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Research Article

Expression of the adhesion molecules CD44, L-selectin (CD62L), and integrin alpha 4 beta 7 by antibody-secreting cells (ASC) was examined in human volunteers after oral, rectal, intranasal, or systemic immunization with cholera toxin B subunit. Almost all blood ASC, irrespective of immunization route, isotype (IgG and IgA), and immunogen, expressed CD44. On the other hand, marked differences were observed between systemically and intestinally induced ASC with respect to expression of integrin alpha 4 beta 7 and L-selectin, adhesion molecules conferring tissue specificity for mucosal tissues and peripheral lymph nodes, respectively. Thus, most ASC induced at systemic sites expressed L-selectin, whereas only a smaller proportion of ASC expressed alpha 4 beta 7. In contrast, virtually all IgA- and even IgG-ASC detected after peroral and rectal immunizations expressed alpha 4 beta 7, with only a minor fraction of these cells expressing L-selectin. Circulating ASC induced by intranasal immunization displayed a more promiscuous pattern of adhesion molecules, with a large majority of ASC coexpressing L-selectin and alpha 4 beta 7. These results demonstrate that circulating ASC induced by mucosal and systemic immunization express different sets of adhesion molecules. Furthermore, these findings provide for the first time evidence for differential expression of adhesion molecules on circulating ASC originating from different mucosal sites. Collectively, these results may explain the anatomical division of mucosal and systemic […]

Differential Expression of Tissue-specific Adhesion Molecules on Human Circulating Antibody-forming Cells After Systemic, Enteric, and Nasal Immunizations

A Molecular Basis for the Compartmentalization of Effector B Cell Responses

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Abstract

Expression of the adhesion molecules CD44, L-selectin (CD62L), and integrin α4β7 by antibody-secreting cells (ASC) was examined in human volunteers after oral, rectal, intranasal, or systemic immunization with cholera toxin B subunit. Almost all blood ASC, irrespective of immunization route, isotype (IgG and IgA), and immunogen, expressed CD44. On the other hand, marked differences were observed between systemically and intestinally induced ASC with respect to expression of integrin α4β7 and L-selectin, adhesion molecules conferring tissue specificity for mucosal tissues and peripheral lymph nodes, respectively. Thus, most ASC induced at systemic sites expressed L-selectin, whereas only a smaller proportion of ASC expressed α4β7. In contrast, virtually all IgA- and even IgG-ASC detected after peroral and rectal immunizations expressed α4β7, with only a minor fraction of these cells expressing L-selectin. Circulating ASC induced by intranasal immunization displayed a more promiscuous pattern of adhesion molecules, with a large majority of ASC coexpressing L-selectin and α4β7. These results demonstrate that circulating ASC induced by mucosal and systemic immunization express different sets of adhesion molecules. Furthermore, these findings provide for the first time evidence for differential expression of adhesion molecules on circulating ASC originating from different mucosal sites. Collectively, these results may explain the anatomical division of mucosal and systemic immune responses in humans as well as the compartmentalization of mucosal immune responses initiated in the upper vs. the lower aerodigestive tract. (*J. Clin. Invest.* 1997. 99:1281–1286.) Key words: cell trafficking • antibody-forming cells • adhesion molecule • human • mucosa

Introduction

Lymphocyte recirculation through lymphoid and nonlymphoid tissues is critical to ensure contact of the immune system with newly encountered antigens and to direct memory and effector cells to sites of immune reactions during tissue injury or infection. The migration of effector and memory lymphocytes is, in contrast with that of naive cells, preferentially directed to peripheral tissues and to a much lesser degree to secondary lymphoid organs (1–3). In addition, these cells preferentially "home" back to the tissue where they were first activated (4–6). Thus, immunoblasts from the peripheral lymph nodes (PLN)[1] tend to home to PLN or to extramucosal sites of inflammation, whereas blast cells originating from mucosal inductive sites, such as Peyer's patches (PP), localize preferentially in the gut wall and in mucosa-associated exocrine glands. However, a certain degree of compartmentalization exists also within mucosa-associated lymphoid tissues (7).

