LFA-1 integrin redistribution during T-cell hybridoma invasion of hepatocyte cultures and manganese-induced adhesion to ICAM-1

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SUMMARY

We have reported previously that the integrin LFA-1 is essential for metastasis of T-cell hybridomas to the liver. We show here that hepatocytes isolated from normal non-inflamed rat liver express intercellular adhesion molecule-1 (ICAM-1) at the dorsal surface and more prominently at the lateral and substratum-adherent surfaces. Anti-rat ICAM-1 mAb inhibited adhesion of TAM8C4 T-cell hybridoma cells to hepatocytes. Invasion between hepatocytes was not affected, but this is probably due to lack of penetration of the mAb between the hepatocytes. In all hepatocyte-adherent TAM8C4 cells, LFA-1 was concentrated at the adhesion site. Redistribution of ICAM-1 to the interacting hepatocyte membrane was also seen, but only for part of the adherent TAM8C4 cells. LFA-1 was highly concentrated on pseudopods of invading TAM8C4 cells inserted between hepatocytes, and on the upper surface of invaded TAM8C4 cells located under the hepatocytes. ICAM-1 was concentrated in the hepatocyte membrane overlying TAM8C4 cells located underneath the monolayer. These results suggest that ICAM-1 is of major importance for liver invasion by these lymphoma cells.

INTRODUCTION

Lymphomas often metastasize preferentially to the liver, which organ is extensively infiltrated by cells spreading diffusely throughout the liver parenchyma (Dingemans, 1973; Roos et al., 1977). This phenomenon can be mimicked in short-term cultures of isolated hepatocytes (Roos et al., 1981). We have proposed that these metastatic lymphomas use similar mechanisms to those used by normal leukocytes for invasion of tissues, on the basis of the observation that activated normal T-cells invade hepatocyte cultures in a similar way to metastatic lymphoma cells (Roos and Van de Pavert, 1983). To support this notion, we generated T-cell hybridomas by fusion of the invasive activated T-cells with non-metastatic BW5147 T-lymphoma cells, and indeed observed that these were highly metastatic and gave rise to extensive and diffuse infiltration of the liver (Roos et al., 1985). Adhesion of migrating normal lymphocytes to endothelial cells is mediated by several adhesion molecules, including the integrins LFA-1 and VLA-4 (Dustin and Springer, 1988; Oppenheimer-Marks et al., 1991; Van Kooyk et al., 1993). Because the T-cell hybridomas do not express VLA-4 (La Rivière et al., 1994), LFA-1 can be activated artificially by Mn²⁺. To study LFA-1 redistribution upon ICAM-1 interaction with higher resolution, we performed immuno-EM on cells before and after Mn²⁺-induced adhesion and spreading on immobilized ICAM-1. By immune fluorescence, LFA-1 was observed to redistribute to the ICAM-1-adherent surface, and to be concentrated in lamellipodia of spreading TAM8C4 cells. By immuno-EM, LFA-1 was localized in microclusters of approximately 10 gold particles. This was seen in cells fixed in suspension, and the size of these clusters did not change upon adhesion to ICAM-1. LFA-1 was present at high density in thin filopodia, but again in microclusters of similar size. Comparable results were obtained with a cytotoxic T-cell clone. We conclude that Mn²⁺-induced activation of LFA-1 is not associated with the formation or enlargement of LFA-1 clusters.

Key words: integrin, activation, clustering
cytotoxic T-lymphocytes adhere to target cells. LFA-1 becomes highly concentrated in the adherent membrane area. Upon adhesion of helper T-cells to antigen-presenting cells, LFA-1 redistribution was only seen in the presence of antigen (Kupfer and Singer, 1989); that is, when the T-cells were activated by triggering of the T-cell receptor. Such triggering causes activation of LFA-1 (Van Kooyk et al., 1989; Dustin and Springer, 1989), so that redistribution apparently occurs only when LFA-1 is activated. We have shown previously that LFA-1 on highly invasive T-cell hybridomas does not bind, or binds poorly, to immobilized ICAM-1, indicating that LFA-1 is not in an active state (La Rivière et al., 1994). Invasion into fibroblast monolayers was blocked by pertussis toxin. This indicated that an extracellular factor is involved that, upon binding to a surface receptor, transmits a signal through a pertussis toxin-sensitive G-protein. ICAM-1 was also concentrated in the hepatocyte surface before they infiltrated. As a measure of invasion we counted intraductal bile-duct epithelial cells that should facilitate invasion between hepatocytes and as a result the diffuse infiltration of liver parenchyma.

