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Role of α_4-Integrins in Lymphocyte Homing to Mucosal Tissues In Vivo[1]

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Lymphocyte recirculation through different organs is thought to be regulated by adhesion molecules ("homing receptors") recognizing tissue-specific vascular addressins on endothelium. Here we show that the α_4/β_7-integrin has a key role in the migration of mouse lymphocytes to mucosal sites. Homing to Peyer's patches but not to peripheral lymph nodes is inhibited by Fab fragments of mAb PS/2 against the α_4-integrin chain, by mAb DATK32 recognizing a combinatorial epitope on the α_4/β_7-integrin, and by mAb FIB30 against the β_7-chain. The Abs significantly reduce homing of lymphocytes to the intestine, as well. The migration of immunoblasts to gut and gut-associated lymphoid tissue also involves the α_4/β_7-integrin heterodimer. Another anti-α_4 Ab, R1-2, which blocks lymphocyte binding to Peyer's patches in the Stamper-Woodruff frozen section assay and lymphocyte adhesion to VCAM-1 and fibronectin, has only minor effects on lymphocyte traffic in vivo. Anti-VCAM-1 Ab as well as the fibronectin peptide CS-1 are without influence on the migration to Peyer's patches or intestine, in contrast to Ab against the mucosal addressin MAdCAM-1. Thus, homing to gut-associated sites is regulated by the α_4/β_7-integrin heterodimer interacting with the vascular addressin, MAdCAM-1, and not with fibronectin or VCAM-1 as counterstructures. Inhibition of homing to Peyer's patches and intestine by the anti-integrin Abs studied was only partial. L-selectin also participates in the homing of small lymphocytes to mucosal sites, especially Peyer's patches, but does not contribute substantially to the localization of blasts into the intestinal wall. The results support a major, but not exclusive role of the α_4/β_7-integrin in lymphocyte traffic to mucosal sites. *Journal of Immunology,* 1994, 152: 3282.

During their continuous recirculation from blood into tissues and back to circulation, lymphocytes cross the endothelial linings of postcapillary venules, especially in lymph nodes and Peyer's patches, where a distinct morphology "high endothelial venules" (HEV)[4] accentuates sites of intense lymphocyte traffic. Interaction of lymphocyte adhesion molecules (partly termed "homing receptors") with epitopes on endothelial molecules expressed in a tissue-restricted manner ("vascular addressins") is thought to enable selective immigration of cell populations into organs. Mainly by use of in vitro assays, a variety of lymphocyte adhesion molecules has been identified and supposed to play a role as organ-specific homing receptors, namely, the L-selectin (1), CD44 (2), and the α_4/β_7- or β_1-integrins also called lymphocyte-Peyer's patch adhesion molecule, LPAM 1 and 2 (3–5).

L-selectin plays a dominant role in directing lymphocyte traffic to peripheral lymph nodes; however, in vivo and in vitro studies have shown that it also contributes to homing into mucosal sites (6–8). α_4-Integrins have been proposed to play a role as homing receptors for mucosal sites, based on the ability of anti-mouse α_4 Ab R1-2 to inhibit adhesion to HEV of Peyer's patches in the frozen

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[4] Abbreviations used in this paper: HEV, high endothelial venules.

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section assay in vitro (3). An anti-rat α_4 Ab (TA-2) has been shown to block spleen lymphocyte entry into Peyer's patches, but not into peripheral lymph nodes in vivo (9). In addition, lymphocyte entry into inflamed skin and into arthritic joints was affected by this Ab (10, 11). In the mouse, in vivo studies on the role of α_4-integrins for migration into mucosal sites have not been carried out to date. More importantly, the relative contribution of α_4/β_1 vs α_4/β_7 to Peyer's patch homing in vivo and the role of these integrins for the migration of blasts to mucosal sites has not been examined.

The α_4-chain can associate with either a β_1-chain (the dimer previously termed VLA-4 in the human) or a β_7 (β_P)-chain. The β_7 heterodimer is responsible for most of Peyer's patch-specific adhesion in vitro (3–5, 12). Recent data demonstrate that mucosal vascular addressin MAdCAM-1 is a ligand for α_4/β_7-integrins (13), and interacts preferentially with α_4/β_7 vs α_4/β_1. MAdCAM-1 is a member of the Ig and mucin-like families of adhesion receptors (14), which is selectively expressed by venules in mucosal tissues; it is an adhesion molecule for mucosa-homing lymphocyte populations (15). Lymphocyte homing to Peyer's patches and the lamina propria has been shown to involve the mucosal vascular addressin (16), thus supporting a unique role of α_4/β_7 in homing to mucosal sites. The β_7-chain also associates with a further α-chain, α_E (M290, or HML), which is expressed predominantly on lymphocytes residing in intestinal sites (12, 17) and seems to be involved in interaction with epithelial cells (18).

Ligands for α_4/β_1-integrins include VCAM-1 and fibronectin. VCAM-1 is induced on endothelium by cytokines and therefore has been suggested to mediate cell entry into inflamed tissue in particular (19–21). α_4-Integrins bind to at least two regions of fibronectin, the CS-1 and the CS-5 region, and the CS-1 peptide is able to block lymphocyte adhesion to cultured rat endothelium (22–25). Although endothelial cells may express fibronectin receptors (26) and hence expose them on the surface, the blocking activity of CS-1 does not necessarily indicate that the ligand for lymphocyte adhesion to Peyer's patch endothelium is fibronectin because CS-1 also blocks α_4 binding to VCAM-1 (27). α_4/β_7 also binds to fibronectin and VCAM-1. In cells expressing both α_4/β_7 and α_4/β_1, binding to VCAM-1 seems to be dominated by α_4/β_1 (13, 28, 29).

In this study we have investigated the role of α_4- and β_7-integrin epitopes in lymphocyte migration to Peyer's patches and other organs in the mouse. The results support a major function of the α_4/β_7 heterodimer for lymphocyte homing into Peyer's patches and the intestine.

