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Research Article

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Neutrophil and Monocyte Cell Surface p150,95 Has iC3b-Receptor (CR_4) Activity Resembling CR_3

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Abstract

Previous investigations of p150,95 (CD11c), the third member of the CD18 membrane glycoprotein family that includes CR_3 (Mac-1 or CD11b) and LFA-1 (CD11a), had demonstrated that solubilized p150,95 bound to iC3b-agarose in a manner similar to isolated CR_3. The current study showed that membrane surface p150,95 also expressed iC3b-receptor activity and was probably the same as the neutrophil receptor for iC3b- or C3dg-coated erythrocytes (EC3bi or EC3dg) that had been previously designated CR_4. Normal neutrophil and macrophage CR_4-dependent EC3bi rosettes were inhibited by monoclonal anti-p150,95, and cells from a patient with CD18 deficiency did not form CR_4-dependent EC3bi rosettes. With neutrophils that bore large amounts of CR_1 and CR_3 and little p150,95, EC3bi were bound primarily via CR_1 and CR_3, and demonstration of p150,95-dependent rosettes required large amounts of fixed iC3b, low-ionic strength buffer, and antibody blockade of CR_1 and CR_3. By contrast, culture-derived macrophages expressed eight times more p150,95 than did monocytes and EC3bi were bound to both p150,95 and CR_3 when EC3bi bore small amounts of fixed iC3b and assays were carried out in isotonic buffer. Comparison of the amounts of CR_1, CR_3, and CR_4 in various tissues by immunoperoxidase staining revealed that CR_4 was the most abundant C3 receptor molecule on tissue macrophages, and suggested that CR_4 might be involved in clearance of C3-opsonized particles or immune complexes.

Introduction

The membrane glycoprotein p150,95 (CD11c; 150 kD α-chain linked noncovalently to 95 kD β-chain) is expressed on phagocytic cells, natural killer (NK)[1] cells, and certain cytotoxic T cells (1–3). The 95 kD β-chain (CD18) is structurally identical to the β-chains of two other related leukocyte membrane glycoproteins, LFA-1 (lymphocyte function-associated antigen 1 or CD11a) and CR_3 (complement receptor type 3, Mac-1, or CD11b) that have structurally distinct α-chains of 175 and 165 kD, respectively (4). Much interest has focused on this family of three surface glycoproteins ("CD18 complex") with the identification of several patients who have an inherited deficiency of the entire CD18 complex and suffer from recurrent bacterial infections (4–6). Recently, nucleotide sequence analysis has indicated that the three members of the CD18 complex are members of a larger supergene family of seven other membrane glycoproteins that includes the five VLA glycoproteins, the vitronectin receptor and platelet glycoprotein GPIIb/IIIa (7–9). All ten of these glycoproteins are either receptors or have adhesion-related functions, and several of the ligands for the receptors that have been characterized are bound via a similar Arg-Gly-Asp (RGD) tripeptide structure (10). Among CD18 family members, CR_3 has been shown to recognize an RGD peptide within iC3b (11).

The functions of p150,95 are just beginning to be defined. Examination of CD18-deficient neutrophils or normal neutrophils treated with antibodies specific for individual α- or β-chains suggests that both CR_3 and p150,95 have common functions in mediating neutrophil and monocyte adherence to protein-coated glass (12) and endothelial cells (13, 14). Furthermore, the adherence step involved in conjugate formation between cytotoxic T cells and target cells has recently been shown to be inhibitable by antibodies to the α-chain of p150,95 (15) as well as by antibodies to the α-chain of LFA-1 (16) or the common β-chain (15, 16). With neutrophils and freshly isolated monocytes that express large amounts of CR_3 and small amounts of p150,95 and LFA-1, CR_3 has been shown to be the major receptor for fixed iC3b (17, 18–20). Recently, however, detergent solubilized p150,95 from U937 cells was shown either to coelute with CR_3 from iC3b-agarose affinity columns (21), or to be purified on iC3b-agarose after selective removal of CR_3 by immunoprecipitation (22), suggesting that it might function as an iC3b-receptor analogous to CR_3. Up until this time, however, attempts to demonstrate

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1. *Abbreviations used in this paper:* BDVA, low-ionic strength rosetting buffer consisting of 1% bovine serum albumin (BSA)/3.2% dextrose/3.5 mM veronal buffer pH 7.2/10 mM sodium chloride/10 mM sodium azide/2 mM each of calcium chloride and magnesium chloride, 4.5 mS conductivity at 22°C; CD18, the common β-chain shared by each of the three members of CD11a/CD11b/CD11c membrane glycoprotein family consisting of LFA-1 (lymphocyte function-associated antigen 1 or CD11a), CR_3 (complement receptor type three, same as Mac-1 or CD11b, specific for iC3b), and CR_4 (complement receptor type four, same as p150,95, Leu M5, or CD11c, specific for iC3b); CR_1, complement receptor type one (specific for C3b, C4b, and iC3b); HBSS/BSA, Hanks' balanced salt solution containing 1% BSA; MAb, monoclonal antibody; NK cells, natural killer cells.

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anti-p150,95 inhibitable iC3b-receptor activity on intact cells had been unsuccessful (2, 22).

In the current study, a receptor for fixed iC3b on sheep erythrocytes (EC3bi) was demonstrated on neutrophils and monocyte-derived macrophages that was not inhibited by a polyclonal mixture of anti-CR_1 and anti-CR_3 antibodies. This receptor activity on neutrophils appears to be the same as the receptor for EC3bi and EC3dg rosettes that had been previously designated CR_4 (23). CR_4 activity was weak and variable on neutrophils and freshly isolated monocytes, and seemed to correspond to low levels of p150,95 expression. By contrast, macrophages generated by 7-d culture of monocytes expressed avid CR_4 rosetting activity and large amounts of p150,95. With both cell types, CR_4 rosetting activity was inhibited by antibodies to the α-chain of p150,95, suggesting that cell surface p150,95 is probably identical to CR_4.

Methods

Neutrophils and monocytes. Blood was obtained from normal volunteers or a patient with inherited deficiency of LFA-1/CR_3/p150,95 (5). Mononuclear cells were isolated by dextran sedimentation and Ficoll-Hypaque centrifugation (24), and cultured for 7 d, either as nonadherent cells in Teflon beakers (25), or as adherent cells in either 24-well plates (24, 26) or 4-well Lab-Tek slide chambers (Miles Scientific, Naperville, IL). For the slide chambers, 2×10^6 mononuclear cells were added to each well. Cells cultured for 7 d in Teflon beakers were transferred to slide chambers coated with either mouse myeloma IgG or monoclonal antibodies (MAbs) (19, 25). Lymphocytes were removed by this adherence step. Neutrophils were prepared either from the Ficoll-Hypaque pellet generated during monocyte isolation (using ammonium chloride for lysis of erythrocytes), or by two-step Ficoll-Hypaque gradients (17). Neutrophils were tested either in suspension or after allowing adherence for 1 h to 8-well slide chambers (5×10^5 neutrophils/well in 200 μl of Hanks' balanced salt solution containing 1% bovine serum albumin; HBSS/BSA).

