Elly Van de Wiel-van Kemenade[●], Anje A. Te Velde[●], Annemiek J. De Boer[●], Ron S. Weening[▽], Alain Fischer[△], Jannie Borst[●], Cornelis J. M. Melief[●◇] and Carl G. Figdor[●]

Division of Immunology[●], The Netherlands Cancer Institute, Antoni van Leeuwenhoek Huis, Amsterdam, Emma Children's Hospital[▼], Department of Pediatrics, Academic Medical Center and Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam and INSERM U 132[△], Hôpital des Enfants Malades, Paris

Both LFA-1-positive and -deficient T cell clones require the CD2/LFA-3 interaction for specific cytolytic activation*

We investigated the capacity of T lymphocytes from a leukocyte adhesion-deficient (LAD) patient to respond to alloantigen. Leukocytes of this patient completely lacked LFA-1 surface expression due to the absence of mRNA coding for the LFA-1 β chain. Despite the absence of LFA-1, T lymphocytes obtained from this patient, cultured with allogeneic stimulator cells (lymphoblastoid B cells JY), were capable of lysing JY cells. Furthermore, two T cell clones (one $CD4^+$ and one $CD8^+$), generated from this lymphocyte culture, specifically lysed the allogeneic lymphoblastoid JY cells. The cytolytic capacity of LFA-1-negative T lymphocytes and T cell clones was comparable to that of control LFA-1-positive T cells with allospecificity against JY. Detailed analysis of the CD4 positive and LFA-1-negative T cell clone demonstrated that it specifically recognized HLA-DQ. Antibody inhibition studies showed that the CTL/target cell interaction was mediated through the CD2/LFA-3 adhesion pathway. LFA-1 expressed by the target cells did not participate in the CTL/target cell conjugate formation and contributed only minimally to the cytotoxic activity. Moreover, when allogeneic LFA-1-deficient B cells, bearing the appropriate HLA-DQ alloantigen, were used as target cells, significant levels of specific cytotoxicity were measured, further excluding a role for LFA-1 in this interaction. The adhesion molecules, VLA-4, CD44 and L-selectin (LECAM1) were not involved.

These results demonstrate that LFA-1-negative T lymphocytes can exert allospecific cytotoxicity and that CTL/target cell contact is mediated through the CD2/LFA-3 route. This observation may explain in part why in LAD patients viral infections, cleared largely by T cells, are less frequently observed than bacterial infections, in which phagocytic cells play a major role.

1 Introduction

Cell-cell interactions among leukocytes and of leukocytes with other cell types play an essential role in immune responses. Multiple cell surface receptors participate in cell adhesion including the glycoproteins that belong to the integrin or to the Ig supergene families [1–3]. LFA-1, CR3, and p150,95 comprise a group of three integrins exclusively expressed by leukocytes. These heterodimeric adhesion molecules are each composed of a unique α chain and share a common β chain, the β_2 chain of the integrins [4]. Various studies demonstrate that the interaction of LFA-1 with its counterstructures ICAM-1 [5] and ICAM-2 [6] plays a major role in antigen-presenting cell/responder cell, and cytotoxic effector cell/target cell interactions [7–9]. The physiological importance of the LFA-1 receptor family is demonstrated in patients with a serious immunodeficiency syndrome, termed leukocyte adhesion deficiency (LAD). This disease is caused by the complete (severe type) or partial (moderate type) absence of cell surface expression of these adhesion receptor molecules [10–13]. The syndrome is characterized by recurrent and often fatal bacterial infections, mainly due to decreased phagocyte functions and poor leukocyte mobilization [10, 14]. The rare reports of viral infections in these patients suggest that, they may be capable of evoking an immune response against viral pathogens, despite the absence of the β_2 integrins. These obervations suggest the involvement of other adhesion molecules in leukocyte function, such as: CD2 – LFA-3, VLA-4, and L-selectin (LECAM1) [15–18].

In this study we have investigated whether specific cytotoxic T lymphocytes can be generated from peripheral blood mononuclear cells of such LAD patients, and which adhesion routes are utilized in interaction of such CTL with target cells. The results demonstrate that cloned LFA-1-negative T cells are capable of exhibiting an allospecific cytotoxic response, and that this target cell adhesion is mediated through CD2 and LFA-3.

[I 10198]

* This work was supported by the Dutch Cancer Society, grant number NKI 87-5.

◇ Present address: Department of Immunohaematology and Bloodbank, Academic Hospital Leiden, The Netherlands

Correspondence: Elly Van de Wiel-van Kemenade, Division of Immunology, The Netherlands Cancer Institute, Plesmanlaan 121, NL-1066 CX Amsterdam, The Netherlands

2 Materials and methods

2.1 T cell cultures

Peripheral blood lymphocytes of an LAD patient (VA) were isolated by centrifugation on Ficoll-Isopaque. PBL

0014-2980/92/0606-1467$3.50+.25/0

were cloned by limiting dilution (5×10^4 – 0.5 cells/well) in round-bottom microtiter plates in 100 µl Iscove's modified Dulbecco's medium (IMDM) supplemented with 5 % pooled human serum, 1 % antibiotic/myotic solution (Flow, Irvine, Scotland) and 0.2 % kanamycin solution (Sigma, St. Louis, MO). The LAD lymphocytes were stimulated with 10^4 irradiated (50 Gy) allogeneic JY cells and 2×10^4 irradiated (40 Gy) allogeneic lymphocytes per well from random donors, together with rIL-2 50 U/ml (Eurocetus, Amsterdam, The Netherlands) and 0.2 µg/ml PHA. T cell cultured in bulk (5×10^4 cells/well) and clones (0.5 cell/well) were expanded and restimulated once a week with this freshly prepared cell mixture together with rIL-2 and PHA. Control clones and T cell cultures (EG) were generated and cultured under the same conditions. T cells had been cultured in bulk for 8–10 weeks. The CD4-positive cytotoxic T cell clone, JS 136 was described previously [19] and specific recognized an HLA-DR determinant exposed by JY cells.

2.2 Cell lines

To determine the specificity of the immune responses the following EBV-transformed B lymphoblastoid cells were used: JY (HLA-: A2; B7; DQw1,DQw3; DR4,DRw6), LAD VA (HLA-: A3,A11; B7,B51; DQw1; DR2), BA (HLA-: A24, A26; B7, B35; Cw3,Cw4; DQw3; DR4), MT (HLA-: A30,A31; Bw60; Cw3; DQw3; DR4; DPw4; Dw14), HO301 (HLA-: A3; B14; Cw8; DQw6; DRw6; DPw5; Dw19,Dw26), AWELLS (HLA-: A2; B44; Cw5; DQw7; DR4; DPw3,DPw4; Dw4), WIN (HLA-: A1; Bw57; Cw6; DQw2,DQw9; DR7; DPw4), APD (HLA-: A1; Bw60; DQw6; DRw13; DPw4; Dw18,Dw25). The LAD VA B cell line was established in our laboratory by EBV infection of B cells from LAD patient (VA) and the BA B cell line by EBV infection of control B cells. The other EBV-transformed B cell lines were kindly provided by Drs. A. Termijtelen and F. Claas, University Hospital of Leiden, The Netherlands. To determine an allospecific cytotoxic activity against LAD EBV-transformed B cells, four previously described LFA-1-negative B cell lines were used: AI, HS, CP, and MC [20]. The B cells and the MHC-negative erythroleukemia cells K562 were cultured in IMDM supplemented with 10 % FCS, 1 % antibiotic/myotic solution and 0.2 % kanamycin solution. All cell lines were free of mycoplasma contamination.