Cell adhesion to ICAM-1 does not require that LFA-1 is redistributed to the interaction site (Kupfer and Singer, 1989; Dustin et al., 1992). However, when cytotoxic T-lymphocytes are activated by Mn²⁺, which activates integrins including LFA-1, it is possible that the mechanism involved is similar to that described for the interaction of T lymphocytes with epithelial target cells (Dransfield et al., 1987; Haverstick and Gray, 1992; Van Kooyk et al., 1988; Landis et al., 1993) enhancing the affinity for their ligands by conformational changes in the integrin molecules (Hynes, 1992; Keizer et al., 1994). We have therefore studied the extent of LFA-1 clustering before and after Mn²⁺-induced adhesion in monolayers of hepatocytes, using the avidity of LFA-1 for ICAM-1 by immuno-EM. Surprisingly, we found that LFA-1 is located in microclusters even in untreated non-adherent cells. The size of these clusters did not change during Mn²⁺-induced adhesion to immobilized ICAM-1. LFA-1 was completely redistributed to the adherent surface, concentrated in lamellipodia, particularly on thin filopodia, but was in all cases located in microclusters. We conclude that activation of LFA-1 by Mn²⁺ is not associated with cluster formation or enlargement.

**MATERIALS AND METHODS**

**Cells**

Generation and culture of the T-cell hybridomas TAM8C4 and TAM2D2 have been reported previously (Roos et al., 1985; La Rivière et al., 1988). We used the same clones as used by La Rivière et al. (1994). BW5147 T-lymphoma cells were cultured similarly. The mouse cytotoxic T-lymphocyte clone CTL 23.21 was cultured as described (La Rivière et al., 1993). Rat hepatocytes were isolated by collagenase perfusion and cultured as described previously (Roos et al., 1985), at 2.5 × 10⁵ cells/well, in Primaria 24-well plates (Becton-Dickinson, Lincoln Park, NJ), or on coverslips in 24-well plates, coated with 2.5 μg bovine plasma fibronectin (Sigma, St Louis, MO).

**Reagents**

We used two rat anti-mouse LFA-1 monoclonal antibodies (mAb), M17/4 (Davignon et al., 1981) and M18/2 (Springer et al., 1982). The hybridomas were gifts from Dr E. Martz and Dr T. Springer, respectively. M17/4 was affinity-purified, and Fab fragments were prepared as described (Roos and Roossien, 1987). M18/2 was used as culture supernatant. Purified anti-rat ICAM-1 mAb (1A29) was purchased from Sekagaku Co. (Tokyo, Japan). Rhodamine-conjugated phalloidin was from Molecular Probes (Junction City, Oregon). Biotinylated sheep anti-mouse IgG, donkey anti-rat IgG and rabbit anti-rat IgG gold-conjugated antibodies were from Amersham UK, and FITC-extravidin was from Sigma (St Louis, MO). Before use, the IgG-gold was centrifuged for 3 minutes at maximum speed in an Eppendorf minifuge. This procedure removed all aggregates, as determined by viewing a drop of the preparation spotted on a Formvar-covered grid in the electron microscope.