Antibodies. Rabbit $F(ab')_2$ anti-CR_1 was used to block CR_1 rosettes (27), and E11 MAb anti-CR_1 was used to quantitate CR_1 expression (28). MAbs to CR_3 α-chain included MN-41 (20, 29), from Dr. Alfred Michael and Dr. Allison Eddy (University of Minnesota, Minneapolis), anti-Mac-1 (18), hybridoma provided by Dr. Timothy Springer (Dana-Farber Cancer Institute, Boston, MA), anti-Mo1 (30), from Dr. Robert Todd (University of Michigan, Ann Arbor), and the IgG_1 MAb 44 (22). MAbs to p150,95 α-chain included anti-Leu M5 (2) and L29 from Dr. Lewis Lanier of Becton Dickinson Monoclonal Center (Mountain View, CA), and the IgG_1 mAb 3.9 (31). L29 is an IgG_1 MAb that was generated by immunization of Balb/c mice with human peripheral blood lymphocytes enriched for NK cells by Percoll density gradient centrifugation (32). Immune splenocytes were fused with SP2/0 Ag14 myeloma cells, and hybridomas were selected in azaserine. L29 has been designated as CD11c reactive (33) on the following basis: (*a*) identical size α- and β-chains precipitated by L29 and anti-Leu M5, (*b*) identical immunofluorescence flow cytometry histogram as anti-Leu M5 with neutrophils and mononuclear cells, (*c*) absent fluorescence staining of cells from two patients with inherited deficiency of LFA-1/CR_3/p150,95, (*d*) inhibition of EC3bi rosettes in a manner similar to both anti-Leu M5 and 3.9. The epitope specificity of anti-p150,95 MAbs was evaluated by cross-blocking experiments in which one MAb labeled with ^{125}I was tested for uptake onto neutrophils or U937 cells that had been incubated with a 100-fold molar excess of unlabeled homologous or nonhomologous MAb (34). Control nonspecific myeloma proteins included IgG_1 MOPC-21 (Litton Bionetics, Charleston, SC) and IgG_{2a} P1.17 (myeloma line obtained from the American Type Culture Collection, Rockville, MD). The murine hybridoma ATCC HB-120 (BB7.5) secreting IgG_1 specific for MHC class I antigens (HLA-A, B, C) was kindly provided by Dr. Jeffrey Frelinger, University of North Carolina at Chapel Hill (35). Each monoclonal immunoglobulin (except for those received as IgG) was purified from ascites fluid or culture supernatant by high performance liquid chromatography using cation exchange (Protein Pak SP-5PW; Waters Associates, Milford, MA) with 10 mM sodium acetate pH 5.5 and a sodium chloride gradient, followed by anion exchange (Protein Pak DEAE-5PW; Waters Associates) with 20 mM Tris/HCl pH 8.5 and a sodium chloride gradient (36).

Preparation of EC3. EC3b and EC3bi were prepared as previously described, followed by analysis of bound C3 fragments with ^{125}I-labeled MAbs to C3c, C3g, and C3d (17). For assays of neutrophils and monocytes, EC3bi with $4.5\text{-}6.5 \times 10^4$ molecules of iC3b/E were used. With monocyte-derived macrophages, EC3bi with $1.2\text{-}1.8 \times 10^4$ molecules of iC3b/E were used.

EC3 rosette assay. Neutrophils in suspension were tested for rosettes as before (17). Adherent neutrophils or monocytes in 8-well slide chambers were washed three times and reconstituted with 140 μl/well of low-ionic strength BDVA buffer (1% BSA/3.2% dextrose/3.5 mM veronal buffer/10 mM sodium chloride-/10 mM sodium azide/2 mM each of calcium chloride and magnesium chloride, pH 7.2, 4.5 mS at 22°C). With macrophages in 4-well chambers, 225 μl of isotonic HBSS/BSA was used instead of BDVA. Next, 100 μl of EC3bi (2×10^8/ml in BDVA or HBSS/BSA) was added to each well, and after 15 min at 37°C, the wells were washed to remove unbound EC3bi, the partitioned top chamber was removed, and a 50×22 mm coverslip was placed over the adherent cells on the glass slide. Rosettes were observed by phase contrast microscopy with cells bearing ≥ 4 bound EC3bi scored as positive. Neutrophil assays included 1 mg/ml of soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) to prevent elastase proteolysis of iC3b on the EC3bi (37).

For studies of antibody blockade of rosettes, adherent phagocytes were incubated for 20 min with antibodies, the volume was brought up to 140 μl (8-well chambers) or 225 μl (4-well chambers) with buffer, and 100 μl of EC3bi were added for rosette assay without washing away unbound antibodies. With neutrophils in suspension, assays were also carried out with excess fluid phase antibodies (20). The amount of $F(ab')_2$ anti-CR_1 used was sufficient to block all monocyte and neutrophil rosettes with EC3b (17). To determine the appropriate amount of each anti-CR_3 MAb, titrations for inhibition of rosette formation were carried out with each phagocytic cell type (results shown for adherent neutrophils only, Fig. 1). For subsequent assays of CR_4 rosetting activity, amounts of anti-CR_3 MAbs were used that were 4- to 10-fold greater than the amounts producing maximum plateau levels of inhibition of EC3bi rosette formation in the presence or absence of excess rabbit $F(ab')_2$ anti-CR_1. As an alternative procedure for demonstration of p150,95-dependent rosettes, surface p150,95 on monocytes cultured for 7 d in Teflon beakers was modulated from the apical surfaces of cells by 45 min cultivation in slide chambers coated with monoclonal anti-p150,95 attached via poly L-lysine and glutaraldehyde (19, 25). Surfaces coated with MAb anti-CR_3, anti-MHC class I, or myeloma IgG served as controls. MAbs to CR_3 and/or p150,95 were then added in the fluid phase prior to EC3bi addition.

