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## TRIGGERING OF THE CD44 ANTIGEN ON T LYMPHOCYTES PROMOTES T CELL ADHESION THROUGH THE LFA-1 PATHWAY<sup>1</sup>

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The CD44 molecule, a molecule which has been previously known as Hermes, Pgp-1, extracellular matrix receptor III, and In(Lu)-related p80, is currently thought to be involved in several steps of normal immune cell function, including lymphocyte adhesion to high endothelial venules and to the extracellular matrix and T cell activation. We now demonstrate that triggering of CD44 on T lymphocytes by anti-CD44 mAb promotes cell adhesion. The induced homotypic adhesion is mediated by lymphocyte function-associated antigen-1 (LFA-1), because it was inhibited by anti-LFA-1 antibodies and not by anti-LFA-3 antibodies. This notion is supported by the temperature and Mg<sup>2+</sup> dependence which is characteristic of LFA-1-mediated adhesion. Moreover, the sensitivity of CD44-induced adhesion to AMG and H7, which both prevent the activation of protein kinase C, and to cytochalasin B, which inhibits microfilament formation, suggests that the activation of the LFA-1 pathway via CD44 involves protein kinase C activation and requires an intact cytoskeleton.

Lymphocytes migrate from the blood to lymphoid organs via specialized HEV<sup>3</sup> (1-3). A primary step in this migration process is the adhesion of lymphocytes to the walls of HEV. This step, which is thought to regulate the preferential migration of lymphocytes (i.e., homing), is mediated by interactions between lymphocyte cell surface molecules termed "homing receptors" (3-5) and their endothelial ligands, so-called "vascular addressins" (6, 7). Recently, several distinct lymphocyte homing receptors were defined by biochemical studies and molecular cloning (8-10).

In humans, an 85 to 95-kDa glycoprotein, the Hermes Ag, is involved in lymphocyte binding to HEV (3, 5, 11, 12). This putative homing receptor is closely related to or identical to the human phagocytic glycoprotein Pgp-1,

the blood group Ag In(Lu), and the extracellular matrix receptor III, all of which fall within the CD44 cluster (13-16). The expression of CD44 Ag is not restricted to lymphocytes; several other hematopoietic and nonhematopoietic cells have also been reported to express CD44 Ag (3, 8, 10, 12). The function of CD44 may thus not be limited to HEV recognition and adhesion, but it may have a much broader role in the biology and pathology of cell adhesion. This possibility is supported by DNA cloning data showing homology to cartilage link proteins (8, 10) and by functional studies which indicate that CD44 participates in hyaluronic acid (17, 18) and collagen recognition (14). Interestingly, several studies have implicated CD44 as a factor in lymphoma metastasis (3, 19, 20, 21). In addition to the CD44 Ag, two members of the integrin family, i.e., the lymphocyte function-associated Ag LFA-1 (CD11a/18) and LPAM-1, molecules that play a role in a wide variety of cell-cell interactions (22), have also been implicated in lymphocyte adhesion to and migration via HEV (23, 24).

The fact that molecules mediating lymphocyte homing apparently may have important unrelated adhesion or receptor functions as well prompted us to study the role of CD44 in various cell-cell interactions. Here we report that mAb directed against CD44 do not inhibit but, in contrast, stimulate homotypic T cell interactions. Our data indicate that the LFA-1 pathway is involved in this CD44-induced adhesion.

### MATERIALS AND METHODS

**mAb and chemicals.** The mAb used were NKI-P1 (IgG1), NKI-P2 (IgG1), Hermes-3 (IgG2a), and 50B4 (IgG2b), all reactive with CD44 (5, 17, 25); CLB-LFA-1/1 (IgG1), specific for the  $\beta$  subunit of LFA-1 (26); CLB-LFA-1/2 (IgG1), specific for the  $\alpha$  subunit of LFA-1 (27); W6/32 (IgG2a), specific for a nonpolymorphic determinant of HLA-ABC (28); R.R1/1 (IgG1), specific for ICAM-1 (29); and TS2/9 (IgG1), specific for LFA-3 (30).

The reagents used were PMA (50 ng/ml; Sigma Chemical Co., St. Louis, MO); sodium azide (0.1%; Merck, Darmstadt, FRG), an inhibitor of oxidative phosphorylation; H7 (20  $\mu$ M; Sigma), which prevents the activation of PKA and PKC (31); AMG (60 nM; Sigma), which prevents the activation of PKC; W7 (20  $\mu$ M; Sigma), which inhibits calmodulin-dependent protein kinase (32); and cytochalasin B (20  $\mu$ M; Sigma), which inhibits microfilament formation (33).

**Cell lines and cell cultures.** JY, an EBV-transformed B cell line, was cultured in RPMI 1640 medium with 25 mM HEPES buffer (GIBCO, Grand Island, NY). T cell lines were cultured in Iscove's medium (GIBCO). Both media were routinely supplemented with 1 mM glutamine, 10% (vol/vol) heat-inactivated FCS (Hyclone Laboratories Inc., Logan, UT), 100 U of natrium penicillin G (Gist Brocades NV, Delft, The Netherlands) per ml, and 100 U of streptomycin sulfate (Pharmachemie BV, Haarlem, The Netherlands) per ml.

