured in the presence of IL-4 at concentrations of 100 and 300 U/ml, respectively, for 72 h were found to contain only 14 and 20% LFA-1 positive cells, respectively, in this experiment, whereas IL-2 added at a concentration of 20 IU/ml or IL-3 were ineffective. Analysis of LFA-1 β-chain-specific mRNA revealed that although in this experiment a low degree of LFA-1 expression on the membranes was observed, LFA-1 β-chain mRNA transcription was induced in a dose-dependent fashion (Fig. 5). LFA-1

TABLE III  
LFA-1 is specifically induced by IL-4<sup>a</sup>

Lymphokines Added	% LFA-1 Positive Cells (Mean ± SD)	
	BL2	UD61
0	0.7 ± 0.6	14.5 ± 2.1
IL-1α	1.0 ± 0.0	12.0 ± 1.4
IL-2	3.0 ± 1.0	16.9 ± 1.5
IL-3	0.4 ± 0.6	11.7 ± 0.4
IL-4	28.6 ± 1.5	48.3 ± 3.8
IFN-α	1.0 ± 1.7	13.8 ± 1.7
IFN-γ	0.7 ± 1.1	17.2 ± 2.5
GM-CSF	3.0 ± 2.0	14.3 ± 1.0
LMW-BCGF	3.0 ± 1.7	13.0 ± 2.8

<sup>a</sup> BL2 (three experiments) and UD61 (two experiments) were cultured for 48 h in the presence of different lymphokines. IL-3 was tested as supernatant of Cos-7 cells transfected with the gene encoding human IL-3 (1% v/v). IL-1α, IL-2, IL-4, IFN-α, IFN-γ were used at concentrations of 10, 20, 200, 250, and 100 U/ml, respectively. GM-CSF was used at 10 ng/ml and LMW-BCGF at 20% v/v. These lymphokine concentrations were found to give optimal effects in stimulation of murine thymocytes in the presence of PHA (IL-1α), T cell proliferation (IL-2, IL-4), B cell proliferation (LMW-BCGF), bone marrow colony formation (IL-3, GM-CSF), and induction of class II MHC Ag (IFN-α, IFN-γ).