Quantitation of CR_1, CR_3, and p150,95. Neutrophils were tested in suspension for uptake of ^{125}I-MAbs (34). Serial dilutions of ^{125}I-MAbs were tested, and the number of antigen sites per cell was determined by Scatchard analysis using the Ligand program (Biomedical Computing Technology Information Centre, Nashville, TN). Monocytes cultured in 24-well plates were examined for uptake of ^{125}I-MAbs within 2 h after adherence and again after cultivation for 7 d. The number of adherent cells per well at the time of assay was determined by Coulter counting of nuclei released with Zapoglobin (Coulter Electronics, Hialeah, FL) from triplicate wells incubated with buffer instead of ^{125}I-MAb. The amount of ^{125}I-MAb bound in the presence of a 100-fold molar excess of homologous unlabeled MAb was subtracted from the total amount of bound ^{125}I-MAb to determine the amount of specifically bound ^{125}I-MAb. With each mAb (E11, MN-41, and 3.9) titration studies showed that 400 ng of ^{125}I-MAb in 300 μl of HBSS/BSA was saturating. Cells were first incubated with either unlabeled MAb or

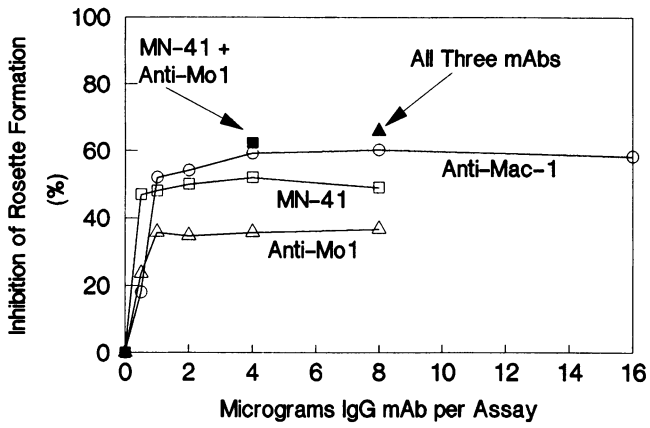

Figure 1. Titration of anti-CR_3 MAbs for inhibition of adherent neutrophil EC3bi rosette formation. The results plotted are the mean of three assays of adherent neutrophils with EC3bi bearing 5.7×10^4 iC3b/E in BDVA low-ionic strength buffer. Neutrophils adhered to eight-well slide chambers ($- 4 \times 10^4$ adherent neutrophils per chamber) were treated with $F(ab')_2$ anti-CR_1 (140 μg) plus the amount of each MAb listed in a total volume of 150 μl for 20 min before addition of 100 μl of EC3bi for assay of rosette formation. A mixture of 4 μg each of MN-41 and anti-Mol (*solid square*) was compared to the same mixture containing 8 μg of anti-Mac-1 (*solid triangle*). Similar results were obtained with each of the other phagocyte types (not shown), and thus the amounts of each anti-CR_3 MAb used in the experiments shown in Tables I–IV were sufficient to produce plateau levels of inhibition of CR_3 rosetting activity.

buffer for 20 min at 4°C, and then incubated with ^{125}I-labeled MAb for 20 min at 4°C. After four washes with HBSS/BSA, cells with bound ^{125}I-MAb were eluted for gamma scintillation counting with 1 ml of 0.05% Triton X-100, 1 ml of HBSS/BSA, and vigorous pipetting.

Immunoperoxidase labeling of monocytes and tissue sections. The following fresh-frozen tissues were obtained from the Imperial Cancer Research Fund Department of Histopathology: tonsil, spleen, thymus, lung, skin, brain, bronchus, and colon. All tissues were judged to be normal by histopathologists, with the exception of reactive tonsils, which were removed after antibiotic therapy of patients. All tissues were obtained from adults except tonsil and thymus obtained from children. Glass-adherent monocytes and 6 μm sections of acetone-fixed fresh-frozen tissues were subjected to indirect immunoperoxidase staining with MAbs as previously reported (28, 31). Briefly, samples were incubated at room temperature for 30 min with 50 μl of various IgG_1 MAb-containing supernatants, followed, after appropriate washing by 40 μl of peroxidase-conjugated goat antimouse immunoglobulin (1:100; Dakopatts, Copenhagen, Denmark). A positive reaction was revealed by a 7-min exposure to hydrogen peroxide (0.012%) and 3,3'-diaminobenzidene (0.6 mg/ml). Controls for the IgG_1 MAbs E11, 44 and 3.9 were a nonspecific IgG_1 MAb and omission of first layer antibodies.

Results

Expression of CR_4-dependent rosettes. CR_4-dependent rosettes were defined as EC3bi rosettes with neutrophils or monocytes that had been treated with amounts of anti-CR_1 and anti-CR_3 that were sufficient to inhibit CR_1- and CR_3-dependent rosettes completely. With neutrophils, such CR_4-dependent rosettes had been previously shown (23) to require > 45,000 molecules of iC3b or C3dg per EC3bi or EC3dg and a low-ionic strength buffer (BDVA). When neutrophils were examined for such CR_4-dependent rosettes in the present study, it was found that some of the rosettes formed in the presence of rabbit anti-CR_1 and saturating amounts of MN-41 anti-CR_3 could be blocked by further treatment of cells with saturating amounts of a second and/or third anti-CR_3 MAb such as anti-Mol and/or anti-Mac-1 (Fig. 1). For this reason, subsequent tests of putative CR_4-dependent rosettes were carried out with a mixture of rabbit anti-CR_1 and three anti-CR_3 MAbs (MN-41, anti-Mac-1, anti-Mol). When tested in this way, neutrophils from 6 of 14 donors formed ≤ 15% CR_4-dependent rosettes, i.e., nearly all rosettes were abolished by the anti-CR_1/CR_3 mixture. The results given in Table I are for the eight remaining neutrophil preparations giving ≥ 23% CR_4-dependent rosettes, as only these preparations were judged satisfactory for tests for inhibition of rosettes by anti-p150,95.

Freshly isolated monocytes tested on four occasions formed only 6–14% CR_4-dependent rosettes and so were not examined further. By contrast, macrophages generated by cultivation of adherent monocytes for 7 d formed much higher proportions of CR_4-dependent rosettes, even when EC3bi contained as few as 12,000 iC3b per E and assays were carried in isotonic buffer (Table II).

Both CR_3 and CR_4 had similar requirements for divalent cations, because all EC3bi rosettes formed with adherent macrophages were blocked by anti-CR_1 in either HBSS/BSA or BDVA containing 20 mM EDTA (data not shown).

Inhibition of CR_4-dependent EC3bi rosettes with anti-p150,95. Each of three available MAbs to the α-chain of p150,95 was found to be specific for a distinct epitope of p150,95, as uptake of each ^{125}I-labeled MAb by neutrophils or U937 cells was blocked only by the unlabeled homologous MAb (data not shown). Each of the anti-p150,95 MAbs inhibited CR_4-dependent EC3bi rosettes with neutrophils and monocyte-derived macrophages (Tables I and II). With neutrophils in suspension, significant inhibition required a mixture of two or three anti-p150,95 MAbs. However, inhibition of adherent neutrophil CR_4-dependent rosettes was demonstrated with each of the IgG_1 anti-p150,95 MAbs individually. By contrast, substitution of an IgG_1 anti-class I MAb for one of the IgG_1 anti-p150,95 MAbs did not inhibit CR_4-dependent rosettes.