2.3 mAb

For phenotypic analysis and for inhibition the functional capacity of the T cell clones the following mAb were used: SPV-L7 (anti-LFA-1 α chain, CD11a) [21]; Bear-1 (anti-CR3 α chain, CD11b) [22]; SAM-1 (anti-VLA-5 α chain, CD49e) [23]; SPV-T3b (anti-CD3) [24]; SPV-L3 (anti-HLA-DQ) [24]; NKI-P2 (anti-CD44) [25]. The following mAb were provided by several laboratories: CLB-LFA-1/1 (anti LFA-1 common β chain, CD18) [26] and CLB-T11/1 (anti-CD2) [27], Dr. R. van Lier, Amsterdam, The Netherlands; CLB-10G11 (anti-VLA-2 α chain, CDw49b) [28], C17 (anti-β_3 chain, CD41) [29], Dr. A. von dem Borne, Amsterdam, The Netherlands; 4399.b (anti-β_4 chain, CD61) [30], and GoH3 (anti-VLA-6 α chain, CD49f) [31], Dr. A. Sonnenberg, Amsterdam, The Netherlands; J143 (anti-VLA-3 α chain, CD49c) [32], Dr. E. Klein, Ulm, FRG; HP2/1, HP1/2 (anti-VLA-4 α chain, CD49d) [33], Dr. F. Sánchez-Madrid, Madrid, Spain; B-5G10 (anti-VLA-4 α chain, CD49d) [34], Dr. M. Hemler, Boston, MA; TS2/9 (anti-LFA-3, CD58) [8] and TS2/16 (anti-β_1 chain, CD29) [35], Dr. T. Springer, Boston, MA; AIIB2 (anti-β_1 chain, CD29) [36], Dr. C. Damsky, San Francisco, CA; K20 (anti-β_1 chain, CD29) [37], Immunotech S.A., Marseille, France; RR1/1 (anti-ICAM-1, CD54) [38], Dr. T. Springer, Boston, MA; RIV-6 (anti-CD4) [39], Dr. F. UytdeHaag, Bilthoven, The Netherlands; WT82 (anti-CD8) [40], Dr. W. Tax, Nijmegen, The Netherlands; B-ly-6 (anti-p150/95 α chain, CD11c) [41], Dr. S. Poppema, Edmonton, Canada; Leu-8 (anti-LECAM1) [18], Becton Dickinson, San Jose, CA.

2.4 Nothern blot analysis

Total RNA was extracted from 5×10^7 cloned LAD T cells and the control T cell clone, JS 136, according the guanidinium isothiocyanate method [42]. Approximately 10 µg/sample of RNA was fractionated on a 1 % agarose gel containing formaldehyde. Ethidium bromide staining showed that comparable amounts of RNA from both T cell clones were applied. RNA was blotted to a nitrocellulose membrane and the filter was hybridized at 65 °C in the presence of 10 % dextran sulfate with a cDNA probe, recognizing the LFA-1 α chain [43] and ^{32}P-labeled by the random priming technique. The filter was washed twice in 1 × SSC, 0.1 % SDS and once in 0.1 × SSC, 0.1 % SDS at 65 °C and exposed to film overnight. Subsequently the filter was stripped in H_2O at 95 °C, and hybridized with the ^{32}P-labeled cDNA probe which recognized the common β_2 chain [44]. Both cDNA probes were kindly provided by Dr. T. Springer, Boston, MA.

2.5 Flow cytometric analysis

Cells were incubated with murine mAb at saturating concentrations for 30 min on ice, washed and stained with polyclonal goat anti-mouse $F(ab')_2$ antibody fragments coupled with FITC for another 30 min on ice. Samples were analyzed on a FACScan (Becton Dickinson, Mountain View, CA).

2.6 Cytotoxicity assay

One thousand labeled target cells (100 µCi ^{51}Cr/10^6 cells, Amersham Int., Amersham, GB) were incubated in V-bottom microtiter plates with various numbers of effector cells in 150 µl IMDM with 0.25 % BSA. The plates were centrifuged for 5 min at 50 × g and incubated at 37 °C and 5 % CO_2 during 4 h. After the incubation period 100 µl of supernatant was collected from each well and counted in a gamma counter. All tests were carried out in triplicate. Specific lysis was determined as percentage of the net maximal ^{51}Cr release by detergent. Spontaneous release in the absence of CTL was <25 % of maximal release by detergent in all experiments.

2.7 Nα-Benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT) esterase secretion

The BLT esterase secretion was determined as described by Spits et al. [45]. Cloned T cells (3×10^5) were mixed with equal numbers of target cells in a round-bottom well of microtiter plates in a total volume of 200 µl IMDM with 5 % FCS and incubated at 37 °C and 5 % CO_2 during 3 h. After incubation supernatants were harvested and BLT secretion was determined. The tests were performed in triplicate. The results were expressed as percentage of total net enzyme activity.

2.8 T cell proliferation assay

Various numbers of cloned T cells were cultured in round-bottom microtiter plates in 150 ml of IMDM with 5 % human serum in the presence of 5×10^4 irradiated (100 Gy) stimulator cells. After 72 h of co-culture at 37 °C and 5 % CO_2 each well was pulsed with 0.4 µCi of [^{3}H] thymidine (New England Nuclear, Boston, MA) for 4 h, after which the DNA was collected using a Titertek multiwell harvester and the incorporated radioactivity was counted. The results were presented as the mean cpm of triplicated cultures.

2.9 Determination of heterotypic cell aggregation

Heterotypic cell aggregation was determined as described previously by Kuypers et al. [46]. T cell clones (2×10^6 cells/ml) were labeled with 100 µM sulfofluorescein diacetate (SFDA), JY and K562 cells (2×10^6/ml) were labeled with hydroethidine (HE) 40 µg/ml in a shaking water bath for 60 min at 37 °C. Labeled cells were washed three times and resuspended in IMDM with 10 % FCS. Equal volumes of labeled cells were mixed and incubated at room temperature. After incubation the cells were fixed in paraformaldehyde (0.5 %) and the number of heterotypic cell aggregation was measured in a FACScan (Becton Dickinson). The results were expressed as the percentage targets/effector conjugates of all analyzed particles containing target cells.

3 Results

3.1 Phenotype of the LFA-1-negative T cell clones

Immunofluorescence analysis of 21 T cell clones derived from LAD patient VA showed that they lacked cell surface expression of the β_2 integrin family, LFA-1, CR3 and p150/95 (Table 1). This suggested a defect in expression of the common β_2 chain. Northern blot analysis of steady-state mRNA isolated from an LAD T cell clone indeed indicated that mRNA encoding the β_2 chain was completely absent, while it could readily be detected in an LFA-1-positive control clone (Fig. 1). LFA-1α mRNA of this LAD clone, however, was expressed at a level comparable to that in the control clone (Fig. 1).

Futher examination of LAD T cell clones by immunofluorescence for expression of other integrins and a number of cell surface molecules, important for T cell functions, revealed no difference between LAD T cell clones and control clones. Table 1 shows the results of 3 LAD clones out of 12 tested. Of the integrin family, only VLA-4 /β_1 complex was expressed at significant levels (Table 1). In addition, all 12 LAD T cell clones expressed the αβ T cell receptor and exhibited similar levels of the activation antigens CD25 (IL-2R) and CD45, and lacked Fcγ receptors (data not shown).

Table 1. Antigen expression on cell surface of LAD and control T cell clones

Antigen		mAb	Fluorescence intensity[a)]				
			LAD 1.1	LAD 6.1	LAD 6.6	JS 136	EG D2
Control			6	3	4	4	6
CD11a	LFA-1α	SPV-L7	5	4	3	449	360
CD11b	CR3	Bear 1	6	6	4	55	39
CD11c	p150,95	B-ly 6	4	3	4	3	11
CD18	β_2	CLB-LFA-1/1	4	4	3	331	347
CDw49b	VLA-2α	10G11	9	14	12	12	16
CDw49c	VLB-3α	J143	11	6	12	17	15
CDw49d	VLA-4α	HP2/1	124	142	129	73	109
CDw49e	VLA-5α	SAM1	12	9	11	13	19
CDw49f	VLA-6α	GoH3	13	9	8	7	14
CD29	β_1	TS2/16	98	113	88	64	86
CD41	β_3	C17	5	3	4	5	5
CD61	β_4	4399.b	4	3	2	3	7
CD54	ICAM-1	RR1/1	47	38	29	24	42
CD58	LFA-3	TS2/9	52	64	41	41	105
CD2		CLB-T11/1	298	481	416	335	351
CD3		SPV-T3b	308	499	360	290	305
CD4		RIV-6	5	402	312	188	286
CD8		WT82	485	7	5	8	7
CD44		NKI-P2	323	421	162	259	363
	LECAM1	Leu 8	7	7	8	5	9

a) Intensity of fluorescence reported as mean channel intensity (arbitrary units).

