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PMA-activation of peripheral blood and tonsillar B lymphocytes induces large adhesive cells reminiscent of large extrafollicular (monocytoid) B cells

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Abstract Extrafollicular (EF) B lymphocytes differ in size and morphology depending on the lymphatic organ involved and the kind of inflammatory reaction. On re-evaluating EF B cells in various sites and conditions we discriminated three forms: a small (lymphoid) and intermediate (centrocytoid), and a large (monocytoid) variant. Immunohistochemically, these variants could be discriminated by their differential expression of adhesion molecules CD62L (L-selectin) and CD11c: small EF B cells were strongly L-selectin$^+$ and CD11c$^-$; intermediate cells were moderately CD62L$^+$ and CD11c$^-$; large cells were faintly CD62L$^+$ or $^-$ but expressed CD11c. In 72 h cultures of normal peripheral and tonsillar B cells, cross-linking surface immunoglobulin in the presence of interleukin-2 or interleukin-4 led to formation of clusters in vitro together with an increase in cell size and a slight up-regulation of CD11c, as determined by flow cytometry. Stimulation with phorbol 12-myristate 13-acetate (PMA), however, gave rise to large, plastic adherent cells which also showed strong homotypic adhesion, expressed CD62L at minimal levels and CD11c at comparably highest levels and altogether mimicked the large cell variant of EF B cells. We conclude that EF B cells are subjected to cytokine-induced metamorphosis and that differences in cell size and morphology reflect their state of activation and activation-associated adhesion properties. Our data suggest that EF B cells in all anatomical sites are functionally closely related cells which – possibly mediated by CD11c/CD18 – may become sessile and proliferate locally once activated by appropriate signals.

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Introduction

At present, B lymphocyte populations around lymph follicles have different names in different organs of the lymphoreticular system. They are designated *marginal zone cells* in the spleen (Kumararatne et al. 1981; Brozman 1985; Timens and Poppema 1985), *sinusoidal* or *monocytoid B cells* in the lymph node (Sheibani et al. 1984; Stein et al. 1984) *intraepithelial B cells* in tonsil (Möller and Mielke 1989), and *centrocyte-like cells* (Spencer et al. 1985) in the dome area of lymphoglandular complexes in the gut and in other mucosa-associated lymphoid tissues. An interrelationship among these cells has been postulated upon several occasions (Spencer et al. 1985; van den Oord et al. 1986; van Krieken et al. 1989). Extensive immunophenotyping of these B cells by CD antibodies lead to the unifying concept of the *extrafollicular B-cell compartment* (Möller and Mielke 1989) further substantiated by subsequent studies (Möller et al. 1991, 1992a, b, 1993; Eichelmann et al. 1992). The examination of the adhesion receptor profiles of B cells in different B cell compartments, however, revealed microheterogeneity among extrafollicular (EF) B cells (Eichelmann et al. 1992; Möller et al. 1992a, b). This was especially true for the expression of *L-selectin* and *CD11c* within this population. *L-selectin*, also known as "lymph node homing receptor" on lymphocytes, Leu-8, LECAM-1 or LAM-1, is a 80–90 kDa glycoprotein with lectin activity (Tedder et al. 1989; Imai et al. 1990). During the 5th International Workshop and Conference on Leukocyte Differentiation Antigens (Boston, November 1993) the L-selectin molecule was designated CD62L. With its lectin domain CD62L (L-selectin) binds to venular endothelium; this binding was shown to be calcium-dependent (Geng et al. 1992). Ligands for CD62L on these cells are sialy-

lated, fucosylated and sulphated glycoproteins of about 50 kDa and 90 kDa (Sgp50 and Sgp90) (Imai et al. 1992). On B cells CD62L is differentially expressed along the differentiation pathway (Lanier and Loken 1984); it is up-regulated when the B cell acquires CD20 (Kansas and Dailey 1989) and is transiently down-regulated and/or shed during the follicular centre blast stage (Kansas et al. 1985) and after activation in vitro (Kansas et al. 1985). In vitro, interleukin-4 inhibits CD62L enhancement in B cells of T/B cell cultures induced by the culture medium itself (Jensen and Prince 1992). Effects of CD62L antibodies suggest that this molecule plays a role not only in the tissue sequestration of B cells by binding to high endothelial venules but also in regulating B cell differentiation (Murakawa et al. 1991).

The CD11c molecule also known as p150,95, Leu-M5, or S-HCL3 (Patarroyo and Makgoba 1989; Schwarting et al. 1985) heterodimerizes with CD18 to form a member of the β2 integrin (syn. Leu-CAM) subfamily. This complex is able to bind C3bi (Patarroyo and Makgoba 1989) and, via the *Gly-Pro-Arg* sequence, the α chain of fibrinogen (Loike et al. 1991). Furthermore, CD11c/CD18 has been shown to bind denatured extracellular proteins (Davies 1992). Since it was found to interact with a yet undefined, interleukin (IL)-1β inducible counter-receptor on stimulated epithelium, it is also regarded as an adhesion molecule (Stacker and Springer 1991). In B cells CD11c/CD18 was induced by IL-4 or via cross-linking of surface IgM in combination with IL-2 or IL-4 (Postigo et al. 1991). Triggering B cells through CD11c confers a co-mitogenic signal to the cell (Postigo et al. 1991). This makes CD11c an activation antigen.

The resumption of our analysis of differences in CD62L (L-selectin) and CD11c expression in EF B cells lead to the question of whether the presence or absence of these molecules might be correlated with cell size and hence with different modes of activation among EF B cells. The present study was designed to address this question.

Materials and methods

Normal lymph nodes, specimens of lymphadenitis associated with primary chronic arthritis and toxoplasmosis, normal juvenile spleens and hyperplastic tonsils obtained from surgery were drawn from our bank of fresh frozen tissues together with gastric specimens showing lymphofollicular gastritis, a thyroid featuring Hashimoto's thyroiditis, one case of myoepithelial sialadenitis, one case of cystadenolymphoma and two Castleman's disease. The corresponding routinely fixed, paraffin-embedded tissue blocks were collected from our files.