T cell lines were initiated by culturing PBMC isolated from normal donors by Ficoll-Isopaque density gradient centrifugation in the presence of the anti-CD3 mAb OKT3 (0.01%; Ortho Diagnostic Systems Inc., Raritan, NJ) and 5% TCGF (34). These cultures were

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<sup>3</sup> Abbreviations used in this paper: HEV, high endothelial venule; H7, 1-(5-isoquinoliny) sulfonyl-2-methyl piperazine; AMG, 1-O-alkyl-2-O-methyl glycerol-3-phosphocholine; PKA, protein kinase A; PKC, protein kinase C; W7, N-(6-aminoethyl)-5-chloro-1-naphthalene sulfonamide; TCGF, T cell growth factor.



restimulated weekly at a concentration of  $2 \times 10^5$  cells/ml by the addition of  $1 \times 10^6$  3000 rad-irradiated PBMC and  $1 \times 10^5$  5000 rad-irradiated JY cells in the presence of OKT3 and 5% TCGF. TCGF was produced as follows. PBMC were stimulated with  $0.1 \mu\text{g}$  of the phorbol ester PMA per ml for 2 h at  $37^\circ\text{C}$  ( $50 \times 10^6$  cells/ml of medium), washed extensively, and cultured for 48 h in the presence of  $15 \mu\text{g}$  of Con A (Sigma) per ml. At the end of the incubation period, Con A was neutralized by adding 50 mM  $\alpha$ -methyl mannoside (30 min,  $37^\circ\text{C}$ ). The supernatant was harvested, filtered (filters from Millipore Corp., Bedford, MA), and stored at  $-20^\circ\text{C}$ .

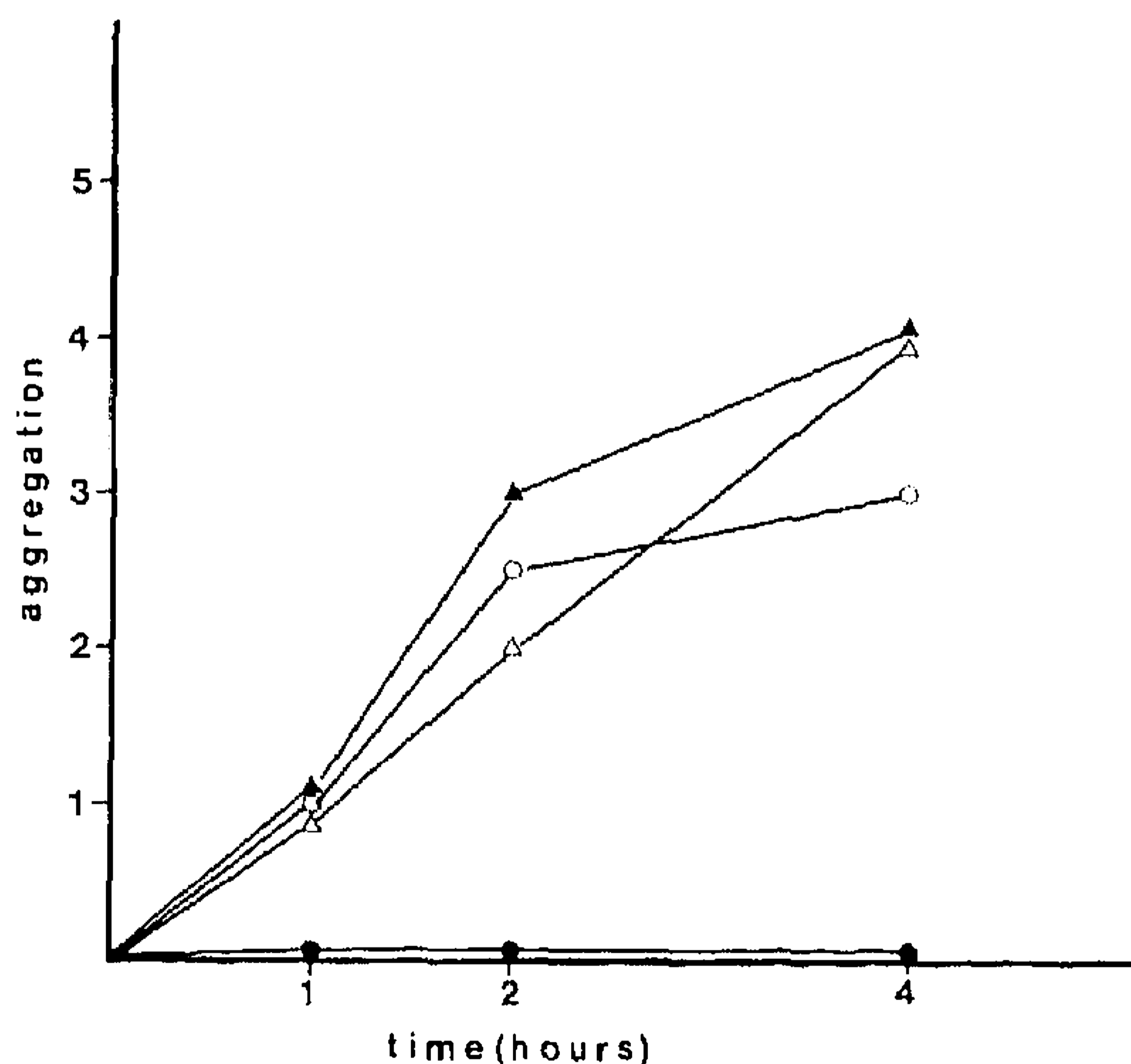
The human cytolytic alloreactive T cell clone JS 136 used in this study is directed against an HLA-DRw6 determinant (35). Cloned T cells were cultured in Iscove's medium supplemented with 2% human serum and stimulated weekly with irradiated allogeneic PBMC and JY cells, PHA ( $0.2 \mu\text{g}/\text{ml}$ ), and IL-2 (50 U/ml). For the aggregation assay, T cells were used 4 to 7 days after stimulation. No residual monocytes were present at this time, as determined by FACS analysis.