The CR_4-dependent rosettes formed with adherent monocyte-derived macrophages were also inhibited by each of the anti-p150,95 MAbs (Table II). However, demonstration of antibody inhibition of rosetting required different assay conditions than those used with monocytes or neutrophils. With EC3bi bearing large amounts of fixed iC3b, 95–99% of adherent monocyte-derived macrophages formed very avid rosettes (frequently with > 40 EC3bi bound per cell), and anti-CR_1/CR_3 inhibition in the proportion of cells forming rosettes was not apparent when rosettes were defined as cells bearing ≥ 4 bound EC3bi. Subsequently, when the amount of fixed iC3b/E was greatly reduced and rosetting was carried out in isotonic buffer, macrophages bore fewer bound EC3bi per cell and antibody-mediated inhibition of rosetting could be more readily demonstrated. Under these conditions, the EC3bi also did not bind to CR_1, so that anti-CR_1 could be omitted from the inhibition mixture. The three anti-p150,95 MAbs had similar rosette inhibiting capacity, and a mixture of the three MAbs produced only slightly more inhibition than the individual MAbs. As with neutrophils, substitution of anti-class I MAb for anti-p150,95 MAb did not inhibit CR_4-dependent rosettes.

Because the anti-p150,95 MAbs produced only partial in-

Table I. Inhibition of Neutrophil EC3bi Rosettes with Monoclonal Anti-p150,95

Antibody treatment*	EC3bi rosette formation	Inhibition of all rosettes	Inhibition of CR_4 rosettes
	%	%	%
Adherent neutrophils			
None	94±3.1	—	—
Anti-CR_1/CR_3‡	38±10§	59	—
Anti-CR_1/CR_3 + anti-Leu M5	14±2.3; $P < 0.004$	85	63
Anti-CR_1/CR_3 + 3.9	12±2.9; $P < 0.004$	87	68
Anti-CR_1/CR_3 + L29	16±5.6; $P < 0.004$	83	58
Anti-CR_1/CR_3 + anti-Leu M5/3.9/L29	6±6.1; $P < 0.001$	94	84
Anti-CR_1/CR_3 + HB-120 anti-class I	40±12	55	0
Neutrophils in suspension			
None	98±1.5	—	—
Anti-CR_1/CR_3‡	45±13§	54	—
Anti-CR_1/CR_3 + anti-Leu M5	40±13; $P > 0.5$	59	11
Anti-CR_1/CR_3 + anti-Leu M5/3.9	35±10; $P < 0.5$	64	22
Anti-CR_1/CR_3 + anti-Leu M5/3.9/L29	14±6.2; $P < 0.01$	86	69

The results are derived from assays of four to eight neutrophil preparations. Mean values±1 standard deviation are given, and P values comparing rosette formation with anti-CR_1/CR_3 versus anti-CR_1/CR_3 plus anti-p150,95 were calculated by the Student's t test. * The following amounts of antibodies were used: 140 μg rabbit anti-CR_1, 10 μg anti-Mac-1, and 5 μg each of MN-41, anti-Mol, anti-Leu M5, 3.9, L29, and HB-120. ‡ Anti-CR_1/CR_3: mixture of rabbit anti-CR_1, MN-41, anti-Mac-1, and anti-Mo-1. § This is the value for CR_4-dependent rosette formation used to calculate the values for inhibition of CR_4 rosettes given in the third column.

hibition of EC3bi rosettes, it appeared possible that none of the MAbs were specific for the iC3b-binding site of p150,95, and that better inhibition of p150,95-dependent function might be achieved by MAb-mediated modulation of p150,95. For this purpose, experiments were carried out with macrophages on which p150,95 had been modulated by cultivation on slides coated with anti-p150,95 (Table III). Partial, but significant inhibition of EC3bi rosette formation was observed with cells cultured on anti-p150,95, and then nearly all of the remaining rosettes were blocked by addition of fluid-phase anti-CR_3. This suggested that p150,95 could mediate EC3bi rosette formation independently of CR_3. When cells were cultured on

Table II. Inhibition of Adherent Monocyte-derived Macrophage EC3bi Rosette Formation with Monoclonal Anti-p150,95

Antibody treatment*	EC3bi rosette formation	Inhibition of all rosettes	Inhibition of CR_4 rosettes
	%	%	%
None	89±7.5	—	—
MN-41 anti-CR_3	48±6.8	46	—
Anti-Mol anti-CR_3	50±7.8	44	—
Anti-Mac-1 anti-CR_3	60±12	33	—
MN-41 + Anti-Mol	45±15	49	—
MN-41 + Anti-Mol + Anti-Mac-1	36±8.7‡	60	—
Anti-CR_3§ + Anti-Leu M5	18±8.8; $P < 0.03$	80	50
Anti-CR_3 + 3.9	21±8.6; $P < 0.05$	76	42
Anti-CR_3 + L29	19±9.1; $P < 0.03$	79	47
Anti-CR_3 + Anti-Leu M5/3.9	18±9.1; $P < 0.03$	80	50
Anti-CR_3 + Anti-Leu M5/3.9/L29	13±5.2; $P < 0.004$	85	64
Anti-CR_3 + Anti-HB-120 (class I)	42±10	53	0

Assays were performed with EC3bi bearing 1.7×10^4 iC3b per E in isotonic HBSS/BSA; conditions that minimize EC3bi binding to CR_1. The results given are the mean values±1 standard deviation for five preparations of monocyte-derived macrophages. In comparisons of rosette formation with anti-CR_3 versus anti-CR_3 plus anti-p150,95, P values were calculated by the Student's t test using the Tadpole program (Elsevier-Biosoft, Cambridge, UK). * The amounts of antibody added were: 10 μg anti-Mac-1, 5 μg each of MN-41, anti-Mol, anti-Leu M5, 3.9, L29, and HB-120. Titrations of the individual anti-CR_3 MAbs with each preparation of macrophages showed that the amounts used were 4- to 10-fold greater than the amounts required to achieve maximum (plateau) values for inhibition of rosette formation (not shown). ‡ This is the baseline value for CR_4-dependent rosette formation used to calculate the values for inhibition of CR_4 rosettes given in the third column. § Anti-CR_3: mixture of MN-41, anti-Mac-1, and anti-Mol.