From the quick-frozen tissues serial frozen sections of about 1 cm^2 in area and 4–6 μm in thickness were air-dried overnight, fixed in acetone for 10 min at room temperature, and immunostained immediately or stored at −20° C for 1–3 weeks. Histological routine stains (H & E, Giemsa) from paraffin sections were made for morphological analysis.

The following monoclonal antibodies (mAb) were used: CD19(HD37) [IgG_1] and anti-IgD(HD9) were kindly supplied by G. Moldenhauer and B. Dörken, Heidelberg, Germany. CD38(OKT10) [IgG_1] were obtained from Ortho, Raritan, NJ. CD3(SK7) [IgG_1], CD62L(anti-Leu8) [IgG_{2a}] (Lanier and Loken 1984), recognizing L-selectin, and CD11c(Leu-M5) [IgG_{2b}] were supplied by Becton-Dickinson, San Jose, Calif. CD10(J5) [IgG_{2a}] was delivered by Coulter, Hialeah, Fl. CD77 (424/3D9) [IgM] was donated by N.T. Brodin to the Leucocyte Typing IV Workshop, Vienna (1989). CD18(BL5) [IgG_1] was supplied by Dianova, Hamburg, Germany.

The method for immonocytochemistry is described in detail elsewhere (Mielke and Möller 1991). Monoclonal antibodies in culture supernatants were used undiluted; ascites preparations were used as 1:200 dilutions, purified reagents were used in a protein concentration of 10 μg/ml. A polyclonal biotinylated sheep antibody to mouse Ig (reactive with all mouse isotypes) and a streptavidin-biotinylated peroxidase complex, all obtained from Amersham, High Wycombe, UK, served as a detection system for the primary antibodies. 3-Amino-9-ethylcarbazole (Sigma, St. Louis, Mo.) was used as substrate for the enzyme; the peroxidase reaction resulted in an intense red precipitate. The sections were faintly counterstained with Harris' haematoxylin.

Negative controls were performed without the primary antibody in each individual case and, in a limited number of cases, by employing several irrelevant monoclonal antibodies of different mouse Ig isotypes directed against non-human antigens. No staining was observed, except for the reaction of granulocytes whose endogenous peroxidase was not destroyed. Strongly stained dendritic stromal cells, histiocytic cells and/or T lymphocytes, always present in various amounts served as intrinsic positive controls. By the same time they were taken as an internal parameter for the maximum reactivity which was regarded as "high antigenic density (+++)". "Very low antigen density (+)" was noted whenever

Table 1 Selected immunoprofile of peripheral B cells compared to PMA-blasts (+++ high; ++ intermediate; + low antigen density; *x/y* admixture of two modes)

Antigen	Follicle center B cells	Follicle mantle B cells	Extrafollicular B cells			Plasma cells	PMA blasts[2]
			small	intermediate	large		
CD19	+++	+++	+++	+++	+++	−	+++
IgD	−	+++	−	−	−	−≫+++[1]	−
CD62L	−	+++	+++	++	+/−	−/++	−
CD11c	−	−	−	−	++	−	++
CD18	−/+++	++	+++	+++	+++	−/++	++
CD10	++	−	−	−	−	−	−
CD38	+++	−	−	−	−	+++	−
CD77	+++/−	−	−	−	−	−	−

[1] A very small subset of plasma cells is IgD-positive
[2] Unfractioned B lymphocytes stimulated with 5 ng/ml PMA for 72 h