**Aggregation assay.** Cells were seeded in 96-well flat-bottomed microtiter plates (Nunc-Immuno Plate Maxisorb; GIBCO). Each well contained  $1 \times 10^5$  cells previously washed and resuspended in RPMI 1640 medium containing 10% FCS and was incubated for various time periods at  $37^\circ\text{C}$  in the absence or presence of mAb. To study the mechanism of CD44-induced cell aggregation, the cells were preincubated with the various inhibitors of cell metabolism for 15 min at  $37^\circ\text{C}$  before adding the mAb. The requirement for bivalent cations was studied by washing the cells in HBSS without calcium and magnesium (GIBCO) but with 1 mM EDTA and 2 mg of D-glucose per ml. The cells were plated, and anti-CD44 antibodies were added.

Homotypic aggregation was measured in a semiquantitative manner by the method described by Rothlein and Springer (29). Scores ranged from 0 to 5, where 0 indicated that essentially no cells were aggregated in clusters; 1 indicated that  $<10\%$  of the cells were found in clusters; 2 indicated that 10 to 50% of the cells were found in clusters; 3 indicated that 50 to 80% of the cells were found in clusters; 4 indicated that  $>80\%$  of the cells were found in small aggregates; and 5 indicated that  $>90\%$  of the cells were found in large, compact clusters. During the experiments, cell viability, as determined by trypan blue exclusion, was 90% or more.

## RESULTS

**CD44 stimulates homotypic adhesion of T cells.** T cell aggregation studies were performed with anti-CD3- and TCGF-stimulated peripheral blood T cells, which were used 4 to 7 days after stimulation, at a time at which they showed no spontaneous aggregation (Fig. 1). The addition of PMA readily induced these T cells to aggregate; this aggregation was not blocked by anti-CD44 mAb. Interestingly, however, the addition of mAb NKI-P1 or NKI-P2 to (non-PMA-stimulated) T cells induced homotypic T cell adhesion (Fig. 1). The induction of adhesion was not observed with the anti-CD44 mAb 50B4 or



**Figure 1.** Induction of T cell aggregation by PMA and NKI-P2. Aggregation of anti-CD3- and TCGF-stimulated peripheral blood T cells was determined after 1, 2, and 4 h of incubation in medium alone (●), medium with PMA (▲), medium with NKI-P2 (○), or medium with PMA and NKI-P2 (Δ). NKI-P2 was added at  $5 \mu\text{g}/\text{ml}$ .

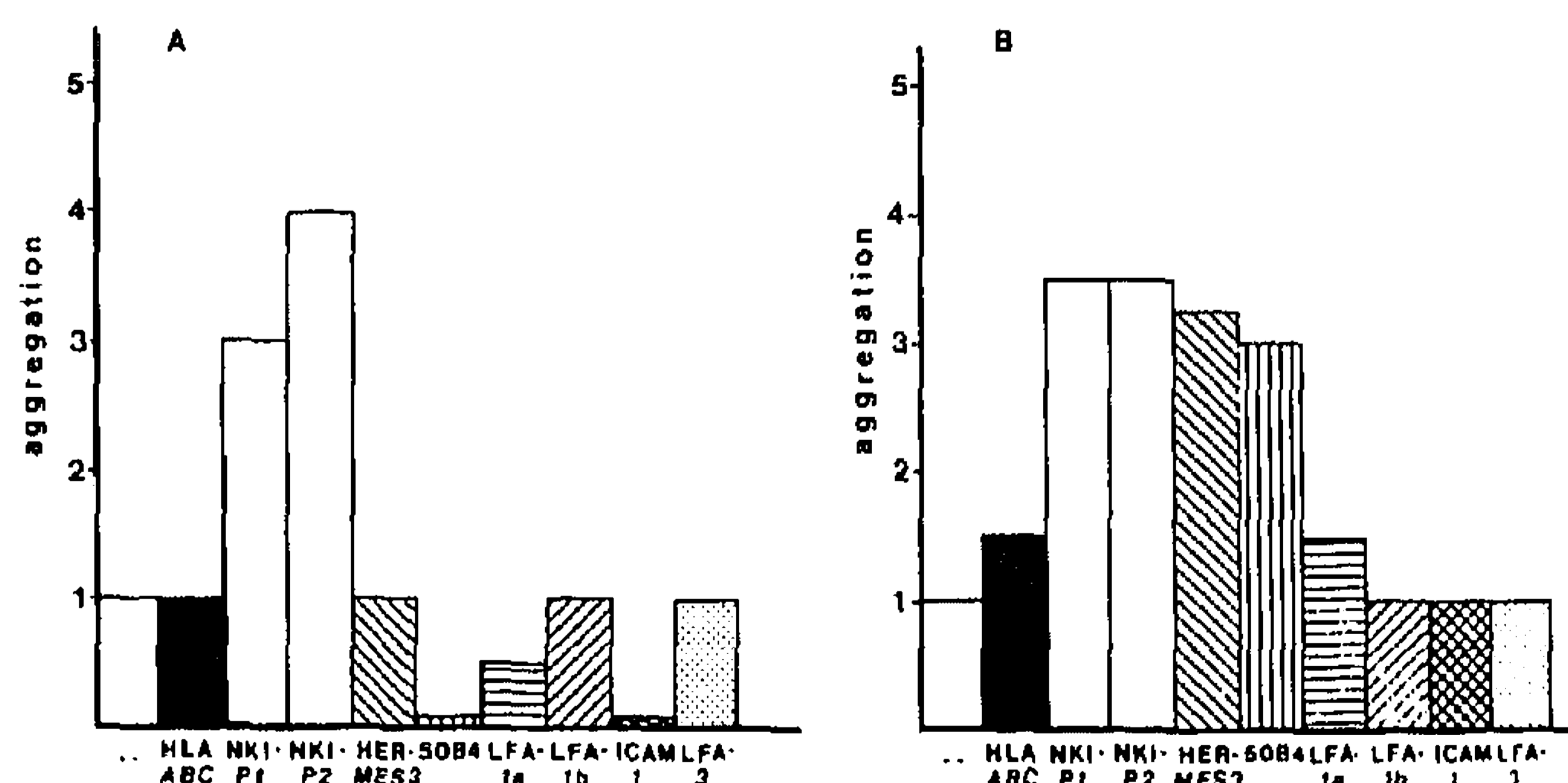
Hermes-3, both of which recognize different epitopes on the CD44 molecule. This failure to induce aggregation did not represent a dose-response problem due to a lack of or a low expression of relevant epitopes on the activated cells or to a low binding affinity, since FACS analysis showed similar binding for all four anti-CD44 antibodies. Moreover, 10-fold-higher concentrations of 50B4 and Hermes-3 were still ineffective at inducing aggregation. Incubation with purified mouse Ig (data not shown) or with antibodies against LFA-1 $\alpha$ , LFA-1 $\beta$ , ICAM-1, LFA-3, and HLA-ABC also did not induce T cell aggregation (Fig. 2A).