Materials and Methods

Reagents and cells

The following rat Abs were used: mAb PS/2 (IgG2b) reacting with the mouse α_4-chain (30) and anti-VCAM-1, MK 2.7.7 (IgG1) (31) were kindly supplied by P. Kincade (Oklahoma Medical Research Foundation, Oklahoma City), anti-α_4 mAb R1-2 (IgG2b) was produced as described (3). mAb DATK32 (IgG2a) recognizes a combinatorial epitope on the α_4/β_7-integrin dimer. It was produced by immunization of rats with the TK-1 cell and characterized by its reaction with α_4/β_7-positive cell lines and 38C13 cells transfected with β_7, but not with β_7-negative cell lines or mock transfected 38C13 cells. It does not react with α_4/β_1 or α_E (α_{M290})/β_7-positive lymphocyte populations or cell lines[5]. FIB 30 reacts with the β_7-chain as shown by its reactivity with β_7-positive and β_1-negative cells lines. Immunoprecipitation and FACS-staining patterns were identical to those of established anti-mouse β_7-Abs. Anti L-selectin mAb MEL-14 (IgG2a) and anti-MAdCAM-1 mAb MECA 367 (IgG2a) were produced as described (1, 16). Anti LFA-1 mAb H-35.89.9 (IgG2b) (32) was obtained from M. Pierres (INSERM-CNRS, Marseille, France). As control, Abs were used: anti-CD4, mAb GK1.5 (IgG2b) (33), anti-CD45, mAb 30G12 (IgG2a) (34) and M/1.89.18 (IgG2b) (35), anti-CD44, mAb IM.7 (IgG2b) (36), anti-MHC I, mAb M1/42.9.8 (IgG2b) (35), anti-HEV, mAb MECA 325, (IgG1) (37) and irrelevant isotype controls. FITC-coupled goat anti-rat-IgG Ab, absorbed against mouse IgG, was from Jackson Immuno Research, Dianova GmbH, Hamburg, FRG.

mAbs were purified by affinity chromatography as described (38) or precipitated by ammonium sulfate from serum-free supernatants. Fab fragments were obtained by cleavage with papain and subsequent purification by HPLC gel permeation chromatography (ZORBAX GF-250, DuPont, Wilmington, DE).

The fibronectin peptide CS-1 (39) was synthesized in the Dept. of Neurobiology, University of Hamburg, Hamburg, FRG, by F. Kullmann and purified by HPLC and gel permeation chromatography. Identity was established by comparison with a standard, kindly supplied by A. Ager and M. Humphries (University of Manchester, Manchester, UK). Purity was checked by reversed phase HPLC (M. Heukeshoven, Heinrich-Pette-Institute for Virology and Experimental Immunology, Hamburg, FRG) and shown to be greater than 95%.

The mouse endothelial cell line TME-3H3 was produced by SV40-transformation as described elsewhere (40). Lymphocytes adhere to this cell line by at least three adhesion pathways, one involving α_4/VCAM-1 as described here and previously (40), one being a CD44-dependent adhesion (41) (H. Uhlig, S. Rebstock, J. Lesley, D. Jablonski-Westrich, and A. Hamann, manuscript in preparation) and a further, small contribution coming from endothelial α_6/β_1-integrin (42). LFA-1 and L-selectin do not play a role in this model.

The mouse lymphoma cell line TK-1 (43) was used as a subline adapted to in vitro growth (3). Lymphocytes were prepared from combined peripheral and mesenteric lymph nodes of specific pathogen-free reared, female BALB/c mice (6 to 12 wk of age, Versuchstierzuchtanstalt Hannover, FRG). In some experiments on blast homing, only mesenteric nodes were used. Dead cells were removed by centrifugation on a cushion of 18% isotonic metrizamide (Nycomed, Oslo, Norway). The cells were suspended in RPMI 1640 (GIBCO, Eggenstein, FRG) supplemented with 10% heat-inactivated FCS.

In vivo homing

The in vivo migration of lymphocytes was analyzed as described (38). In short: total lymphocytes were labeled with 20 μCi chromium-51/ml, lymphoblasts with 1 μCi [^{125}I]iododeoxyuridine/ml. Dead cells were removed by centrifugation on an isotonic 18% metrizamide density cushion. A total of 5×10^6 lymphocytes, suspended in 0.25 ml, were injected after 15 min of preincubation at room temperature together with the respective Ab into the tail vein of one 8- to 12-wk-old BALB/c mouse. In general, Abs were used in the form of Fab preparations, but in case of most anti-α_4, anti-β_7, or anti-endothelial Abs, IgG could be used with similar results. Each experiment consisted of four control and four sample animals and was repeated at least once. Mice were killed after 1 h and the distribution of radioactivity in different organs and the remaining body was measured. Peripheral lymph nodes consisted of a pool of the superficial inguinal nodes, the brachial and axillary nodes, and the superficial cervical nodes. All Peyer's patches were collected. Blood values are computed for 2-ml volume. Radioactivity recovered amounted to

[5] D. P. Andrew, C. Berlin, S. Honda, T. Yoshino, A. Hamann, P. Kilshaw, and E. Butcher. Distinct but overlapping epitopes are involved in $\alpha_4\beta_7$ mediated adhesion to VCAM-1, MAdCAM-1, fibronectin, and in lymphocyte aggregation. Submitted for publication.

about 80 to 90% of injected activity at this time point. Each sample was counted to 3% statistical error. The mean values and their SD were determined from four animals. For values given as percentage of control, the SD was computed from combined variances of sample and control. A variety of control experiments with more than 10 mAbs was carried out showing that injected Fab fragments of Abs against molecules not involved in lymphocyte/endothelium recognition do not influence significantly the migration of lymphocytes.

Frozen section assay

The frozen section assay was performed as described (38). In short, Hoechst 33342-labeled (44) reference cells (rat lymphocytes) were mixed with mouse lymphocytes and overlaid with or without prior addition of the rat mAb on sections of mouse Peyer's patches or peripheral lymph nodes at 7°C and 90 rpm for 30 min. All rat monoclonals used do not react with rat lymphocytes. After incubation, the slides were fixed in 0.5% glutardialdehyde at 4°C and the ratio of HEV-bound mouse sample cells vs rat reference cells was determined microscopically. Relative binding was expressed as percent of control. The mean and SD of four to five slides (15 to 30 patches or lymph nodes) were determined per experiment. For values expressed as percent of control a combined SD was computed from the sum of control and sample variances. A variety of control Abs (anti-MHC, anti-CD44, anti-HEV (mAb MECA 325) (37) or irrelevant isotype controls) used were without effects on lymphocyte binding (data not shown). Anti-VCAM-1 Ab and CS-1 were tested in a modified assay: unlabeled lymphocytes were overlaid onto frozen sections, together with FITC-labeled mAb MECA 325 to stain HEV, and the absolute number of cells adhering to HEV in the presence or absence (control) of Ab or CS-1 was counted together with the number of HEV. The mean number of lymphocytes bound per HEV or per patch section was determined from five or six slides.

Binding assay on cultured endothelial cells

Binding of lymphocytes to the endothelial cell line The-3H3 grown as monolayer in microtiter-plates was assessed as described elsewhere (40). In short, Hoechst 33342-labeled (44) lymphocytes were pretreated for 30 min at 4°C with the Abs indicated. After washing, the lymphocytes were centrifuged onto the endothelial cells and unbound cells removed by a following centrifugation of the plate upside down (45). The percentage of total lymphocytes bound was determined by measurement of fluorescence before and after removal of the unbound cells in a microtiter-fluorimeter (Dynatech, Billinghurst, UK). The fluid volume was kept to 0.2 ml for the measurement.