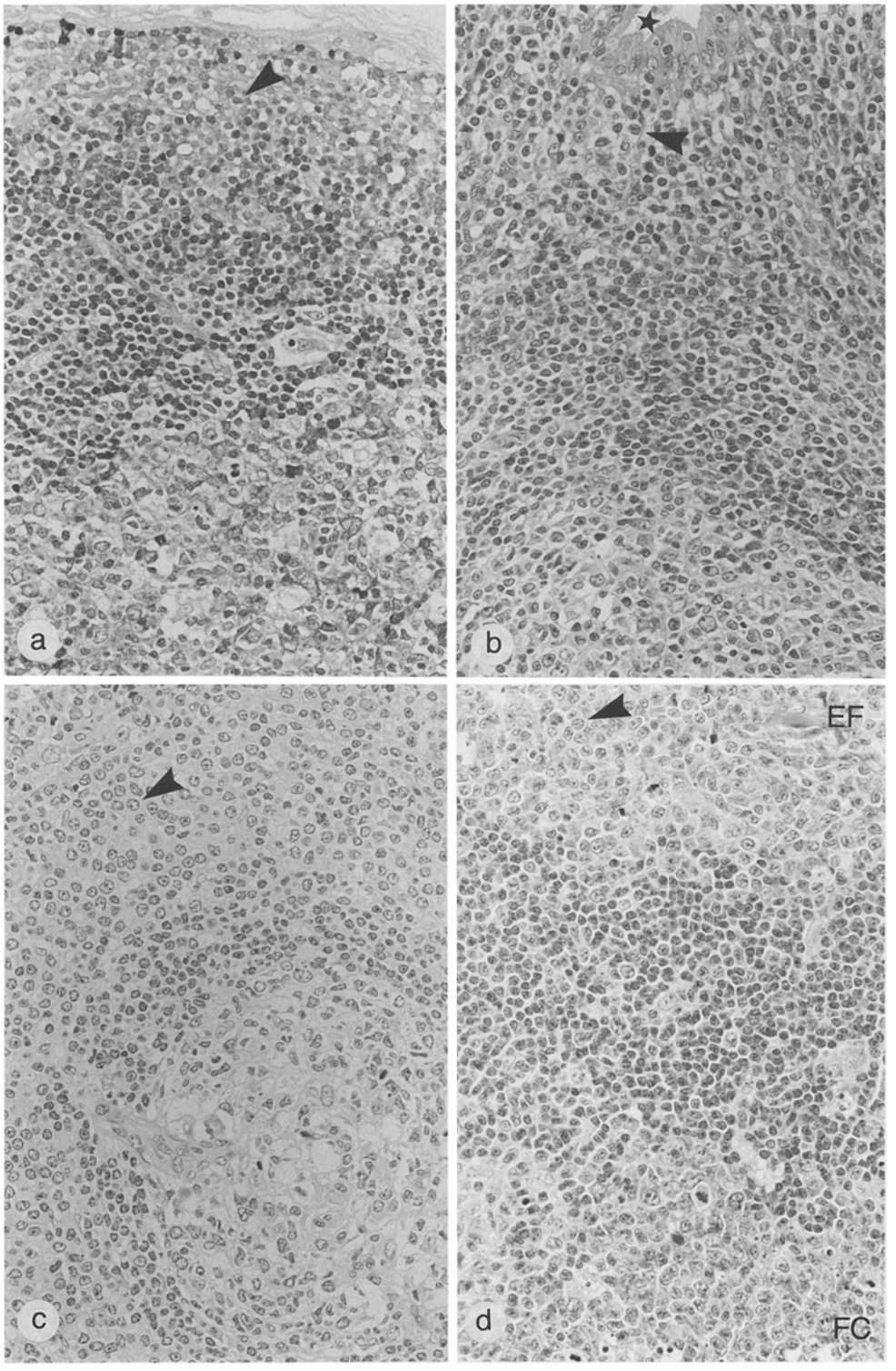


Fig. 1a–d Morphological spectrum of extrafollicular B lymphocytes (*FC*: follicle centre, *FM*: follicle mantle; *EF*: extrafollicular B-cell compartment; in each case a cytologically typical EF cell is marked by an *arrowhead*; haematoxylin/eosin, ×343). **a** Lymphadenitis associated with rheumatoid arthritis. EF B cells in peri- and intrasinusoidal position are slightly larger than mantle cells due to an increase in cytoplasmic volume. **b** Ileal dome area (*asterisk* mark the gut lumen). The FM merges into the EF compartment. There is a steady increase in cell size towards the mucosal surface. The cells in direct subepithelial position have the size of larger centrocytes but can be discriminated from those through the broad and pale cytoplasmic rim. **c** Marginal zones in spleen have distinct borders. Marginal zone cells have increased amounts of pale cytoplasm, enlarged, bean-shaped or ovoid nuclei with delicate chromatin, and small nucleoli. **d** Toxoplasmic lymphadenitis with its typical peri- and intrasinusoidal infiltrates of pale-staining monocytoid B cells. These highly transformed EF cells with very irregular nuclei nearly match the size of FC cells

the immunostaining was indeed very faint; staining intensities ranging between these extremes were scored as "intermediate antigen density (++)". To visualize the entire B cell compartment for comparison with CD11c expression among B cells, a subsequent tissue section was stained with CD19(HD37) (cf. Table 1).

For cell culture, tonsillar B lymphocytes of five pairs of hyperplastic tonsils and peripheral blood B cells (PBL) from a patient with chronic B lymphocytosis were used. All cells were maintained in RPMI 1640 medium (Gibco, Grand Island, N.Y.) supplemented with 10% heat inactivated fetal calf serum (Gibco), 1 mM glutamine, 100 U/ml penicilline, 100 µg/ml streptomycine and 2,5 µg/ml amphotericine B and cultured at 37° C in humified 5% carbon dioxide atmosphere. Tonsils were obtained from routine tonsillectomy and cell suspensions were obtained by mincing the tissue and pressing it through a stainless steel sieve. Mononuclear cells from tonsils and peripheral blood were isolated by Ficoll-Hypaque (Pharmacia, Piscataway, N.J.) gradient centrifugation, followed by depletion of monocytes/macrophages by treatment with

2.5 mM L-leucine methyl ester for 40 min at room temperature. B lymphocytes were prepared by two subsequent cycles of rosetting with 2-aminoethylisothiouronium bromide-treated sheep red blood cells and Ficoll-Hypaque gradient separation. This procedure led to residual (CD3-positive) T cell contaminations that were regularly below 3% (data not shown). The enriched B lymphocytes were resuspended in 3 ml of 80% Percoll (Biochrom, Berlin, Germany) at approximately 5×10^7 cells and layered under Percoll step gradients in 15 ml conical tubes with 3 ml of 80% (1.099 g/ml), 55% (1.070 g/ml), 50% (1.064 g/ml) and 30% (1.040 g/ml) Percoll. In several experiments tonsillar B cell isolates were separated using a density gradient. After centrifugation at 1600 g for 25 min, cells from the 55/80% Percoll interfaces were isolated and referred to as "D55/80" (dense B cells). B cell purity of dense B lymphocytes assessed by CD19-surface immunofluorescence was 97–99% (data not shown). PBL, unfractioned and dense B cells were stimulated with 0,001% formalinized *Staphylococcus aureus* Cowan I, (SAC) (Calbiochem, San Diego, Calif.) in combination with 50 U/ml rIL-2 or 500 U/ml rIL-4 (Boehringer Mannheim, Mannheim, Germany) for 72 h or alternatively, with 100 U/ml recombinant interferon-γ (rIFN-γ, Boehringer Mannheim) in combination with 100 ng/ml recombinant tumour necrosis factor α (rTNF-α, Boehringer) for 72 h. Stimulation was carried out applying 5 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma) for 72 h. Stimulated B lymphocytes were analysed for CD62L and CD11c surface expression in immunofluorescence, PMA-blasts were additionally analysed for surface antigens listed in Table 1.

For flow cytometry immunofluorescence staining was performed in polystyrene round-bottom tubes (Falcon, San Jose, Calif.). Throughout, dilutions and washings were carried out in RPMI 1640 medium (Gibco) containing 2% heat-inactivated FCS, 0.1% sodium azide and 10 mM HEPES. Approximately 10^6 cells/sample, suspended in 50 µl of medium, were incubated at 4° C with an equal volume of the appropriate dilution of each mAb. After 45 min, cells were washed twice in 500 µl of cold medium, and 2 µg of $F(ab')_2$ goat anti-mouse IgG and IgM fluorescein isothiocyanate (FITC) conjugate (Jackson Immunoresearch, West Grove, Pa.) was added for 45 min at 4° C. Cells were washed again twice and resuspended in 300 µl of medium containing 1 µg/ml propidium iodide (Sigma). From each sample the green fluorescence of 10^4 cells was analysed. Dead cells were removed from analysis by selectively gating on propidiumiodine fluorescence, forward and side scatter parameters. Flow cytometry was performed on a FACScan cytometer with the LYSYS II software (Becton Dickinson).