**Interactions of T-cell hybridoma cells with hepatocytes**

The interaction of TAM8C4 cells with hepatocytes was assessed as previously described (Roos and Van de Pavert, 1982; Roos et al., 1985). Briefly, 5 × 10⁵ cells in Dulbecco’s modified Eagle’s medium [DMEM] with 10 mM HEPES were added to hepatocyte cultures in wells of a 24-well plate, and incubated at 37°C in 5% CO₂. After 2 hours, the cultures were washed with phosphate-buffered saline (PBS), fixed, dehydrated, detached in propylene oxide and embedded in Epon. Toluidine Blue-stained sections of 1 μm were observed under a light microscope. We counted hepatocyte nuclei. TAM8C4 cells adherent to the dorsal hepatocyte surface (adherent cells), and TAM8C4 cells located between and under the hepatocytes (infiltrated cells). The interaction index: number of adherent + infiltrated cells divided by number of hepatocyte nuclei, was used as a measure of adhesion (since infiltrated cells obviously have adhered to the hepatocyte surface before they infiltrated). As a measure of invasion we used the infiltrated fraction: number of infiltrated cells divided by number of adherent + infiltrated cells.

For inhibition experiments, we pre-incubated TAM8C4 cells for 30 minutes at room temperature with Fab fragments of M17/4 anti-LFA-1 mAb (15 μg/ml) at double cell density and this cell suspension was diluted 1:1 when added to the hepatocytes so that the final concentration was 7.5 μg/ml. The hepatocyte cultures were pre-incubated with 1A29 anti-ICAM-1 mAb (20 μg/ml) at 37°C. The medium was then diluted 1:1 by addition of the T-cell hybridoma cells so that the final concentration during incubation was 10 μg/ml. Controls were treated similarly but in medium without antibodies.

**Immune fluorescence**

Cells were fixed with 2% paraformaldehyde in PBS for 20 minutes, permeabilized with 0.5% Triton X-100 in PBS for 2 minutes, followed by a 10 minute incubation in PBS with 1% bovine serum albumin (BSA). All immune reactions were performed at room temperature for 45 minutes, all antibodies were diluted in PBS with 1% BSA. Cells were incubated with primary antibodies, subsequently with bio-
tinylated anti-rat or anti-mouse IgG antibodies and then with extravidin-FITC. Actin filaments were visualized by decoration with rhodamine-conjugated phalloidin. Preparations were viewed using standard immune fluorescence microscopy or in a Bio-Rad MRC-600 confocal laser scanning microscope (CSLM).

**Results**

**ICAM-1 expression on hepatocytes**

On most cell types, ICAM-1 is present at low levels, unless the cells have been stimulated with cytokines (Dustin et al., 1986; De Fougerolles et al., 1991). However, ICAM-1 is expressed by hepatocytes and endothelial cells in normal human liver (De Fougerolles et al., 1991). We confirmed this for isolated rat hepatocytes in culture. ICAM-1 was present on freshly isolated hepatocytes as detected by FACScan analysis (not shown), and on hepatocytes that had been cultured for 24 hours as observed by immune fluorescence (Fig. 1). ICAM-1 is located on the dorsal surface in small clusters, and abundantly present on both the lateral surfaces of adjacent hepatocytes and the ventral surface, which are the sites where infiltrating lymphoma cells accumulate (Roos et al., 1977, 1985).

**Involvement of ICAM-1 in invasion**

To study the involvement of ICAM-1 in invasion, we tested the effect of an anti-rat ICAM-1 mAb on the interaction between rat hepatocytes and TAM8C4 T-cell hybridoma cells, which are highly invasive. This effect was determined by counting cells in sections of embedded cultures (Roos and Van de Pavert, 1982). As a measure of adhesion to the hepatocyte surface, we used the interaction index. This is the total number of interacting TAM8C4 cells, i.e. both those seen to adhere to the hepatocyte surface and those that have infiltrated (obviously after they have first adhered to the outer surface),
divided by the number of hepatocyte nuclei. Fig. 2 shows the results of two experiments. We confirmed previous results obtained with TAM2D2 T-cell hybridomas and hepatocytes, determined by counting cells in sections of embedded cultures. The interaction index (inter. index) and the infiltrated fraction (infil. fract.) are measures of adhesion and invasion, respectively (see Materials and Methods). x axis: percentage of controls. The results of two independent experiments are shown. Interaction time was 2 hours. Control values for the interaction index were 1.35 and 1.40, and for the infiltrated fraction 0.50 and 0.46, in exp. 1 and 2, respectively.