Determination of relative binding affinity and of cross-blocking of anti-α_4 Fab

The relative affinity of the Fab fragments used toward α_4 was measured by indirect immunofluorescence and quantification of bound Ab in the FACS (FACScan flow cytometer, Becton and Dickinson, Heidelberg, FRG). The TK-1 lymphoma cell line expressing uniformly high amounts of α_4/β_7, but not α_4/β_1 (4) was used as target. A total of 10^6 cells was incubated in 100 μl RPMI 1640 and different concentrations of the Fab fragments for 30 min at 37°C. Subsequently the cells were washed at 0°C and stained with a saturating amount of FITC-coupled second stage Ab for 30 min at 0°C. After staining, cells were fixed with 1% paraformaldehyde and analyzed in the FACS using a logarithmic detector setting. The median of the specific fluorescence intensity in linear units was used, obtained by converting the log fluorescence intensity (channel number) mathematically to a linear fluorescence. The background (median of the control) was subtracted.

When the incubation with Fab was carried out at 4°C, half-maximal binding was at concentrations two- to fourfold lower than that required at 37°C for all three Abs. To investigate whether R1-2 and PS/2 recognize overlapping epitopes, TK-1 cells were stained with biotinylated R1-2 Fab or PS/2 Fab followed by streptavidin-phycoerythrin after preincubation with or without competing Ab in 10-fold excess. Both Abs were able to suppress binding of each other under these conditions to nearly 100%.

Results

Anti-α_4-chain Ab PS/2 partially inhibits homing of lymphocytes into Peyer's patches and intestine

We examined the role of α_4-integrins in lymphocyte trafficking by analyzing the effects of mAbs to α_4-integrins on the short term migration of lymphocytes in vivo. Chromium-51-labeled cells were pretreated with Ab (Fab fragments, if not otherwise stated) and injected together with a given amount of Fab into the tail vein to prevent possible loss of Ab from the receptors during the in vivo stay. A 1-h period of homing was chosen, because during this time the accumulation of radioactivity reflects the direct entry of lymphocytes into the different tissues after passing through the lung (46), and this period does not extend much above the mean half life of Fab fragments in vivo (47).

Fab fragments of mAb PS/2 directed to the murine α_4-chain (30) inhibited lymphocyte accumulation in Peyer's patches by 50 to 65% (Fig. 1, Table I). Within this range, the higher doses of Ab appeared to be slightly more efficient. Uncleaved IgG of PS/2 caused the same degree of inhibition. Presence of the Ab also reduced entry of cells into the intestine (Peyer's patches removed) by 40 to 50%. Concomitantly, cells prevented from entering the Peyer's patches and gut were redistributed predominantly into spleen and peripheral lymph nodes. The rate of accumulation in skin, lungs, or liver was not changed by the Ab. Control Abs were without effect on the distribution of cells.

Because the majority of the lymph node lymphocytes are T cells (60 to 70%), we wished to rule out the possibility that the incomplete inhibition by the anti-α_4 Ab is caused by a differential use of the molecules by B and T cells. However, the inhibitory effect of PS/2 Ab on the migration of pure B cells was only slightly different from that on the T cell-dominated unseparated population (Table I).

The α_4/β_7-heterodimer mediates the α_4-dependent migration to mucosal sites

Recently a mAb, DATK32, directed to an epitope displayed by α_4/β_7 dimers, but not by α_4/β_1 or α_E/β_7, has been produced. This Ab reacts with a combinatorial epitope of α_4/β_7, does not stain β_7-negative/β_1-positive cells, and inhibits lymphocyte binding to several ligands[5]. Fab fragments of this Ab reduced lymphocyte entry into Peyer's patches to the same degree or, at high doses, slightly better than Fab fragments of mAb PS/2 (Fig. 1, Table I). Also Abs directed against epitopes on the β_7-chain, FIB30 (Table I), and FIB22 (data not shown), inhibited homing to the Peyer's patches to the same degree. The β_7-specific Abs also impaired the migration into the intestine itself. Thus, lymphocyte traffic to mucosal sites involves the integrin α_4 dimer containing the β_7 chain. In