The process of selective lymphocyte homing is dependent on binding of organ-specific adhesion molecules, so-called homing receptors, expressed on lymphocytes, to their ligands, addressins, on postcapillary high endothelial venules (HEV) in the target organ. It is generally believed that transient and reversible interactions between selectins and their carbohydrate ligands mediate primary binding of leukocytes and their rolling onto HEV (8, 9). These signals, in concert with binding of endothelial surface molecules and/or surface bound chemokines, induce rapid activation of integrins. Integrin binding to endothelial counter receptors results in strengthened adhesion and triggers extravasation. However, it is now apparent that the different adhesion molecules involved in lymphocyte homing have overlapping functions, as illustrated by the finding that β1 and β7 integrins mediate initial lymphocyte binding and rolling under shear flow (10).

In humans, the best characterized leukocyte adhesion molecules conferring tissue specificity are L-selectin (CD62L) and

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1. *Abbreviations used in this paper:* ASC, antibody-secreting cell; CTB, cholera toxin B subunit; HEV, high endothelial venule; i.n., intranasal; MAdCAM-1, mucosal addressin cellular adhesion molecule; MLN, mesenteric lymph node; MNC, mononuclear cell; p.o., peroral; PLN, peripheral lymph node; TT, tetanus toxoid.

integrin α4β7. On lymphocytes, the α4 integrin chain can associate with two different β chains, β1 and β7. α4β7 is a receptor for the mucosal addressin cell adhesion molecule (MAdCAM-1) (11), and is thereby involved in selective lymphocyte trafficking to mucosal lymphoid tissues (12). α4β1 binds to endothelial cells via the vascular cell adhesion molecule, VCAM-1, and is mainly mediating lymphocyte migration into sites of inflammation (13) but not to PLN (14). L-Selectin has been identified as a lymphocyte homing receptor mediating binding to and entry into PLN (15). Its ligand, the PLN addressin, is a tissue-specific endothelial cell carbohydrate antigen found on HEV of PLN in adults and at sites of chronic inflammation (16). In addition, it has been shown that the hyaluronate-binding molecule CD44 may be involved in lymphocyte binding to mucosal HEV in vitro (17, 18).

In this study, we have examined whether the known differences in migratory behavior of circulating B cell blasts originating from systemic vs. mucosal sites could be explained by the use of distinct organ-specific adhesion molecules. We now report that human circulating antibody-forming cells induced by intestinal, systemic, and intranasal immunization express distinct combinations of integrin α4β7 and L-selectin.

Methods

Volunteers and immunizations. The study was performed with due approval from the Human Research Ethical Committee of the Medical Faculty at the University of Göteborg, and comprised 44 healthy volunteers, aged 18–51, who gave informed consent to participate.

Peroral (p.o.) immunizations: 14 volunteers received two doses of an oral cholera vaccine (19), given 2–3 wk apart, each dose consisting of 10^{11} killed *Vibrio cholerae* organisms and 1 mg of purified recombinant cholera toxin B subunit (CTB) (SBL Vaccine, Stockholm, Sweden) given in 150 ml of a bicarbonate buffer.

Rectal immunizations: seven volunteers were immunized twice, 2 wk apart, with the above CTB-containing cholera vaccine, given by means of a syringe in 3 ml of physiological saline. After administration of the immunogen, the volunteers stayed in horizontal position for 30 min with one rotation after 15 min.

Intranasal (i.n.) immunizations: six volunteers were immunized twice with 0.5 mg of purified CTB (Institut Merieux, Lyon, France) 2 wk apart. After a nasal wash with physiological saline, 250 μg of CTB was introduced into each nostril in aliquots of 25 μl. Four additional volunteers received 100 μg of recombinant CTB (SBL vaccine), in the form of an aerosol sprayed onto the nasal mucosa. The vaccine was administered in saline without bicarbonate to minimize the risk of any CTB reaching the intestine in an active (acid-labile pentameric) form. These doses were selected from preliminary clinical studies and found to give consistent blood antibody-secreting cell (ASC) responses without inducing untoward reactions, whereas higher doses (1 mg) gave rise to transient local nasal hypersecretion and repeated sneezings.

Systemic immunizations: four volunteers received a total of 50 μg rCTB, administered by intracutaneous injections at two sites in the arm, each injection consisting of ~ 25 μg rCTB in 50 μl physiological saline. These immunizations caused a local erythematous reaction, which appeared 7–10 d after injection and disappeared within 3–4 wk. Seven additional volunteers received an s.c. injection of a tetanus toxoid (TT)/diphtheria toxoid vaccine (SBL) containing two Floculation Unit of TT.