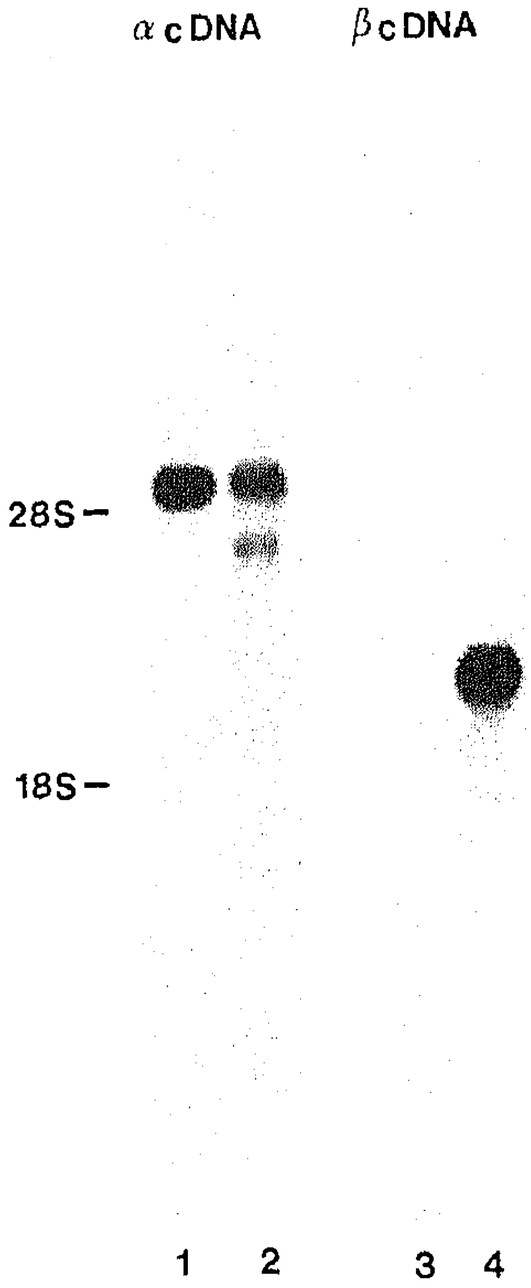

Figure 1. Northern blot analysis of LFA-1 α and β chain expression in LAD and control T cell clones. Total mRNA was extracted from LAD 6.1, a T cell clone lacking LFA-1 surface expression, that was generated from LAD patient VA (lanes 1 and 3), and JS 136 an LFA-1-positive T cell clone (lanes 2 and 4), generated from a healthy donor. LFA-1 transcripts were detected by hybridization with ^{32}P-labeled probes specific for the LFA-1 α chain (lanes 1 and 2), and for the common β chain (lanes 3 and 4).

3.2 Cytotoxic activity of LFA-1-negative T cells

We investigated the responsiveness of LAD T cells after stimulation with allogeneic EBV-transformed JY cells. LAD T cells, cultured in bulk, showed a cytotoxic capacity as high as control T cells (Fig. 2). Furthermore, 2 LAD T cell clones, out of 21 tested, showed cytolytic activity against JY, despite the absence of LFA-1. One of the clones was $CD8^+$ (LAD 1.1), the other $CD4^+$ (LAD 6.6). The results in Fig. 2 demonstrate that the cytolytic activity of LAD T cell clones against the stimulator cells JY was similar to that of the control T cell clones (JS and Eg). No cytotoxic activity of both $CD4^+$ T cell clones (LAD 6.6, JS 136, and Eg D2) and the in bulk cultured T cells (LAD; control) against the nonspecific target K562 (the erythromyeloid cells that lack HLA class I and class II expression) could be observed, whereas the $CD8^+$ T cell clone (LAD1.1) exhibited low cytotoxic activity against K562 (data not shown). To examine this LFA-1-independent cytotoxic activity and its specificity in more detail, we studied LAD 6.6, the $CD4^+$ LAD T cell clone, since this clone showed the highest target specificity. The cytotoxic capacity of both clones (LAD 6.6 and the control clone JS 136) could be inhibited by mAb against CD3 and CD4 (Fig. 3A), indicating that the target cell is specifically recognized by the TcR. This notion is supported by the BLT serine esterase release which was observed after co-culturing of the T cells with JY cells but not with K562 cells

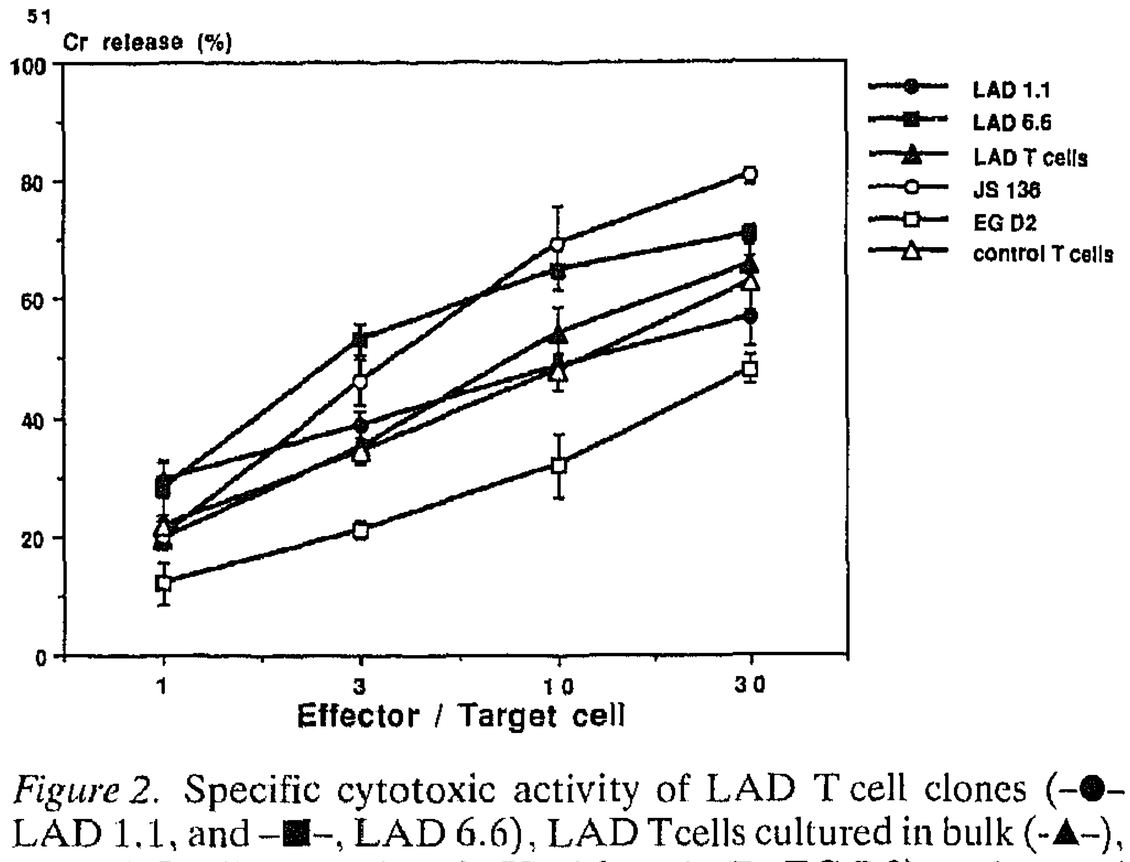

Figure 2. Specific cytotoxic activity of LAD T cell clones (–●– LAD 1.1, and –■–, LAD 6.6), LAD Tcells cultured in bulk (-▲–), control T cell clones (–○–), JS 136, and –□– EG D2), and control T cells cultured in bulk (–Δ–). Various concentrations of effector cells were added to 1×10^3 specific target cells (JY).

(Fig. 3B). Release of BLT serine esterase as a result of CTL degranulation is thought to be specially triggered by TcR ligation [45].

The lytic activity of LAD 6.6, but not that of the HLA-DR specific T cell clone JS 136 [19], could be inhibited by mAb specific for HLA-DQ suggesting that the cytolytic activity of LAD 6.6 is directed against a determinant on the HLA-DQ molecule (Fig. 3A). To analyze further the specificity of the T cell clone LAD 6.6 we used a panel of well-characterized EBV-transformed B cell lines. JY and 3 other EBV-transformed B line cells out of 16 were lysed by LAD 6.6 and stimulated proliferation of LAD 6.6. (Fig. 4 shows 6 B cell lines out of 16 tested). Out of the four lines recognized, three shared the HLA-DQw3 allotype. Again, lysis could be inhibited by addition of mAb against HLA-DQ but not by mAb against HLA class I (data not shown).

From these results we conclude that LAD T cell clones can exert specific cytotoxic activity in spite of the lack of the LFA-1 antigen at the cell surface. The LAD 6.6 T cell clone most likely recognized a determinant on the HLA-DQw3 molecule.