![Graph showing interaction index and infiltrated fraction](image)

To measure the effect of the antibodies on the invasion step, i.e. the migration into the monolayer, we determined the infiltrated fraction. This is the fraction of the interacting cells that have infiltrated. We observed a reduction of this parameter by M17/4 anti-LFA-1 Fab fragments to 30-40% of controls (Fig. 2), indicating that LFA-1 is involved in both the initial adhesion and the subsequent infiltration between the hepatocytes. This inhibition was not seen in previous experiments (Roos and Roossien, 1987). The reason for this discrepancy is not clear. The effect of the anti-ICAM-1 mAb was variable, as shown in Fig. 2, but on average no inhibition was observed. This is probably due, however, to lack of penetration of the antibody between hepatocytes. In fact, the labeling of ICAM-1 on the lateral membranes, as shown in Fig. 1, could only be obtained after permeabilization of hepatocytes before reaction with the antibody. Lack of inhibition therefore does not argue against a role of ICAM-1 in invasion.

**Cell surface distribution of LFA-1 during invasion**

Next, we investigated possible changes in LFA-1 distribution during the invasion process. Upon adhesion of the T-cell hybridoma cells to hepatocytes, we observed a clear increase in LFA-1 density at the lymphoma-hepatocyte interface (Fig. 3), although some LFA-1 was still present at other parts of the TAM8C4 cell surface. During invasion most, if not all, LFA-1 was located at the site of interaction with the lateral hepatocyte membranes, between which the TAM8C4 cells migrate (Fig. 3). On cells that had moved to a location under the hepatocytes (Fig. 3H), LFA-1 was present only on the upper surface in contact with hepatocytes, as could be judged from stereo CSLM images similar to Fig. 3H (not shown).

**Cell surface distribution of ICAM-1 during invasion**

As described above, the ICAM-1 density on the dorsal hepatocyte surface was not very high. It was therefore quite a surprise to see a high density of ICAM-1 at the site of attachment of some lymphoma cells. Note that this is not due to ICAM-1 on the hybridomas, because they do not express ICAM-1 and, moreover, the anti-rat ICAM-1 antibody does not react with mouse cells. This ICAM-1 accumulation was observed at the interaction site with only approximately 25% (11/46) of the adherent TAM8C4 cells. This was revealed by double staining for ICAM-1 and actin (Fig. 4E-F), the latter to delineate cell circumferences. When TAM8C4 cells had invaded, ICAM-1 was located predominantly at the TAM8C4-hepatocyte interaction site, as shown in Fig. 4G,H for a T-cell hybridoma cell underneath a hepatocyte.

**Immuno-EM studies of LFA-1 distribution**

We next used immuno-electron microscopy (EM) to study LFA-1 distribution before and after ICAM-1 interaction in more detail. For this we used the M18/2 mAb, directed against the LFA-1 β-subunit (Springer et al., 1982), which still reacts after paraformaldehyde fixation of cells, and does not inhibit the binding of LFA-1 to ICAM-1. To assess the distribution in non-adherent cells, TAM8C4 cells were fixed in suspension, treated with M18/2, followed by gold-labeled secondary antibodies, and then attached to a poly-L-lysine-coated substratum. Next, cells were dry-cleaved as described previously by applying adhesive tape to the dorsal surface of critically point-dried cells (Mesland et al., 1981). Upon removal of the tape, most of the cell body is torn away, leaving behind a surface-adherent
plasma membrane with associated cytoskeleton, a preparation that is thin enough to be examined by transmission EM.