In all groups, only the volunteers responding to the immunizations with sufficient numbers of ASC were included in further analyses of adhesion molecule expression (see Results).

Isolation of mononuclear cells. Heparinized venous blood was collected 1 wk after the last immunization, and mononuclear cells (MNC) were isolated by gradient centrifugation on Ficoll-Hypaque™ (Pharmacia Diagnostics AB, Uppsala, Sweden). Interface MNC were collected, washed three times with cold PBS, and then resuspended in cold PBS supplemented with 1% (vol/vol) fetal calf serum or in Iscove's medium with 5% of fetal calf serum and 100 μg/ml of gentamicin (Gibco Europe, Edinburgh, UK) (complete medium). Cell suspensions were kept on ice before being further fractionated and/or assayed for ASC numbers.

Fractionation of MNC. Cell surface expression of CD44, integrin α4β7, and L-selectin by isolated ASC was determined using a combination of immunomagnetic cell sorting and ELISPOT techniques (20). Briefly, paramagnetic microspheres (Dynabeads; Dynal, Oslo, Norway) coated with sheep antibodies to mouse immunoglobulins were incubated overnight at 4°C with mouse monoclonal antibodies Hermes-3, ACT-1, or Dreg-56, specifying CD44, integrin α4β7, and L-selectin, respectively (17, 21, 22). Coated beads were washed and mixed with the MNC suspensions at optimal bead to cell ratios of 10:1 (CD44, α4β7) or 5:1 (L-selectin). Beads and cells were pelleted by centrifugation and incubated at 4°C for 45 min, and then gently resuspended and incubated for another 20 min at 4°C. Cells attached to beads and free beads were retained by applying a magnetic field along the side of the tubes and unbound cells were removed from the tube by aspiration. The resulting positively and negatively selected cell suspensions were resuspended in equal volumes of complete medium and assayed for numbers of ASC by the ELISPOT test described below. This procedure resulted in depletion of 96% of $CD44^+$ and L-$selectin^+$ cells, and of 93% of $\alpha4\beta7^+$ cells from the original cell suspension, as determined by comparative flow cytometric analyses of unfractionated and negatively selected MNC suspensions using a Facscan® (Becton-Dickinson & Co., San Jose, CA). Cell surface expression of CD44, α4β7, and L-selectin was determined by stepwise exposure for 30 min at 4°C of MNC suspensions to Hermes-3, ACT-1, or Dreg-56, followed by FITC-labeled $F(ab')_2$ fragments of rabbit antibodies to mouse Ig (Dakopatts AS, Glostrup, Denmark).

To ascertain that unspecific binding of ASC to beads did not occur, cells bound to beads coated with isotype-matched irrelevant mAbs (~ 6–10% of the starting MNC population) were analyzed in ELISPOT assays as described below. These cells did not contain any detectable ASC, attesting to the specificity of the separation method employed.

Enumeration of ASC. Fractionated and unfractionated MNC suspensions were assayed for numbers of vaccine-specific IgG and IgA ASC by a two-color micromodification of the ELISPOT technique (23). Briefly, various numbers of MNC were incubated for 14 h at 37°C in 100 μl of complete medium in nitrocellulose-bottomed 96-well plates (Millipore Corp., Bedford, MA) previously coated with 100 μl of PBS containing 20 μg/ml of purified TT (SBL) or 2.5 μg/ml of cholera toxin (List Biological Laboratories, Inc., Campbell, CA). For the latter antigen, wells were first coated with 3 μm of GM1 ganglioside (Sigma Chemical Co., St. Louis, MO) to facilitate adsorption of cholera toxin. Solid phase-bound immunoglobulins secreted by individual ASC were revealed as spots by stepwise addition of combinations of horseradish peroxidase– or alkaline phosphatase–labeled goat antibodies to human IgG, IgA, or IgM (Southern Biotechnology Associates, Birmingham, AL), and suitable chromogen substrates (23).

The percentage of ASC expressing a certain marker was calculated by dividing the number of ASC in the positive fraction with the total number of ASC in the positive and negative fractions assayed at the same frequency. Unfractionated cells were assayed in parallel to positively and negatively selected cell suspensions, and the sum of spots in the positive and negative ASC fractions consistently corresponded (within 80–120%) to the sum of spots in wells incubated with unfractionated cells.