3.3 Role of adhesion molecules in cytotoxicity

Blocking experiments with mAb demonstrated that the specific cytolytic activity of LAD 6.6 against the LFA-1-positive target cell, JY, could be inhibited (±55 %) by anti-LFA-3, anti-CD2 mAb, or by a combination of these mAb (Fig. 5). A combination of mAb against LFA-1 and ICAM-1 had only a minor inhibitory effect (22 %), caused by the fact that JY cells express normal levels of LFA-1 and LAD 6.6 cells express ICAM-1. Blocking of both adhesion pathways caused an inhibition of the cytotoxic activity of 83 %. Three mAb directed against VLA-4 α chain (HP1/2, HP2/1, B-5G10), and three against the β chain (TS2/16, AIIB2, K20) of the integrin adhesion receptor which had previously been shown to participate in cytotoxic T cell interactions [47] were ineffective, despite the fact that some of them (HP1/2, and AIIB2) showed a high inhibitory effect

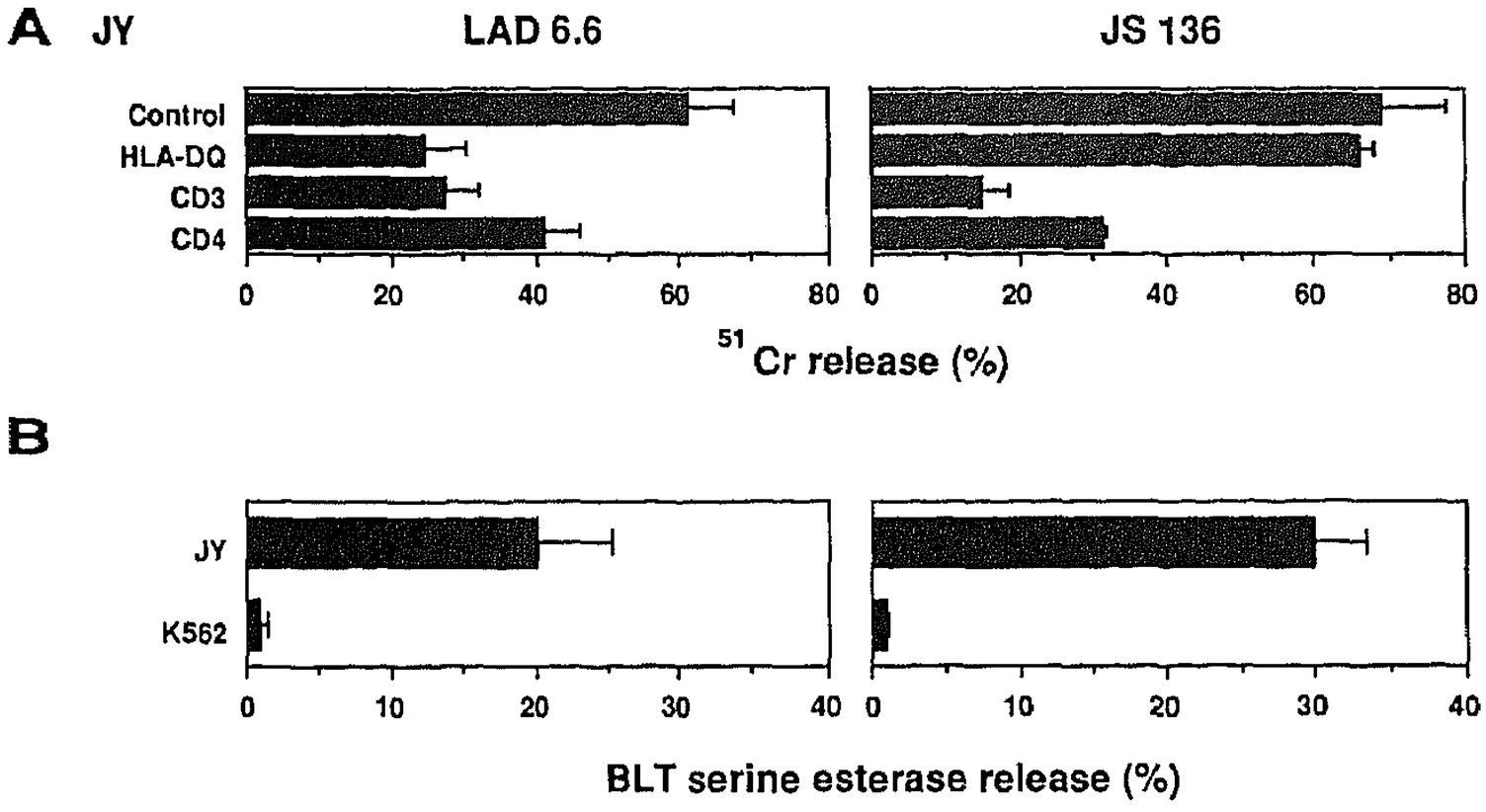

Figure 3. Specific cytotoxicity of T cell clones LAD 6.6 (LFA-1 negative) and JS 136 (LFA-1 positive) with the target cells (JY). The effector: target cell ratio was 30:1. (A) Inhibition of cytotoxicity by mAb: purified IgG (10 μg/ml) was present during the assay. mAb against HLA class I (W6/32) was used as control mAb. One representative experiment out of four is shown. (B) BLT serine esterase release from LAD 6.6 and JS 136 after stimulation with JY cells and cells of the erythromyeloid cell line K562.

on VLA-4-mediated T cell adhesion to endothelial cells [48, 49]. In addition, mixtures of anti-VLA-4 mAb with anti-CD2 and anti-LFA-3 or anti-LFA-1 and anti-ICAM-1 mAb did not result in enhanced inhibition of lysis. Further, mAb against CD44 were also ineffective (Fig. 5). Interestingly, we found that even when LFA-1 is expressed on both, the effector cells (JS 136) and target cells (JY), the LFA-1/ICAM-1 adhesion pathway was subserved by the CD2/LFA-3 pathway in this allospecific cytotoxic activity (Fig. 5).

