ISOLATED HUMAN FOLLICULAR DENDRITIC CELLS DISPLAY A UNIQUE ANTIGENIC PHENOTYPE

By FOLKE SCHRIEVER, ARNOLD S. FREEDMAN, GORDON FREEMAN, ELLEN MESSNER, GRACE LEE, JOHN DALEY, AND LEE M. NADLER

From the Division of Tumor Immunology, Dana-Farber Cancer Institute; and the Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115

Lymphoid follicles are primary sites for the humoral immune response. Within the complex microenvironment of the lymphoid follicle, B cells receive a multitude of signals that influence their migration and differentiation. In the germinal center, follicular dendritic cells (FDCs)[1] are physically associated with B cells by forming a dense three-dimensional network. These cells play a central role by trapping and delivering immune complexes to B cells.

Although morphologically similar, FDCs can be distinguished from the lymphoid dendritic cells (interdigitating reticulum cells), which were first described by Steinman et al. (1, 2). Whereas FDCs are localized to primary and secondary follicles, lymphoid dendritic cells are found in T cell-rich interfollicular areas. The latter cells are functionally distinct in that they are potent stimulators of T cells in autologous and allogeneic mixed lymphocyte reactions (3–6). Consistent with their function to fix immune complexes, FDCs express all three receptors for the third complement component (CR1, CR2, and CR3), whereas lymphoid dendritic cells do not (6).

The cellular origin of FDCs is still unknown. Attempts have been made to define the lineage of these cells by in situ and in vitro staining with mAbs directed against lineage-restricted and -associated antigens (7–9). These investigations demonstrated that FDCs express CR1, CR2, and CR3, as well as the marker DRC-1. However, the expression of other B and myeloid markers has been more controversial. Since germinal center B cells are in such close proximity to FDCs, it is not possible to conclusively distinguish in situ whether staining is localized to FDCs or to B cells. Similarly, partially enriched FDCs form dense clusters with lymphoid cells in vitro (7) and, therefore, it has been difficult to precisely determine their antigenic phenotype. In the present study, unclustered FDCs were isolated and phenotypically characterized. These cells display a unique phenotype in that they expressed both B cell and myeloid markers, as well as many adhesion molecules. Using their antigenic and morphologic characteristics, FDCs were isolated to homogeneity by flow cyto-

This work was supported by National Institutes of Health grants CA-34183 and CA-40216. F. Schriever is supported by post-doctoral fellowship grant I3-Schr 318/1-1 from the Deutsche Forschungsgemeinschaft (DFG). A. Freedman is supported by Public Health Service grant 5K08 CA-01105-03 awarded by the National Cancer Institute, Department of Health and Human Services. Address correspondence to Folke Schriever, Division of Tumor Immunology, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115.

[1] *Abbreviations used in this paper:* CALLA, common acute lymphoblastic leukemia antigen; CR, complement receptor; EMA, epithelial membrane antigen; FDCs, follicular dendritic cells; ICAM, intercellular adhesion molecule; LFA, leukocyte function-associated antigen; PCR, polymerase chain reaction.

metric cell sorting. These highly purified FDCs did not express IL-6 mRNA. It may now become possible to further clarify the role of these cells in cell-cell interaction and cytokine production within the microenvironment of the germinal center.

Materials and Methods

Antibodies. Antibodies used in the present study are listed Table I. Many of the reagents were provided by the Fourth International Workship on Human Leukocyte Differentiation Antigens. Other antibodies were either produced in our laboratory, obtained through companies (listed in Table I), or were generous gifts of the following investigators: Dr. A. Albino (J143; Memorial Sloan-Kettering Cancer Center, New York); Dr. C. Damsky (BIE5; UCSF, San Francisco); Dr. C. Dinarello (rabbit anti-IL-6; Tufts University, Boston); Dr. S. Gillis (Immunex, Seattle); Dr. J. Griffin (904, Mol, Mo2, AML-2-23, 322, My7, IV-3 D3, My9, L4F3, 12-8; Dana-Farber Cancer Institute, Boston); Dr. M. Hemler (TS2/7; Dana-Farber Cancer Institute); Dr. R. Hynes (rabbit anti-VLA-β 1; Massachusetts Institute of Technology, Cambridge); Dr. J. Johnson (63D3; Institut für Immunologie, München, FRG); Dr. T. Kishimoto (rabbit anti-rhIL-6; Institute for Molecular and Cellular Biology, Osaka, Japan); Dr. D. Mason (R4/23; John Radcliffe Hospital, Oxford, England); Dr. C. Morimoto (2H1, 4B4, GAP P3, 2H4, 8F2; Dana-Farber Cancer Institute); Dr. J. Ritz (J2, J5, 2F12, 10F12, S6F1, NKH-1; Dana-Farber Cancer Institute); Dr. C. Rudd (A1A5; Dana-Farber Cancer Institute); Dr. A. Sonnenberg (GOH3; Central Laboratory for the Netherlands, Amsterdam); Dr. T. Springer (RR.1; Center for Blood Research, Boston); Dr. A. Wardlow (rabbit anti-CD18; Center for Blood Research). Biotinylated B1 (CD20) and B2 (CD21) were obtained from Coulter Immunology (Hialeah, FL). mAb 543 (CD35) was produced from a hybridoma cell line (American Type Culture Collection, Rockville, MD). The working concentration of the antibodies was confirmed by positive control stainings of reactive cell populations. Biotinylation of mAbs and preparation of phycoerythrin-streptavidin conjugations were done according to Daley (10). In all experiments mouse and rat Igs of the corresponding isotype and preimmune rabbit serum, respectively, were used as negative controls.

Immunophenotyping. Reactivity of a large panel of antibodies with FDCs was determined by immunoperoxidase staining on cytospin preparations of tonsillar low density cells. Cells were spun onto microscopic slides with a cytocentrifuge (Shandon Southern Instruments Inc., Sewickley, PA), air dried, and fixed with acetone for 3 min. Mouse, rat, and rabbit antibody binding was detected using affinity-purified peroxidase-conjugated antiserum of rabbit anti-mouse Ig, (P260; Dako Corp., Glostrup, Denmark), rabbit anti-rat Ig (P162; Dako Corp.), and swine anti-rabbit Ig (P399; Dako Corp.), respectively. Antibody reactivity was visualized with 3-amino-9-ethylcarbazole (Sigma Chemical Co., St. Louis, MO) (0.25 mg/ml in 0.1 M acetate buffer, pH 5.0) with 0.003% H_2O_2. Nonspecific antibody binding to Fc receptors was inhibited by diluting the antibodies in 10% human serum in PBS. Cells were counterstained with Meyer's hematoxylin. To quantitate the percentage of FDC clusters among total FDCs, immunoperoxidase staining with mAbs against CR1, CR2, and CR3 was used. Briefly, in all cell preparations equal numbers of FDCs were counted over a representative microscopic field. Clustered FDCs were defined as cells that contained one or more lymphocytes trapped in their cytoplasmic processes. In some experiments specific antibody binding to FDCs was confirmed by double labeling FDCs using a combination of immunoperoxidase and immunofluorescence. Briefly, after incubation with antibodies and peroxidase-coupled antiserum, cytospin preparations were labeled with biotinylated mAbs against CD21 (mAb B2) and CD35 (mAb 543), followed by incubation with streptavidin/phycoerythrin complex. Immunoperoxidase staining was subsequently developed as described above, which did not affect the immunofluorescence staining. Antibody binding to the cell surface of viable FDCs in suspension was visualized by using fluoresceinated goat anti-mouse Ig (Tago Inc., Burlingame, CA).