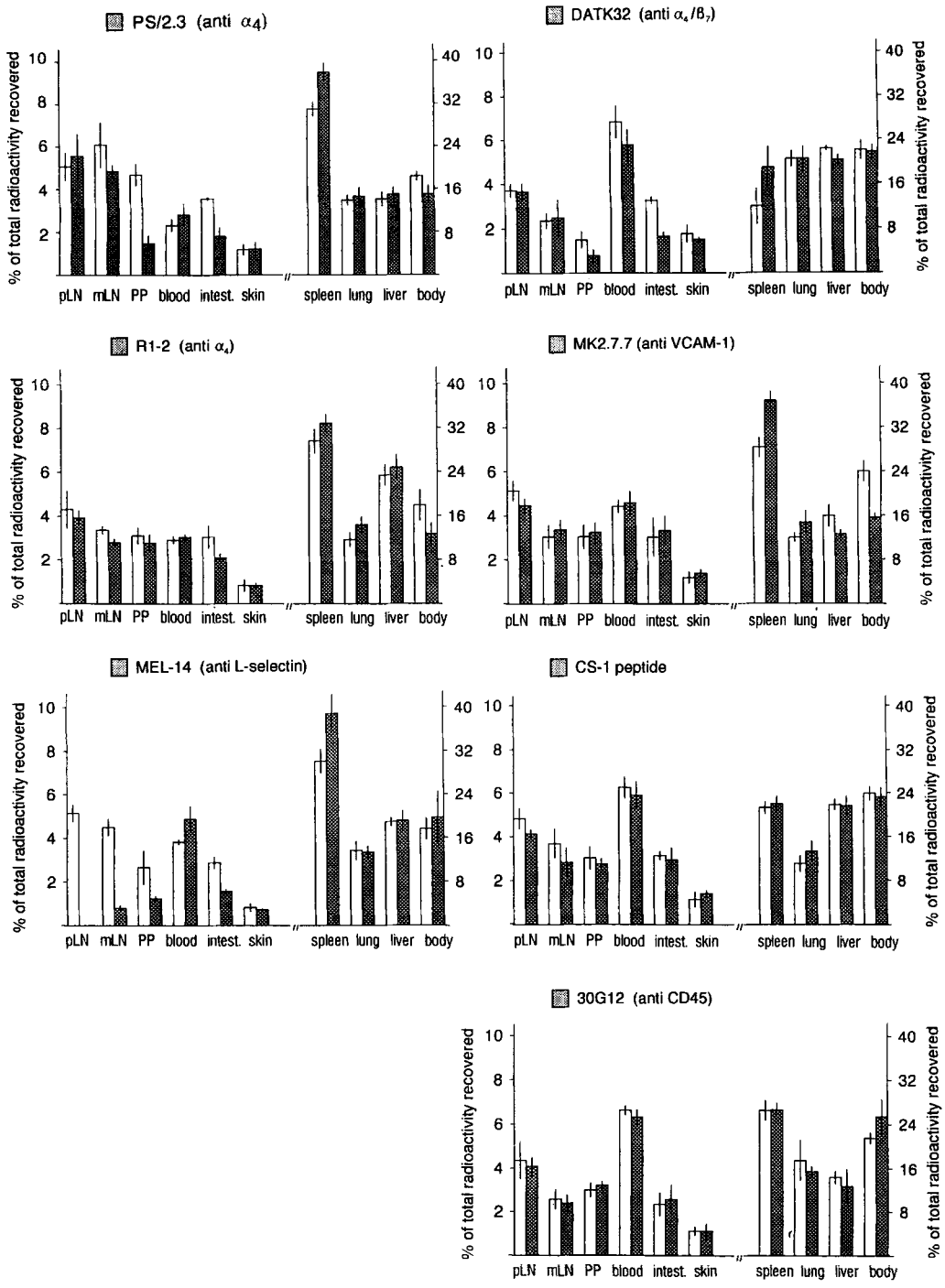

FIGURE 1. Effect of various Abs and CS-1 peptide on short term lymphocyte traffic. Chromium-51-labeled lymphocytes were pretreated with 100 μg Fab (in 250 μl PBS) or with PBS alone (controls) and injected together with the Ab into the tail vein. Radioactivity was counted in the organs 1 h after injection. Open columns, control mice (no Ab); dark columns, Ab or CS-1-injected animals. In case of CS-1 peptide, lymphocytes were preincubated with 2 mg CS-1/250 μl and, additionally, 2 mg of CS-1 were injected i.p. every 15 min. Bars: SD ($n = 4$). Note the different scale for the organs on the right part of the figure. The absolute number of cells distributing to the different organs varies between experiments, depending on the age of the animals and other factors.

Table I. *Effects of various Abs on short term lymphocyte homing to peripheral lymph nodes (pLN), Peyer's patches (PP), and intestine*[a]

Treatment: mAb	Ag	Radioactivity in the Organs (% of Control)		
		pLN	PP	Intestine
PS/2 Fab, 100 μg	α_4	128 ± 28	44 ± 7	52 ± 10
PS/2 Fab, 500 μg		98 ± 15	35 ± 6	37 ± 8
PS/2 IgG, 500 μg		94 ± 14	38 ± 8	55 ± 12
PS/2 Fab, 100 μg, B cells only		110 ± 24	32 ± 28	51 ± 28
R1-2 Fab, 100 μg (mean of 3 experiments)	α_4	107 ± 16	84 ± 13	91 ± 18
R1-2 Fab, 300 μg		124 ± 30	82 ± 12	96 ± 27
DATK32 Fab, 100 μg	α_4/β_7	98 ± 13	52 ± 22	51 ± 6
DATK32 Fab, 500 μg		85 ± 17	25 ± 13	56 ± 9
FIB30 IgG, 500 μg	β_7	90 ± 18	56 ± 20	76 ± 26
MEL-14 Fab, 100 μg	L-selectin	1 ± 0.2	46 ± 14	63 ± 8
PS/2 Fab + MEL-14 Fab, 100 μg		2 ± 0.4	1 ± 0.5	24 ± 8
H-35.89.9 Fab, 300 μg	LFA-1	21 ± 4	56 ± 10	84 ± 7
MK 2.7.7 IgG, 300 μg	VCAM-1	96 ± 21	101 ± 11	113 ± 18
30G12 Fab, 100 μg	CD45	94 ± 20	107 ± 13	109 ± 27
M1/89.18. Fab, 100 μg	CD45	95 ± 27	98 ± 25	97 ± 18
M1/42.9 Fab, 100 μg	MHC I	101 ± 19	99 ± 32	79 ± 31
CS-1 peptide, 4×2 mg/mouse	Not applicable	86 ± 9	91 ± 17	94 ± 18
MECA367 IgG, 300 μg	MAdCAM-1	75 ± 13	34 ± 7	4 ± 1
MECA367 IgG+ PS/2 Fab, 300 μg		50 ± 16	28 ± 11	3 ± 1

[a] Chromium-51-labeled lymphocytes were pretreated with the Abs (given amount of Fab or IgG in 250 μl) and injected together with the mAb into the tail vein. Radioactivity was counted in the organs 1 h after injection. Values are expressed as percent of control (no Ab). Mean of four animals each, ± combined SD.

contrast, the homing of lymphocytes into peripheral lymph nodes is not affected by the β_7-specific Abs.

The α_4/β_7 dimer is involved in the migration of blasts to mucosal sites

Lymphoblasts (labeled by incorporation of the DNA precursor [^{125}I]iododeoxyuridine) have by themselves a lower preference to enter lymphoid tissues including the Peyer's patches compared with resting lymphocytes. Instead, they display a prolonged stay in the lung and, at later time points, home to other nonlymphoid tissues including the lamina propria (48, 49). The low number of blasts entering the Peyer's patches as well as that fraction of blasts arriving within the first hour in the intestine was reduced in the presence of anti α_4-Ab, similar to the effects found with resting lymphocytes (Fig. 2). In other organs studied significant effects were not observed. This shows that α_4-integrins are also involved in the migration of activated lymphocytes into gut-associated sites.

The same effects were found with anti-α_4/β_7 mAb DATK32 (Fig. 2) or anti-β_7 mAb FIB 30 (not shown), suggesting that the integrin heterodimer α_4/β_7 plays the prime role in homing to the gut mucosa (Fig. 2). The results were similar whether blasts originating from all lymph nodes or only mesenteric lymph node blasts were used (not shown).

Ab against L-selectin, which inhibits the localization of resting lymphocytes into Peyer's patches and intestine as well (8) (Fig. 1; Table I), reduces also the low degree of homing of blasts to Peyer's patches, but only marginally affects their entry into the intestine (Fig. 2). This applies to blasts from mesenteric lymph nodes as well as to peripheral lymph node blasts (data not shown).

Anti-α_4-chain Ab R1-2 is inefficient in blocking homing in vivo

A role for α_4-integrins as homing receptors for mucosal tissue was proposed because of the blocking effect of the R1-2 Ab on lymphocyte adhesion to HEV of Peyer's patches. R1-2 recognizes an epitope related or adjacent to the epitope defined by mAb PS/2, because both Abs cross-block each other almost completely (data not shown). However, when R1-2 was applied to in vivo homing experiments, this Ab had only weak effects on lymphocyte homing to Peyer's patches and intestine (Fig. 1, Table I). A higher dose of Fab also failed to be more efficient.

Functional activity of the R1-2 Fab fragments used was confirmed by its capacity to block lymphocyte adhesion to Peyer's patch HEV in the frozen section assay down to concentrations of 5 μg/ml (Table II). One hour after injection, levels of Fab in the serum as determined by ELISA were in the range of 5 to 10 μg (data not shown).