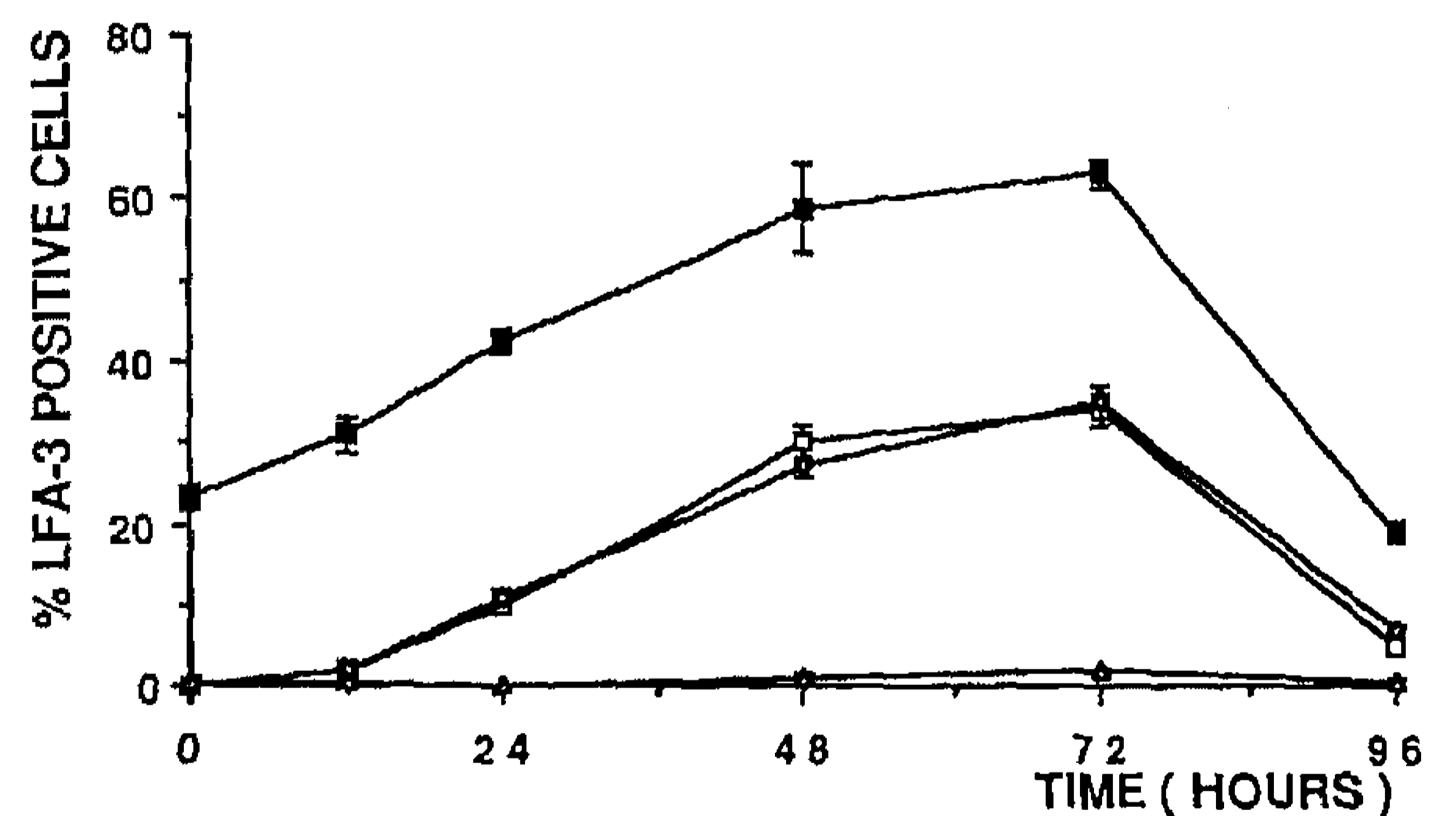


Figure 4. Kinetics of IL-4-induced LFA-3 expression on BL2 and UD61 cells and its inhibition by anti-IL-4 antiserum. IL-4 was added at a concentration of 200 U/ml. □, BL2 + IL-4; ◇, BL2 + IL-4 + preimmune rabbit serum (1/250); △, BL2 + IL-4 + anti-IL-4 antiserum (1/250); ■, UD61 + IL-4. Mean ± SD of two experiments. IL-4-induced LFA-3 expression was also not affected by a rabbit anti-human erythrocyte antiserum (1/250, not shown).

β-specific mRNA could not be detected when the BL2 cells cultured in the absence of IL-4, in the presence of IL-2, or IL-3 transfection supernatant. IL-4 did not affect the constitutive expression of mRNA specific for GAPDH. These results indicate that the induction of LFA-1 membrane expression could be confirmed at the specific mRNA level.

*Effects of IL-4 and anti-LFA-1 mAb on aggregate formation.* LFA-1 Ag have been shown to act as adhesion molecules in heterotypic and homotypic cell interactions, including PMA induced formation of homotypic aggregates of EBV-LCL (12, 13). Therefore, we investigated aggregate formation of BL2 cells in the presence of IL-4 and PMA. BL2 grows as single cells (Fig. 6A). In Figure 6B, it is shown that although IL-4 induced detectable