Cytochemical Analyses. Specific and nonspecific esterase staining was performed using naphthol AS-D chloroacetate esterase and α-naphtylacetate esterase (Sigma Chemical Co.), respectively, as substrate. Cytoplasmic peroxidase was detected with the substrate shown above.

Phagocytosis Assay. To test FDCs for their ability to phagocytize, low density cell prepara-

TABLE I
Antibodies Used for the Present Study

CD/specificity	mAb	Source*	CD/specificity	mAb	Source*
CD1a	T6A, T6	Coulter	CD35	543	ATCC
CD1b	T009, T074, T070	WS	CD37	HD28; G28-1 HH1	WS
CD1c	T042	WS	CD38	5D2; GR7A4;	
CD2	T11(2); T11(3)			HB7 T16; IC0-20	WS
CD3	T3		CD39	G28-8; AC2	WS
CD4	T4	Coulter	CD40	G28-5; EA-5	WS
CD5	T1	Coulter	CDw10	Gp IIb/IIIa	Dako
CD6	2H1		CD45	GAP P3	
CD7	T159, T160, T161	WS	CD45R	2H4	
CD8	T8	Coulter	NK	NKH-1	
CD9	J2		B5		
CD10	J5		B7		
CD11a	2F12		Surface IgG		
CD11b	904; M01		Surface IgD		
CD11c	p150/95	Dako	Surface IgM		
CD13	My7		Surface κ		
CD14	AM1-2-23, 322, Mo2		Surface λ		
CD16	Leu-11b	BD	ICAM-1	RR.1	
CD18	10F12; S6F1		VLA 1	TS2/7	
CD19	B4; B-C3; J3-119;	Coulter	VLA 3	J143	
	CLB-CD19	WS	VLA 4	8F2	
CD20	B1; 1F5; 2H7; B-ly1	Coulter	VLA 5	BIE5	
		WS	VLA 6	GOH3	
CD21	B2; BU-35; 21A/5;		VLA β chain	4B4; A1A5	
	BU-36	WS	DRC-1	R4/23	Dako
CD22	HD6; HD39; HD239;		rhIL-	Rabbit serum	
	G28-7	WS	PCA-I		Coulter
CD23	Blast 2; H107; M-L47;		Factor VIII		Dako
	BU-38; 3-5;	WS	Epithelial		
CD24	LC66; 32D12; ML5	WS	membrane		
	HB8		antigen		Dako
CD25	Tac		63D3		ATCC
CDw29	4B4; A1A5		HLA class I	W6.32	
CD30	RSC1	Dako	HLA class II	6/7; 6; 332	
CD32	IV-3 D3				
CD33	My9; L4F3				
CD34	12-8				

* Antibodies were provided by other investigators (see Materials and Methods) or from the following companies: ATCC, American Type Culture Collection, Rockville, MD; BD, Becton Dickinson & Co., Mountain View, CA; Coulter Immunology, Hialeah, FL: Dako Corp., Santa Barbara, CA; WS, 4th International Workshop on Human Leukocyte Differentiation Antigens.

tions were incubated 2 h at 37°C with opsonized human RBCs. Plastic adherent monocytes from human spleen served as positive control cells.

Isolation and Purification of Follicular Dendritic Cells. Human tonsils were obtained immediately after nonurgent tonsillectomy, placed in a Petri dish, and gently teased apart using forceps, knife, and stainless steel mesh. Tissue was digested with RPMI media (Gibco Laboratories, Grand Island, NY) containing 2 mg/ml collagenase (type IV; Worthington Biochemical Corp., Freehold, NJ), 5 mM EDTA (pH 7.4), 10% FCS, 4 mM glutamine, 10 U/ml penicillin, 10 U/ml streptomycin, 1 mM sodium pyruvate, and 0.1 mg/ml DNase (type I; Sigma Chemical Co.). To investigate cluster formation of FDCs with lymphocytes under different conditions,

equal small tonsil pieces were digested separately for 1 h on ice, at room temperature and at 37°C, or at room temperature in the presence of 2, 10, and 50 mM EDTA (pH 7.4). Cell suspensions were centrifuged at 1,800 rpm for 25 min over a discontinuous gradient of 15% (1.02 g/ml density) and 35% (1.04 g/ml density) Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden), and low density cell preparations enriched for FDCs were collected from the 15%/35% interphase.

Final FDC purification was achieved by labeling low density cell suspensions with mAb 322 (CD14), subsequent staining with fluoresceinated goat anti-mouse Ig (Tago Inc.), and sorting with an EPICS cell sorter (Coulter Electronics Inc., Hialeah, FL). FDCs were sorted using parameters of log fluorescence and log 90° light scatter, and gate windows were set to exclude cell clusters. Cells were sorted twice to reach the highest purity possible. Both antibodies were ultracentrifuged at 4°C with 15,000 rpm for 30 min immediately before use to eliminate protein aggregations. Nonspecific binding to cell surface Fc receptors was blocked by diluting the antibodies in 10% human serum in media. Purity of the sorted FDC preparations was determined by re-analysis with flow cytometry. For two-color analyses, sorted FDCs were labeled with biotinylated mAbs against B1 (CD20) or B2 (CD21), stained with streptavidin/phycoerythrin complex, and analyzed by FACS.