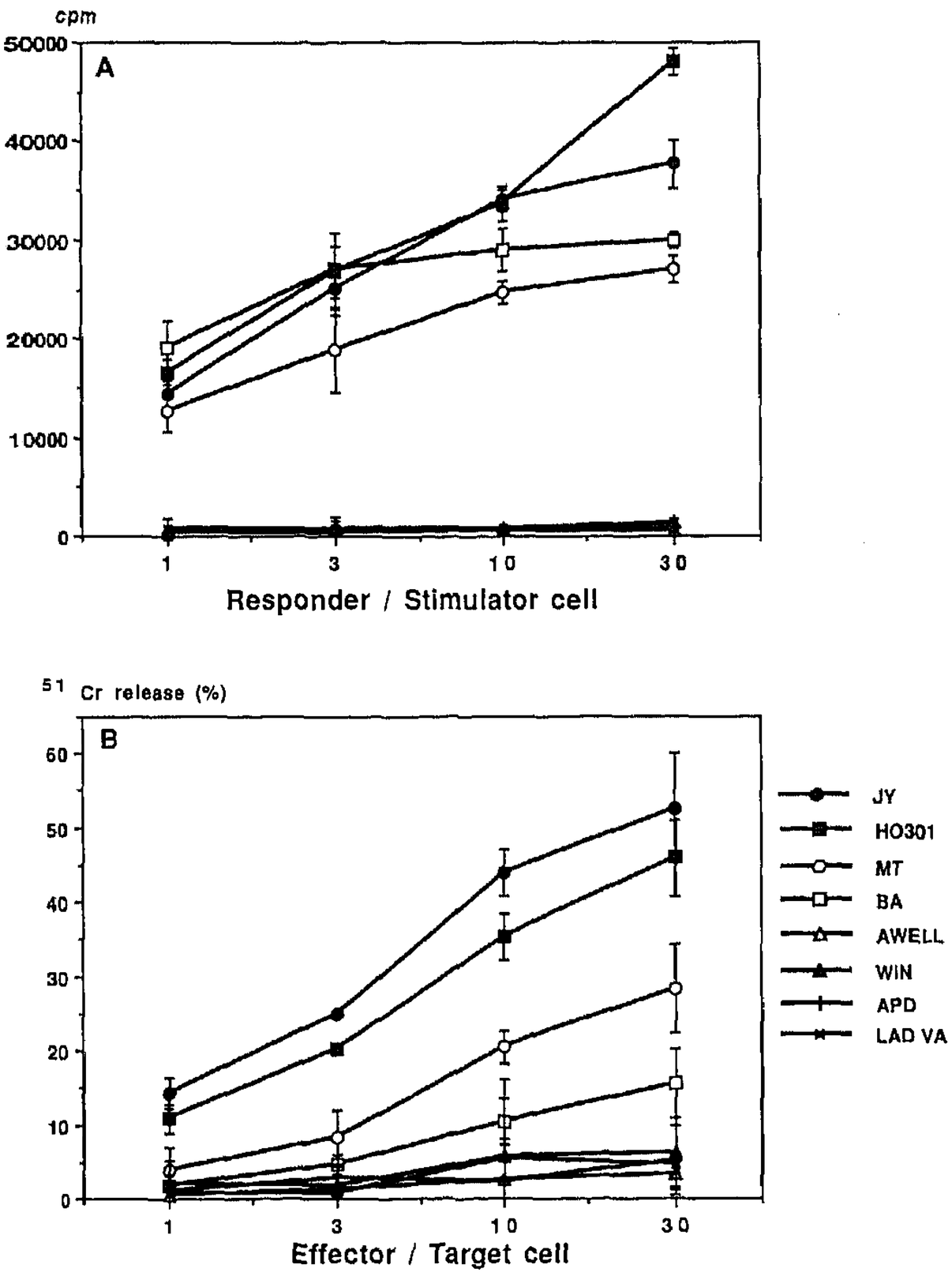

Figure 4. Response of LAD 6.6 T cell clone to a panel of HLA-typed B cell lines and to autologous EBV-transformed B cells (LAD VA). (A) Proliferation of LAD 6.6 responder cells; various concentrations of responder cells were added to 5×10^4 stimulator cells well. (B) Specific cytotoxicity of LAD T cells; various concentrations of effector cell were added to 1×10^3 target cells/well.

From these results we conclude that the CD2/LFA-3-mediated CTL/target cell interaction plays a prominent role in the TcR-mediated cytolytic process against JY cells for both, the LFA-1-negative and the LFA-1-positive T cell clone. LFA-1 expressed on T cells seems less essential for this allospecific cytolytic interaction. Whereas LFA-1 expressed on target cells may contribute to a minor extent.

3.4 Role of adhesion molecules in heterotypic conjugate formation

To examine the role of both adhesion pathways during the initial conjugate formation phase of the cytolytic process in more detail, we investigated binding of the T cell clones to specific target cells (JY). LAD 6.6 and JS 136 formed similar numbers of conjugates with the specific target JY (Fig. 6). Furthermore, we performed mAb blocking studies to determine which molecules participated in specific CTL/target cell binding. mAb directed against CD3, CD4, and HLA-DQ did not inhibit conjugate formation (data not shown), confirming previous reports demonstrating that TcR-Ag/HLA interaction did not contribute significantly to the maintenance of cell/cell interaction. The results in Fig. 6 show that anti-CD2 and anti-LFA-3 mAb completely blocked conjugate formation between LAD T cells and JY target cells. In contrast, blocking of conjugates of JS 136 with JY cells was only obtained when the LFA-1/ICAM-1 pathway was blocked simultaneously with the CD2/LFA-3 pathway (Fig. 6), demonstrating that both adhesion pathways are involved.

These results support the conclusion that in specific target cell binding, both the CD2/LFA-3 and the LFA-1/ICAM-1 adhesion pathways are comparably involved. They act independently from each other and when one pathway is absent (LAD) or blocked by mAb (JS 136), the other adhesion route is sufficient to form conjugates.

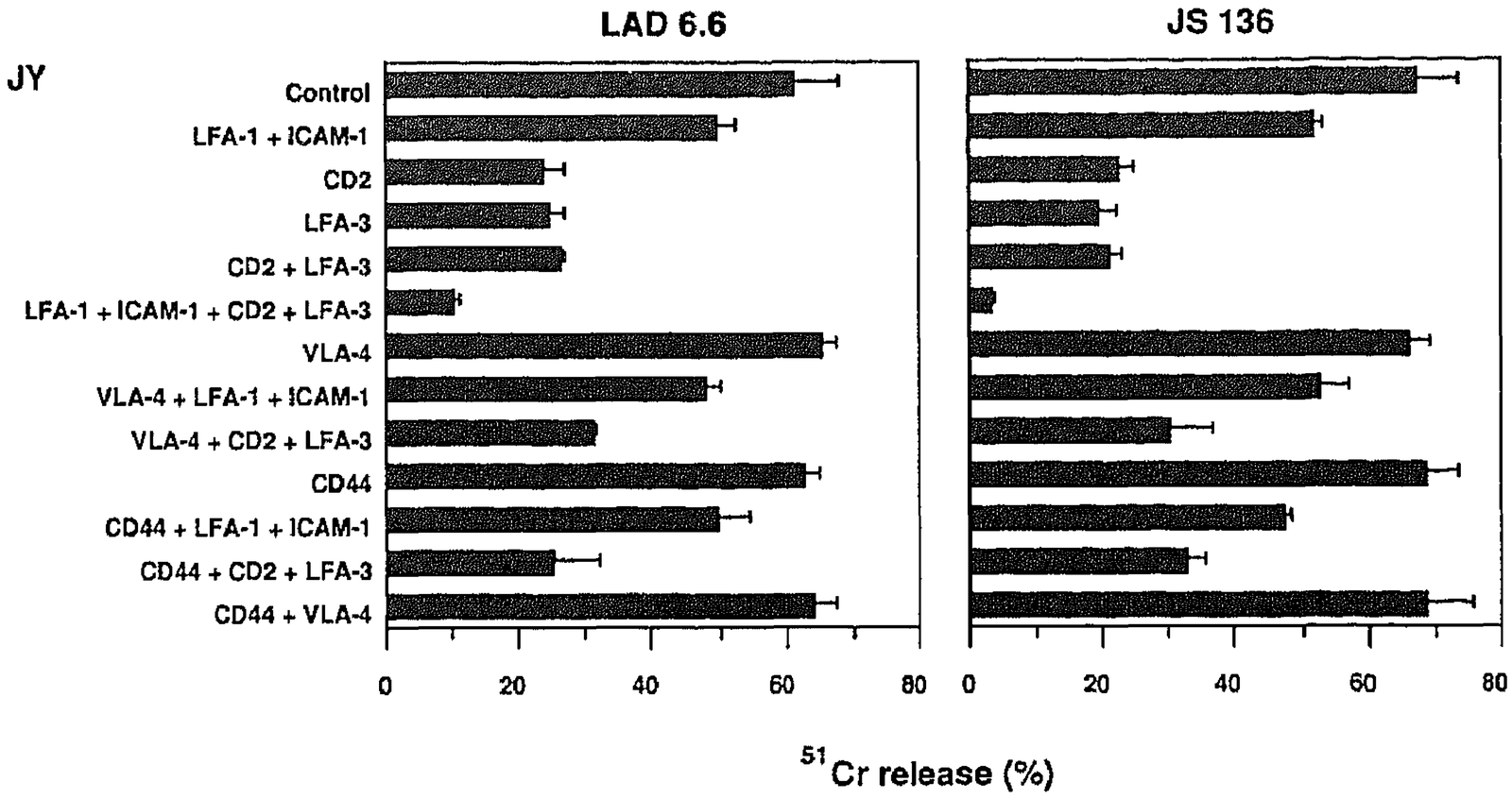

Figure 5. Inhibition of cytotoxic activity of LFA-1-negative (LAD 6.6) and LFA-1-positive (JS 136) T cell clones against allogeneic B cells JY. Purified IgG (10 μg/ml) of each mAb was present in the assay except for anti-VLA-4 mAb and anti-CD44 mAb which were used in a dilution of 1/100 ascites fluid. mAb against MHC class I (W6/32) was used as control mAb. The effector: target cell ratio was 30:1. One representative experiment out of three is shown.

3.5 Role of LFA-1 expressed by the target cell in cytotoxicity

To determine further the contribution of LFA-1 expressed by the target cells, we investigated the cytotoxic capacity of the LFA-1-negative T cell clone (LAD 6.6) against previously described, EBV-transformed B cells of four patients suffering from LAD [20]. Cells of one patient (AI) were lysed (Fig. 7A). The cells of this patient completely lack surface expression of LFA-1 [50]. Lysis of AI cells was blocked by mAb against HLA-DQ, CD3, and CD4 demonstrating that HLA-DQ interaction with TcR/CD3 complex and CD4 was involved (Fig. 7B). Furthermore, mAb against the LFA-1/ICAM-1 adhesion pathway could not inhibit lysis of the target cells, whereas mAb blocking the CD2/LFA-3 adhesion pathway caused inhibition of lysis (Fig. 7B). All tested mAb against VLA-4α and β1 were were incapable of inhibiting lysis (data not shown). Allotyping of AI cells revealed that the HLA-DQw3 is expressed, which explains specific recognition by LAD 6.6. From these data we conclude that allospecific cytotoxic activity can exist without involvement of LFA-1.