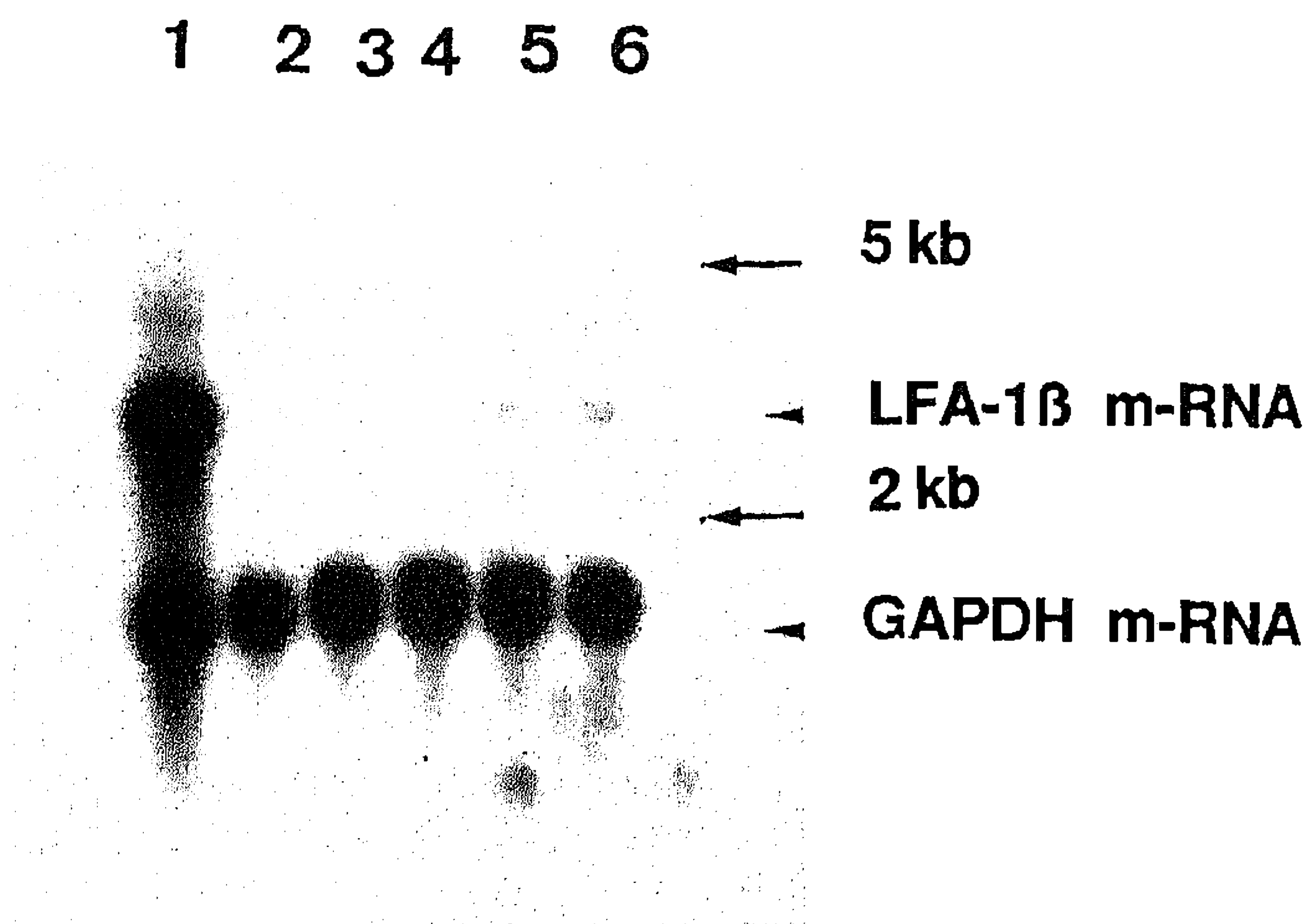


Figure 5. IL-4 induces specific LFA-1  $\beta$ -chain mRNA expression in BL2. LFA-1  $\beta$ -chain mRNA induction in BL2 after incubation with medium only (lane 2); IL-2: 20 IU/ml (lane 3); IL-3, 1% transfection supernatant (lane 4), IL-4 100 U/ml (lane 5), IL-4 300 U/ml (lane 6) for 72 h. HSB2 (T leukemia cell line) was used as control (lane 1). Expression of GAPDH mRNA was not affected by IL-4. Percentage of LFA-1<sup>+</sup> BL2 cells after incubation with medium 0%; IL-2 1%; IL-4 (100 U/ml) 13%; IL-4 (300 U/ml) 20%.

levels of LFA-1 expression on 22% of the BL2 cells after 16 h of incubation, it did not affect the growth characteristics of BL2. Also culturing of BL2 cells in the presence of PMA (1 ng/ml) did not result in detectable levels of LFA-1  $\alpha/\beta$  expression or aggregate formation (Fig. 6C). However, BL2 cells cultured in the presence of both IL-4 and PMA for 16 h formed aggregates (Fig. 6D). No differences in aggregate formation were observed when PMA was added at 1 or 10 ng/ml (not shown). Addition of PMA (1 ng/ml) to BL2 cells that had been cultured in the presence of IL-4 for 18 h resulted in aggregate formation within 2 h (Fig. 6E).

The aggregate formation is mediated by LFA-1, because adhesions induced by combinations of IL-4 and PMA did not occur in the presence of anti-LFA-1  $\beta$ -chain mAb CLB54 (Fig. 6F). Anti-LFA-1  $\alpha$ -chain mAb were less effective. Moderate or slight reductions in aggregate formation were observed in the presence of the anti-LFA-1  $\alpha$ -chain mAb IOT 16 and SPV-L7, respectively, whereas the anti-LFA-1  $\alpha$ -chain mAb SPV-L12 had no effect (not shown). Also, the anti-class I MHC mAb W6/32 (Fig. 6G), the anti-HLA-DR mAb L243, the anti-HLA-DQ mAb SPV-L3, or the anti-CD23 mAb 25 did not affect aggregate formation, despite the fact that IL-4 upregulates the expression of class II MHC and CD23 expression (26) (not shown). The notion that LFA-1 is associated with aggregate formation was furthermore supported by the finding that aggregates of BL2 cells formed by incubation with combinations of IL-4 and PMA, or after the sequential addition of IL-4 and PMA were dispersed 4 h after the addition of mAb CLB54 (Fig. 6H, Table IV). The anti-LFA-1  $\beta$  mAb IOT 16, SPV-L7, and SPV-L12 induced only a weak or a nonsignificant dispersion of existing aggregates 4 h after addition, whereas anti-class I MHC (W6/32) or anti-class II MHC mAb L243 (HLA-DR) and SPV-L3 (HLA-DQ) were ineffective (Table IV). Taken together, these data indicate that LFA-1 Ag are involved in adhesions between BL2 cells. However, induction of LFA-1 expression by IL-4 is insufficient for aggregate formation and that a second activation signal delivered via PMA is required.