Representative examples of results are shown in Fig. 5A-C. For comparison, the LFA-1 distribution as revealed by immune fluorescence is shown in the inset to Fig. 5A. Fig. 5D-F are drawings based on similar but somewhat larger areas in other micrographs showing only cell borders and the location of gold particles represented as black dots. We found that LFA-1 molecules were always located in small clusters. On TAM8C4 cells (Fig. 5A,D) clusters consisted on average of 10 gold particles (range 6-15). We recently reported that invasive T-cell hybridomas adhere poorly or not at all to ICAM-1, and that for optimal invasion LFA-1 requires activation (La Rivière et al., 1994). However, TAM8C4 cells adhered to some extent, indicating that part of the LFA-1 was constitutively active, and the observed clustering might be due to this activated state. We therefore observed another invasive T-cell hybridoma, TAM2D2, which did not bind to ICAM-1 at all (La Rivière et al., 1994). As shown in Fig. 5E, the clusters were similar: with on average 9 particles (range 5-14). To assess whether this pattern of distribution is a peculiarity of these T-cell hybridomas, we have also studied a normal T-cell, i.e. a cytotoxic T-cell clone that is also invasive (La Rivière et al., 1993). The results, shown in Fig. 5B, were similar. Furthermore, we investigated a non-invasive lymphoma cell line, BW5147, which was the lymphoma fusion partner used to generate these hybridomas. BW5147 cells express similar levels of LFA-1 as invasive T-cell hybridomas and yet do not interact with hepato-

Fig. 3. LFA-1 distribution on TAM8C4 cells after 2 hours interaction with hepatocytes. (A,B,C) Three CLSM images, 2 µm apart in a downward direction, of adherent (arrowheads) and infiltrating (asterisk) TAM8C4 cells. (D,E) Adherent; and (F,G) invading TAM8C4 cells. (H) Flattened TAM8C4 cell (arrow) located beneath a hepatocyte. In all conditions, LFA-1 is concentrated at the interface between TAM8C4 cells and hepatocytes. Bar, 5 µm.

Fig. 4. (A,C,E,G) Distribution of ICAM-1 on hepatocytes during interaction with TAM8C4 cells. To delineate the cells, the preparations were double stained for F-actin with rhodamine-phalloidin (B,D,F,H). (A,C,E) ICAM-1 is accumulated at the interaction site of hepatocytes with some TAM8C4 cells (filled arrows), but not with others (open arrows). (G) At the ventral surface, ICAM-1 is concentrated at the interaction site with a flattened infiltrated TAM8C4 cell (arrow). Bar, 5 µm.
tocytes. Also in these cells (Fig. 5C,F) LFA-1 molecules were located in clusters. However, these were somewhat larger, with on average 25 particles (range 21-30).

The clustered distribution observed was not due to aggregates in the gold-IgG preparation, as determined by electron microscopy. It was also not induced by sample preparation, since clusters were also observed in thin sections (Fig. 5G), although obviously less clearly than in the large membrane areas visible in the cleaved cells. To show that such microaggregation is not a general feature of surface proteins, we would like to have used mAb against fixation-resistant epitopes on other proteins, but none were available to us. However, in other lymphoma cells we have previously studied H-2 antigens (Feltkamp et al., 1987), which are not expressed by the T-cell hybridomas. The majority of the H-2 antigens was found to be dispersed.

**LFA-1 distribution upon interaction with immobilized ICAM-1**

As referred to above, invasive T-cell hybridomas adhere poorly or not at all to ICAM-1, and for optimal invasion by these cells LFA-1 requires activation (La Rivière et al., 1994). TAM8C4 cells do adhere to some extent to immobilized ICAM-1, but this can be increased by artificial activation of LFA-1 by 2 mM Mn²⁺ (La Rivière et al., 1994). This concentration was higher than required for human T-cells, as described by Dransfield et al. (1992), for unknown reasons. In the presence of 2 mM Mn²⁺, the cells adhered and spread extensively within 15