RNA Purification and Polymerase Chain Reaction. SRBC receptor negative cells from human tonsil were stimulated for 10 h with anti-Ig beads and highly purified FDCs were obtained by cell sorting. RNA from these cells was extracted by a modification of the acid guanidinium thiocyanate-phenol-chloroform isolation procedure designed for small quantities of cells (11). Since the number of highly purified FDCs after the sort was very small (10^5), gene expression of these cells was analyzed using the very sensitive technique of polymerase chain reaction (PCR) (12). RNA (250–500 ng) was reverse transcribed using 200 U of Moloney murine leukemia virus reverse transcriptase and 0.8 μg oligo-dT as primer for 1 h at 37°C. One third of the resulting cDNA was analyzed for IL-6 or aldolase A expression by amplifying with 5 U of Thermus aquaticus (Taq) polymerase (Perkin-Elmer Corp., Norwalk, CT) and primers (50 pmol each) during 40 cycles of PCR (12) on a PHC-1 Programmable Dri Block (Techne, Inc., Princeton, NJ). The sense primer for IL-6 consisted of nucleotides 200–223 and the antisense primer consisted of nucleotides 542–517 (13). The sense primer for aldolase consisted of a 21 nucleotide-long sequence and the antisense primer was a 23 nucleotide-long sequence (14) (both primers as well as an oligonucleotide probe were a generous gift of Dr. Daniel Bergsagel, Dana-Farber Cancer Institute, Boston). Every cycle included denaturation at 94°C, annealing of primers and fragments at 37°C, and primer extension at 72°C. 16 μl of the product DNA obtained by PCR was applied to a 3% NuSieve GTG/1% Sea-Kem GTG agarose gel. Gels were run in tris acetate EDTA buffer containing ethidium bromide at 50 V for 3–5 h and Southern transferred onto a Zeta-Probe membrane (Bio-Rad Laboratories, Richmond, CA). Hybridization was performed using ^{32}P-labeled cDNA for IL-6 (kindly provided by Dr. Steven Clark, Genetics Institute, Cambridge, MA) and a ^{32}P-labeled 23-base oligonucleotide probe specific for the amplified aldolase A fragment (14).

Results

Morphological Identification of FDCs. Using characteristic morphological features of FDCs, it was possible to distinguish them from other dendritic-shaped cells, i.e., macrophages and interdigitating reticulum cells. As seen in Fig. 1, FDCs had four fundamental morphologic characteristics. They were very large in size (70–100 μm) (Fig. 1 *A*) and often contained two nuclei (Fig. 1, *A* and *B*). In addition, FDCs formed intense clusters with lymphocytes (Fig. 1, *C* and *D*) by a network of fine processes (Fig. 1 *C*, *arrows*). Although Wright Giemsa staining demonstrated large cell size, binuclear appearance, and acidophilic cytoplasm, it was impossible to definitively conclude by cytochemical staining alone that a cell was an FDC. Therefore, the reactivity of mAbs directed against CRs (CR1, CR2, CR3) was used to provide additional evidence that cells were FDCs. All anti-CR mAbs bound homogeneously to the cell surface and the cytoplasm of FDCs and, therefore, the prominent cytologic

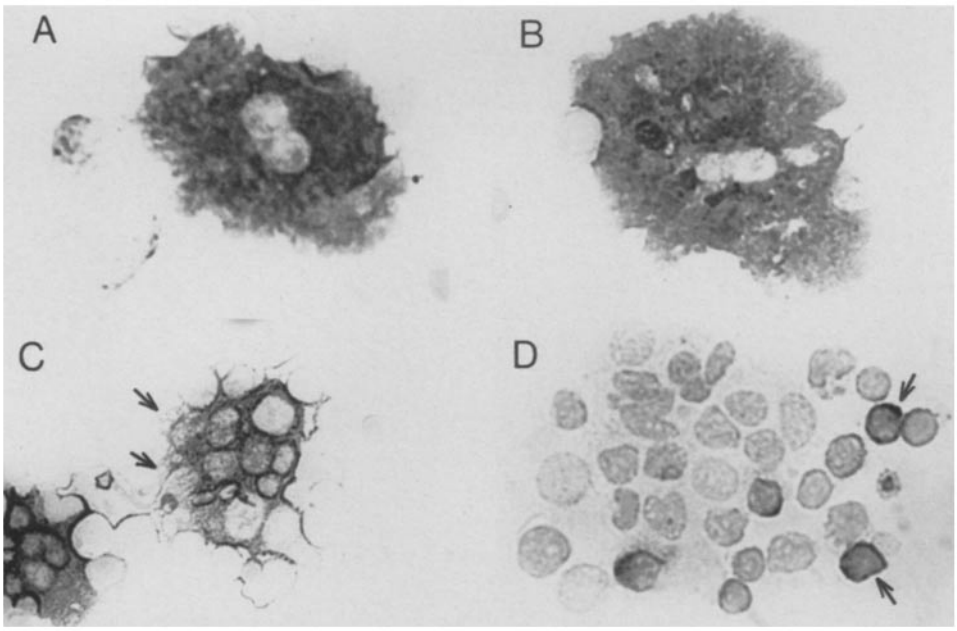

FIGURE 1. Immunoperoxidase staining of FDCs in low density cell preparations from human tonsil. (*A*) Anti-CD14 on single FDC; (*B*) anti-CD35 (CR1) on single FDC; (*C*) anti-CD21 (CR2) reveals the cellular processes of FDCs (*arrows*) that retain adherent lymphocytes; (*D*) anti-surface IgD as negative control shows no reactivity with FDCs but stains adherent lymphocytes (*arrows*).

features of these cells could be revealed by those antibodies (Fig. 1, *B* and *C*). Similarly, mAbs directed against other antigens expressed on FDCs, including anti-CD14 (Fig. 1 *A*), demonstrated the identical staining pattern seen with anti-CR mAbs. Fig. 1 *D* depicts an example for a negative control antibody. Antisurface IgD bound to clustered B cells (*arrows*) but lacked reactivity with the FDC.

Inhibition of Lymphocyte Adherence to FDCs. During preparation of single cell suspensions and density sedimentation, lymphocytes consistently adhered to FDCs, forming tight clusters. Attempts to disrupt these aggregations by vigorous vortexing or by incubating the cells with EDTA or cold media did not diminish the number of bound lymphocytes. This cluster formation presented a major problem for further phenotypic analyses since it was extremely difficult to distinguish mAb staining of lymphocytes from that of FDCs.