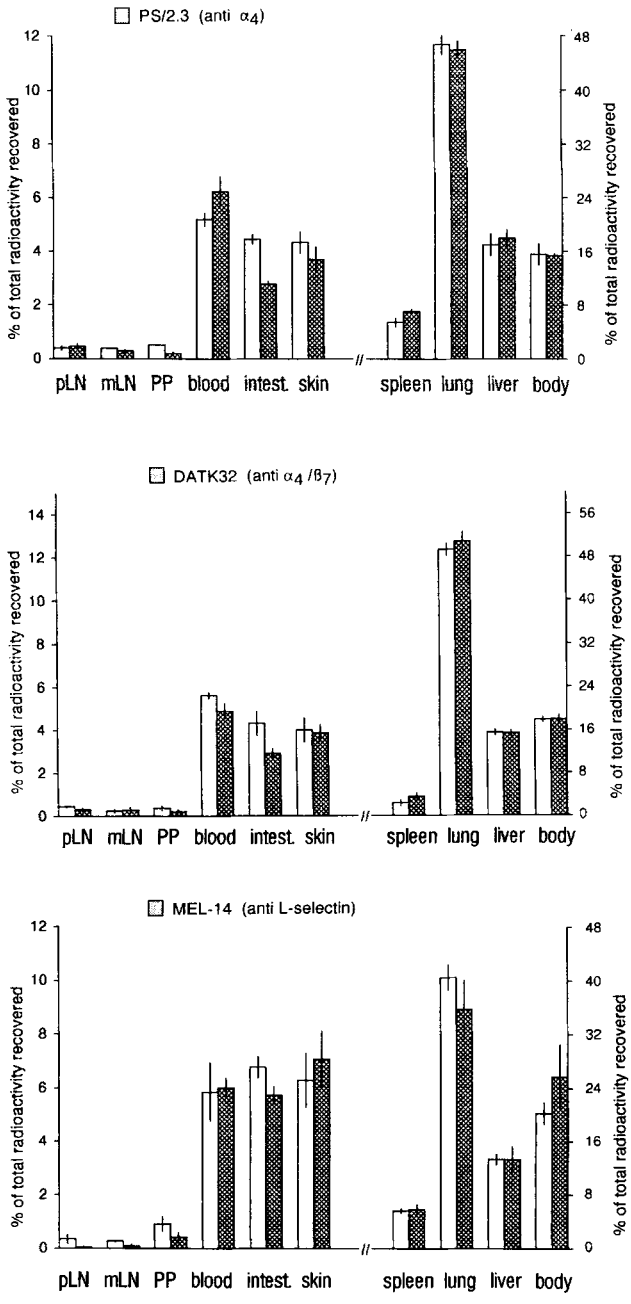

FIGURE 2. Effect of different Abs on the migration of lymphoblasts. Blasts were labeled with [^{125}I]iododeoxyuridine , pretreated with Ab (Fab, 100 μg) or PBS (controls) and injected together with the Ab into the tail vein. Radioactivity was counted in the organs 1 h after injection. Open columns, control mice (no Ab); dark columns, Ab-injected animals. A variety of control Abs tested had no effect on blast homing (not shown). Bars, SD (n = 4). Note the different scale for the organs on the right part of the figure.

Even more conclusively, serum from mice given 100 μg R1-2 Fab i.v. 1 h before blood withdrawal still contained Fab concentrations high enough to inhibit the adhesion of lymphocytes to Peyer's patch HEV in vitro (Fig. 3). A certain inhibitory effect of normal mouse serum at the high concentrations used was also observed consistently.

Because these in vitro assays are performed at 7°C, a low affinity of the Ab at 37°C could explain its lack of an in vivo effect. However, R1-2 inhibited the binding of lymphocytes to the endothelial cell line TME-3H3 (40) at 37°C, which exhibits an α_4-integrin/VCAM-1-mediated adhesion component in addition to CD44- and α_6/β_1-integrin-mediated components as mentioned above (Fig. 4).

In order to compare directly the relative affinity of the used Fab fragments at 37°C, we labeled the lymphoma cell line TK-1 expressing the α_4/β_7 dimer, but not α_4/β_1 dimer (4), with varying Fab concentrations at 37°C and measured the amount of Ab bound after staining at 0°C with a FITC-coupled second Ab in the FACS. Although this method is not able to determine the affinity in absolute values because of a possible dissociation of the Ab from the receptor during the second stage labeling, it is a useful method to rank the Abs according to their binding strength. As shown in Figure 5, R1-2 Fab required concentrations 5 to 10 times higher than PS/2 Fab for half-maximal binding. However, the DATK32 Fab, being the most effective inhibitor in vivo, displayed an even lower affinity than R1-2. This argues against a role of Ab affinity in determining the effect on lymphocyte migration in vivo.

No evidence for a role of VCAM-1 and fibronectin-related epitopes in homing to mucosal sites in vivo

We analyzed a potential role of the two established ligands for α_4-integrins, VCAM-1 and the CS-1 region of fibronectin, for lymphocyte migration. The MK 2.7.7 mAb against VCAM-1 (31) was able to inhibit lymphocyte binding to the endothelial cell line TME-3H3 (Fig. 4). However, adhesion to Peyer's patch HEV was not affected (Table II). Likewise, anti-VCAM-1 Ab did not influence the in vivo homing of lymphocytes to Peyer's patches or intestine (Fig. 1, Table I). A reduction by 30 to 40% of lymphocyte accumulation in the presence of anti-VCAM-1 Ab was observed in the residual body, comprising mainly bones, muscle, kidney, stomach, and brain as major compartments.

Because blocking Abs against the CS-1 region of mouse fibronectin are not available, we studied the role of this ligand for α_4-integrins by inhibition studies with the CS-1 peptide. Inclusion of 20 to 100 μM of CS-1 into the frozen section assay greatly reduced lymphocyte binding to Peyer's patch HEV (Fig. 6) or, in some experiments using a different pool of Peyer's patches, even abolished binding (not shown). The variable inhibition seems to be caused by local variations in the expression of ligands, because consistently the same Peyer's patches in a given pool of patches were susceptible to inhibition by CS-1. To test the role of CS-1-related ligands for homing in vivo, the chromium-labeled lymphocytes were preincubated in CS-1 (2 mg/250 μl = 3 mM) before injection and three further

Table II. *Influence of various Abs on lymphocyte binding to HEV in vitro*[a]

Tissue	Ab	Condition	Ag	Binding (% of control)
Peyer's patches	PS/2	Sup[b]	α_4	1 ± 0
	R1-2	Sup	α_4	25 ± 8
	R1-2	Fab, 25 μg/ml	α_4	20 ± 2
	R1-2	Fab, 5 μg/ml	α_4	26 ± 3
	MK 2.7.7	Sup	VCAM-1	96 ± 14
	MEL-14	Sup	L-selectin	95 ± 14
	H-35.89.9	Fab, 25 μg/ml	LFA-1	98 ± 7
	Control (GK1.5)	Sup	CD4	103 ± 9
Peripheral lymph nodes	R1-2	Sup	α_4	96 ± 15
	MEL-14	Sup	L-selectin	6 ± 1
	H-35.89.9	Fab	LFA-1	62 ± 4

[a] Relative binding of mouse lymphocytes to HEV of Peyer's patches or peripheral lymph nodes in the frozen section assay. Given is the mean ± SD from four to five sections (representative experiments); control: no Ab present during pretreatment.
[b] Hybridoma supernatant.