NKI-P1 and NKI-P2 were also found to evoke the aggregation of the CTL clone JS 136. Unlike anti-CD3-stimulated peripheral blood T cells, JS 136 was also induced to aggregate by the anti-CD44 mAb Hermes-3 and 50B4 (Fig. 2B). Finally, the mAb NKI-P1 and NKI-P2 also induced homotypic adhesion of freshly isolated peripheral blood T cells. However, despite the fact that these cells expressed levels of CD44 comparable to those expressed by the activated T cells and the CTL clone, this interaction required long (4 to 18 h) incubation times and the mAb Hermes-3 and 50B4 were ineffective (data not shown).

**CD44-induced homotypic T cell adhesion is energy dependent and requires an intact cytoskeleton.** Subsequently, we assessed the metabolic requirements for CD44-induced T cell aggregation. In these experiments, the anti-CD44 mAb NKI-P2, being the most potent inducer of aggregation, was used. The addition of sodium azide, an inhibitor of oxidative phosphorylation, prevented CD44-induced T cell aggregation (Fig. 3), indicating that CD44-induced cell aggregation is metabolic energy dependent. This notion is supported by the observation that aggregation could not be induced at  $4^\circ\text{C}$  (data not shown).

The addition of cytochalasin B, which prevents the formation of microfilaments, also blocked CD44-induced aggregation. Furthermore, AMG and H7, which interfere with PKC and PKA (Fig. 3), and W7, which inhibits calmodulin-dependent protein kinase, all were found to prevent CD44-induced cell aggregation. In contrast, cycloheximide, an inhibitor of protein synthesis, did not affect aggregation (data not shown). Hence, CD44-induced cell aggregation requires an intact cell metabolism and cytoskeleton and seems to depend on intracellular protein kinase activity but not on protein synthesis.

**CD44-induced adhesion requires magnesium.** To



**Figure 2.** Induction of T-cell aggregation by several anti-CD44 mAb. Aggregation of anti-CD3- and TCGF-stimulated peripheral blood T cells (A) and of the CTL clone JS 136 (B) was assayed in medium alone or in medium with anti-HLA-ABC; the anti-CD44 mAb NKI-P1, NKI-P2, Hermes-3, or 50B4; anti-LFA-1 $\alpha$ ; anti-LFA-1 $\beta$ ; anti-ICAM-1; or anti-LFA-3. All mAb were used at  $5 \mu\text{g}/\text{ml}$ .