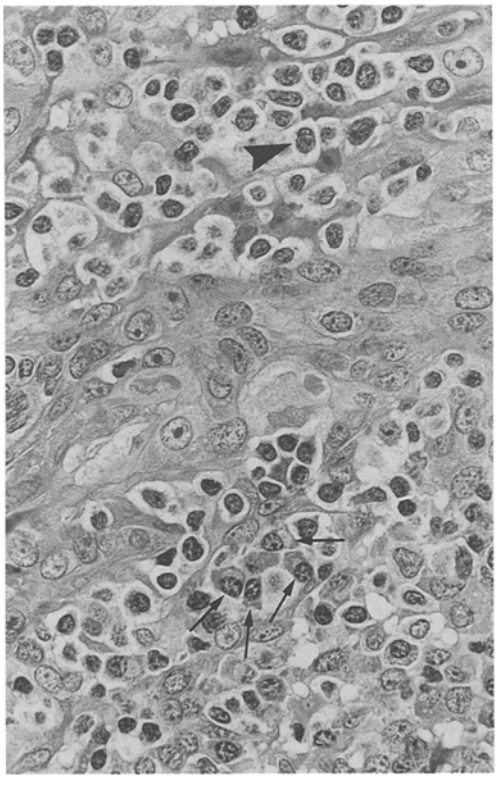


Fig. 2 The meshwork of tonsillar crypt epithelium is the microenvironment of EF tonsillar B cells. In hyperplastic tonsillitis, EF B cells are transformed to large cells with a broad rim of pale cytoplasm and irregular nuclei reminiscent of nuclei of centrocytes (a cytologically typical EF cell is marked by an *arrowhead*). They are clearly discernible from plasma cells (*arrows*) that predominantly are located beneath the epithelial basement membrane (haematoxylin/eosin, × 540)

Results

In normal lymph nodes, extrafollicular (EF) B cells were only slightly larger and morphologically very similar to

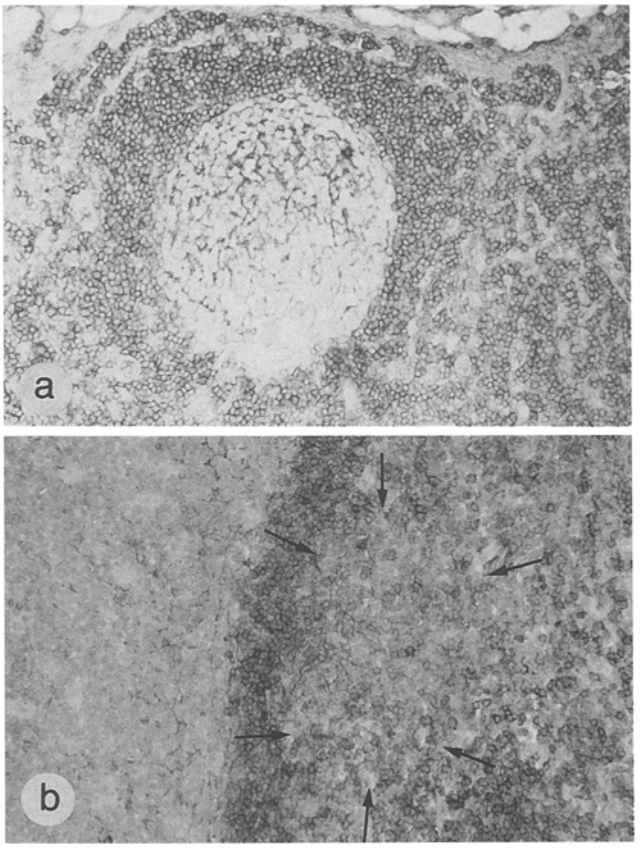
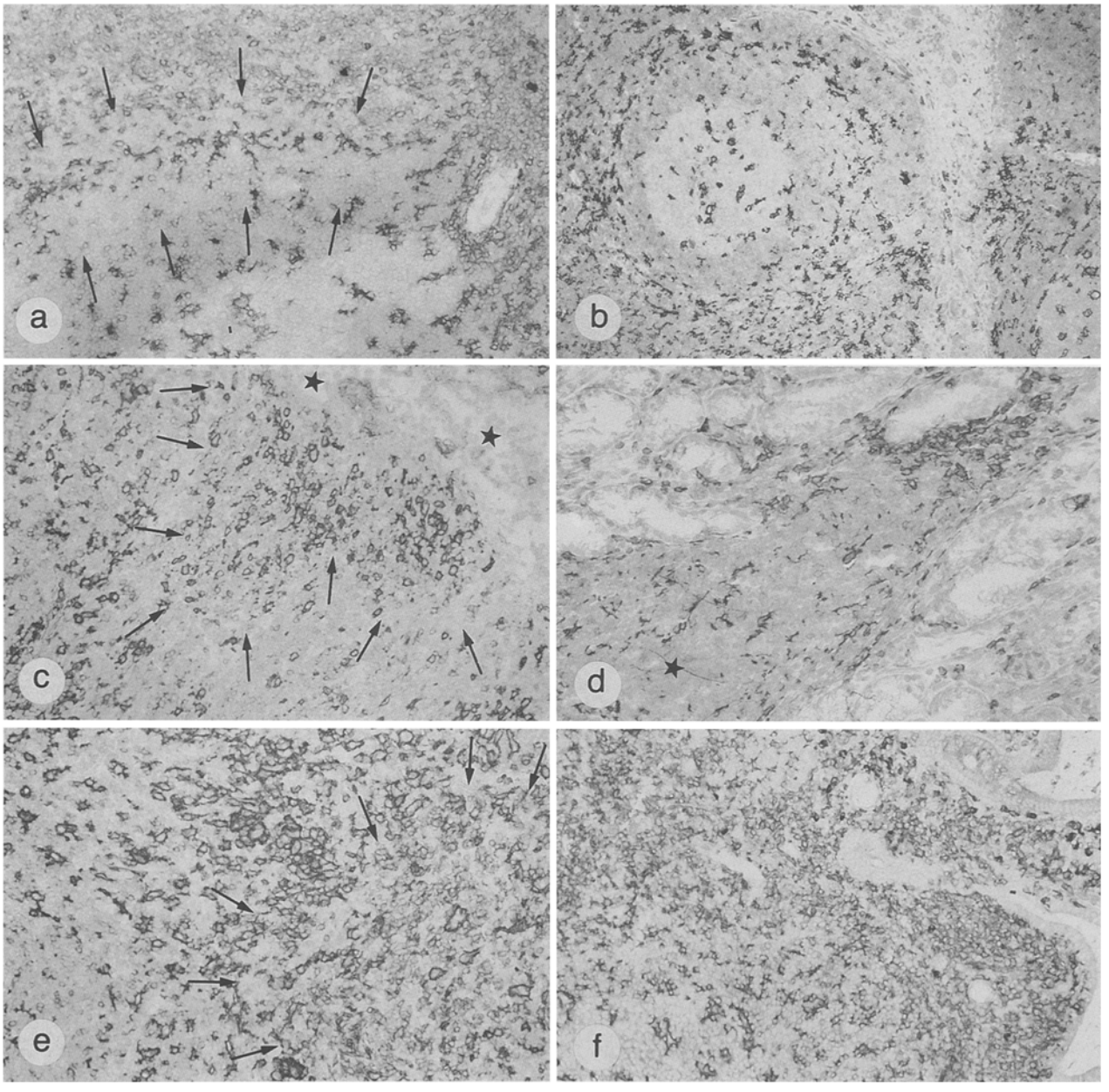