*Kinetics of PMA-induced aggregate formation and its*

*inhibition by anti-LFA-1 mAb.* To determine the kinetics of aggregate formation induced by PMA more precisely, BL2 cells that had been cultured with IL-4 for 16 h were subsequently incubated with PMA (1 ng/ml) for various time periods. In Figure 7A, it is shown that significant aggregate formation was already observed after 30 min of incubation, indicating that conversion of LFA-1 to its activated state occurred rather rapidly. Maximal levels of aggregate formation were obtained 4 h after PMA was added. Aggregate formation induced by PMA was strongly blocked in the presence of the anti-LFA-1  $\beta$ -chain mAb CLB54, whereas the anti-LFA-1  $\alpha$ -chain mAb IO 16 or SPV-L7 had moderate or weak blocking activity (Fig. 7A). The control mAb W6/32 and L243, which are directed against class I and class II MHC Ag respectively, did not affect the PMA induced aggregate formation. Interestingly, BL2 cells incubated for 16 h with PMA and subsequently incubated with IL-4 for various time periods started to form low numbers of aggregates after 6 h of incubation with IL-4 (Fig. 7B), whereas continuous incubation with PMA for 22 h failed to induce aggregate formation. This aggregate formation was inhibited by the anti-LFA-1  $\beta$  mAb CLB54.

*IL-4 is unable to induce LFA-1 expression on EBV-LCLs of LAD patients.* To determine whether IL-4 could induce the expression of LFA-1 in LAD patients, EBV-LCL from two LAD patients were incubated with IL-4. The EBV-LCL line of patient A expressed strongly reduced levels of LFA-1 Ag (1%) when compared to normal EBV-LCL, whereas LFA-1 was could not be detected on the EBV-LCL of patient B (Table V). Incubation of the cell lines with IL-4 at a wide range of concentrations (10 to 2000 U/ml) for up to 72 h did not enhance or induce LFA-1 expression. The results with 500 U/ml of IL-4 are shown in Table V. In addition, culturing of both EBV-LCL in the continuous presence of IL-4 (500 U/ml) for 3 wk (by changing the medium and by adding 500 U/ml of IL-4 twice per week) did not induce or enhance the expression of surface LFA-1 (not shown). In contrast, a small increase in both the percentage and MFI of CD23<sup>+</sup> cells was obtained (Table V). It is of importance to note that these two LCL of the LAD patients predominantly grow as single cells with some small loose clumps of maximally 10 cells. LCL of the LAD patients cultured in the presence of IL-4 (500 U/ml) in combination with PMA (10 ng/ml) failed to form aggregates, confirming the fact that aggregate formation cannot be induced in the absence of the LFA-1 expression (not shown).

#### DISCUSSION

The majority of the BL cell lines tested in this study has a reduced or a lack of expression of LFA-1 family Ag. In contrast by screening a large series ( $n = 55$ ) of EBV-LCL, we detected only one LCL (UD61) that consistently displayed a reduced expression of LFA-1. Only a proportion of the UD61 cells (maximal 15%) had detectable levels of LFA-1 expression whereas all the other EBV-LCL tested had high levels of LFA-1 expression on virtually all cells.

There seems to be a correlation between LFA-1 expression and growth characteristics of the BL cell lines. With the exception of Jijoye which is LFA-1 negative, but grows in aggregates, the majority of the BL cell lines that lack or express very low levels of LFA-1 grow as single cells and small loose aggregates. In contrast, BL cells that