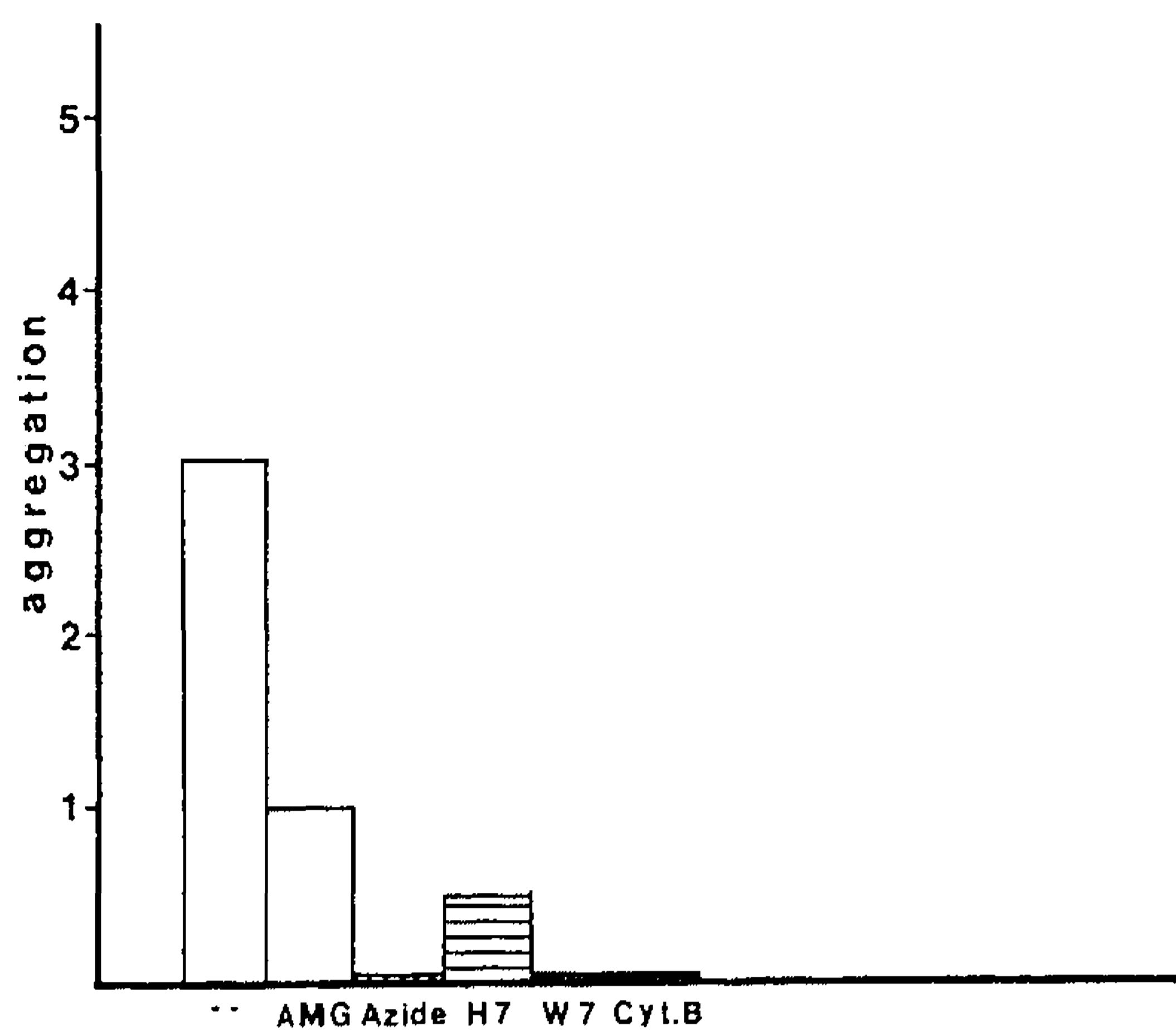


Figure 3. Metabolic requirement for CD44-induced cell aggregation. Aggregation of anti-CD3- and TCGF-stimulated peripheral blood T cells was determined after the addition of NKI-P2 at 5  $\mu$ g/ml alone (leftmost bar) or in combination with AMG, azide, H7, W7, or cytochalasin B (Cyt.B).

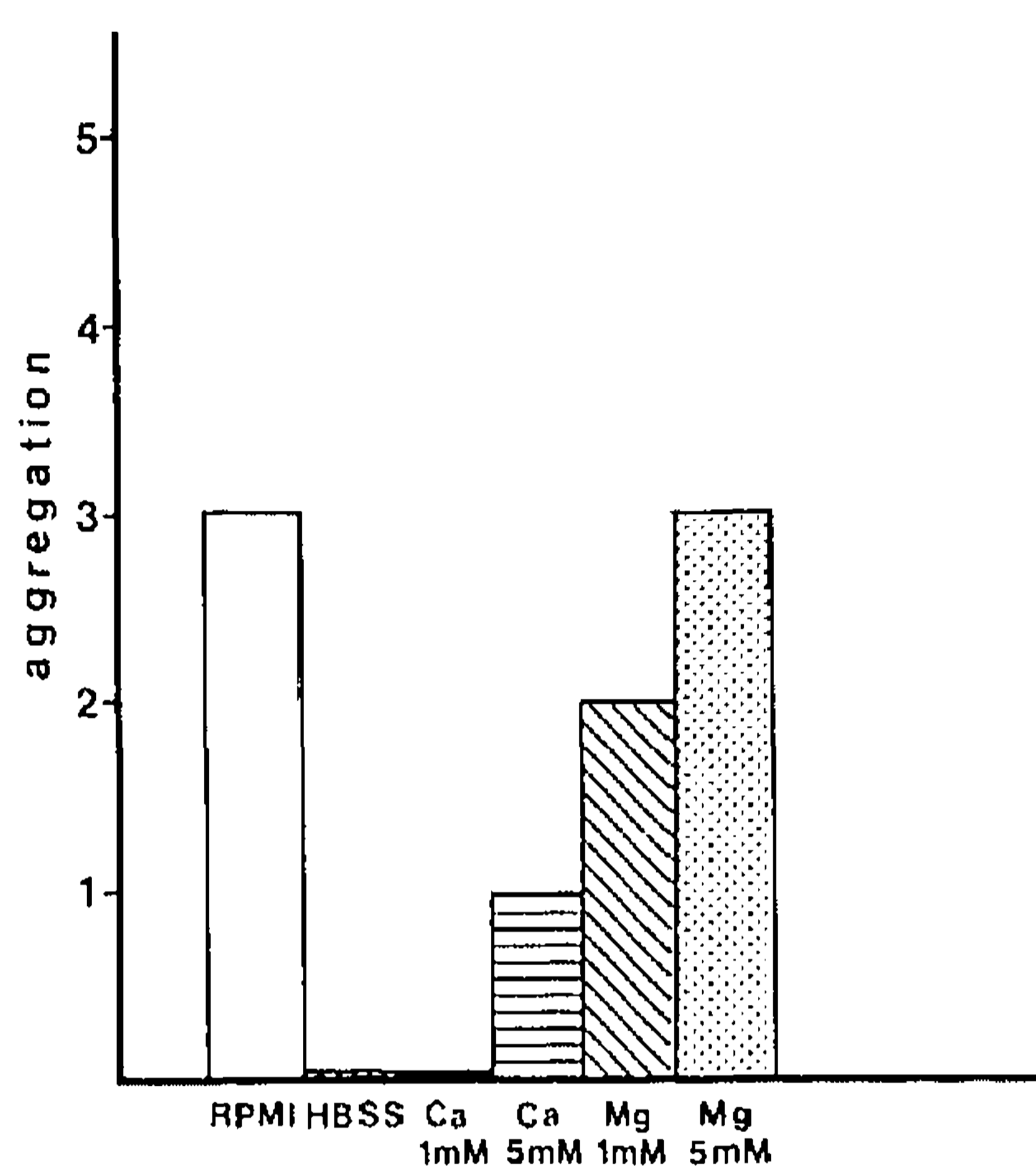


Figure 4. Cation requirement for CD44-induced cell adhesion. Induction of aggregation of anti-CD3- and TCGF-stimulated peripheral blood T cells with NKI-P2 (5  $\mu$ g/ml) was determined in RPMI 1640 medium, in HBSS free of calcium and magnesium, or in HBSS with 1 mM calcium, 5 mM calcium, 1 mM magnesium, or 5 mM magnesium.

study the cation requirement of CD44-induced cell adhesion, cells were washed in HBSS without calcium or magnesium but with 1 mM EDTA to remove all remaining bivalent cations. No aggregation occurred in bivalent cation-depleted medium (Fig. 4). Aggregation was completely restored by 5 mM magnesium, whereas the addition of 5 mM calcium caused only minimal cell aggregation.