Table III. Blockade of Macrophage CR_4-dependent Rosette Formation by Cultivation of Cells on Surfaces Coated with Anti-p150,95

Antibody on culture surface	Fluid-phase antibody added	EC3bi rosette formation	Inhibition of total rosettes	Inhibition of CR_4 rosettes
		%	%	%
Mouse myeloma IgG	None	86±8.6	—	—
	Anti-CR_3*	23±3.1‡	73	—
	Anti-CR_3 + Anti-p150,95§	9.3±3.4; $P < 0.002$	83	60
MAb 3.9 anti-p150,95	None	59±15; $P < 0.02$	31	—
	Anti-CR_3	2.8±1.6; $P < 0.001$	97	88
MN-41 anti-CR_3	None	24±2.8	72	—
	Anti-p150,95	6.3±4.1	93	73
HB-120 anti-class I	None	88±7.5	0	—
	Anti-CR_3	25±4.1	65	—
	Anti-CR_3 + Anti-p150,95	9.3±0.9	83	60

The results given are the mean values±1 standard deviation from five preparations of nonadherently cultured monocyte-derived macrophages. Inhibition of rosette formation on MAb 3.9 coated surfaces versus nonspecific myeloma IgG-coated surfaces (31%) was statistically significant. Modulation on surfaces coated with MAb 3.9 anti-p150,95 versus modulation on either myeloma IgG or anti-class I coated surfaces allowed significantly more inhibition of rosette formation by fluid phase anti-CR_3 (P value for comparison between MAb 3.9 versus P1.17 myeloma IgG surface is given). P values were calculated as in Table II. * Mixture of three anti-CR_3 MAbs: MN-41, anti-Mac-1, and anti-Mol; same amounts shown in Table II to produce plateau values for inhibition of rosette formation. ‡ By definition, this is the control level of CR_4-dependent rosette formation; i.e., the level of EC3bi rosettes in the presence of anti-CR_3. The inhibition of CR_4-dependent rosette formation calculated in the fourth column is based on this value. § Mixture of three anti-p150,95 MAbs: 3.9, anti-Leu M5, and L29, 5 μg of each.

control surfaces coated with myeloma IgG or HB-120 anti-class I instead of MAb anti-p150,95, a comparable blockade of rosettes required fluid phase anti-p150,95 in addition to fluid phase anti-CR_3. Another important finding was that modulation on a surface coated with anti-CR_3 MAb produced reciprocal results to those obtained with MAb anti-p150,95 coated surfaces. Surface modulation with a single MAb anti-CR_3 was as effective in blocking EC3bi rosettes as was the addition of three fluid phase anti-CR_3 MAbs to cells on myeloma IgG or anti-class I coated surfaces. The majority of the remaining CR_4-dependent rosettes expressed after CR_3 modulation were blocked by further addition of fluid phase anti-p150,95 MAbs.

To exclude the possibility that rosette inhibition might be have been produced by the MAb-triggered release of proteolytic enzymes that converted EC3bi to unreactive EC3d, supernatants of rosette inhibition mixtures of neutrophils or macrophages were incubated with EC3bi for 1 h at 37°C. Analysis with ^{125}I-labeled anti-C3c MAb showed no loss of C3c antigens from these EC3bi as compared to control EC3bi incubated in parallel in HBSS/BSA buffer.

Absence of CR_4-dependent rosettes with neutrophils and monocyte-derived macrophages from a patient with partial deficiency of the LFA-1/CR_3/p150,95 membrane antigen family. Normal neutrophils, tested in parallel with the patient's neutrophils, formed 23% CR_4-dependent EC3bi rosettes that were reduced to 8 and 2% by anti-Leu M5 and 3.9, respectively (Table IV). The patient's neutrophils did not express CR_4 activity, because all of the EC3bi rosettes formed in low-ionic strength BDVA buffer were blocked by the anti-CR_1/CR_3 mixture. Tests of this patient's neutrophils revealed 6,300 CR_3 per neutrophil (~ 10% of normal) by assays for uptake of ^{125}I-anti-CR_3, whereas no expression of p150,95 antigens was observed (data not shown).

Monocytes from the patient and a normal control were analyzed after being cultured nonadherently for 7 d. As found before (Table III), the CR_4 rosetting activity of the normal cells was inhibited by anti-p150,95 present on surfaces onto which nonadherently grown monocytes were allowed to attach (Table IV). By contrast, no EC3bi rosettes were observed when the patient's monocytes were examined under the same assay conditions that generated 99% EC3bi rosette formation with the normal monocyte control (Table IV).

Comparison of the amounts of CR_1, CR_3, and p150,95 on neutrophils, monocytes, and macrophages. The relative CR_4 dependence of EC3bi rosette formation appeared to correlate with the level of surface p150,95 versus the levels of CR_1 and CR_3 (Table V). Neutrophils and monocytes expressed 3-12 times more CR_1 and CR_3 than p150,95, whereas monocyte-derived macrophages expressed increased amounts of p150,95 that approached levels of CR_1 and CR_3.

Comparison of isolated monocytes with tissue macrophages by immunoperoxidase staining demonstrated that tissue macrophages from eight organs exhibited abundant staining for p150,95 in combination with weak or absent staining for CR_1 and CR_3 (Table VI). Figs. 2–4 illustrate the indirect immunohistochemical staining patterns obtained on a variety of tissues using MAbs E11 (CR_1), 44 (CR_3), and 3.9 (p150,95). In Fig. 2, the p150,95-positive labeling of tissue macrophages in thymic medulla (Fig. 2 *a*) and spleen white pulp (Fig. 2 *d*) is compared with the lack of staining for CR_1 and CR_3 on the same cells (Fig. 2 *b, c, e, f*). Strong expression of CR_1 was evident in lymphoid tissues, but it was restricted to lymphocytes (probably B cells, Fig. 2 *f*) and dendritic reticulum cells. CR_3 was also readily detectable on tissue granulocytes (polymorphonuclear cells, Fig. 2 *e*). In Fig. 3, the p150,95 expressing spleen white pulp macrophages (Fig. 3 *a*) are compared to the CR_3-positive granulocytes found within the spleen marginal zone (Fig. 3 *b*). Another comparable situation was seen in the tonsil where p150,95 expressing macrophages were found in interfollicular areas (Fig. 3 *c*) and germinal centers (Fig. 3, *c, e*), whereas CR_3-expressing granulocytes were seen in blood vessels (Fig. 3, *d:* see arrow), or in situations of inflammation

Table IV. Absence of CR_4 Rosette Formation with Neutrophils and Monocyte-derived Macrophages from a Patient with Partial Deficiency of LFA-1/CR_3/p150,95 (CD18)