Fig. 3 Frozen sections stained for CD62L (Amino-ethylcarbazole and a faint haematoxylin counterstain, × 137). **a** Lymphadenitis associated with rheumatoid arthritis. Mantle cells and EF cells strongly express CD62L while follicle centre B cells are negative. Immunostaining in the follicle centre is confined to the apical part of the dendritic network. **b** Normal spleen. The FC is CD62L-negative; MZ B cells are strongly CD62L-positive while the staining of marginal zone (EF) B cells is definitely weaker. **c** Toxoplasmic lymphadenitis. The follicle centre cells (left) are devoid of CD62L, mantle cells are strongly positive. The monocytoid B cells express CD62L at very low levels (area indicated by *arrows*)



mantle zone B cells. They were situated in a narrow, cap-like region on top of the follicle and extending into the marginal sinus (Fig. 1a). EF cells were also hardly discriminable from mantle zone B cells in cystadenolymphoma and Hashimoto's thyroiditis. EF cells were distinctly enlarged in the marginal zones of spleens featuring minor nuclear irregularity, a reduction of chromatin density and a narrow rim of leptochrome cytoplasm (Fig. 1c). The marginal zone B cells closely resembled centrocyte-like cells in the dome region of the terminal ileum (Fig. 1b). The EF B cell compartment of hyperplastic tonsils is situated suprafollicularly extending into the crypt epithelium. In this site the average cell size was larger due to a balanced nuclear and cytoplasmic enlargement and nuclear outlines were ovoid or bean-shaped (Fig. 2). This phenotype was also encountered within myoepithelial proliferations in myoepithe-

Fig. 4a–f Frozen sections stained for CD11c (Amino-ethylcarbazole and a faint haematoxylin counterstain, × 137). **a** Spleen. Lymphoid cells of the marginal zone (*arrows*) are essentially CD11c-negative; only some scattered cells express CD11c very weakly. In the centre of the marginal zone there is a thin garland of strongly CD11c-positive interstitial dendritic cells. **b** Lymph node with mild follicular hyperplasia. The EF cell compartment is devoid of CD11c expressing lymphoid cells; the strongly stained cells are perisinusoidal dendritic cells. **c** Hyperplastic tonsil (crypt lumen marked by *asterisk*). The intraepithelial B cells (region marked by *arrows*) express CD11c at intermediate levels and are intermingled with strongly stained histiocytic cells. **d** Lymphofollicular gastritis (follicle centre marked by *asterisk*). EF B cells in immediate vicinity to a crypt express CD11c at intermediate levels. **e** Toxoplasmic lymphadenitis (FC: follicle centre; FM: follicle mantle). The monocytoid B cells (region marked by *arrows*) express CD11c at intermediate levels. Strongly stained histiocytic cells and epithelioid cells are scattered throughout the lesion. **f** Ileal lymphoglandular complex. The lymphoid cells in the dome area are predominantly CD11c-positive, note that the antigen density increases towards the gut lumen