CD44-induced T cell adhesion is inhibited by anti-LFA-1 mAb. To further analyze the mechanism of CD44-induced homotypic T cell interactions, we performed mAb inhibition studies. In these experiments, potentially inhibitory or control mAb were added simultaneously with NKI-P2 to the T cells. mAb directed against the LFA-1 $\alpha$  and LFA-1 $\beta$  chains blocked CD44-induced aggregation of both the anti-CD3-stimulated peripheral blood T cells and the CTL clone JS 136 (Fig. 5), cell populations that express high levels of LFA-1. mAb R.R1/1 directed against ICAM-1, a ligand of LFA-1, did not significantly inhibit the aggregation of anti-CD3-stimulated peripheral blood T cells, although some inhibitory effect was occasionally observed (Fig. 5A). However, CD44-induced aggregation of JS 136 was completely blocked by mAb R.R1/1 (Fig. 5B). These different effects of mAb R.R1/1 on aggregation paralleled ICAM-1 expression on these

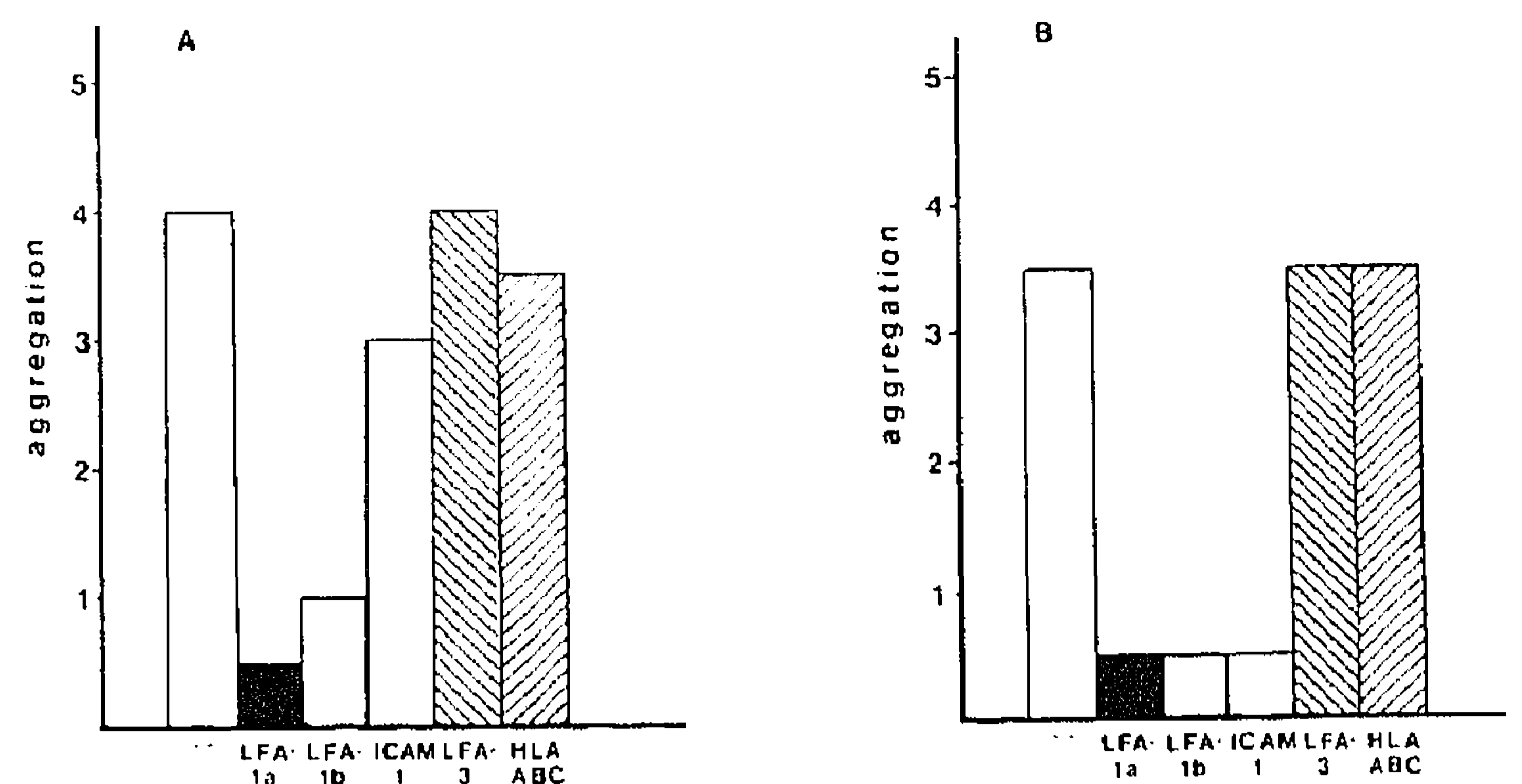


Figure 5. mAb inhibition studies of CD44-induced cell adhesion. Induction of aggregation of anti-CD3- and TCGF-stimulated peripheral blood T cells (A) or of the CTL clone JS 136 (B) by NKI-P2 alone (leftmost bars) or in combination with anti-LFA-1 $\alpha$ , anti-LFA-1 $\beta$ , anti-ICAM-1, anti-LFA-3, or anti-HLA-ABC was studied. All mAb were applied at 5  $\mu$ g/ml.