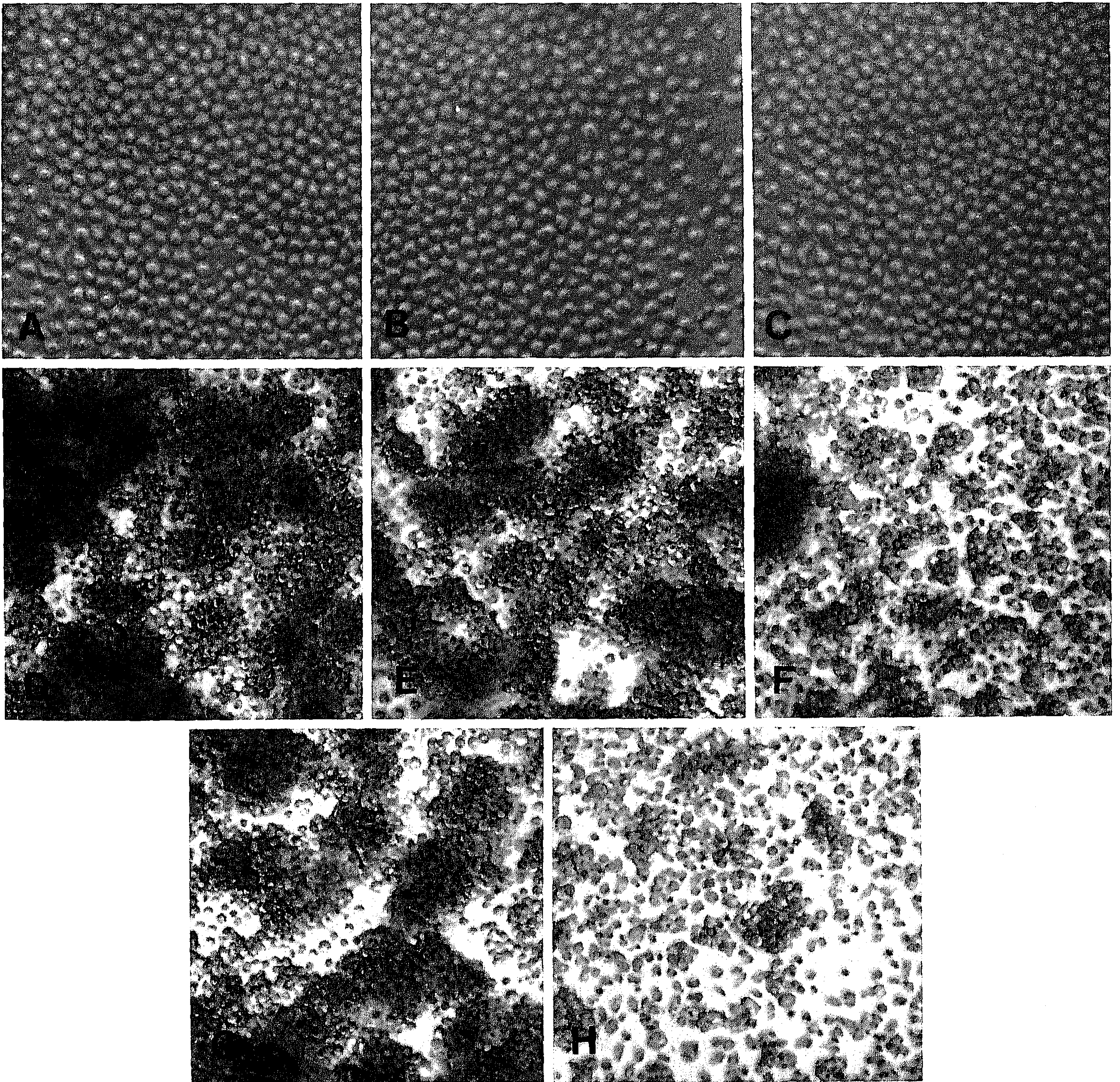


Figure 6. Growth characteristics of BL2 cells. A, medium; B, IL-4 for 16 h; C, PMA for 16 h; D, IL-4 + PMA for 16 h; E, IL-4 for 16 h, followed by PMA for 2 h; F, IL-4 + PMA + mAb CLB54 (1  $\mu$ g/ml) for 16 h; G, IL-4 + PMA  $\pm$  mAb W6/32 (1/200) for 16 h; H, IL-4 for 16 h, followed by PMA for 2 h, followed by mAb CLB54 for 4 h. IL-4 was added at concentrations of 200 U/ml, PMA at 1 ng/ml.

express considerable levels of LFA-1 grow generally in intermediate to large aggregates.

Interestingly, culturing of BL2 and UD61 in the presence of IL-4 resulted in induction or enhancement of LFA-1 expression on BL2 and UD61 cells, respectively. Induction of LFA-1 expression seems to be specific for IL-4, because IL-1- $\alpha$ , IL-2, IL-3, GM-CSF, LMW-BCGF, IFN- $\alpha$  and IFN- $\gamma$  were ineffective. In addition, the LFA-1-inducing effect of IL-4 was completely inhibited by the anti-IL-4 antiserum. Although IL-4 has growth promoting activity on activated B cells (41), it could be excluded that induced/enhanced expression of LFA-1 reflects the selective outgrowth of a minor subpopulation of LFA-1 cells, because no differences in proliferation were observed between BL and LCL cultured in the presence or absence of IL-4 (Table II). The notion that IL-4 induced de novo

LFA-1 expression was confirmed at the specific mRNA level. IL-4 added at concentrations of 100 and 300 U/ml induced transcription of LFA-1 $\beta$ -specific mRNA in a dose-dependent fashion, whereas the constitutive mRNA transcription of the house keeping gene GAPDH was not affected. In the absence of IL-4, no constitutive LFA-1  $\beta$ -chain-specific mRNA expression could be detected.