Therefore, in an attempt to block the formation of cell clusters, tonsils were manipulated under a number of different conditions. Table II summarizes the results of two representative experiments investigating if aggregate formation of FDCs is temperature dependent and if it can be influenced by chelation of Ca^{2+} and Mg^{2+}. FDCs were identified by their characteristic morphology and reactivity with anti-CR mAbs. As seen in Table II, by lowering the incubation temperature during tonsillar digestion, the percentage of clusters could be decreased consistently. In addition, cluster formation was inhibited progressively by increasing concentrations of EDTA, indicating that the aggregation of FDCs with lymphocytes is Ca^{2+} and Mg^{2+} dependent. Furthermore, it was examined if adherence of lymphocytes to FDCs could

TABLE II

Inhibition of Cluster Formation of FDCs with Lymphocytes

Exp.	FDCs	Conditions during digestion of tonsil					
		37°C	Room temperature	4°C	EDTA 2 mM	EDTA 10 mM	EDTA 50 mM
		%					
1	Single cells	30	49	74	55	65	70
	Clusters	70	51	26	45	35	30
2	Single cells	53	73	90	66	74	87
	Clusters	47	27	10	34	26	13

Single and clustered FDCs were determined by immunoperoxidase stainings of low density cell preparations.

be blocked with mAbs directed against adhesion molecules (anti-leukocyte function-associated antigen 1 [anti-LFA-1] and anti-intercellular adhesion molecule 1 [anti-ICAM-1]) or against CD14 and class I. Tonsils were digested in the presence of each of these antibodies, however, no significant decrease in cluster formation was observed.

Antigen Expression of Follicular Dendritic Cells. Using conditions that significantly decrease cluster formation, it was possible to precisely examine the expression of cellular antigens on FDCs. These studies were performed with the objective to define lineage and to detect functionally relevant cell surface molecules. Single cell suspensions prepared in the presence of EDTA contained <0.1% FDCs (Table III). By centrifuging over a low density gradient, FDCs were enriched to 4–6% (Table III). In these cell preparations, 50–70% of FDCs showed no adherent lymphocytes. Staining with lineage-restricted mAbs directed against B cells (anti-CD20, anti-CD24) and T cells (anti-CD2) demonstrated that the overwhelming majority (95%) of the remaining clustered lymphocytes were B cells, whereas <5% were T cells. By immunoperoxidase staining of single unclustered FDCs, mAb binding to these cells could be confidently determined. To exclude that the binding pattern of some of the antibodies was due to the expression or lack of certain epitopes, some of the markers were tested with a series of different mAbs. In addition to immunoperoxidase staining of fixed cytospin preparations, viable cells in suspension were also ex-

TABLE III

Purification Steps and Enrichment of FDCs from Human Tonsil

Preparation	FDCs
	%
Physical isolation	
Single cell suspension	<0.1*
Preparation of low density cells (35% Percoll gradient)	4–6*
Antigen-specific purification	
Sort for highly CD14 positive cells by FACS	70‡
Sort of cells obtained from above sort	98.8‡

* Determined by immunoperoxidase staining.
‡ Assessed by FACS.

amined for expression of cell surface antigens. All FDC$^+$ mAbs (Table IV) demonstrated reactivity with the cytoplasm of FDCs as well as with their cell surface.

FDCs demonstrated a unique lineage-restricted antigen pattern by coexpressing B and myeloid markers. As seen in Table IV, FDCs were stained with mAbs directed against the B lineage-restricted CD19 and CD21 antigens and mAbs against myeloid-restricted antigens CD14, CD11b, and 63D3. However, other B cell lineage-restricted molecules, including CD20, CD22, and sIg (IgM, IgD, IgG, κ and λ), were negative (Table V). In addition, several myeloid markers, including CD13 and CD33, were not expressed (Table V). Antigens found on both B and some myeloid cells, including HLA class II, CD23, CD37, and CDw40, were also expressed on FDCs, whereas CD24 was not. In contrast to the finding that some of the B and myeloid antigens were found on FDCs, all T lineage-restricted antigens, including CD3, CD6, and CD8, were negative. In addition, anti-NKH1, which uniquely stains resting and activated NK cells, did not bind to FDCs.

An interesting observation was that although FDCs shared antigenic properties with B and myeloid cells, they did not express the leukocyte common antigen (CD45 and CD45R). This molecule is a marker for most hematopoietic cells and is found on virtually all B, T, myeloid, and NK cells. Stein and colleagues (7) have previously

TABLE IV

Positive Reactivity of Antibodies with FDCs

Antigens	Example antibody
Lineage-restricted antigens:	
Myeloid lineage	
CD14	Mo2
CR3 (CD11b)	Mo1
63D3	63D3
B cell lineage	
CD19	B4
CR2 (CD21)	B2
Complement receptors, adhesion molecules, and Fc receptors:	
Complement receptors	
CR1 CD35 (C3bR)	543
CR2 CD21 (C3dR)	B2
CR3 CD11b (C3biR)	Mo1
Adhesion molecules	
VLA 3	J143
VLA 4	8F2
VLA 5	BIE5
VLA 6	GOH3
VLA β chain	4B4
ICAM-1	RR1
Fc receptors	
Low affinity IgE Fc receptor (CD23)	Blast 2
Not lineage restricted:	
DRC-1	R4/23
CD37	G28-1
CDw40	G28-5
HLA Class I	W6/32
HLA Class II	6/7

TABLE V

Negative Reactivity of Antibodies with FDCs

Lineage-restricted antigens	Example antibody	Adhesion molecules and Fc receptors	Example antibody
T cell lineage		LFA-1α (CD11a)	2F12
CD1a	T6	p150/95 (CD11c)	
CD1b	T009	β chain of CD11a,	
CD2	T11	CD11b and CD11c	10F12
CD3	T3	gpIIb/IIIa (CDw41)	
CD6	2H1	VLA 1	TS
CD7	T159	40-kD FcR (CD32)	IV-3 D3
CD8	T8	Low affinity FcR	
B cell lineage		(CD16)	Leu-11b
CD20	B1		
CD22	G28-7, HD39		
B5	B5		
B7	B7		
Surface IgG			
Surface IgD			
Surface IgM			
Surface κ			
Surface λ			
PCA-1			
Myeloid lineage			
CD13	My7		
CD33	My9		
CD34	12-8		
CD38	5D2		
CD39	G28-8		
NK lineage			
NKH-1			
Lymphoid lineage			
CD45 (LCA)	T200		
CD45R (LCAr)	2H4		
Not lineage restricted			
CD1c	T024		
CD4	T4		
CD5	T1		
CD9	J2		
CD10	J5		
CD24	HB8		
CD25	IL-2R		
CD30	RSCI		
Other lineages			
Factor VIII (endothelial)			
EMA			

described an antigen, termed DRC-1, that is found on all FDCs as well as on a subset of B cells. We observed that DRC-1 was strongly expressed on all FDCs, confirming the identity of these cells. In addition, some low density B cells were positive for DRC-1. Anti-CD30 directed against Reed-Sternberg cells was also unreactive with FDCs. This observation is of interest since the Reed-Steinberg cells have a density

and morphology similar to FDCs (15). Evidence that FDCs were not of epithelial or endothelial origin is derived from their lack of reactivity with mAbs against the epithelial membrane antigen (EMA) and factor VIII, respectively. Anti-CALLA has been previously shown to stain a population of cells in the germinal center (16) which to date has not been identified. Isolated FDCs were common acute lymphoblastic leukemia antigen (CALLA) negative. These data provided support for the idea that FDCs, although expressing B and myeloid markers, represent a unique cell type.