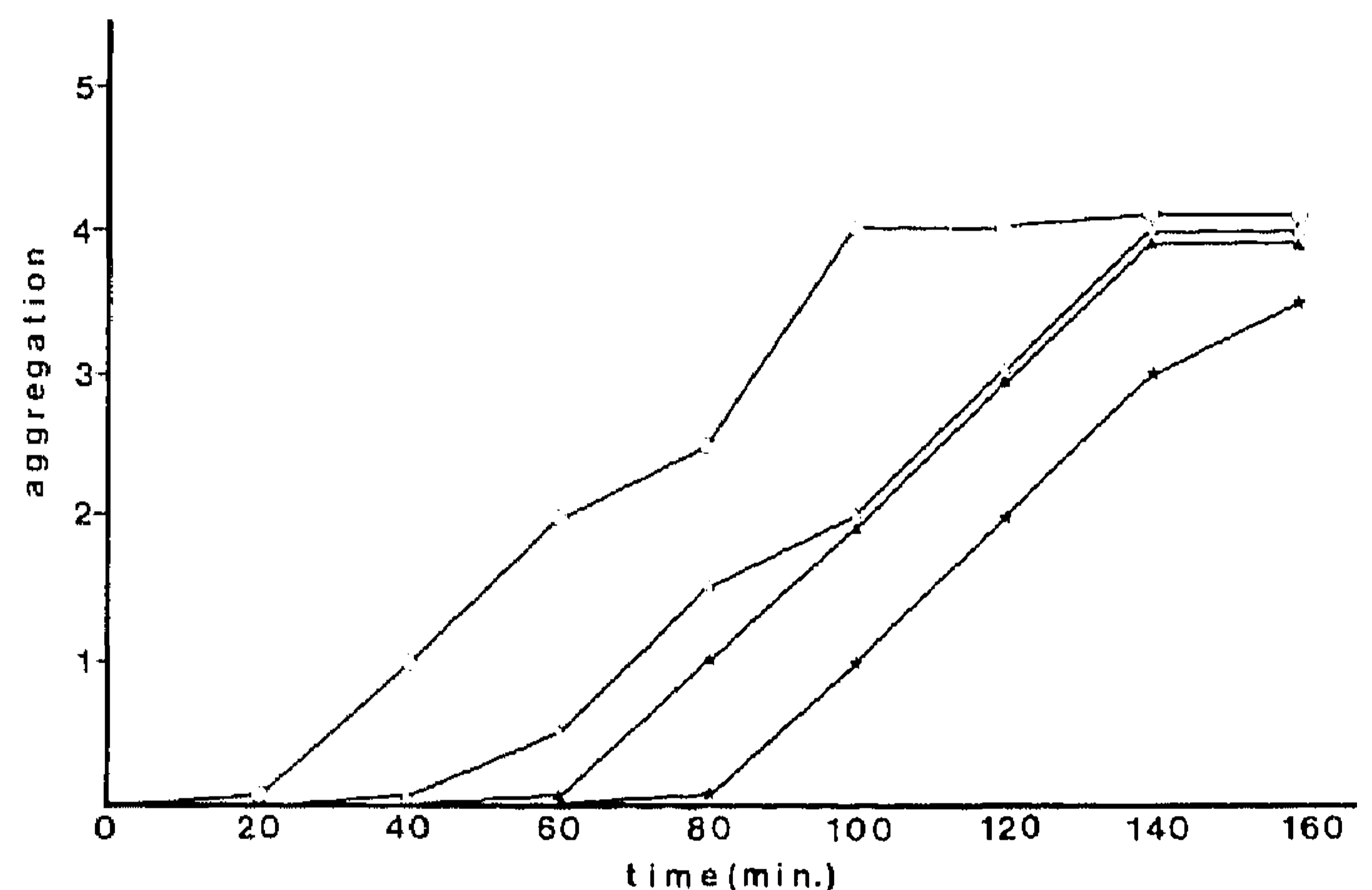


Figure 6. Aggregation and reaggregation kinetics of CD44-induced cell clustering. NKI-P2 was added to all cell cultures at the start of the experiment. Cells were either allowed to aggregate undisturbed (O) or resuspended after 20 min ( $\Delta$ ), 40 min ( $\blacktriangle$ ), or 60 min ( $\star$ ). For this experiment, anti-CD3- and TCGF-stimulated peripheral blood T cells were used and NKI-P2 was added at 5  $\mu$ g/ml.

two cell types, anti-CD3-stimulated peripheral blood T cells and JS 136 cells having low and high levels of ICAM-1 expression, respectively (data not shown). mAb against LFA-3 and HLA-ABC could not inhibit CD44-induced T cell aggregation (Fig. 5). Importantly, the stimulation of CD44 did not influence the cell surface levels of LFA-1 and/or ICAM-1 in either of the cell populations, as tested in FACS double-staining experiments.

T cell reaggregation after disruption of CD44-induced adhesion. The time course of NKI-P2-induced T cell adhesion is shown in Figure 6. During this time course, CD44 was not modulated from the cell surface, as determined by FACS analysis (data not shown). In separate cultures, cells were resuspended 20, 40, or 60 min after the addition of NKI-P2. These resuspended cells readily reaggregated in the presence of NKI-P2, and the kinetics of aggregation and reaggregation were similar.

#### DISCUSSION

The LFA-1 molecule plays a key role in lymphocyte adhesion (22). Lymphocytes do not adhere spontaneously, but activation of PKC by phorbol esters gives rise to strong LFA-1-dependent adhesion, suggesting that the activation of LFA-1 is required to induce cell adhesion (36). Recently, stimulation of the functionally important

CD2 and CD3 surface structures of T lymphocytes has been shown to promote LFA-1-dependent adhesion (37, 38). We now demonstrate that triggering of the CD44 molecule also enhances the adhesion of T lymphocytes. The induced adhesion is mediated by LFA-1 because it can be inhibited by anti-LFA-1 antibodies but not by anti-LFA-3 antibodies. This notion is supported by the  $Mg^{2+}$  and temperature dependence which is characteristic of LFA-1-mediated adhesion (22, 39).