4 Discussion

In the present study we have investigated the role of LFA-1/ICAM-1, CD2/LFA-3, and other surface glycoproteins in the cytolytic activity of T cell clones. Numerous studies have demonstrated that LFA-1 is particularly involved in the conjugate binding step that precedes lysis of the target cells [3, 7–9, 51]. Several investigations carried out with peripheral blood lymphocytes from LAD patients, whose leukocytes lack LFA-1 at the cell surface, demonstrate that T cell functions are impaired [52, 53]. However, Mazerolles et al. [54] and Krensky et al. [55] showed that after repeated antigenic stimulation, T cells of LAD patients were capable of responding to alloantigen. Our results confirm and extend these observations, and show that T cells, and cloned T cells derived from peripheral blood of

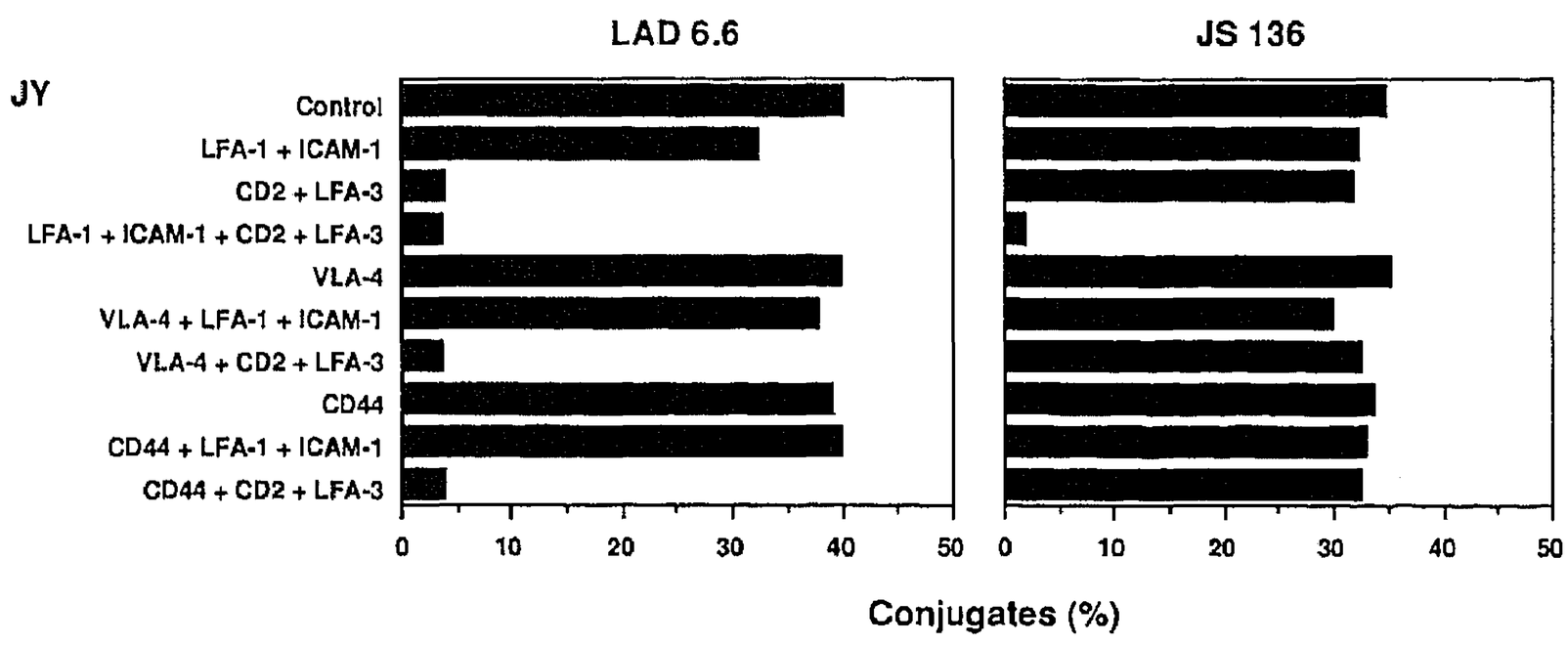

Figure 6. Inhibition by mAb of conjugate formation of the T cell clones LAD 6.6 (LFA-1 negative) and JS 136 (LFA-1 positive) with the allogeneic target B cells JY. Purified IgG (5 μg/ml) of each mAb was present in the assay, except for anti-VLA-4 mAb and anti-CD44 mAb which were used in a dilution of 1/200 ascites fluid. mAb against MHC class I (W6/32) was used as control mAb. LAD T cells were incubated for 120 min with their targets, and JS 136 cells were incubated for 60 min. One representative experiment out of four is shown.

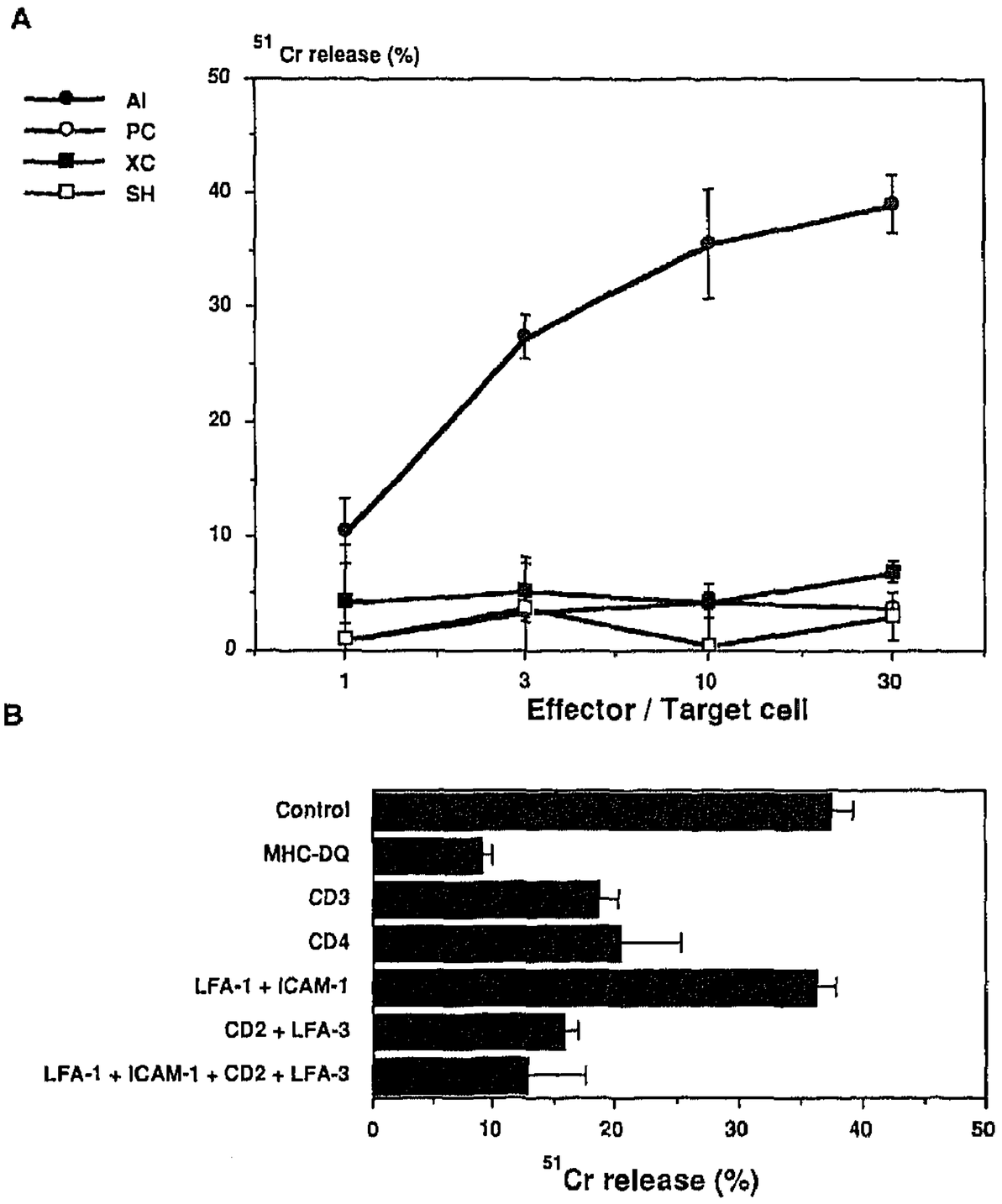

Figure 7. Specific cytotoxic activity of the LFA-1-negative T cell clone, LAD 6.6 against LFA-1-negative EBV-transformed B cells. (A) B cell lines of four patients with LAD. (B) mAb inhibition of cytotoxic activity of LFA-1 negative (LAD 6.6) T cell clones against allogeneic B cell AI. Purified IgG (10 μg/ml) of each mAb was present in the assay. mAb against MHC class I (W6/32) was used as control mAb. The effector: target cell ratio was 30:1. One representative experiment out of three is shown.