Cell type	Fluid-phase antibody addition	EC3bi rosette formation	
		Normal control	CD18 deficient patient
		%	%
Adherent neutrophils (BDVA)	None	98	81
	Anti-CR_1	99	45
	Anti-CR_1/CR_3*	23‡	0
	Anti-CR_1/CR_3 + anti-Leu M5	8	Not done
	Anti-CR_1/CR_3 + MAb 3.9	2	Not done
Teflon 7-d cultured macrophages (HBSS/BSA)			
Tested on myeloma IgG surface	None	99	0
	Anti-CR_3	52	0
Tested on MAb 3.9 surface	None	47	0
	Anti-CR_3	4	0

* Anti-CR_1/CR_3: mixture of rabbit anti-CR_1, MN-41, anti-Mac-1, and anti-Mol. With the Teflon-cultured macrophages, EC3bi with reduced amounts of fixed iC3b in isotonic HBSS/BSA were used as in the experiments shown in Table III so that anti-CR_1 was not required and only the anti-CR_3 mixture of three MAbs was used. ‡ By definition, this is the control level of CR_4-dependent rosette formation.

where they were found in large numbers in the tonsil, particularly in the trabecular areas. The general tissue macrophage pattern of strong expression of p150,95 compared to CR_3 is in contrast to the expression of these molecules by monocytes and neutrophils which exhibited only moderate expression of p150,95 (Fig. 3 *f*) compared to CR_3 (Fig. 3 *g*). Various tissues that contained p150,95-expressing macrophages are illustrated in Fig. 4. Both alveolar macrophages and skin macrophages expressed CR_3 at much lower levels than p150,95. Although macrophages from bronchus stained predominantly for CR_3 rather than for p150,95, additional staining for CD14 suggested that these were probably immature cells (38) despite their large size (data not shown).

Discussion

The current study showed that membrane surface p150,95 expressed the same type of iC3b-receptor activity that had been previously demonstrated with the detergent-solubilized molecule (22). This iC3b-receptor activity of p150,95 is probably identical to the neutrophil receptor for EC3bi and EC3dg rosettes that had been previously designated CR_4 (23). Because neutrophils and monocytes express the iC3b-receptors CR_1 and CR_3 in addition to CR_4, demonstration of p150,95 (CR_4) dependent EC3bi rosette formation required prior blockade of CR_1 and CR_3 with a polyclonal mixture of antibodies to CR_1 and CR_3. Under these conditions, EC3bi rosettes were blocked by each of three MAbs directed to unique epitopes on the α-chain of p150,95. Neutrophils and monocytes that expressed very small amounts of p150,95 exhibited only low levels of CR_4-dependent rosette formation, the detection of which required large amounts of fixed iC3b and low-ionic strength buffer. By contrast, culture-derived macrophages expressed greatly increased amounts of p150,95 and avid CR_4-dependent rosette formation that was readily detectable with small amounts of fixed iC3b and isotonic buffer. Normal neutrophil and macrophage CR_4-dependent rosettes were inhibited by anti-p150,95, whereas neutrophils and macrophages from a patient with inherited deficiency of the entire LFA-1/CR_3/p150,95 membrane antigen family failed to form CR_4-dependent rosettes.

Table V. Quantitation of CR_1, CR_3, and CR_4 on Neutrophils, Freshly Isolated Monocytes, and Monocyte-derived Macrophages (Mϕ)

	Molecules/cell determined by uptake of ^{125}I-mAbs*		
	CR_1	CR_3	CR_4
Neutrophils	41,772±6,552	57,496±12,428	5,941±363
Monocytes‡	25,365±8,954	24,097±14,836	7,747±5,898
Adherently cultured Mϕ§	36,458±29,330	126,796±60,924	63,095±24,935

* Mean numbers±1 SD are given for four different preparations of each cell type.
‡ Monocytes were assayed within 2 h of adherence.
§ Adherently cultured Mϕ were generated from the same preparations of monocytes by culture for 7 d in 24-well plastic plates. Many of these cells are much larger than monocytes, so that the receptor number increase reflects both an increase in cell size as well as a probable increase in receptor density per unit area of membrane.

Table VI. Immunoperoxidase Staining of CR_1, CR_3, and p150,95 on Monocytes versus Tissue Macrophages

Cell type or source	Relative intensity of staining		
	MAb E11 (CR_1)	MAb 44 (CR_3)	MAb 3.9 (p150,95)
Blood monocytes	Moderate	Strong	Very weak
Alveolar macrophages	Negative-weak	Weak-moderate	Moderate
Brain macrophages	Negative	Negative	Moderate
Bronchus macrophages	Weak	Strong	Negative
Colon macrophages	Negative	Negative	Moderate
Skin macrophages	Negative	Weak	Moderate
Splenic macrophages	Weak	Weak	Strong
Thymus macrophages	Negative	Negative	Strong
Tonsil macrophages	Negative	Negative	Strong