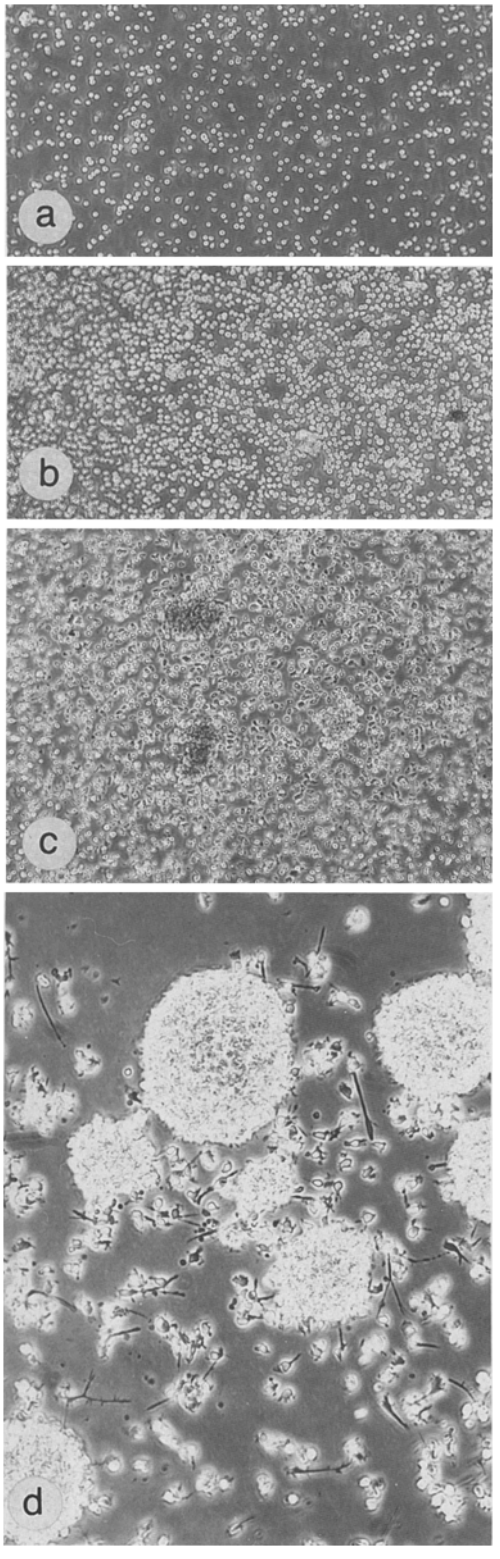


Fig. 5a–d Cultures (72 h) of dense tonsillar B cells (D55/80); (× 80). **a** Cells grown in medium alone were small and round. **b** Cells grown in interferon γ (IFNγ) plus tumour necrosis factor α (TNFα) were small and occasionally formed small clusters. **c** Staphylococcus aaureus Cavain I (SAC) together with IL-4 induced short pseudopod-like cytoplasmic processes; cells increased in size and clusters were larger and more numerous. **d** Phorbol 12-myristate 13-acetate (PMA) induced long cellular processes; cells were predominantly large, adhered to plastic and grew in large clumps

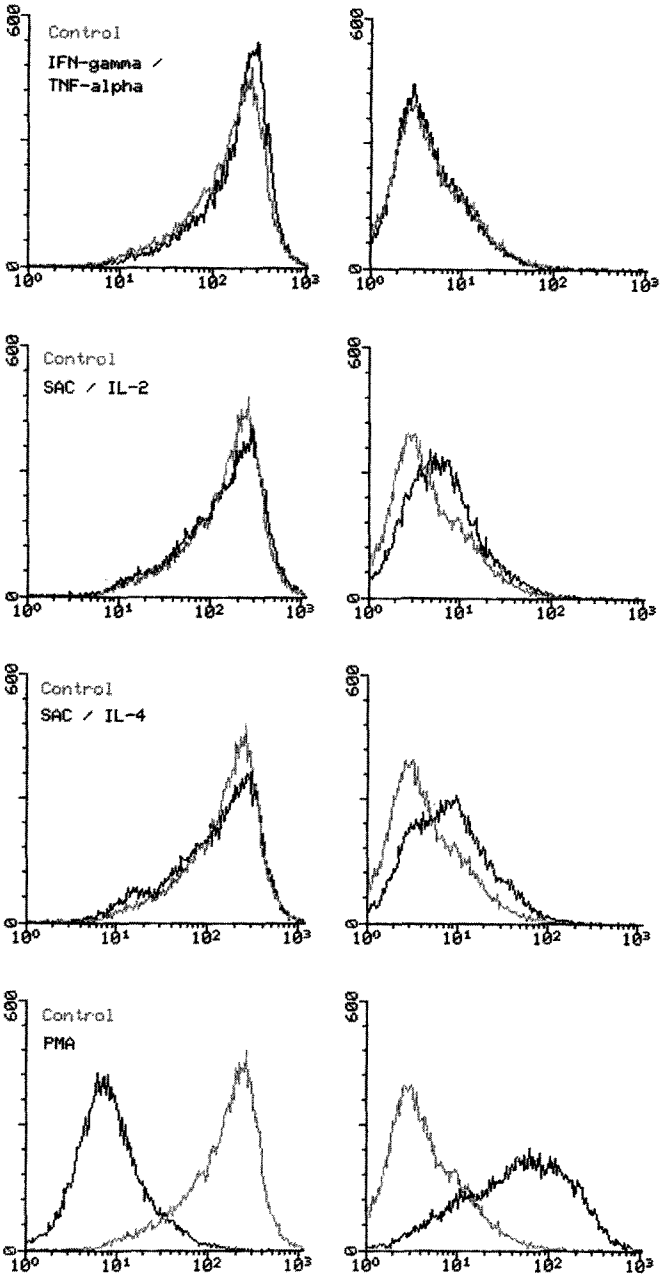


Fig. 6 Surface expression of CD62L (left column) and CD11c (right column) in unfractionated tonsillar B cells (tonsil No. 1) after 72 h culture. Cultures kept in medium without stimulating agents were used as controls. INFγ plus TNFα did not lead to changes in CD62L or CD11c on the cell surface. Treatment with SAC and IL-2 or IL-4 slightly increased the number of CD11c-positive cells, while CD62L expression was essentially unchanged. PMA lead to a substantial increase of CD11c expression and to a dramatic down-modulation of CD62L

lial sialadenitis. Lymph nodes affected by toxoplasmosis displayed the well-known aspect of monocytoid B cells (Fig. 1 d). These cells were situated peri- and intrasinusoidal, corresponded to centrocytes in cell size and had very similar nuclei. However, the cytoplasmic rim was distinctly broader and characteristically pale. Mitotic figures were always present, however, in small numbers. Comparing EF B cells in all conditions described above, three forms can be discriminated: a small (lymphoid)

Table 2 Expression of CD62L in peripheral blood B cells (PBL, unfractioned and in the dense (D55/80) fraction of tonsillar B cells (T_1–T_7 number of tonsil specimen; *mF* mean fluorescence; *%Pos* percentage positive cells; *n.a.* not analysed)