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**Fig. 5.** LFA-1 distribution on cells fixed in suspension before incubations with M18/2 mAb and gold-labeled secondary antibodies. Immune-incubated cells were attached to poly-L-lysine-coated grids and dry-cleaved. Inset in A: CLSM images of LFA-1 on TAM8C4 cell focused at top and central planes, respectively. (A,D) TAM8C4; and (E,G) TAM2D2 T-cell hybridoma cells; (B) CTL 23.21 cytotoxic T-cells; and (C,F) BW5147 lymphoma cells. On all cells, LFA-1 is distributed in clusters (arrows). (D-F) Drawings of LFA-1-detecting gold particles and cell margins, based on micrographs similar to (A-C) but at lower magnification. Bars: (A-C) 60 nm; (D-G) 200 nm.
ICAM-1 induces large-scale redistribution of LFA-1, there is no apparent change in the extent of microclustering of LFA-1 molecules.

DISCUSSION

We have shown previously that LFA-1 is essential for metastasis formation by T-cell hybridomas (Roossien et al., 1989). The liver is massively invaded by cells spreading diffusely between the hepatocytes. LFA-1-deficient mutants do not form liver metastases at all. LFA-1 can bind to three distinct counterstructures, ICAM-1, -2 and -3 (Dustin et al., 1986; Staunton et al., 1989; Springer, 1990; De Fougerolles et al., 1992). ICAM-3 is expressed only on lymphoid cells (De Fougerolles et al., 1992), and is therefore probably not the relevant counterstructure in the liver. ICAM-2 differs from ICAM-1 in that it is constitutively expressed, particularly by endothelial cells, and that ICAM-2 levels are not enhanced by inflammatory cytokines (De Fougerolles et al., 1991). ICAM-1 expression is usually low or undetectable in cells other than lymphoid or inflamed tissues (De Fougerolles et al., 1992). In humans, one of the exceptions is the liver, where ICAM-1 is expressed by both endothelial cells and hepatocytes at higher levels than ICAM-2 (De Fougerolles et al., 1991). In rats, ICAM-1 was reported not to be expressed in liver (Tamatani and Miyasaka, 1990), but we found ICAM-1 to be present on freshly isolated rat hepatocytes and short-term rat hepatocyte cultures, in keeping with the data on human liver. The reason for this discrepancy is not clear.

ICAM-1 is probably the major, if not the only, counterstructure that LFA-1, on invading T-cell hybridoma cells, binds to in the liver. We were unable to determine ICAM-2 expression on these rat hepatocytes, since antibodies against rat ICAM-2 are not available, but in human liver ICAM-2 levels are very low (De Fougerolles et al., 1991). Adhesion to the dorsal surface of hepatocytes is inhibited by anti-ICAM-1 mAb, albeit less extensively than anti-LFA-1 mAb. This may be due to a difference in blocking efficacy. During adhesion to the hepatocytes, LFA-1 was redistributed to the contact site, as was described for CTL-target cell interactions (Kupfer and Singer, 1989). This is not required for LFA-1-mediated adhesion, however, as shown for large granular lymphocytes adhering to purified ICAM-1 in lipid bilayers (Dustin et al., 1992), or helper T lymphocytes adhering to antigen-presenting cells in the absence of antigen (Kupfer and Singer, 1989). In the latter case, LFA-1 redistribution to the interacting membrane did occur in the presence of cognate antigen; that is, upon triggering of the T-cell receptor. The same process obviously occurs during CTL-target cell interactions. Such triggering has been shown to induce the activation of LFA-1 that causes an increase in LFA-1 affinity or avidity (Van Kooyk et al., 1989; Dustin and Springer, 1990; De Fougerolles et al., 1992). ICAM-1 is probably the major, if not the only, counterstructure that LFA-1, on invading T-cell hybridoma cells, binds to in the liver. We were unable to determine ICAM-2 expression on these rat hepatocytes, since antibodies against rat ICAM-2 are not available, but in human liver ICAM-2 levels are very low (De Fougerolles et al., 1991). Adhesion to the dorsal surface of hepatocytes is inhibited by anti-ICAM-1 mAb, albeit less extensively than anti-LFA-1 mAb. This may be due to a difference in blocking efficacy. During adhesion to the hepatocytes, LFA-1 was redistributed to the contact site, as was described for CTL-target cell interactions (Kupfer and Singer, 1989). This is not required for LFA-1-mediated adhesion, however, as shown for large granular lymphocytes adhering to purified ICAM-1 in lipid bilayers (Dustin et al., 1992), or helper T lymphocytes adhering to antigen-presenting cells in the absence of antigen (Kupfer and Singer, 1989). In the latter case, LFA-1 redistribution to the interacting membrane did occur in the presence of cognate antigen; that is, upon triggering of the T-cell receptor. The same process obviously occurs during CTL-target cell interactions. Such triggering has been shown to induce the activation of LFA-1 that causes an increase in LFA-1 affinity or avidity (Van Kooyk et al., 1989; Dustin and Springer, 1990). We have reported previously that LFA-1 on highly invasive T-cell hybridoma cells needs to be activated before it can participate in the invasion process. The available evidence indicates that cells in the invaded monolayers produce factors that activate LFA-1 via a signal transmitted by a pertussis toxin-sensitive G-protein (La Rivière et al., 1994). Also, invasion into hepatocyte cultures and metastasis to the liver are inhibited by pertussis toxin pretreatment (Roos and Van de Pavert, 1987).
suggesting that LFA-1 is also activated during interaction with hepatocytes. The observed LFA-1 redistribution strongly supports this notion.