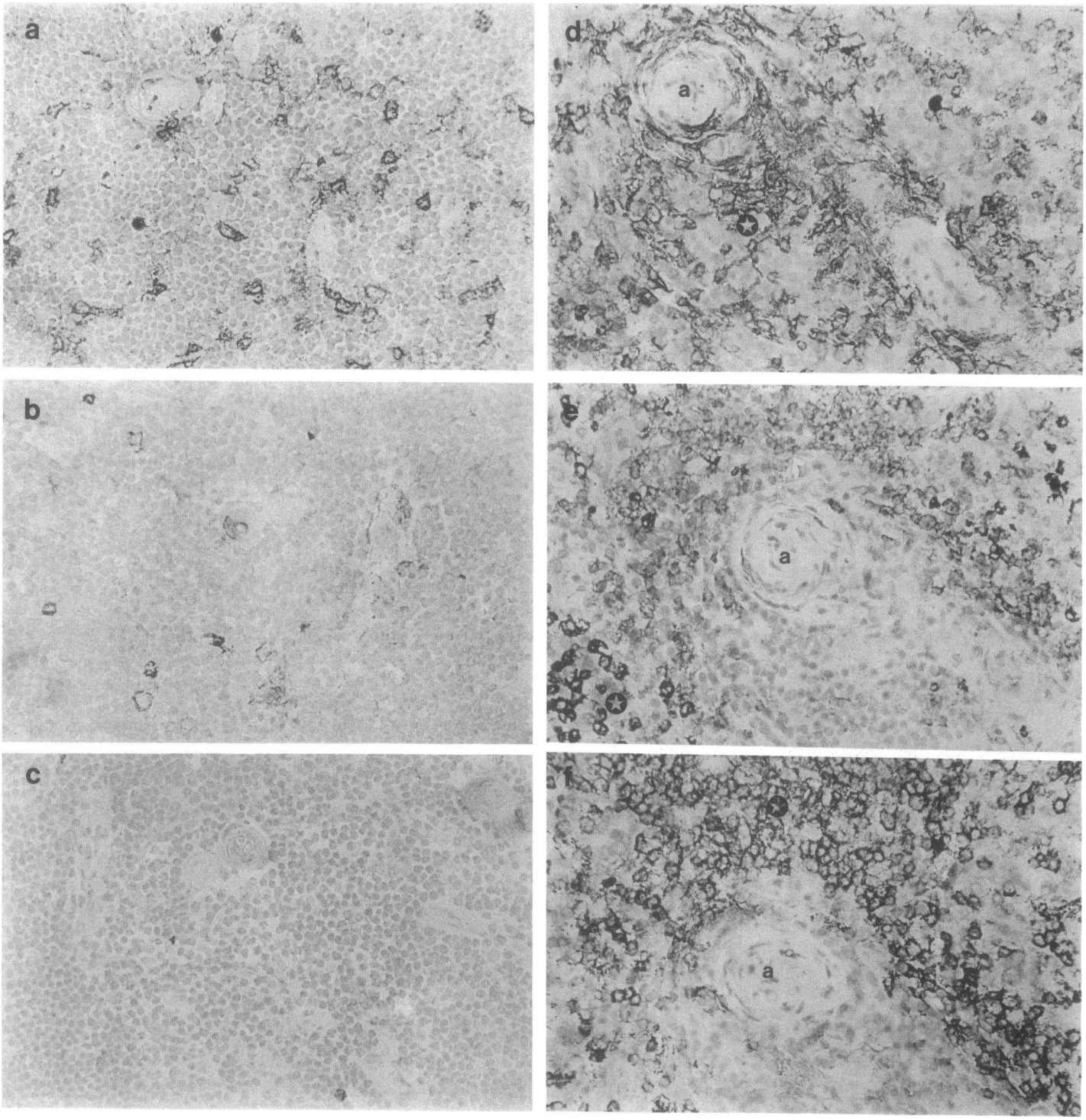

Figure 2. Immunoperoxidase labeling of thymus and spleen with mAbs specific for CR_1, CR_3, and p150,95. Sections of thymus medulla labeled with: (*a*) MAb 3.9 (p150,95) showing many reactive macrophages, versus sections (*b*) MAb 44 (CR_3), (*c*) MAb E11 (CR_1) showing little positive staining; magnification, 680. Consecutive sections of spleen white pulp labeled with: (*d*) MAb 3.9 illustrating aggregations of macrophages within the white pulp; area labeled with star below the arteriole (*a*); (*e*) MAb 44 showing chiefly positive granulocyte labeling in the marginal zone area at the edge of the white pulp (*see* star), (*f*) MAb E11 showing strong staining of lymphocytes (B cells) within an active germinal center adjacent to the arteriole (*see* star above arteriole); magnification 680.

The p150,95 molecule was originally discovered by immunoprecipitation with MAbs to the common β-subunit of the LFA-1/Mac-1 family (1). Tests of its function were not possible until MAbs to its unique α subunit were generated (2, 3, 31), and these studies were complicated by similar functions expressed by CR_3 on the same cell. Evidence that p150,95 functions along with CR_3 and LFA-1 in promoting neutrophil adherence to protein-coated glass surfaces was obtained by examining both MAb-treated normal neutrophils and neutrophils from patients with an inherited deficiency of the entire