Results

Frequencies of vaccine-induced ASC. The frequencies of vaccine-specific ASC occurring in the circulation after mucosal

Table I. Frequencies of Circulating Vaccine-specific ASC after Systemic, p.o., i.n., and Rectal Administration of CTB or TT

Administration	Antigen	Vaccine-specific ASC/10^6 MNC	
		IgG	*IgA*
p.o.	CTB	41 (8–516)*	44 (4–406)
Rectal	CTB	10 (0–26)	10 (2–21)
i.n.	CTB	10 (0–86)	25 (1–292)
s.c.	TT	134 (5–872)	5 (0–37)
Intracutaneous	CTB	23 (2–162)	12 (2–70)

*Frequencies of vaccine-specific ASC, determined 7 d after the last immunization. Data are expressed as geometric mean and the range of individual data is given in brackets.

and parenteral immunizations with the prototype immunogens CTB and TT were determined before the first and 7 d after the last immunization. None of the volunteers had any detectable ASC reacting with CTB or TT before the immunizations. All routes of immunization did, however, give rise to substantial ASC responses. These responses differed with regard to both magnitude and isotype distribution, depending on administration route. Thus, CTB-specific ASC induced by p.o. immunization consisted of approximately equal numbers of IgG- and IgA-secreting cells. Rectal immunization led to lower ASC responses, which also comprised similar numbers of IgG- and IgA-secreting cells. After i.n. immunization, on the other hand, vaccine-specific IgA-secreting cells dominated over IgG-secreting cells (Table I).

In contrast, the anti–TT ASC population induced by s.c. immunization comprised large numbers of IgG-secreting cells and small, yet significant, numbers of IgA-secreting cells (Table I). When CTB was administered by intracutaneous injection, IgG-secreting cells dominated over IgA-secreting cells. Vaccine-specific IgM ASC were virtually absent, irrespective of immunization route and immunogen.

Because of the major differences in magnitude and isotype composition of blood ASC responses after different immunization routes and marked individual variations, cell suspensions were assayed at different densities to allow accurate comparisons. All subsequent results presented were obtained from volunteers having responded to the vaccines with at least 10 IgA or IgG ASC/10^6 MNC.

Adhesion molecules on ASC induced after immunization by various mucosal routes. Adhesion molecule expression by circulating spontaneous ASC induced by different immunization routes was examined by collecting peripheral blood MNC 7 d after the last immunization, sorting the cells according to cell surface expression of CD44, L-selectin, and integrin α4β7, and then immediately assaying the cell fractions for numbers of specific IgA and IgG ASC.

Virtually all vaccine-specific ASC, irrespective of mucosal immunization route, expressed CD44. Expression of the PLN homing receptor L-selectin, on the other hand, varied considerably between ASC populations induced by different routes of mucosal immunization. Thus, the mucosal homing receptor integrin α4β7 was expressed by the large majority of IgA and IgG ASC induced by p.o. immunization, whereas only a minor fraction of these ASC coexpressed L-selectin (Fig. 1 *A*). Similar to this, the large majority of circulating CTB-specific IgA