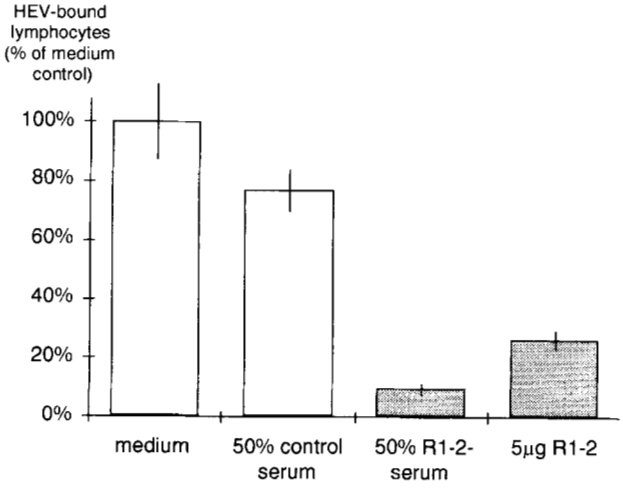

FIGURE 3. Inhibitory levels of R1-2 Fab are detectable in the serum of mice 1 h after injection. The adhesion of lymphocytes to Peyer's patch HEV in the frozen section assay was tested in the presence of medium, 50% normal mouse serum (control serum), 50% serum of mice injected with 100 μg of R1-2 Fab 1 h before killing (R1-2-serum), and 5 μg/ml R1-2 Fab. The serum of four animals each was pooled. Mean of five sections, ± SD.

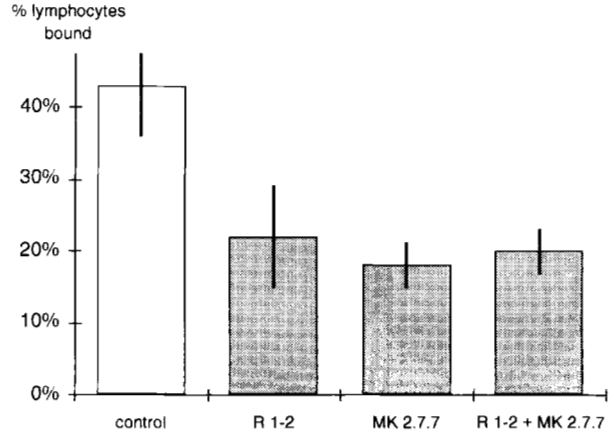

FIGURE 4. Anti-α_4 (R1-2) and anti-VCAM-1 (MK 2.7.7) block one pathway of lymphocyte binding to cultured endothelial cells (TME-3H3) at 37°C. After pretreatment of the lymphocytes with the indicated Abs or control supernatant at 4°C for 30 min, lymphocyte adhesion to the endothelial cells was measured at 37°C as described in *Materials and Methods*. *Bars*, SE of sixfold determinations.

injections of 2 mg CS-1/mouse were given every 15 min to allow for loss of peptide caused by metabolism and excretion. A CS-1 serum level showing at least partial inhibition in the frozen section assay was retained until termination of the homing experiments (Fig. 7). As mentioned above, serum alone already had a certain inhibitory effect. In spite of the inhibitory effect observed in the in vitro assay, an influence of CS-1 on lymphocyte migration in vivo was not detectable (Fig. 1, Table I).

In contrast, the mAb MECA 367 recognizing the mucosal addressin MAdCAM-1 very efficiently blocked lymphocyte migration into Peyer's patches and significantly inhibited entry into the gut wall (Table I). The small degree of lymphoblast homing to Peyer's patches was further reduced and the entry of blasts into the gut was inhibited by about 50%, when elevated blood levels are taken into account (data not shown). A combination of anti MAdCAM-1 and anti-α_4-integrin mAbs showed similar effects; in addition, a significant degree of inhibition was found in the peripheral nodes (Table I).

Discussion

The large family of integrins includes receptors for extracellular matrix as well as for ligands expressed on a variety of cells including endothelium (50). Abs against the α_4-chain were shown to inhibit selectively lymphocyte binding to HEV of Peyer's patches in vitro (3, 4) and, in the rat, lymphocyte homing to Peyer's patches and to sites of inflammation in vivo (9). The in vivo role of the two types of β-chains associated with α_4, β_7, and β_1 has not been established so far.

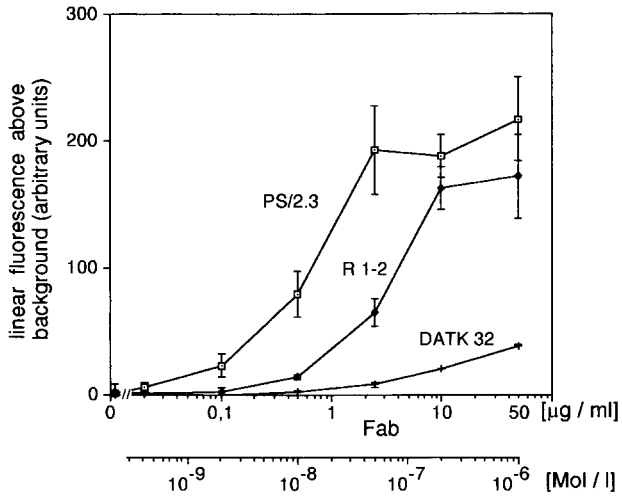

FIGURE 5. Relative affinities of anti-α_4 and anti-α_4/β_7 Fab Abs at 37°C. TK-1 cells were incubated with the different Fabs at various concentrations for 30 min at 37°C, washed, and stained with FITC-coupled secondary Ab at 0°C. Fluorescence was measured in the FACS. Given is the fluorescence (arbitrary units) above background (isotype control) on a linear scale. Mean ± SD of three experiments.