All four anti-CD44 mAb used in this study promoted homotypic adhesion of T lymphocytes but were not equally effective, and T cells from different sources varied in sensitivity to their aggregation-inducing effects. NKI-P1 and NKI-P2 were the most potent mAb and induced homotypic aggregation of the CTL clone JS 136, activated T cells, and nonstimulated peripheral blood T cells, while Hermes-3 and 50B4 only induced the aggregation of JS 136. This difference between the anti-CD44 mAb did not represent a dose-response problem due to a lack of or a low expression of relevant epitopes on the cells or to a low binding affinity. Presumably, it was related to the fact that NKI-P1 and NKI-P2 recognize closely related epitopes that are known to differ functionally from the epitope recognized by Hermes-3 (5, 17).

As mentioned above, the different T cells expressed comparable levels of CD44 but were not equally sensitive to the adhesion-promoting effects of each of the anti-CD44 mAb. Moreover, they aggregated at different rates. These observations most likely reflect variations in the initial activation state of the cells. Nonactivated peripheral blood T cells and cells of the alloreactive T cell clone JS 136 were the least and the most sensitive to adhesion induction via CD44, respectively, while activated T cells had an intermediate sensitivity. Cell adhesion was thus most markedly stimulated in T cell populations that were already activated to a certain level. As with these costimulatory effects on T cell adhesion, CD44 has recently been found to enhance T cell proliferation when added in combination with a suboptimal concentration of immobilized anti-CD3 mAb or stimulatory concentrations of anti-CD2 mAb (40–42). Furthermore, anti-CD44 mAb in combination with stimulatory anti-CD2 mAb were found to enhance T cell-monocyte aggregation (42, 43). On the basis of these data, a general picture emerges in which triggering of CD44 in combination with other (suboptimal) T cell stimulatory signals can provide the initial step in T cell adhesion and activation. Once a certain level of activation has been reached, stimulation of CD44 alone, either by mAb or by binding to a ligand, further activates T cells and enhances T cell adhesion. This sequence of events could have important implications for the pathogenesis of chronic inflammatory diseases, such as rheumatoid arthritis, in which CD44 molecules on (pre)activated T cells interact with HEV and extracellular matrix constituents in the inflamed synovium. These interactions via CD44 would increase T cell adhesion and activation and, consequently, might enhance and perpetuate the inflammatory response. In this context, the recent finding that hyaluronic acid, a major constituent of the extracellular matrix of connective tissues and cartilage, is a ligand of CD44 is of great interest (17, 18).

How does the triggering of CD44 activate the LFA-1 pathway? Although the present data cannot definitively answer this question, the finding that both AMG, which selectively prevents the activation of PKC, and H7, which

prevents the activation of both PKC and PKA, effectively inhibited the aggregation-promoting effect of anti-CD44 suggests an important role for PKC-dependent mechanisms. PKC activated via CD44 might activate LFA-1 by direct phosphorylation of the  $\beta$ -chain or, alternatively, by phosphorylation of a cytoskeletal protein such as talin, which might then bind and activate LFA-1 (44). Furthermore, the fact that cytochalasin B, an inhibitor of microfilaments, blocked CD44-induced cell aggregation indicates that the connection of CD44 and/or LFA-1 to the cytoskeleton (36, 45) is important to its function in cell activation. Alternatively, the induction of adhesion by anti-CD44 antibodies might result from a direct interaction of CD44 and LFA-1, leading to a conformational change in the LFA-1 molecule, as has been suggested for the induction of adhesion by the anti-LFA-1 mAb NKI-L16 (46).

CD44-induced cell aggregation of the CTL clone JS 136 was blocked by anti-ICAM-1. However, anti-ICAM-1 did not block the CD44-induced aggregation of anti-CD3-stimulated peripheral blood T cells. These differences in blocking effects paralleled a difference in ICAM-1 expression between the two cell types, JS 136 and anti-CD3-stimulated peripheral blood T cells, having high and low levels of ICAM-1 expression, respectively. It is possible that the adhesion of anti-CD3-stimulated peripheral blood T cells is largely mediated through an interaction of LFA-1 with a ligand distinct from ICAM-1. Consistent with our observation, several LFA-1-mediated adhesion phenomena involving activated lymphocytes have also been described to be ICAM-1 independent (47, 48).

CD44-triggered adhesion was persistent, because cells readily reaggregated after disruption of the aggregates. Recently, studies of CD2- and CD3-induced cell adhesion (37, 38) have revealed that enhanced adhesion induced by CD2 is also persistent. In contrast, triggering of CD3 only transiently promotes T cell adhesion. Although further studies are needed to clarify these differences between CD44- and CD2-triggered adhesion versus CD3-induced adhesion, the fact that anti-CD3 mAb are readily modulated from the cell surface, whereas anti-CD2 mAb and anti-CD44 mAb remain present, suggests that differences in the kinetics of receptor turnover play a role.

In conclusion, our data indicate that triggering of CD44 can induce T cell aggregation via the LFA-1 pathway. However, in other systems, different adhesion pathways might be activated through CD44, since Denning et al. (40) and Haynes et al. (13) have recently provided evidence that antibodies against CD44 increase T cell-monocyte binding via CD2–LFA-3. Furthermore, we have observed that T cell clones from an LFA-1-deficient patient can also be induced to aggregate via CD44 (our unpublished observation). This LFA-1-independent adhesion, which does not require cations or metabolic energy, probably involves CD2–LFA-3 or direct binding of CD44 to (one of) its ligands (14, 17, 18).

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