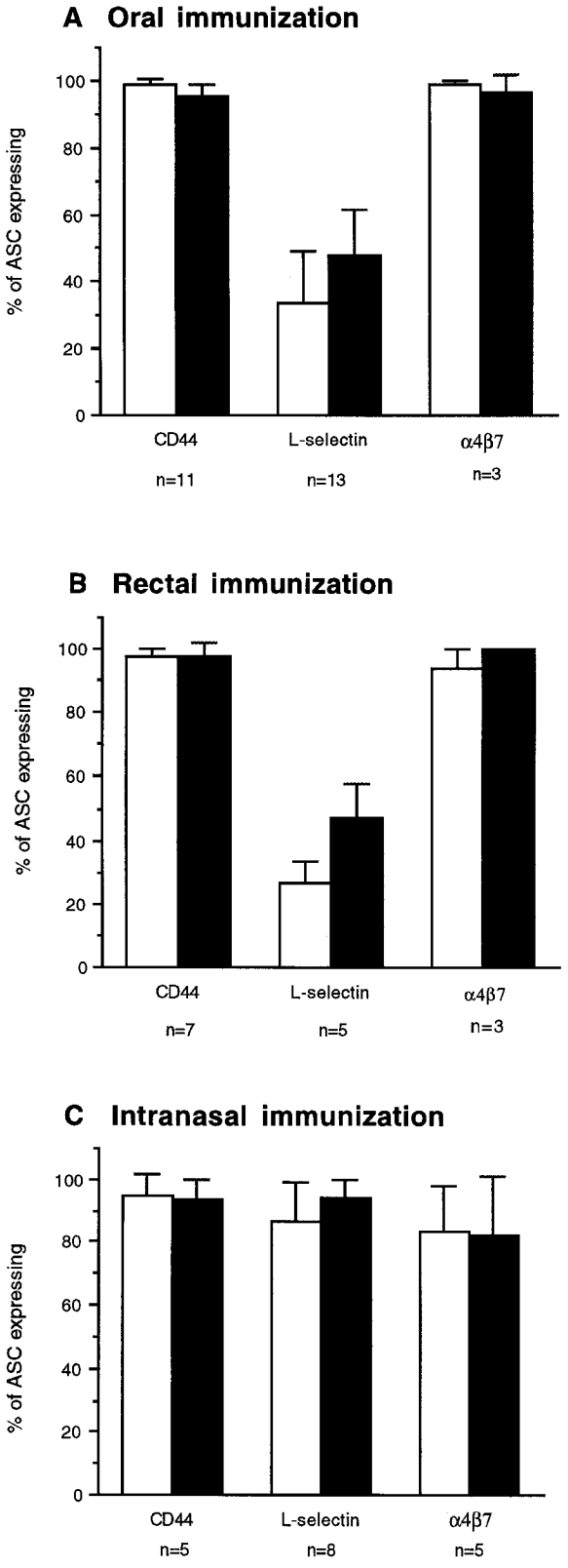

Figure 1. Expression of adhesion molecules by human circulating ASC after immunization by different mucosal routes. Blood ASC were collected and fractionated 7 d after peroral (*A*), rectal (*B*), and intranasal (*C*) immunizations with CTB and were assayed for frequencies of specific IgA- (*open bars*) and IgG-secreting (*filled bars*) cells. Data are expressed as mean percentage of ASC expressing the indicated adhesion molecules + SD.

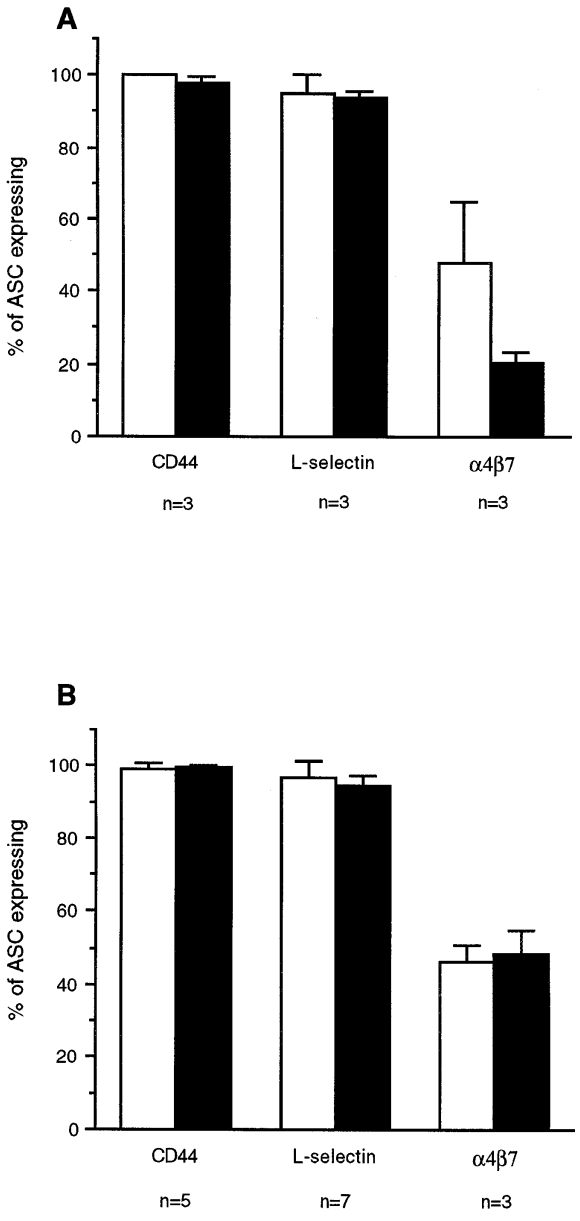

Figure 2. Expression of adhesion molecules by circulating ASC after systemic immunization. Blood ASC were collected and fractionated 7 d after intracutaneous immunization with CTB (*A*) and subcutaneous immunization with TT (*B*) and were assayed for frequencies of specific IgA- (*open bars*) and IgG-secreting (*filled bars*) cells. Data are expressed as mean percentage of ASC expressing the indicated adhesion molecules + SD.