Since FDCs fix and retain immune complexes, we examined these cells for the expression of complement and Fc receptors. As stated above, FDCs demonstrated very strong reactivity with mAbs against the three receptors for the third complement component, CR1, CR2, and CR3 (Table IV). In addition, the low affinity IgE Fc receptor (CD23) was strongly positive on FDCs, which was of interest since this receptor is expressed on activated B cells and monocytes. However, it was surprising to observe that the low affinity and the 40-kD Fc receptors (CD16 and CDw32) were not found on FDCs.

To investigate if FDCs have cell surface structures that might regulate their adhesion properties in the germinal center microenvironment, mAbs against intercellular and cell-matrix adhesion molecules were examined. As seen in Tables IV and V, the majority of molecules belonging to the three subgroups of the integrin superfamily were tested. FDCs expressed most of the adhesion proteins of the VLA family, including VLA-3, -4, -5, -6, and their common β chain, whereas VLA-1 was negative. The second subgroup of the integrins includes LFA-1, Mol, and p150/95, which form a group of heterodimer proteins with a homologous α chain, CD11a, CD11b, and CD11c, and a common β chain (CD18). In contrast to the broad expression with VLA proteins, only the α chain of Mol (CD11b) was positive. A very interesting observation was that antibodies against the corresponding β chain of this molecule did not bind to FDCs, whereas reactivity was seen with other lymphocytes. This negative result is based upon lack of staining with two anti-CD18 mAbs and a heteroantisera against CD18. Although LFA-1 was not expressed on FDCs, its natural ligand ICAM-1 was strongly positive. GpIIb/IIIa, which is a member of the third subgroup of integrins, was also not detected on FDCs. However, this observation was not surprising since this molecule is selectively expressed on platelets.

Phagocytic Activity and Cytochemical Staining. To determine if FDCs not only express some monocyte antigens but also behave functionally like monocytes, the ability of these cells to phagocytize opsonized human RBCs was examined. FDCs incubated with RBCs for 3 h at 37°C failed to ingest RBCs, whereas control monocytes demonstrated avid phagocytosis. The cytochemical pattern of FDCs, however, was similar to that of monocytes. FDCs were weakly stained for monocyte-associated nonspecific esterase, whereas they did not contain granulocyte-restricted specific esterase and peroxidase.

Isolation of Homogeneous Populations of FDCs. To obtain homogeneous cell populations that would allow to study the gene expression of these cells, an isolation procedure based on the unique antigenic phenotype of FDCs was performed. Using CD14 as a marker for flow cytometric cell sorting, FDCs were further purified from low density cells (4–6% FDCs). This antigen was selected because it was strongly positive on all FDCs of the 45 tonsil preparations examined by in situ staining. In con-

trast, mAbs against CD14 showed a significantly weaker binding to monocytes, macrophages, and granulocytes and were completely negative on lymphocytes.

Using flow cytometry, FDCs could be detected by their very strong staining with anti-CD14. Moreover, by examining both parameters of fluorescence intensity and log 90° light scatter, a well-demarcated FDC population could be identified (Fig. 2 *A*). As seen in Fig. 2 *A*, brightly stained CD14$^+$ FDCs (4–7%) could be distinguished from a population (4–5%) expressing lower levels of CD14 (Fig. 2 *A*). By flow cytometry cell sorting with parameters specific for FDCs, an enriched cell population (~70%) was obtained (Table III). To isolate a homogeneous FDC preparation, these partially purified cells were resorted using the identical parameters. As seen in Table III and depicted in Fig. 2 *B*, this procedure resulted in a 98.8% pure cell preparation. As shown in Fig. 3, the highly purified cells had a large cytoplasm and some of the cells contained two nuclei, which is a morphology identical to the one of FDCs in tonsillar low density cell preparations. This also demonstrated that the sorted cells were FDCs. In an attempt to confirm the purity and antigenic phenotype of this homogeneous FDC preparation, cells were labeled with biotinylated anti-CD21 (Fig. 2 *C*) or anti-CD20 (Fig. 2 *D*) mAbs and stained with streptavidin-phycoerythrin. As seen in Fig. 2 *C*, these cells uniformly expressed CD21, whereas they were totally unreactive with anti-CD20 (Fig. 2 *D*). This demonstrates that the FDC preparation did not contain monocytes or B cells. Moreover, these findings confirm the in situ observation that FDCs coexpress CD14 and CD21 yet lack CD20.

Analysis of IL-6 Expression in FDCs. To further examine the role of FDCs within the lymphoid follicle, it was of interest to examine whether these cells produce IL-6. This cytokine was selected since it has been shown to be important in terminal B cell differentiation (17). To test if FDCs express the IL-6 protein, immunostaining with rabbit antiserum to IL-6 was performed. The antibody strongly stained the cytoplasm of activated tonsillar B lymphocytes but was unreactive with FDCs (data

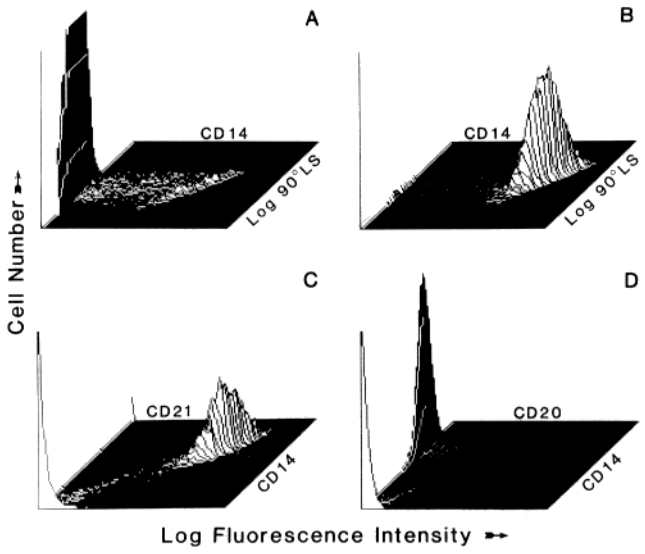

FIGURE 2. Cell sorting of FDCs to homogeneity and double labeling of highly purified FDCs. (*A*) Staining profile of tonsillar low density cell preparation with anti-CD14. (*B*) 98.8% pure FDC preparation obtained after cell sorting of highly CD14$^+$ low density cells shown in *A*. (*C*) Coexpression of CD14 and CD21 on highly purified FDCs. (*D*) Lack of reactivity of homogeneous FDCs with anti-CD20.