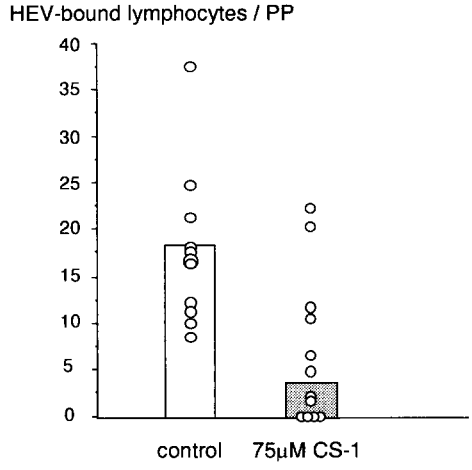

FIGURE 6. Inhibitory effect of CS-1 peptide on lymphocyte binding to Peyer's patch HEV in the frozen section assay. Lymphocytes were overlaid onto sections of Peyer's patches in the presence or absence of CS-1 peptide. The columns represent the mean numbers (median) of cells adhering to HEV in individual Peyer's patches. Each dot represents one individual patch section out of a pool of patches. Control peptides were without effect.

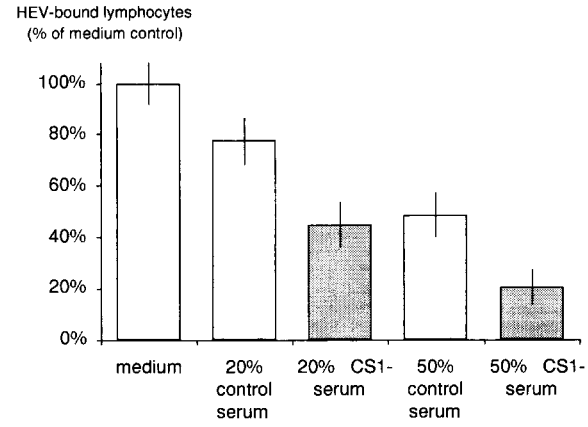

FIGURE 7. Inhibitory levels of CS-1 peptide are detectable in the serum of mice 1 h after injection. The adhesion of lymphocytes to Peyer's patch HEV in the frozen section assay was tested in the presence of medium, 50% or 20% normal mouse serum (control serum) and 50% or 20% serum of mice injected four times with 2 mg CS-1 every 15 min (CS1-serum). The serum of four animals each was pooled. Mean of five sections ± SD.

Here we show that the migration of lymphocytes to mucosal sites is mediated by the α_4/β_7 heterodimer. PS/2 Ab against mouse α_4 inhibits lymphocyte entry into Peyer's patches, confirming a major role of this integrin chain for homing to mucosal sites. Homing into these regions is inhibited to the same degree by the mAb DATK32,[5] a mAb directed to an epitope located on mouse α_4/β_7 dimers but not α_4/β_1 dimers, and by anti-β_7 mAb FIB 30[5]. The pattern of inhibition is similar to that of the α_4 Ab. In contrast, an Ab recognizing an activation epitope of the β_1-chain and blocking lymphocyte adhesion to cultured endothelium in vitro, mAb 9EG7 (42), was not found to influence lymphocyte homing to mucosal sites or other tissues studied (A. Hamann, M. Lenter, and D. Vestweber, unpublished observations), suggesting that at least the respective activation-dependent binding site of β_1 is not involved in recirculation. These data assign a selective role for the β_7-chain in lymphocyte homing to mucosal sites.

Abs against α_4 or β_7 were not found to influence the immigration of lymphocytes into normal skin. The inhibitory effect of anti α_4-Ab on lymphocyte entry into inflamed skin as well as into other sites of inflammation in the rat (9, 10) therefore indicates that α_4-integrins have a dual role in lymphocytes homing into either mucosal tissues or into sites of inflammation, respectively. The role of the different β-chains in immigration into inflamed sites has not been studied so far.

In contrast to the findings in the rat, the anti-α_4-Abs did not completely inhibit the entry of lymphocytes into Peyer's patches in the mouse. Most data indicate that incomplete inhibition is not caused by an insufficient Ab affinity, because PS/2 Ab has a high affinity for α_4 and very high doses of Fab and bivalent IgG block to a similar degree. The mAb DATK32 has an affinity approximately two orders of magnitude lower and blocks even better than PS/2. Two mAbs against the β_7-chain, FIB 30 and FIB 22 affected lymphocyte homing to mucosal sites to the same degree as the former Abs. Only application of mAb against the ligand, the mucosal addressin MAdCAM-1, resulted in

a higher degree of inhibition in Peyer's patch homing. Because MAdCAM-1 can be decorated with carbohydrates recognized by L-selectin (51), an effect on L-selectin as well as α_4/β_7-mediated adhesion to MAdCAM-1, by e.g. steric hindrance, could explain the more efficient inhibition observed. Side effects due to the use of complete IgG instead of Fab might also play a role. These factors may also help explain the slight inhibition found in migration to peripheral lymph nodes by MECA 367 Ab, where MAdCAM-1 is expressed at low levels in young mice.

How do other molecules contribute to the homing of resting lymphocytes into mucosal sites? We have previously implicated L-selectin and LFA-1 in this context (6–8, 38). Partial inhibition of Peyer's patch homing by anti LFA-1 and L-selectin mAbs is reproduced here. L-selectin dominates in lymphocyte homing to peripheral lymph nodes. It was originally thought to participate exclusively in that site, because lymphocytes pretreated with MEL-14 Ab and washed before injection for short term homing were not significantly impaired in their localization in Peyer's patches (1). Significant inhibition of Peyer's patch homing is consistently observed, however, when Ab is co-injected as in the present and a previous study (8). Immigration into lymph nodes and Peyer's patches could be the result of a concerted action of these molecules, as it has also been suggested for the interaction of lymphocytes with endothelium of inflamed tissue (52). In support of this we observed a complete prevention of lymphocyte entry into Peyer's patches with a combination of anti-α_4 and anti-L-selectin Abs.

The partial inhibition by each single and complete inhibition by a combination of both Abs could point to a differential role of these molecules within distinct subpopulations of resting lymphocytes. This appears unlikely given the relatively uniform expression of α_4/β_7 on the majority of lymph node B and T cells (12, 53, and [5]), and the lack of major deviations in the effect of anti-α_4 or anti-L-selectin Abs when pure B or T cells instead of unseparated cells were studied in these or previous homing experiments (8).

The involvement of both L-selectin and α_4/β_7 in lymphocytes homing to Peyer's patches is consistent with recent proposals that lymphocyte-HEV recognition involves an active multi-step process in which specificity is determined by combinatorial association of adhesion and activating receptors involved in initiating binding under flow, in triggering rapid G-protein-linked activation, and in activation-dependent adhesion (54, 55). In this regard, it is relevant that recent in situ studies have shown that lymphocyte interactions with Peyer's patch-HEV involved an initial transient rolling interaction, a pertussis toxin-sensitive G protein signaling event, and a secondary but rapid activation-dependent triggering of sticking and firm arrest (56). The parallels between these observations and previous studies demonstrating sequential rolling and sticking of neutrophils (57–59) are striking, and in the context of the current demonstration of involvement of both L-selectin and α_4/β_7 in lymphocyte binding Peyer's patch HEV, raises the possibility that the L-selectin plays a critical role in lymphocyte attachment and rolling along HEV of Peyer's patches, whereas α_4/β_7 may play an important role in activation-dependent sticking and arrest.