Experiment	Control		IFN-γ+TNFα		SAC+IL-2		SAC+IL-4		PMA	
	mF	%Pos.	mF	%Pos.	mF	%Pos.	mF	%Pos.	mF	%Pos.
PBL	55	77.2	63	83.7	33	61.9	n.a.	n.a.	7	8.2
T1 unfract.	192	97.2	218	98.2	193	96.4	178	94.5	13	24.9
T2 unfract.	157	94.2	n.a.	n.a.	131	93.0	104	88.7	n.a.	n.a.
Dense tonsillar B cells										
T4	108	93.5	169	97.5	147	95.3	108	91.9	33	63.7
T5	84	88.8	n.a.	n.a.	84	91.5	75	85.9	n.a.	n.a.
T6	99	91.8	131	94.5	74	93.2	83	95.0	19	53.7
T7	123	94.5	121	96.4	108	92.0	101	93.9	14	25.5

Table 3 Expression of CD11c in peripheral blood B cells (PBL, unfractioned and in the dense (D55/80) fraction of tonsillar B cells (T_1–T_7 number of tonsil specimen, *mF* mean fluorescence; *%Pos* percent positive cells; *n.a.* not analysed

Experiment	Control		IFN-γ+TNFα		SAC+IL-2		SAC+IL-4		PMA	
	mF	%Pos.	mF	%Pos.	mF	%Pos.	mF	%Pos.	mF%	Pos.
PBL	3	2.5	2	1.6	4	4.8	n.a.	n.a.	9	27.6
T1 unfract.	7	12.6	7	11.1	10	18.4	13	27.6	87	78.7
T2 unfract.	11	14.4	n.a.	n.a.	15	26.5	14	25.3	n.a.	n.a.
Dense tonsillar B cells										
T3	3	0.9	3	0.6	n.a.	n.a.	n.a.	n.a.	27	37.6
T4	5	5.9	5	4.0	15	32.3	19	42.9	120	88.8
T5	6	5.2	n.a.	n.a.	16	24.6	13	18.5	n.a.	n.a.
T6	5	2.1	6	3.1	10	18.4	12	24.2	37	72.4
T7	4	2.5	4	1.7	12	17.8	16	23.8	58	61.6

variant, an intermediate (centrocytoid) variant, and a large (monocytoid) variant. Whereas in spleens and toxoplasmosis the boundaries of the mantle cell and the EF compartment is quite distinct, it is less so in tonsil, Peyer's patch and lymphofollicular gastritis. Actually, there is a continuous enlargement and cytological metamorphosis from the follicular mantle to the epithelial surface.

The compiled results of the immunostainings are given in Table 1. CD62L (L-selectin) was expressed at high levels in mantle zone B cells but is lacking in follicle centre cells. EF B cells expressed CD62L differentially, small cells at high (Fig. 3a), intermediate cells at intermediate levels (Fig. 3b) and large cells at very low levels (Fig. 3c). CD11c was undetectable in mantle zone B cells and follicle centre cells but was differentially expressed in EF B cells (Fig. 4a–f). Small and intermediate EF cells were CD11c-negative. Large EF cells expressed CD11c. The antigen density on large EF cells was low to intermediate compared to that of histiocytic cells. These findings are suggestive of down-regulation of CD62L in parallel with an induction of CD11c during the metamorphosis of small EF cells to large EF cells. Since large EF B lymphocytes were the only B cells expressing CD11c (Table 1), this antigen can be regarded as a marker for the large extrafollicular B lymphocyte.

B cell isolates from peripheral blood and hyperplastic tonsil were kept in short term culture for 72 h and were subjected to different activation protocols. Compared to cells that were kept in culture medium alone, the combination of IFNγ and TNFα did not induce remarkable changes. The average cell size as revealed by invert microscopy (Fig. 5a, b) and forward scatter in flow cytometry did not change nor did the surface expression of CD62L (Table 2) and CD11c (Table 3). After cross-linking of surface immunoglobulin (with SAC) in the presence of IL-2, PBL and cells of the dense fraction increased in size around 25% (cell size increment of unfractioned B cells was around 10%) and formed small clusters (not shown). Along with this, surface expression of CD62L was essentially unchanged, yet, some degree of up-regulation of CD11c could be observed. Enhancement of CD11c was more distinct upon stimulation with SAC plus IL-4. Under these conditions a minimal down-regulation of CD62L surface expression was detectable in most experiments. Cells of the dense fraction formed tiny pseudopod-like cytoplasmic protrusions (Fig. 5c), increased in size and formed small clusters comparable to IL-2 stimulated cells.

PMA-stimulation induced dramatic changes in all B cell isolates. The mean cell size increased around 40% in cultures from dense and unfractioned B cells while the increment was around 30% for PBL (data not shown). Cells of all preparations developed long cytoplasmic processes, became plastic adherent and developed a high degree of homotypic adhesion (Fig. 5d). Furthermore, PMA-induced B cell activation lead to a general, severe down-regulation of CD62L and a significant up-regulation of CD11c (Fig. 6). The immunophenotype of PMA blasts resembled to the phenotype of large EF B lymphocytes. PMA blasts expressed CD11c, lacked CD62L and the germinal centre markers CD10, CD77 and CD38. The corresponding immunophenotype of large EF B cells and PMA blasts suggests that PMA

stimulation may drive B cells into EF stage of differentiation.

The combination of IFNγ and TNFα did not influence the variables studied. SAC in combination with IL-2 or IL-4 led to an increase in cell size about 20%, formation of small cell clusters and a slight up-regulation of CD11c-surface expression. In addition, SAC/IL-4 lead to a minimal decrease in expression of CD62L. PMA-stimulation, however, gave rise to a phenotype morphologically and serologically very reminiscent of the large cell variant of EF B lymphocytes. i.e., monocytoid B cells.

Discussion

Our re-evaluation of EF B cell morphology in various sites and under various culture conditions strongly favours the concept that an activation-associated continuous transformation of a small lymphoid EF B cell leads to a large, transformed B blast, long known as monocytoid B cell (Sheibani et al. 1984), which may enter the cell cycle (Stein et al. 1984). The large cell variant either forms aggregates ("sinusoidal B cell reaction") (Stein et al. 1984) suggesting homotypic adhesion or is in close association with sinus endothelium (Möller and Lennert 1984) or epithelial cells (Möller and Mielke 1989) suggesting additional heterotypic adhesion capacity.