We also observed a high density of ICAM-1 in the contact area of approximately 25% of the hepatocyte-adherent TAM8C4 cells. This can be explained by assuming that LFA-1 activation and redistribution occur first, and that diffusing ICAM-1 molecules then get trapped at the interaction site because of their binding to activated LFA-1. Cells adherent to membrane areas without ICAM-1 concentration had probably not been attached long enough for ICAM-1 to accumulate. Alternatively, this ICAM-1 concentration may have occurred only at specific sites, for instance near the boundaries between adjacent hepatocytes, but we have no indications for that. It is also not clear whether this ICAM-1 concentration is a prerequisite for subsequent invasion.

Activation of LFA-1 and the interaction between LFA-1 and ICAM-1 are probably major determinants of the actual invasion step, i.e. the migration between hepatocytes. This notion is supported by the high ICAM-1 density at the lateral hepatocyte surfaces, the inhibition of invasion by anti-LFA-1 mAb and the extensive redistribution of LFA-1 to the pseudopodia inserted between the hepatocytes. In addition, in the cells that had accumulated under the monolayer, LFA-1 was located predominantly on the upper surface in contact with the overlying hepatocyte membrane, in which area ICAM-1

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**Fig. 7.** EM of ICAM-1-adherent cells. (A-C) Low magnification of whole mounts of (A) TAM8C4 and (B) CTL 23.21 cells, both spread in the presence of Mn²⁺; and (C) CTL 23.21 cells spread upon treatment with PMA. The Mn²⁺-treated TAM8C4 and CTL 23.21 cells extend long filopodia, which are not present upon PMA treatment of CTL cells. (D-F) Immuno-EM of LFA-1. (D) In TAM8C4 cells, wet-cleaved after adhesion to ICAM-1, LFA-1 is detected in clusters of equal size (arrows) as seen on cells in suspension (Fig. 5A). On filopodia of TAM8C4 (E) and CTL 23.21 (F) many LFA-1 clusters (arrows) are present. The number of gold particles per cluster is not increased. Bars, 200 nm.
density was higher than in the remainder of the substratum-adherent hepatocyte membrane. Invasion was not inhibited by anti-ICAM-1 mAb, but this was probably due to lack of penetration of the mAb between the hepatocytes, as demonstrated by the lack of lateral surface labeling when this antibody was used without prior permeabilization of the hepatocytes. Given the apparently higher affinity of LFA-1 for ICAM-1 than for the other ICAMs (De Fougerolles and Springer, 1992), the substantial ICAM-1 expression may be one of the factors determining the extensive liver metastasis of T-cell hybridomas and other lymphomas (Dingemans, 1973; Roos et al., 1977, 1985). Furthermore, the tendency of lymphomas to spread diffusely in the liver parenchyma may be due to the concentration of ICAM-1 at the lateral hepatocyte surface, providing for a haptotactic ICAM-1 gradient (McCarthy and Furcht, 1984) that may direct migrating lymphoma cells to a location between the hepatocytes.