In addition to Peyer's patches, migration into the intestine itself is also affected by anti-α_4 and anti-β_7 Abs. The inhibition of lymphocyte homing into Peyer's patches as well as into gut tissue is not surprising because the distribution of an endothelial ligand shown to play a role as tissue-specific recognition element, the mucosal addressin MAdCAM-1, detected by mAb MECA 367 (16), predicted an overlap in homing mechanisms guiding cells into gut-associated lymphoid tissue and into the intestinal mucosa. In both cases, the α_4/β_7 heterodimer seems to play a key role as lymphocyte "homing receptor." The degree of inhibition by Abs against α_4/β_7 is slightly lower for homing to the intestine as compared with the Peyer's patches, which may suggest that the relative role of α_4/β_7 and other adhesion molecules is not identical for homing to Peyer's patches and the intestine, respectively. However, we cannot exclude a significant nonspecific component to the residual counts localized in the intestine. In situ studies of lymphocyte interactions with lamina propria venules are planned to further elucidate these issues.

In addition to the anti-α_4/β_7-integrin, anti-L-selectin displayed a detectable effect on lymphocyte localization to the intestine from which Peyer's patches were removed. Whereas binding of L-selectin and reactivity with mAb MECA 79 against the peripheral lymph node addressin-associated carbohydrate ligands for L-selectin has been detected at low levels in Peyer's patch HEV (7, 60), its presence in vessels of the lamina propria has not been reported. This may suggest either that its expression on these endothelia is very low or, alternatively, that a small number of "HEV-like" vessels not associated with macroscopically identifiable Peyer's patches may account for much of the immigration of resting lymphocytes into the intestine.

It is important to emphasize, however, that resting lymphocytes migrate only at a low rate into the lamina propria, especially if the comparison with Peyer's patch homing is made on a per gram of tissue basis (46, 48). Lymphoblasts display a bias for nonlymphoid tissue and, in contrast to naive lymphocytes, may acquire selective homing properties ("organ-specific" homing) which is most impressive in case of gut-derived thoracic duct blasts, which accumulate nearly exclusively in the intestine with time (reviewed in Refs. 49 and 61). The molecular basis for the specific homing of such blasts has not been elucidated so far. Although at the early time points studied here the majority of gut-seeking blasts still is held up in the lung, our data clearly show that immigration of blasts into the intestine also involves the integrin α_4/β_7 dimer and MAdCAM-1

(see also Ref. 16), although other molecules may be required in addition. The effect of anti-L-selectin mAb on blast homing into the intestine was negligible.

A role of α_4-integrins for lymphocyte homing into mucosal sites was first inferred from inhibitory effects of the mAb R1-2 in the Stamper-Woodruff frozen section assay (3, 4). However, the effect of this Ab on lymphocyte migration into Peyer's patches in vivo is weak and does not reach significance within a single experiment, regardless of the dose applied. Although the affinity of R1-2 Fab is four- to fivefold lower than that of PS/2 Fab, this difference is not sufficient to explain the inability of R1-2 to block migration in vivo, because the Ab DATK32 has a much lower affinity and is able to inhibit lymphocyte migration as well as PS/2. In addition, serum from mice given R1-2 Fab 1 h before blood withdrawal still contained Fab concentrations high enough to inhibit the adhesion of lymphocytes to Peyer's patch HEV in vitro, indicating that the serum levels of Ab during the duration of a homing experiment did not fall below functional levels. Consistent with the results presented here, R1-2 also fails to block lymphocyte binding to purified MAdCAM-1 (13). Interestingly, a recent in vivo study has shown that R1-2 efficiently blocks the effector phase in a model of contact hypersensitivity, but does not affect lymphocyte traffic to the skin (62).

α_4-Integrins are known to recognize several different ligands. VCAM-1 is a ligand expressed on certain types of endothelium, especially after cytokine induction (19–21). α_4-Integrins also bind to fibronectin and this interaction as well as binding to other ligands is inhibited by a 25-amino acid peptide from the alternatively spliced CS-1 region (22–25, 27). The mAbs R1-2 and PS/2 both have been shown to block α_4-mediated binding to VCAM-1 and fibronectin (28).

Ab to VCAM-1 was not found to influence lymphocyte migration into Peyer's patches or intestine, although an effect on the localization of cells in the residual body provided evidence for its functional activity. The residual body contains several tissues, notably the bone marrow in which VCAM-1 mediated interactions have been described (31). Consistently, anti-VCAM-1 also failed to inhibit in the Peyer's patch frozen section assay. In contrast, we show here and have previously shown (16) that Ab against MAdCAM-1 inhibits efficiently the immigration of lymphocytes into Peyer's patches in vivo.

The CS-1 peptide is able to block the Peyer's patch frozen section assay efficiently, although the heterogeneity in its effect related to individual Peyer's patches points to the importance of other ligands. CS-1 was also found to block adhesion to human endothelium in inflamed synovia (52). CS-1, injected together with the chromium-labeled cells and every 15 min afterward to compensate for the rapid excretion and metabolism of peptides in the circulation, was not able to influence lymphocyte migration. Again, the serum of mice that had received CS-1 still contained functionally active levels of CS-1 after termination of the experiment. Lymphocyte binding to Peyer's patch HEV is already reduced in the presence of higher concentrations of normal mouse serum. It is tempting to speculate that this inhibition is caused by a modulating effect of serum fibronectin. As mentioned above, the ability of mAb R1-2 to inhibit lymphocyte adhesion to Peyer's patch HEV in the frozen section assay may reflect a contribution of fibronectin to binding in this in vitro model. The failure of the Ab to block homing in vivo indicates that the frozen section assay on Peyer's patch HEV, as is true for other in vitro models, mirrors accurately only certain components of the in vivo situation. In this instance it appears to underestimate the contribution of L-selectin, and perhaps overemphasize the contribution of fibronectin-related structures.

In conclusion, our results suggest that MAdCAM-1, but neither VCAM-1 nor CS-1-related recognition structures, plays a role in lymphocyte migration into noninflamed mucosal sites. We have confirmed the hypothesized role of α_4/β_7-integrin for lymphocyte homing to Peyer's patches in vivo, and have demonstrated for the first time its involvement in early lymphoblast trafficking to small intestinal lamina propria. The in vivo effects of anti-α_4/β_7 Abs parallel those of the inhibitory effects of anti-MAdCAM-1 mAb MECA 367 on lymphocyte homing (16), consistent with the recent demonstration that α_4/β_7 and MAdCAM-1 represent a specific receptor/counterreceptor pair (13). Our results suggest that α_4/β_7 will represent an important target for the modulation of lymphocyte trafficking to mucosal sites.

Acknowledgments

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