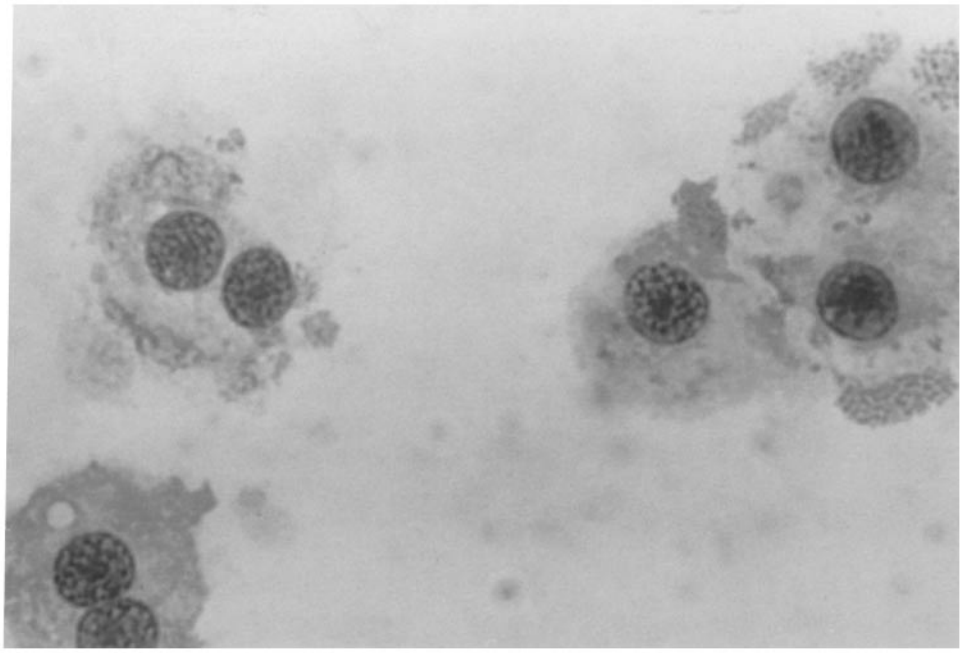

FIGURE 3. Morphology of highly purified FDCs with Wright Giemsa stain.

not shown). In an attempt to determine whether FDCs expressed IL-6 mRNA, PCR-amplified cDNA from highly purified FDCs was examined. Activated B cells were used as a positive control cell preparation (18). Because of its abundant and uniform cellular expression, aldolase A was selected as a positive control. Fig. 4 shows the ethidium bromide staining pattern and the corresponding Southern blot analysis of PCR product DNA obtained for IL-6 and aldolase A. Activated B cells were IL-6^+ (Fig. 4, *A* and *B*, lane *3*), whereas FDCs were negative (Fig. 4, *A* and *B*, lane *4*). As seen in Fig. 4, *A* and *B* (lanes *1* and *2*), both activated B cells and FDCs expressed aldolase A PCR product DNA.

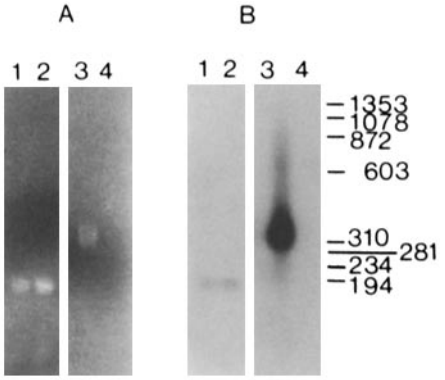

FIGURE 4. IL-6 and aldolase A gene expression of highly purified FDCs. cDNA produced by PCR from activated tonsillar B cells (lanes *1* and *3*) and highly purified FDCs (lanes *2* and *4*). cDNA was amplified using primers specific for aldolase A (lanes *1* and *2*) and for IL-6 (lanes *3* and *4*). (*A*) Electrophoretic analysis of the PCR products. (*B*) Southern analysis of the gel using a ^{32}P-labeled oligonucleotide probe for aldolase A (lanes *1* and *2*) and a ^{32}P-labeled cDNA probe for IL-6 (lanes *3* and *4*). The molecular sizes of a Hae III digest of ϕX174 are given on the right side of the figure.

Discussion

In the present report, FDCs were purified in an attempt to examine the expression of cellular antigens that define lineage and function. In addition, these cells were isolated to homogeneity in order to study their gene expression. FDCs were identified by their characteristic morphology and by their expression of receptors for the third complement component, the myeloid-restricted antigen CD14, and the FDC antigen DRC-1. Unclustered FDCs displayed a unique antigenic phenotype since they expressed several B and myeloid lineage-restricted antigens, but lacked T and NK cell antigens as well as the leukocyte common antigen. FDCs expressed adhesion molecules, including most of the VLA proteins, ICAM-1, and CD11b. FDCs could be isolated to homogeneity by their intense staining with anti-CD14 using flow cytometric cell sorting. These highly purified FDCs expressed CD14 and CD21 but lacked CD20. This antigen pattern and characteristic morphology confirmed that these cells were, in fact, homogeneous FDC preparations. Analysis of the cDNA from these highly purified cells demonstrated no mRNA for IL-6. The study suggests that FDCs may belong to a unique cellular population.

Immunostaining of FDC clusters, both in situ and in suspension, have provoked controversy. Gerdes et al. (7) observed that human FDC clusters were positive for CRs, class I and II molecules, and monocyte markers, and therefore concluded that these cells were most closely related to monocytes or macrophages. Humphrey et al. (19) challenged these findings by the observation that murine FDCs do not express Ia and macrophage markers and that they do not originate from the bone marrow. In the present study FDCs could be separated from adherent B cells, and therefore, it was possible to more precisely demonstrate the expression of lineage-restricted antigens. FDCs had an antigenic phenotype uncommon to any of the known hematopoietic cells. They shared several myeloid (CD14, CD11b, 63D3) and B (CD19, CD21) lineage-restricted antigens and did not express any of the known T or NK cell markers. However, FDCs lacked several other important B- (sIg, CD20, CD22, B5, B7) and myeloid (CD13, CD33, CD34, CD38, CD39)-restricted antigens. Therefore, the expression of B- and myeloid-restricted antigens may not be sufficient to define the lineage of these cells. Cytochemical staining and lack of phagocytosis further suggests that these cells only partially resemble myeloid cells. The finding that FDCs were not positive for the common leukocyte marker CD45 supports the hypothesis that human FDCs do not originate from bone marrow-derived hematopoietic cells. Moreover, FDCs do not appear to be of epithelial or endothelial origin. Therefore, our results are consistent with the hypothesis that FDCs may not belong to a classical hematopoietic cell lineage.