and IgG ASC induced by rectal immunization expressed α4β7, with a minority of ASC coexpressing L-selectin (Fig. 1 *B*).

In contrast with the circulating cells induced by peroral or rectal immunizations, most CTB-specific circulating ASC induced by i.n. immunization coexpressed CD44 and α4β7, as well as L-selectin (Fig. 1 *C*).

Adhesion molecules on ASC induced after systemic immunizations. The distribution of L-selectin and α4β7 on blood ASC induced by systemic administration of CTB or TT was almost reciprocal to that of ASC induced by intestinal vaccination. Virtually all vaccine-specific ASC appearing after intracutaneous CTB-immunization expressed CD44 and L-selectin, while α4β7 expression was observed on < 50% of IgA- and < 20% of IgG-secreting cells (Fig. 2 *A*). Similarly, almost all TT-specific ASC isolated after s.c. immunization expressed CD44 and L-selectin, whereas much fewer TT-specific ASC expressed α4β7 (Fig. 2 *B*).

Discussion

This study demonstrates that human circulating ASC induced by different immunizations express a distinct pattern of tissue-specific adhesion molecules, depending on the route of initial immunization.

It has long been known that parenteral immunization results in the transient appearance of vaccine-specific ASC in peripheral blood (24). The presence of specific ASC in blood after enteric immunization has also been reported (25, 26), an observation supporting the notion of a common mucosal immune system (27). According to this concept, progenitors of B cell immunoblasts recruited at mucosal inductive sites are disseminated via the circulation to their final destination, mainly in the intestinal mucosa, but also in remote mucosa-associated tissues and exocrine glands. Recently, however, a number of studies have indicated a certain degree of compartmentalization of immune responses within mucosa-associated lymphoid tissues (7, 28–30). The restricted redistribution of migrating B cell immunoblasts from different inductive sites is predicted to be the result of differential expression of homing receptors on the circulating cells as well as of their ligands on HEV in different tissues (8, 9).

In this study, CD44 was expressed by virtually all circulating ASC, irrespective of immunization route. CD44 is a widespread hyaluronate-binding cell surface molecule that has been reported to play an important role in lymphocyte attachment to mucosal HEV in vitro (17, 18). However, its importance in tissue-specific lymphocyte homing has not yet been documented in vivo (31). The results of this study, together with the observation that virtually all circulating immunoglobulin-secreting cells, irrespective of antigen specificity, express CD44 (M. Quiding-Järbrink and C. Czerkinsky, unpublished data) indicate that CD44 is not a useful marker of ASC origin or destination.

In contrast with CD44, expression of the tissue-specific adhesion molecules integrin α4β7 and L-selectin varied considerably between ASC populations induced at different sites. Systemic immunizations led to blood ASC responses where almost all ASC expressed L-selectin, whereas L-selectin was expressed only by a minority of ASC induced by peroral immunizations. That in vitro activation of circulating B cells results in rapid downregulation of surface L-selectin is well documented (22, 32). In addition, L-selectin is differentially expressed during B cell maturation and differentiation (33). We believe, however, that the presence or absence of L-selectin on circulating ASC is relevant to their migratory behavior rather than a reflection of activation or maturation stage. In a recent study, we could demonstrate that almost all circulating ASC induced by p.o. or s.c. immunization were at a similar, late stage of differentiation (34). This was particularly evident for ASC induced by p.o. immunization, which were also the most heterogeneous with respect to L-selectin expression.