Immunohistology revealed that cells of the EF compartment are heterogenous in their expression of CD62L (L-selectin) and CD11c. This was already noted in previous studies (Möller et al. 1992b; Eichelmann et al. 1992). We show here that the small cell variant is CD62-L-positive/CD11c-negative while the large cell variant is only very weakly CD62L-positive but expresses CD11c at intermediate levels. This observation suggests a down regulation of CD62L in parallel to a CD11c induction along with EF B cell metamorphosis.

Phenotypic changes leading in this direction were observed after stimulating peripheral and tonsillar B cells through cross-linking of surface immunoglobulin (via SAC) in the presence of IL-2 or IL-4. Cells increased in size, developed cellular protrusions and tended to aggregate while levels of CD11c surface expression changed from very low to low and CD62L levels slightly decreased. These observations are in line with published data (Banchereau et al. 1991; Postigo et al. 1991; Björk et al. 1992; Jensen and Prince 1992). Since follicular centre B cells are devoid of detectable CD11c, it can be concluded that, at least in the presence of IL-4, triggering through surface immunoglobulin drives a considerable subset of PBL (resting) and dense tonsillar (early activated) B cells into the EF stage of differentiation. However, CD11c-positive B blasts closely resembling large EF cells in situ are achieved only upon PMA-stimulation. Dense (small) tonsillar B cells do not only consist of sIgD-positive mantle zone lymphocytes. In this fraction about 30 percent of cells are sIgD-negative while also lacking germinal centre markers like CD38 and CD10 (data not shown). These cells, therefore, represent the small lymphoid form of EF B lymphocytes. We thus conclude that PMA-stimulation and to a lesser extent stimulation with SAC + IL-4 drives peripheral and dense tonsillar B cells to the large EF-blast differentiation stage.

PMA-treatment of B-chronic lymphocytic leukaemia (CLL) induces morphological features resembling hairy cell leukaemia (Gazitt and Polliack 1987) together with CD11c neo-expression (Polliack et al. 1988). The B-acute lymphoblastic leukaemia (ALL) line REH was induced for further maturate, to express CD11c and to become monocytoid in morphology (Patel et al. 1990). CD11c induction in normal B cells was shown on day 2 upon PMA-administration (Visser and Poppema 1990; Postigo et al. 1991). Along with morphological changes, PMA caused plastic adherence in hairy cell leukaemia (Tokumine et al. 1988; Visser and Poppema 1990) and CD11c/CD18-mediated fibronectin binding capacity in normal B lymphocytes (Postigo et al. 1991). PMA causes homotypic adhesion in B-CLL cells (Visser and Poppema 1990) and, as we confirm, in normal B cells.

PMA (sometimes referred to as *tetradecanoyl phorbol acetate*, TPA) is one of the most potent cell stimulating agents. PMA mimics intracellular diacylglycerol and acts through activation of protein kinase C (reviewed by Berridge 1993). Via this route of intracellular signalling, PMA also induces the DNA-binding protein NF-κB (Iwasaki et al. 1992) which, in turn, acts on the promoter region of numerous genes (reviewed by Gilmore 1990). Thus, PMA initiates a wide range of physiological activities within the cell. By-passing the level of G protein-linked and tyrosine kinase-linked membrane receptors and phospho-lipase C, PMA might nevertheless be quite an artificial activator. Thus, the physiological (set of) signal(s) inducing the large transformed (monocytoid) phenotype of EF B cells in vivo is still unknown.

Schwarting et al. (1985) were the first to describe the well-known CD11c expression in hairy cell leukaemia. CD11c was further detected in a minority of B-CLLs (De la Hera et al. 1988) and immunocytomas (lymphoplasmacytoid lymphoma) (Visser et al. 1989). Monocytoid B cell lymphoma (Sheibani et al. 1986) was shown to express CD11c in most but not all reported cases (Sheibani et al. 1988; Ngan et al. 1991; Davies et al. 1992), leukaemic ones included (Traweek et al. 1989). Corresponding with what we show for reactive EF cells, the cytomorphological spectrum within the monocytoid lymphoma category ranges from small, lymphoid (leukaemic) (Traweek and Sheibani 1992) to large, transformed cells (a-leukaemic, nodal and extranodal tumours) (Nathwani et al. 1992). Mielke and Möller (1991) demonstrated CD11c-expression in cases of primary gastro-intestinal B-cell lymphomas which had an immunoprofile essentially or in parts corresponding to EF B cells. Also in this series, CD11c-positive tumours were either centrocytoid or had large-anaplastic cytology. Furthermore, CD11c is inconsistently expressed in pri-

mary mediastinal (thymic) B cell lymphoma, which in our experience comprises large cell and medium sized cellular variants (Möller et al. 1986). Consequently, CD11c expression was taken as one argument among others favouring the relationship of this type of tumour with large EF B cells (Möller et al. 1989 b). Interestingly, presence of CD11c and of CD62L in mediastinal B cell lymphoma were, on the level of individual tumours, quite often mutually exclusive (Eichelmann et al. 1992). All in all, there is good evidence that the activation-associated transformation of reactive EF cells shown in this study has its equivalent in B cell neoplasia and that CD11c is the best marker currently known to positively define EF B lymphomas.

Experimental systems in rat suggest that the EF compartment is colonized by memory B cells that do not recirculate once they have entered this microarea (Liu et al. 1988; MacLennan and Chan 1993). In line with this concept we have shown antigen-driven, activation-associated transformation to occur in this cell type leading to substrate (for example plastic) and intercellular adhesion. This transformation goes in parallel to down-regulation of the venular endothelium binding molecule CD62L and up-regulation of an alternative (sinusoidal?) endothelium/fibronectin/iC3 binding adhesion molecule, that is the β2 integrin CD11c/CD18. B cells were shown to receive a mitogenic signal through CD11c (Postigo et al. 1991). CD11c might thus account for the low proliferative activity which can be observed in monocytoid B cell clusters. One net effect of the monocytoid B cell transformation seems thus to be a *localized* multiplication of antigen triggered, adhesive EF B cells.

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