Since FDCs create a three-dimensional network with B cells within the germinal center and in tissue culture, we attempted to examine those cellular antigens that might be involved in interactions between these cells. The ability to bind and present antigen-antibody-complement complexes to B lymphocytes appears to be an important function of the FDCs (19–22). FDCs displayed a unique antigenic pattern by expressing all three receptors for the third complement component. In addition, one major feature of the FDCs appears to be their ability to bind to germinal center B lymphocytes. FDCs expressed the cell-cell adhesion molecules ICAM-1 and CD11b, which is consistent with previous in situ observations (23, 7). Our results imply that the binding of FDCs to B cells is a dynamic, temperature-dependent process that

is regulated by Ca^{2+} and Mg^{2+}. These conditions are crucial for the function of cell-cell adhesion molecules, as shown for binding of LFA-1^+ lymphocytes to ICAM-1 (24). Although cluster formation could not be inhibited by using mAbs against these molecules, it can not be excluded that these receptors, in addition with other adhesive proteins, might indeed regulate B-FDC adhesion.

The observation that FDCs were stained with mAbs against VLA proteins demonstrates that FDCs apparently express receptors for laminin, fibronectin, and collagen. These proteins belong to a group of heterodimers with unique α chains and a common β chain (25), and some of them are found on a large variety of cell types (26). One of their major functions seems to be to mediate the adhesion of leukocytes to extracellular matrices (27). VLA-4 differs from the other members of the VLA family since it may have cell-cell adhesion functions (28). The fact that FDCs were positive for nearly all of the VLA proteins suggests that these receptors might play a role for FDC function.

FDCs and lymphoid dendritic cells (interdigitating reticulum cells) are morphologically similar, low density cells. Both cell types provide in vivo and in vitro a complex microenvironment for lymphoid cells by forming extensive networks with cellular processes (29–31). This report, however, demonstrated that FDCs could be identified as a unique cell type with phenotypic characteristics that were distinct from those of lymphoid dendritic cells. In contrast to lymphoid dendritic cells, which contain mostly T cells in their in vitro aggregations (31), FDCs had B cells bound within their processes. In addition, the immunophenotypic profile of tonsillar FDCs differs significantly from that of isolated lymphoid dendritic cells. The latter cells were negative for most of the markers that are expressed on FDCs. As reported by Hart and McKenzie (6), lymphoid dendritic cells were not stained, for example, by mAbs against the complement receptors CR1, CR2, and CR3, the myeloid markers CD14 and 63D3, and the FDC antigen DRC-1. Moreover, lymphoid dendritic cells, in contrast to FDCs, did express the common leukocyte antigens CD45 and CD45R. This observation supports the notion that these cells might represent distinct cell entities with different cellular origins. This hypothesis is supported by the finding that lymphoid dendritic cells originate from the bone marrow (1), in contrast to FDCs, as observed by Humphrey et al. (19).

Several observations deserve further comment. Other investigators have reported that FDC clusters express surface Ig and CALLA (7), whereas in this study, singly dispersed FDCs were negative for these markers. It was of great interest that CD11b was clearly expressed on FDCs, but its corresponding β chain (CD18) could not be detected. A possible explanation for this observation might be that the level of CD18 was too low to be detected by in situ staining technique. It was also surprising to note that although FDCs fix immune complexes, they did not express the 40-kD Fc receptor and the low affinity Fc receptor. However, FDCs did have low affinity Fc IgE receptors (CD23). The importance of this Fc receptor to FDC function is still unknown.

Finally, isolation of homogeneous populations of FDCs will facilitate functional characterization of these cells. It should now be possible to determine whether these cells produce cytokines important for B cell differentiation. Although highly purified FDCs do not seem to produce IL-6, these cells might secrete this or other cytokines after appropriate stimulation. In addition to the examination of the cytokine produc-

tion of these cells, it will be important to study the structures that regulate FDC function and that are involved in cell-cell adhesion. In the future, it will also be necessary to confirm the antigenic phenotype of the FDCs by detecting the expression of the corresponding genes.

Summary

In the present study, follicular dendritic cells (FDCs) were purified to homogeneity in order to define the lineage and function of these cells. FDCs were identified by their characteristic morphology and by their expression of receptors for the third complement component, the myeloid-restricted antigen CD14, and the FDC antigen DRC-1. Unclustered FDCs displayed a unique antigenic phenotype since they expressed several B- and myeloid lineage-restricted antigens, but lacked T and NK cell antigens as well as the leukocyte common antigen. FDCs expressed adhesion molecules, including most of the VLA proteins, intercellular adhesion molecule 1 (ICAM-1), and CD11b. FDCs could be isolated to homogeneity by their intense staining with anti-CD14 using flow cytometric cell sorting. These highly purified FDCs expressed CD14 and CD21 but lacked CD20. This antigen pattern and characteristic morphology confirmed that these cells were, in fact, homogeneous FDC preparations. Analysis of polymerase chain reaction-amplified cDNA from highly purified FDCs showed no transcripts for IL-6. The isolation of homogeneous FDC populations will be important for the analysis of the functional role of FDCs within the lymphoid follicle.

The very helpful discussions with M. Hemler and H. Stein are greatly acknowledged. We thank H. Levine for his excellent help and Jeffrey Segil and James Whitman for their technical assistance. We thank G. Wang for preparing the manuscript.

Received for publication 23 February 1989.

Note added in proof: FDCs were also tested for reactivity with the blind panel antibodies of the Fourth International Workshop on Human Leukocyte Differentiation Antigens. The corresponding antigens were revealed after submission of the manuscript. In contrast to the findings presented in this study, FDCs were stained by the blind panel antibodies against surface κ and surface IgM.