a severe type of LFA-1-deficient patient can exert high and specific cytolytic activity against allogeneic JY cells after repeated stimulation. The fact that the cytolytic activity of these T cells and T cell clones is similar to that of LFA-1-positive control T cells and T cell clones indicates that efficient lysis of allogeneic target cells can occur in the absence of LFA-1. We show that the adhesion of LAD T cells (LAD 6.6) to its target cells is mediated by CD2/LFA-3 interaction. Although the CD2 cell surface expression of LAD T cell clones was similar to that of LFA-1-positive T cell clones (Table 1), we can not completely exclude that selection occurred for T lymphocytes with a high CD2 expression. However, the cloning frequency was similar to that of LFA-1-positive lymphocytes (±60%) with an allospecific cytotoxic response of about 10%. Furthermore, we show that LFA-1-negative B cells (AI) can be lysed in the complete absence of LFA-1, both on target and effector cells (Fig. 7). Even if LFA-1 is expressed on the target cells (JY), the contribution of the LFA-1/ICAM-1 interaction is limited. Since the allospecific LAD T cell clones were raised in the presence of LFA-1-positive JY cells, we can not completely rule out that LFA-1 expressed by JY cells, played a role in the initiation of an immune response.

Our results are partially in contrast with those reported by Mentzer et al. [52] and Krensky et al. [55]. Mentzer et al. have shown that LFA-1 expressed by the target cells significantly contributes to lysis by allospecific CTL, whereas Krensky et al. have shown that anti-LFA-1 mAb partially inhibited the cytotoxic activity of bulk cultures of CTL derived from moderate and severe type of LAD patients in one case but not in another. However, the role of the CD2/LFA-3 adhesion pathway was not investigated in these studies. An explanation for the stronger dependency on the LFA-1 adhesion route in these experiments may be due to the fact that the T lymphocytes used in these studies were not absolutely deficient in LFA-1, or to differences in the level of cytotoxic activity of the lymphocytes of the various LAD patients.

In contrast with findings of Clayberger et al. [47], we were unable to demonstrate that VLA-4 is involved in lymphocyte cytotoxicity, although the anti-VLA-4 α and anti-β1 antibodies used effectively inhibit binding of T cells to activated endothelial cells [48, 56]. Furthermore, L-selectin (LECAM1) which is involved in homing of resting lymphocytes [18], is not involved in the cytolytic process since it is not expressed by cloned CTL. Finally the CD44 molecule that may participate in cell adhesion and lymphocyte homing [57], and is expressed at high levels on both LFA-1-negative and -positive CTL (Table 1), does not contribute to T cell mediated cytotoxicity, despite the fact that anti-CD44 mAb can enhance homotypic T cell aggregation [58].

Spits et al. [9] have shown that LFA-1 is particularly important in conjugate formation but does not seem to be involved in later phases of the cytolytic process such as delivery of the cytolytic hit. Our results now demonstrate that, at least for some lymphocytes, CTL/target interactions can take place in the complete absence of LFA-1 on the CTL. In addition, analysis of the LFA-1-positive control T cells also demonstrates that LFA-1 is not indispensable since CTL/target cell interactions could only be inhibited if both the LFA-1/ICAM-1 and LFA-3/CD2 adhesion pathways are blocked. These observations suggest that, although LFA-1 may be of importance in the initiation of an immune response, at least in some cases, antigen-specific CTL can bind and lyse their target cells in an LFA-1-independent manner. Possibly the enhanced expression of CD2, as reported on memory and activated peripheral T lymphocytes by Sanders et al. [59] may explain why binding of targets, by these activated cells, depends less on the LFA-1/ICAM-1 pathway.

Our results indicate that specific lysis, not only by LFA-1-deficient, but also by the LFA-1-positive CTL used in this study, is primarily mediated by the CD2/LFA-3 adhesion pathway, whereas the LFA-1/ICAM-1 adhesion pathway is subserved (Fig. 5). In conjugate formation, however, between specific LFA-1-positive CLT and target cells, both the LFA-1/ICAM-1 and the CD2/LFA-3 adhesion route are involved. In the absence of LFA-1 (LAD) or after blocking one route by mAb, CTL/target cell binding is regulated by the other adhesion pathway (Fig. 6).

Although LAD patients suffer frequently from severe bacterial infections, that are probably due to impaired migration and phagocytosis by macrophages and granulocytes, only two cases of unusually severe viral infections have been reported [14]. These observations together with

our findings support the notion that T cells in such patients can at least partially compensate for the LFA-1 defect. The results of this study show that cytolytic activity can occur after CTL/target cell binding mediated via the CD2/LFA-3 adhesion route whereas other observations show that VLA-4/VCAM interaction mediates binding of LAD T and B cells to activated endothelium [56, 60]. These observations indicate that a lymphocyte seems better equipped with supplementary adhesion receptors than myelomonocytic cells, that apparently lean heavily on the three receptors comprising the LFA-1 family to migrate and to carry out their function [10, 14].

We thank Willeke van de Kasteele for help with T cell clones cultures, Remco Brandt for RNA isolation, Mark Dessing for help with FACScan analysis, Dr. L. de Waal for the HLA-DQ typing of the LAD EBV transformed B cells, Dr. H. Spits for critically reading the manuscript, and Marie Anne van Halem for secretarial help.

Received December 6, 1991; in revised form February 18, 1992.