The expression of α4β7 on vaccine-specific blood ASC induced by systemic immunizations was restricted to less than

half of the ASC pool. Since all circulating TT-specific ASC express the α4 integrin chain (34), the $\alpha4\beta7^-$ ASC subset detected in this study probably express the integrin α4β1. Together with L-selectin and LFA-1, α4β1 may facilitate lymphocyte homing to inflamed nonlymphoid sites and antigen-challenged lymph nodes (9). The fact that systemic immunization with either CTB or TT resulted in ASC with a very similar expression of L-selectin and α4β7 demonstrates that the immunization route, rather than the immunogen, determines the type(s) of adhesion molecules expressed by the corresponding ASC.

Enteric (p.o. and rectal) immunizations resulted in a reciprocal distribution of L-selectin and α4β7 as compared with systemic immunization. Almost all circulating ASC activated by p.o. or rectal immunization expressed α4β7, whereas less than half of these cells coexpressed L-selectin. A recent study also indicates, although indirectly, a similar pattern of α4β7 expression on ASC induced by diarrheal disease (35). It is possible that the $\alpha4\beta7^+$ and L-$selectin^-$ ASC are bound to extravasate through flat-walled venules in the intestinal lamina propria, which constitutively express MAdCAM-1 but only little PLN addressin (8, 10, 36). $\alpha4\beta7^+$ and L-$selectin^+$ ASC, on the other hand, may leave the circulation and migrate to more organized mucosal lymphoid tissues, such as mesenteric lymph nodes (MLN) and Peyer's patches, whose HEV express both MAdCAM-1 and PLN addressin (36, 37), and to which L-selectin–dependent homing has been documented (38). In accordance with this notion, our previous studies have shown that MLN harbor large numbers of vaccine-specific ASC after enteric immunization in monkeys (39).

Preliminary data from our laboratory also indicate that a majority of blood IgA ASC from oral cholera vaccinees display an "intestinal" homing receptor profile even after systemic reexposure to the CTB component of the oral vaccine (results not shown). Although preliminary, these results could explain earlier findings indicating that systemic immunization can "boost" a mucosal IgA response (40, 41).

In contrast with ASC detected after enteric immunization, which mainly express α4β7 and little L-selectin, almost all blood ASC induced by nasal immunization coexpressed α4β7 and L-selectin. In keeping with this observation, recent studies have indicated a certain degree of compartmentalization of immune responses induced in different mucosa-associated lymphoid tissues (7, 28, 29). Thus, the palatine tonsils serve poorly as expression sites for B cell responses induced by intestinal immunization. Furthermore, an immune response induced by tonsillar or i.n. antigen exposure and giving rise to large numbers of ASC in tonsils and in blood, is not reflected in the duodenal mucosa (7). The fact that blood ASC induced by p.o. and i.n. immunization differ with respect to cell surface adhesion molecule expression may at least partly explain the compartmentalization of immune responses initiated in the upper as opposed to the lower aerodigestive tract. We cannot exclude, however, that the differences in adhesion molecule expression documented may have resulted from the different amounts of antigen administered by different routes, rather than from the immunization route per se.

Interestingly, the relative distribution of α4β7 and L-selectin was similar on both IgA- and IgG-secreting cells, irrespective of immunization route. It has previously been argued that IgG responses induced by p.o. immunizations might largely result from "leakage" of antigen into the systemic compartment. The observation that a large proportion of CTB-specific IgG ASC expressed α4β7 but no L-selectin after p.o. and rectal immunization suggests that the IgG response after enteric vaccination may actually be derived from mucosal inductive sites including but not limited to the MLN (39). In spite of the similarities in adhesion molecule expression between IgG and IgA ASC, our earlier studies have shown considerable differences in the distribution of IgA- and IgG-secreting cells induced by p.o. immunization. IgA-secreting cells clearly dominate in the duodenal mucosa and in salivary glands (42, 43), whereas IgG-secreting cells actually dominate in the colonic mucosa and MLN (39). Therefore, additional recognition events involving molecules other than α4β7 and L-selectin and their counter receptors are likely also to govern the migration of ASC to particular effector sites.

In conclusion, this study demonstrates that human circulating ASC induced by intestinal and systemic immunization, respectively, have distinct surface expression of L-selectin and integrin α4β7. This study thus provides the first experimental evidence that effector B cells resulting from mucosal vs. nonmucosal immune responses are segregated into homing receptor–defined subsets. In addition, a differential expression of adhesion molecules on circulating ASC after oral and nasal immunization may provide a molecular basis to the compartmentalization of mucosal immune responses initiated in the upper and lower aerodigestive tract.

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