References

1. Steinman, R. M., and Z. A. Cohn. 1973. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J. Exp. Med.* 137:1142.
2. Steinman, R. M., G. Kaplan, M. D. Witmer, and Z. A. Cohn. 1979. Identification of novel cell type in peripheral lymphoid organs of mice. *J. Exp. Med.* 149:1.
3. Inaba, K., M. D. Witmer-Pack, M. Inaba, S. Muramatsu, and R. M. Steinman. 1988. The function of Ia^+ dendritic cells and Ia^- dendritic cell precursors in thymocyte mitogenesis to lectin and lectin plus interleukin 1. *J. Exp. Med.* 167:149.
4. Inaba, K., R. M. Steinman, W. C. Van Voorhis, and S. Muramatsu. 1983. Dendritic cells are critical accessory cells for thymus-dependent antibody responses in mouse and in man. *Proc. Natl. Acad. Sci. USA.* 80:6041.
5. Steinman, R. M., B. Gutchinov, M. D. Witmer, and M. C. Nussenzweig. 1983. Den-

dritic cells are the principal stimulators of the primary mixed leukocyte reaction in mice. *J. Exp. Med.* 157:613.

6. Hart, DN., and J. L. McKenzie. 1988. Isolation and characterization of human tonsil dendritic cells. *J. Exp. Med.* 168:157.
7. Gerdes, J., H. Stein, D. Y. Mason, and A. Ziegler. 1983. Human dendritic reticulum cells of lymphoid follicles: their antigenic profile and their identification as multinucleated giant cells. *Virchows Arch. B.* 42:161.
8. Buckley, P. J., M. R. Smith, M. F. Braverman, and S. A. Dickson. 1987. Human spleen contains phenotypic subsets of macrophages and dendritic cells that occupy discrete microanatomic locations. *Am. J. Pathol.* 128:505.
9. Wood, G. S., R. R. Turner, R. A. Shiuba, L. Eng, and R. A. Warnke. 1985. Human dendritic cells and macrophages. *Am. J. Pathol.* 119:73.
10. Daley, J. F. 1988. Immunofluorescent reagents: preparation and analysis. *Lect. Notes Coastal Estuarine Stud.* 25:167.
11. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156.
12. Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science (Wash. DC).* 239:487.
13. Hirano, T., K. Yasukawa, H. Harada, T. Taga, Y. Watanaba, T. Matsuda, S.-I. Kashiwamura, K. Nakajima, K. Koyama, A. Iwamatsu, S. Tsunasawa, F. Sakiyama, H. Matsui, Y. Takahara, T. Taniguchi, and T. Kishimoto. 1986. Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. *Nature (Lond.).* 324:73.
14. Chelly, J., J.-C. Kaplan, P. Maire, S. Gautron, and A. Kahn. 1988. Transcription of the dystrophin gene in human muscle and non-muscle tissues. *Nature (Lond.).* 333:858.
15. Cotner, T., A. Das Gupta, T. Papayannopoulou, and G. Stamatoyannopoulos. 1988. Isolation of Reed-Steinberg cells from lymph nodes of Hodgkin's disease patients. *Blood.* 73:222.
16. Nadler, L. M. 1986. B cell/leukemia panel workshop: summary and comments. *In* Leukocyte Typing II: Human B Lymphocytes. E. L. Reinherz, B. F. Haynes, L. M. Nadler, and I. D. Bernstein, editors. Oxford University Press, Oxford. 3–43.
17. Muraguchi, A., T. Hirano, B. Tang, T. Matsuda, Y. Horii, K. Nakajima, and T. Kishimoto. 1988. The essential role of B cell stimulatory factor 2 (BSF-2/Il-6) for the terminal differentiation of B cells. *J. Exp. Med.* 167:332.
18. Freeman, G. J., A. S. Freedman, S. N. Rabinowe, J. M. Segil, J. Horowitz, K. Rosen, J. F. Whitman, and L. M. Nadler. 1989. Interleukin 6 gene expression in normal and neoplastic B cells. *J. Clin. Invest.* In press.
19. Humphrey, J. H., D. Grennan, and V. Sundaram. 1984. The origin of follicular dendritic cells in the mouse and the mechanism of trapping of immune complexes on them. *Eur. J. Immunol.* 14:859.
20. Szakal, A. K., M. H. Kosco, and J. G. Tew. 1988. A novel in vivo follicular dendritic cell-dependent iccosome-mediated mechanism for delivery of antigen to antigen-processing cells. *J. Immunol.* 140:341.
21. Schnizlein, C. T., M. H. Kosco, A. K. Szakal, and J. G. Tew. 1985. Follicular dendritic cells in suspension: identification, enrichment, and initial characterization indicating immune complex trapping and lack of adherence and phagocytic activity. *J. Immunol.* 134:1360.
22. Heinen, E., N. Braun, P. G. Couile, J. Van Snick, M. Moeremans, N. Cormann, C. Kinet-Denoel, and L. J. Simar. 1986. Transfer of immune complexes from lymphocytes to follicular dendritic cells. *Eur. J. Immunol.* 16:167.

23. Dustin, M. L., R. Rothlein, A. K. Bhan, C. A. Dinarello, and T. A. Springer. 1986. Induction by IL 1 and interferon-γ: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). *J. Immunol.* 137:245.
24. Marlin, S. D., and T. A. Springer. 1987. Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). *Cell.* 51:813.
25. Hemler, M. E. 1987. The VLA Protein Family. *J. Biol. Chem.* 262:3300.
26. Hemler, M. E., C. Huang, Y. Takada, L. Schwarz, J. L. Strominger, and M. L. Clabby. 1987. Characterization of the cell surface heterodimer VLA-4 and related peptides. *J. Biol. Chem.* 262:11478.
27. Hemler, M. E. 1988. Adhesive protein receptors on hematopoietic cells. *Immunol. Today.* 9:109.
28. Takada, Y., M. J. Elices, C. Crouse, and M. E. Hemler. 1989. The primary structure of the α^4 subunit of VLA-4: homology to other integrins and a possible cell-cell adhesion function. *EMBO (Eur. Mol. Biol. Organ.) J.* In press.
29. Heinen, E., C. Lilet-Leclercq, D. Y. Mason, H. Stein, J. Boniver, D. Radoux, C. Kinet-Denoel, and L. J. Simar. 1984. Isolation of follicular dendritic cells from human tonsils and adenoids II. Immunocytochemical characterization. *Eur. J. Immunol.* 14:267.
30. Lilet-Leclercq, C., D. Radoux, E. Heinen, C. Kinet-Denoel, J. Defraigne, M. Houben-Defresne, and L. J. Simar. 1984. Isolation of follicular dendritic cells from human tonsils and adenoids. I. procedure and morphological characterization. *J. Immunol.* 66:235.
31. Tsai, V., and N. J. Zvaifler. 1988. Dendritic cell-lymphocyte clusters that form spontaneously in rheumatoid arthritis synovial effusions differ from clusters formed in human mixed leukocyte reactions. *J. Clin. Invest.* 82:17314.