To study the redistribution of LFA-1 upon interaction with ICAM-1 we used immuno-electron microscopy on cells adhering to immobilized recombinant ICAM-1. As discussed above, LFA-1 on T-cell hybridomas requires activation, and therefore these cells adhere poorly or not at all to ICAM-1. The TAM8C4 cells that we used in this study adhere to some extent, but adhesion is enhanced by Mn²⁺ (La Rivière et al., 1994). The cells spread on the ICAM-1-coated surface, but again this spreading was more rapid and more extensive in the presence of Mn²⁺. We first studied suspension cells and found LFA-1 to be located in small, widely separated clusters. Concentration of integrins in microclusters has been reported to occur upon PMA-induced activation (Detmers et al., 1987) or upon interaction with ligand (Kornberg et al., 1992; Samuelsson et al., 1993; Meijne et al., 1994), but to our knowledge has never before been observed in unstimulated non-adherent cells. These clusters were not a peculiarity of these T-cell hybridomas, since they were also seen in normal T-lymphocytes, i.e. a cytotoxic T-cell clone, which is also invasive (La Rivière et al., 1993). However, their presence was not associated with invasive capacity, since LFA-1 in non-invasive BW5147 lymphoma cells was also located in clusters, albeit of somewhat larger size.

Upon adhesion and spreading on ICAM-1, LFA-1 was redistributed as in hybridoma-hepatocyte interactions. All LFA-1 was translocated to the contact area, i.e. the basal surface, and in addition was concentrated in spreading lamellipodia. Thus, when LFA-1 is in the active state, all signals required for LFA-1 redistribution and spreading are generated upon interaction of LFA-1 with ICAM-1. This is in agreement with a recent report that engagement of β2 integrins causes actin polymerization, possibly due to production of phosphatidylinositol trisphosphate (Löfgren et al., 1993). Quite remarkably, the cells extended long thin filopodia that contained high concentrations of LFA-1. Such filopodia have also been observed in other cell types upon spreading induced by activation of integrins (Arroyo et al., 1992).

Changes in the avidity of integrins have been ascribed to an altered conformation (Hynes, 1992; Keizer et al., 1988; Landis et al., 1993), but also to formation of integrin clusters (Detmers et al., 1987; Haverstick and Gray, 1992; Van Kooyk et al., 1994). The increased LFA-1-mediated adhesion induced by Mn²⁺ is apparently not due to enhanced cluster formation, since the LFA-1 clusters in adherent cells did not differ in size from those in suspended cells, and were still quite separated, even on the filopodia. Thus, this enhancement of adhesion is not due to cluster formation.

We conclude that interaction of LFA-1 with ICAM-1 plays a pivotal role in invasion of hepatocyte cultures by T-cell hybridoma cells, and probably in invasion of the liver, also by other types of lymphoma cells. Furthermore, the presence of an ICAM-1 gradient in the liver may be responsible for the directed migration of lymphoma cells into the liver parenchyma. This invasion is associated with substantial redistribution of LFA-1 on the hybridoma cells and also with ICAM-1 redistribution on the hepatocyte surface. Similar LFA-1 redistribution occurs during adhesion and spreading of the hybridoma cells on immobilized ICAM-1 induced by activation of LFA-1 by Mn²⁺. Finally, we conclude that LFA-1 is located in microclusters in non-adherent T-cell hybridoma cells as well as normal T-cells and that the size of the clusters does not change when the cells adhere and spread on ICAM-1 in the presence of Mn²⁺.

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