In addition to the induction of LFA-1 expression, IL-4 was also found to induce the expression of LFA-3 which recently has been shown to represent one of the natural ligands of CD2 (42-45). Inasmuch as limited quantities of anti-LFA-3 antibodies were available, induction of LFA-3 by IL-4 was not studied in detail, but the data obtained thusfar indicate that it followed similar kinetics as that shown for LFA-1 suggesting that the expression of LFA-1 and LFA-3 are induced in a coordinated fashion.



TABLE IV

Dispersion of preformed aggregates of BL2 cells induced by IL-4 in presence of PMA<sup>a</sup>

Aggregates incubated with:	Aggregation Score
Medium	3+
CLB54 (LFA-1 $\beta$ )	<1+
SPV-L7 (LFA-1 $\alpha$ )	2+
SPV-L12 (LFA-1 $\alpha$ )	3+
IOT 16 (LFA-1 $\alpha$ )	2+
25 (CD23)	3+
W6/32 (HLA-A,B,C)	3+
L243 (HLA-DR)	3+
SPV-L3 (HLA-DQ)	3+

<sup>a</sup> BL2 cells were cultured with IL-4 (200 U/ml) and PMA (1 ng/ml) for 40 h. mAb CLB54, SPV-L7, SPV-L12, IOT-16, SPV-L3, and L243 were added at a concentration of 1  $\mu$ g/ml. W6/32 was added at a dilution of 1/200. Aggregates were scored after 4 h of incubation at 37°C and 5% CO<sub>2</sub> in the presence of the various mAb. The mAb CLB54, SPV-L7, SPV-L12, IOT-16, SPV-L3, and L243 tested at 5  $\mu$ g/ml and W6/32 diluted 1/50 gave similar blocking results (not shown).

Taken together these data indicate that IL-4 in addition to its capacity to induce CD23 (26, 46) and class II MHC Ag (26) on normal B cells and BL cell lines, also induces the induction of LFA-1 and LFA-3 Ag. IL-4 did not induce the expression of detectable levels of the other members of the LFA-1 family, despite the fact that CR3bi and p150,95 share the same  $\beta$ -chain. The mechanisms underlying the preferential induction of LFA-1 expression by IL-4 remain to be determined, but it may be possible that it is related to the cell type studied, because recently we demonstrated that induced the expression of CR3bi and p150,95 on human monocytes, whereas LFA-1 expression remained unaltered (47). Also Springer and Anderson (48) have shown that individual members of the LFA-1 family can be independently modulated upon cell activation.

BL2 is growing in single cells and does not form aggregates and therefore can be classified to belong to the group I BL, which according to their cell surface markers are identical to biopsy obtained BL cells (49). Clayberger et al. (24) reported that a proportion of lymphomas, including the majority of biopsy obtained BL, failed to express LFA-1, indicating that the lack or reduced LFA-1 expression on BL cell lines studied is not the result of *in vitro* culture conditions. These LFA-1-deficient BL cells were very poor stimulators of both autologous and allogeneic T cell responses, and an initial survey indicated that LFA-1 expression on these lymphoma cells correlated with relapses (24). Therefore these authors suggested that the tumor cells lacking LFA-1 cannot initiate

efficient immune responses *in vivo*, which might contribute to the escape of these tumors from immunosurveillance. However, we demonstrate here that BL2 cells also lacked expression of LFA-3, which is a natural ligand for CD2 that is involved in the alternative T cell activation pathway (50–52). In addition, recently it has been demonstrated that BL (including BL2) have defective expression of ICAM-1 (25), which is one of the natural ligands of LFA-1 (16–18). We and others (11, 51) have demonstrated that destruction of target cells by allospecific CTL clones is preceded by Ag nonspecific adhesions that are mediated via two different adhesion pathways in which effector cell LFA-1 and target cell ICAM-1, and effector cell CD2 and target cell LFA-3 are involved (51–52). Therefore, the general absence or very low expression of adhesion molecules may account for the failure of BL to form adhesions with T cells and subsequent induction of T cell responsiveness. This notion is supported by our findings that conjugate formation between CD4<sup>+</sup> T cell clones and BL2 cells is reduced when compared to that between these T cell clones and the EBV-LCL of patient BL2 which expressed normal levels of LFA-1 and LFA-3 respectively (D. Blanchard and H. Spits, unpublished data). Furthermore, recent murine studies have demonstrated that IL-4 indeed augmented adhesions between Ag-specific T and B cells after B cells had been incubated with IL-4 (53), but the increased conjugate formation was attributed to enhanced class II MHC expression. Although IL-4 also enhances class II MHC expression on BL2 cells (26) anticlass II MHC antibodies failed to prevent the formation of homotypic aggregates. In contrast, preliminary data obtained with human B cells indicated that also enhances LFA-1 expression on normal purified tonsillar B cells as judged by an increase of the mean fluorescence intensity. In addition, the increased expression of LFA-1 observed upon activation of the B cells was further enhanced in the presence of IL-4 (F. Rousset, unpublished).