5 References

1 Springer, T. A., Dustin, M. L., Kishimoto, T. K. and Marlin, S. D., *Annu. Rev. Immunol.* 1987. *5:* 223.
2 Hynes, R. O., *Cell* 1987. *48:* 549.
3 Martz, E., *Hum. Immunol.* 1987. *18:* 3.
4 Sanchez-Madrid, F., Nagy, J. A., Robbins, E., Simon, P. and Springer, T. A., *J. Exp. Med.* 1983. *158:* 1785.
5 Marlin, S. D. and Springer, T. A., *Cell* 1987. *51:* 813.
6 Staunton, D. E., Dustin, M. L. and Springer, T. A., *Nature* 1989. *339:* 61.
7 Krensky, A. M., Robbins, E., Springer, T. A. and Burakoff, S. J., *J. Immunol.* 1984. *132:* 2180.
8 Sanchez-Madrid, F., Krensky, A. M., Ware, C. F., Robbins, E., Strominger, J. L., Burakoff, S. J. and Springer, T. A., *Proc. Natl. Acad. Sci. USA* 1982. *79:* 7489.
9 Spits, H., Van Schooten, W., Keizer, H., Van Seventer, G., Van de Rijn, M., Terhorst, C. and De Vries, J. E., *Science* 1986. *232:* 403.
10 Anderson, D. C. and Springer, T. A., *Annu. Rev. Med.* 1987. *38:* 175.
11 Kishimoto, T. K., Hollander, N., Roberts, T. M., Anderson, D. C. and Springer, T. M., *Cell* 1987. *50:* 193.
12 Wardlaw, A. J., Hibbs, M. L., Stacker, S. A. and Springer, T. A., *J. Exp. Med.* 1990. *172:* 335.
13 Miedema, F., Tetteroo, P. A. T., Terpstra, F. G., Keizer, G., Roos, M., Weening, R. S., Weemaes, C. M. R., Roos, D. and Melief, C. J. M., *J. Immunol.* 1985. *134:* 3075.
14 Todd III, R. F. and Freyer, D. R., *Hematol. Oncol. Clin. North Am.* 1988. *2:* 13.
15 Fischer, A., Lisowska-Grospierre, B., Mazerolles, F., Le Deist, F., Perez, N., Dimanche-Boitrel, M. T. and Griscelli, C., in Springer, T. A., Anderson, D. C., Rosenthal, A. S. and Rothlein, R. (Eds.), *Leukocyte Adhesion Molecules.* Springer Verlag, New York 1988, p. 95.
16 Takada, Y., Elices, M. J., Crouse, C. and Hemler, M. E., *EMBO J.* 1989. *8:* 1361.
17 Miyake, K., Underhill, C. B., Lesley, J. and Kincade, P. W., *J. Exp. Med.* 1990. *172:* 69.
18 Camerini, D., James, S. P., Stamenkovic, I. and Seed, B., *Nature* 1989. *342:* 78.
19 Borst, J., Spits, H., Voordouw, A., De Vries, E., Boylston, A. and De Vries, J. E., *Hum. Immunol.* 1986. *17:* 426.
20 Dimanche-Boitrel, M. T., Guyot, A., De Saint-Basile, G., Fischer, A., Griscelli, C. and Lisowska-Grospierre, B., *Eur. J. Immunol.* 1988. *18:* 1575.
21 Keizer, G. D., Borst, J., Figdor, C. G., Spits, H., Miedema, F., Terhorst, C. and De Vries, J. E., *Eur. J. Immunol.* 1985. *15:* 1142.
22 Spits, H., Borst, J., Giphart, M., Coligan, J., Terhorst, C. and De Vries, J. E., *Eur. J. Immunol.* 1984. *14:* 229.
23 Keizer, G. D., Te Velde, A. A., Schwarting, R., Figdor, C. G. and De Vries, J. E., *Eur. J. Immunol.* 1987. *17:* 1317.
24 Spits, H., Keizer, G., Borst, J., Terhorst, C., Hekman, A. and De Vries, J. E., *Hybridoma* 1983. *2:* 423.
25 Pals, S. T., Hogervorst, F., Keizer, G. D., Thepen, T., Horst, E. and Figdor, C. G., *J. Immunol.* 1989. *143:* 851.
26 Miedema, F., Tetteroo, P. A. T., Hesselink, W. G., Werner, G., Spits, H. and Melief, C. J. M., *Eur. J. Immunol.* 1984. *14:* 518.
27 Van Lier, R. A. W., Brouwer, M. and Aarden, L. A., *Eur. J. Immunol.* 1988. *18:* 167.
28 Giltay, J. C., Brinkman, H. J. M., Modderman, P. W., Von dem Borne, A. E. G. Kr. and Van Mourik, J., *Blood* 1989. *73:* 1235.
29 Tetteroo, P. A. T., Lansdorp, P. M., Leeksma, O. C. and Von dem Borne, A. E. G. Kr., *J. Haematol.* 1983. *55:* 509.
30 Falcioni, R., Sacchi, A., Resau, J. and Kennel, S. J., *Cancer Res.* 1988. *48:* 816.
31 Sonnenberg, A., Modderman, P. W. and Hogervorst, F., *Nature* 1988. *336:* 487.
32 Fradet, Y., Cordon-Cardo, C., Thomson, T., Daly, M. E., Whitmore, Jr., W. F., Lloyd, K. O., Melamed, M. R. and Old, L. J., *Proc. Natl. Acad. Sci. USA* 1984. *81:* 224.
33 Campanero, M. R., Pulido, R., Ursa, M. A., Rodriguez-Moya, M., De Landázuri, M. O. and Sánchez-Madrid, F., *J. Cell. Biol.* 1990. *110:* 2157.
34 Hemler, M. E., Huang, C., Takada, Y., Schwarz, L., Strominger, J. L. and Clabby, M. L., *J. Biol. Chem.* 1987. *262:* 11478.
35 Hemler, M. E., Sanchez-Madrid, F., Flotte, T. J., Krensky, A. M., Burakoff, S. J., Bhan, A. K., Springer, T. A. and Strominger, J. L., *J. Immunol.* 1984. *132:* 3011.
36 Hall, D. E., Reichardt, L. F., Crowley, E., Holley, B., Moezzi, H., Sonnenberg, A. and Damsky, C. H., *J. Cell. Biol.* 1990. *110:* 2175.
37 Amiot, M., Huet, S., Azogui, O., Dastot, H., Bernard, A. and Boumsell, L., in: Du Pont, B. (Ed.), *Immunobiology of HLA, Vol. II. Immunogenetics and Histocompatibility,* Springer Verlag, Berlin 1988, p. 556.
38 Rothlein, R., Dustin, M. L., Marlin, S. D. and Springer, T. A., *J. Immunol.* 1986. *137:* 1270.
39 Leerling, M. F., Vaessen, L. M. B., Reubsaet, C. H. K., Weimar, W., Ettekoven, H., Marsman, F. R. and Kreeftenberg, J. G., *Dev. Biol. Stand.* 1990. *71:* 191.
40 Tax, W. J. M., Leeuwenberg, H. F. M., Willems, H. W., Capel, P. J. A. and Koene, R. A. P., in Bernard, A., Boumsell, L., Dausset, J., Milstein, C. and Schlossman, S. F. (Eds.), *Leucocyte Typing,* Springer Verlag, Berlin 1984, p. 721.
41 Visser, L., Shaw, A., Slupsky, J., Vos, H. and Poppema, S., *Blood* 1989. *74:* 320.
42 Chomczinsky, P. and Sacchi, N., *Anal. Biochem.* 1987. *162:* 156.
43 Larson, R. S., Corbi, A. L., Berman, L. and Springer, T., *J. Cell Biol.* 1989. *108:* 703.
44 Kishimoto, T. K., O'Connor, K., Lee, A., Roberts, T. M. and Springer, T. A., *Cell* 1987. *48:* 681.
45 Spits, H., Paliard, X. and De Vries, J. E., *J. Immunol.* 1989. *143:* 1506.
46 Kuypers, T. W., Koenderman, L., Weening, R. S., Verhoeven, A. J. and Roos, D., *Eur. J. Immunol.* 1990. *20:* 501.
47 Clayberger, C., Krensky, A. M., McIntyre, B. W., Koller, T. D., Parham, P., Brodsky, F., Linn, D. J. and Evans, E. L., *J. Immunol.* 1987. *138:* 151.
48 Pulido, R., Elices, J. J., Campanero, M. R., Osborn, L., Schiffer, S., Garcia-Pardo, A., Lobb, R., Hemler, M. E. and Sánchez-Madrid, F., *J. Biol. Chem.* 1991. *266:* 10241.
49 Van de Wiel-van Kemenade, E., Van Kooyk, Y., De Boer, A. J., Huijbens, R. J. F., Weder, P., Van de Kasteele, W., Melief, C. J. M. and Figdor, C. G., *J. Cell Biol.*, in press.

50 Fischer, A., Seger, R., Durandy, A., Grospierre, B., Virelizier, J. L., Le Deist, F., Griscelli, C., Fischer, E., Katatchkine, M., Bohler, M. C., Descamps-Latscha, B., Trung, P. H., Springer, T. A., Olive, D. and Mawas, C., *J. Clin. Invest.* 1985. *76:* 2385.
51 Shaw, S., Ginther Luce, G. E., Quinones, R., Gress, R. E., Springer, T. A. and Sanders, M. E., *Nature* 1986. *323:* 262.
52 Mentzer, S. J., Bierer, B. E., Anderson, D. C., Springer, T. A. and Burakoff, S. J., *J. Clin. Invest.* 1986. *78:* 1387.
53 Anaout, M. A., Spits, H., Terhorst, C., Pitt, J. and Todd III, R. F., *J. Clin. Invest.* 1984. *74:* 1291.
54 Mazerolles, F., Lumbroso, C., Lecomte, O., Le Deist, F. and Fischer, A., *Eur. J. Immunol.* 1988. *18:* 1229.
55 Krensky, A. M., Mentzer, S. J., Clayberger, C., Anderson, D. C., Schmalstieg, F. C., Burakoff, S. J. and Springer, T. A., *J. Immunol.* 1985. *135:* 3102.
56 Vennegoor, C. J. G. M., Van de Wiel-van Kemenade, E., Huijbens, R. J. F., Sánchez-Madrid, F., Melief, C. J. M. and Figdor, C. G., *J. Immunol.*, in press.
57 St. John, T., Meyer, J., Idzerda, R. and Gallatin, W. M., *Cell* 1990. *60:* 45.
58 Huet, S., Groux, H., Caillou, B., Valentin, H., Prieur, A. and Bernhard, A., *J. Immunol.* 1989. *143:* 798.
59 Sanders, M. E., Makgoba, M. W., Sharrow, S. O., Stephany, D., Springer, T. A., Young, H. A. and Shaw, S., *J. Immunol.* 1988. *140:* 1401.
60 Schwartz, B. R., Wayner, E. A., Carlos, T. M., Ochs, H. D. and Harlan, J. M., *J. Clin. Invest.* 1990. *85:* 2019.