Despite the fact that IL-4 induces LFA-1 expression on BL2 cells, this did not result in adhesion between BL2 cells and aggregate formation. Homotypic cell/cell interactions were only induced when IL-4 was added simultaneously with PMA. Also addition of PMA to BL2 cells cultured in IL-4 for 16 h resulted in induction of aggregate formation within 30 min, without enhancing LFA-1 expression. PMA added alone for 18 h was ineffective in inducing LFA-1 expression or aggregate formation. Fur-

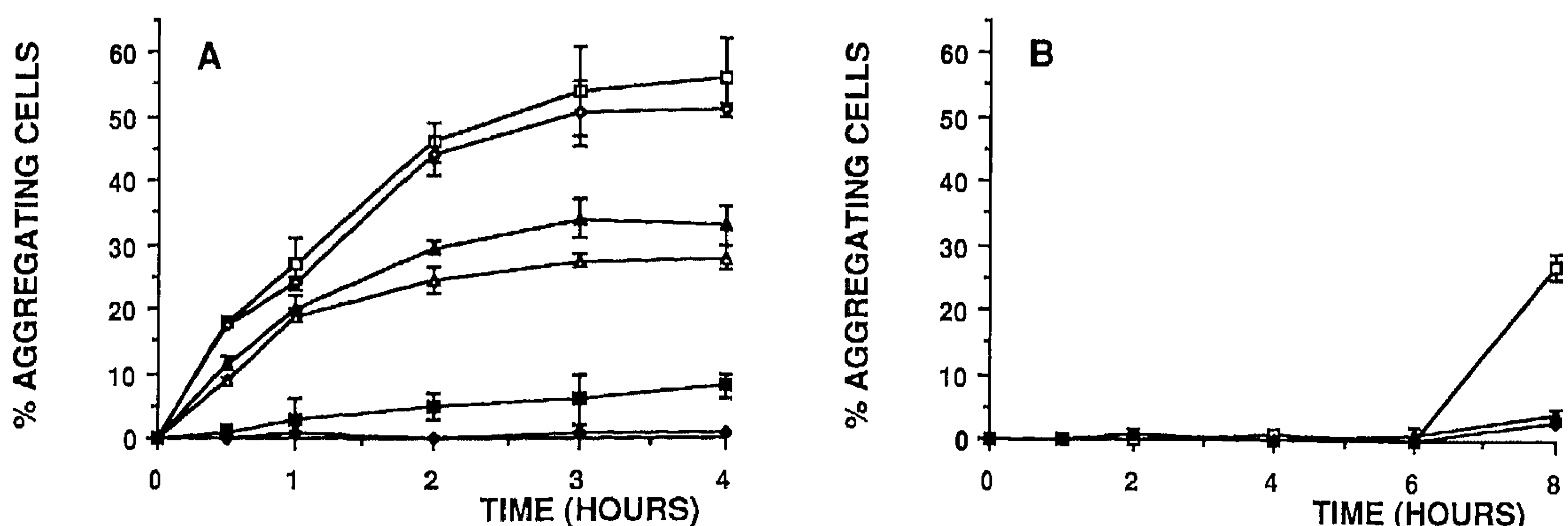


Figure 7. Kinetics of aggregate formation (mean  $\pm$  SD of two experiments). A, BL2 cells were preincubated with IL-4 (200 U/ml) for 16 h; PMA or mAb were added at time 0.  $\square$ , PMA (1 ng/ml);  $\diamond$ , PMA + W6/32 (1/200);  $\blacktriangle$ , PMA + SPV-L7 (1  $\mu$ g/ml);  $\blacktriangle$ , PMA + IOT 16 (1  $\mu$ g/ml);  $\blacksquare$ , PMA + CLB54 (1  $\mu$ g/ml);  $\diamond$ , medium. B, BL2 were preincubated with PMA (1 ng/ml) for 16 h. IL-4 was added at time 0.  $\Delta$ , PMA (1 ng/ml);  $\square$ , PMA (-16 h) + IL-4;  $\blacksquare$ , PMA + IL-4 + CLB54 (1  $\mu$ g/ml).



TABLE V

Effects of IL-4 on LFA-1 $\alpha$ , $\beta$  expression on LCL from LAD patients<sup>a</sup>

Cell Line	Incubated with	% Positive Cells			
		LFA-1 $\alpha$	LFA-1 $\beta$	CD23	Class I-MHC
Patient A	none	1 $\pm$ 1	2 $\pm$ 1	67 $\pm$ 5	95 $\pm$ 1
	IL-4	3 $\pm$ 2	3 $\pm$ 1	83 $\pm$ 4	94 $\pm$ 2
Patient B	none	0 $\pm$ 0	0 $\pm$ 0	81 $\pm$ 2	98 $\pm$ 1
	IL-4	0 $\pm$ 1	2 $\pm$ 1	91 $\pm$ 4	95 $\pm$ 2

<sup>a</sup> Cells of LCL of LAD patients A and B were incubated with IL-4 (500 U/ml) for 72 h. LFA-1 $\alpha$ , LFA-1 $\beta$ , CD23, and class I MHC Ag were determined by using mAb SPV-L7, CLB54, 25, and W6/32, respectively. Mean  $\pm$  SD of six experiments.

thermore, aggregate formation induced by IL-4 and PMA was blocked effectively by the anti-LFA-1  $\beta$ -chain mAb CLB54 and, to a lesser extent by the anti-LFA-1  $\alpha$ -chain mAb SPV-L7, SPV-L12 and IOT 16. The anti-LFA-1  $\beta$ -chain mAb CLB 54 dispersed aggregates that were already formed more efficiently than anti-LFA-1  $\alpha$ -chain mAb, which had only weak effects. These results indicate that the LFA-1 molecules were involved in aggregate formation.

The LCL of the LAD patients which could not be induced to express LFA-1 Ag also did not form aggregate in the presence of IL-4 or in the presence of IL-4 in combination with PMA, confirming the notion that aggregate formation can not be induced in the absence of LFA-1 expression. The defective LFA-1 expression in LAD patients varies and can reflect defects at the DNA or RNA level or can be attributed to posttranslational protein modifications (22, 38, 54–56). The actual defects in the patients studied here are not known, but our data indicate that IL-4 added at a large concentration range and for prolonged periods cannot restore LFA-1 expression. These data are in line with results that were reported by Rothlein and Springer (13) who showed that PMA induced a strong aggregation of normal LCL, but was unable to induce aggregate formation by LCL of LAD patients. PMA did not induce LFA-1 expression on BL2 cells, addition of IL-4 to BL2 cells that had been incubated in PMA for 16 h resulted in a low degree of LFA-1 expression and aggregate formation after 6 h, whereas continuous incubation in PMA for 22 h did not result in aggregate formation. These results confirm the notion that both IL-4 and PMA are required for aggregate formation and indicate that LFA-1 is more rapidly induced on PMA-treated BL2 cells.

Collectively, our data indicate that IL-4 induces LFA-1 expression, but that a second signal provided by PMA is required to convert the LFA-1 molecule from an inactive to an active configuration, thereby allowing binding of LFA-1 to ICAM-1. Inasmuch as PMA activates PKC (57), it may be possible that addition of PMA results in an intracellular signal that activates PKC, resulting in subsequent phosphorylation of the LFA-1  $\beta$ -chain. Molecular cloning of LFA-1  $\beta$ -chain revealed that it contained various possible phosphorylation sites including four serine residues (38). The notion that LFA-1 can become phosphorylated via PKC is supported by the finding that only the serine residues of the  $\beta$ -chain were found to be phosphorylated (58).

Our data indicate that LFA-1 is not an adhesion initiating molecule, but requires a structural modification or activation before it contributes to adhesion strengthening. Such an adhesion strengthening role of LFA-1 has also been proposed by others (13, 59). The natural signal

that would induce the activated state remains to be determined, but LFA-1 activation may be induced via other, LFA-1-independent adhesion pathways, such as those between CTL and target cells mediated via CD2 and LFA-3 or by the weak LFA-1-independent cell-cell interactions that precede LFA-1-dependent adhesions (60–62). Finally, it remains to be determined whether the low levels of ICAM-1 in type I BL cell lines like BL2 (25) are sufficient to allow aggregate formation or whether IL-4 also simultaneously up-regulates ICAM-1 expression.

**Acknowledgments.** We wish to thank Miss I. Durand for flow microfluorimetry assistance and Mrs. N. Courbière for typing the manuscript.

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