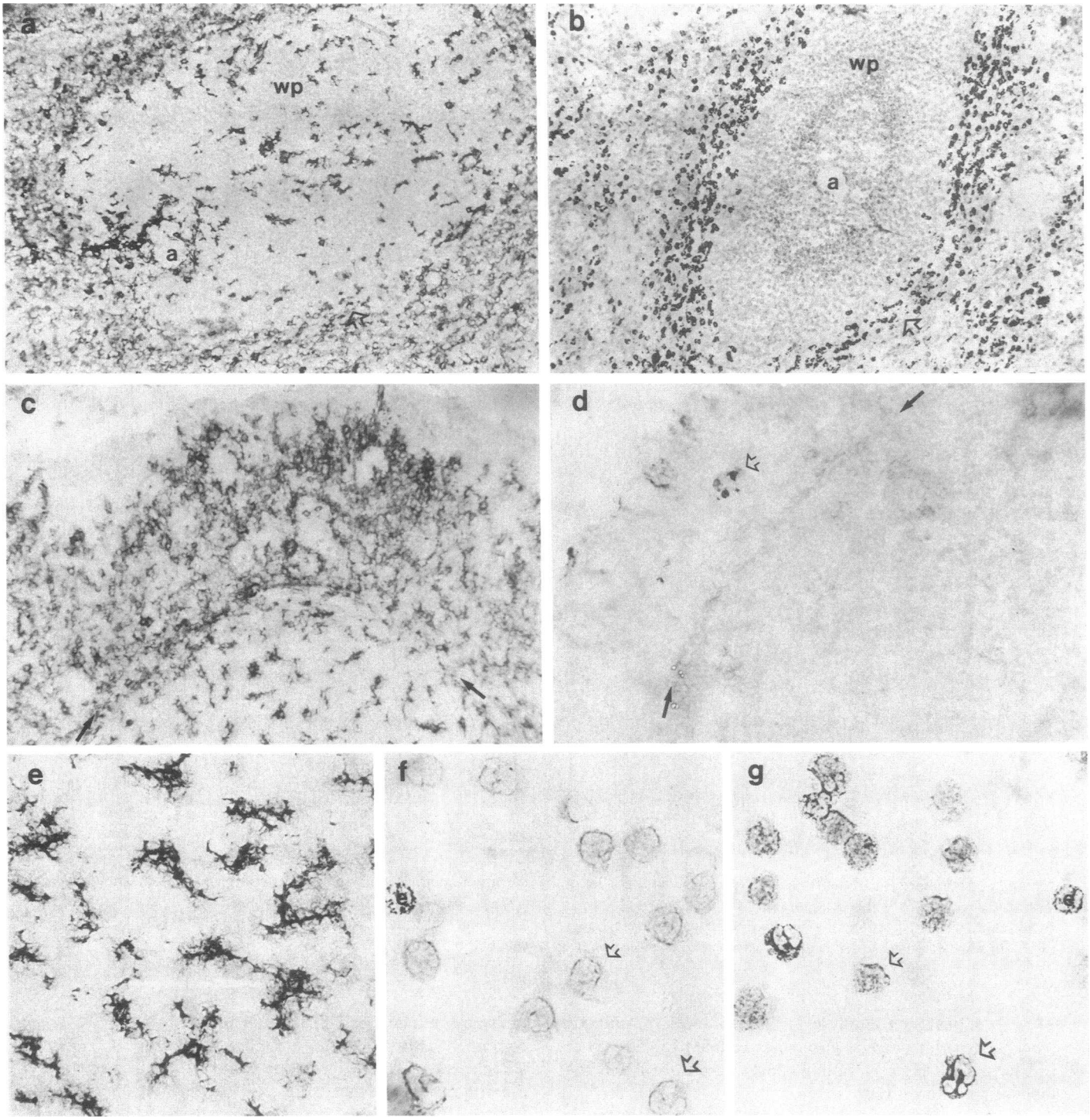

Figure 3. Immunoperoxidase labeling of monocytes, spleen, and tonsil with MAbs specific for CR_3 and p150,95. Sections of spleen labeled with: (*a*) MAb 3.9 (p150,95) showing macrophage staining in the white pulp (wp) area and in the surrounding marginal zone (*open arrow*) and red pulp; (*b*) MAb 44 (CR_3) showing strong staining of granulocytes in the marginal zone (*open arrow*) and red pulp; magnification, 270. Sections of tonsil labeled with: (*c*) MAb 3.9 showing extensive labeling of interfollicular and germinal center macrophages, and (*d*) MAb 44 showing labeling of a few granulocytes within a blood vessel (*open arrow*); magnification 440. In sections *c* and *d*, the border of the germinal center is indicated by two opposing arrows. Section *e* shows higher magnification (1,090) of tonsil germinal center macrophages with branched processes stained with MAb 3.9. Peripheral blood monocytes (small open arrow) and neutrophils (*large open arrow*) labeled with: (*f*) MAb 3.9 and (*g*) MAb 44. Two eosinophils, positively stained because of endogenous peroxidase activity are labeled *e* in the cell center and are seen at the left border of section (*f*) and the right border of section (*g*); magnification 1,090.

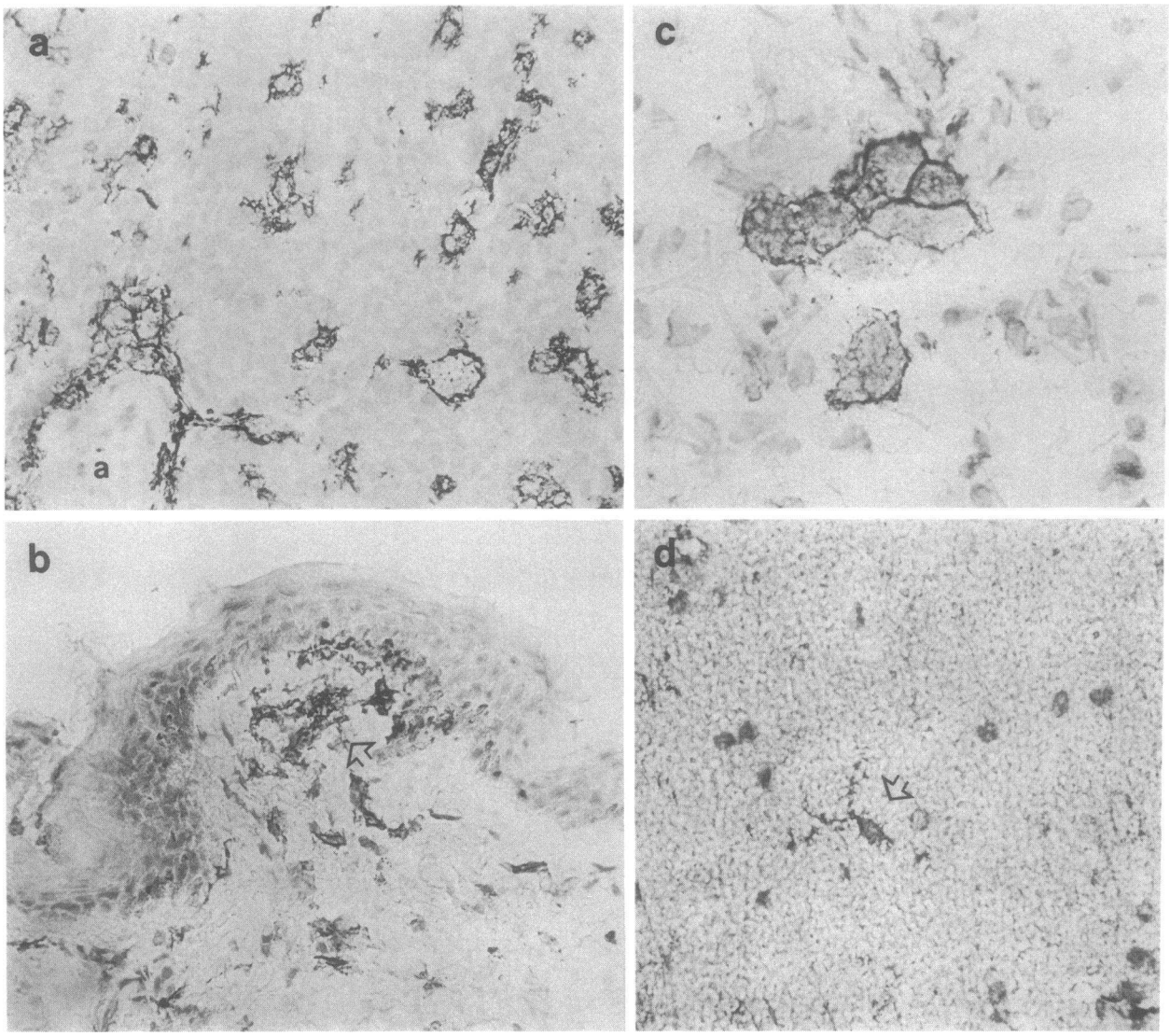

Figure 4. Immunoperoxidase labeling of spleen, lung, skin, and brain with MAb 3.9 (p150,95). Tissue section of (*a*) spleen, showing macrophages adjacent to arteriole in white pulp at higher magnification (680); (*b*) skin, showing positively stained macrophages within dermis at interface with epidermis (*open arrow*); 680 magnification; (*c*) lung, showing a cluster of p150,95 positive alveolar macrophages, 1,090 magnification; (*d*) brain, showing a p150,95 positive microglial cell; 680 magnification.

LFA-1/CR_3/p150,95 family (12). Similar types of antibody blocking experiments have also indicated that p150,95 may be involved in conjugate formation between cytotoxic T cells and their sensitive target cells (15).

Evidence that p150,95 might be a complement receptor has come from several sources. First, monocyte rosetting with EC3d was inhibited by modulation with anti-β-chain but not by modulation with anti-CR_3 or anti-LFA-1 (39). Second, an iC3b/C3dg receptor activity (CR_4) was demonstrated on neutrophils that was not inhibited by anti-CR_1 and anti-CR_3, and which was absent on neutrophils from patients with inherited deficiency of the LFA-1/CR_3/p150,95 (23). Third, detergent-solubilized p150,95 from U937 cells was shown to bind and elute from iC3b-Sepharose after selective removal of CR_3 by immunoprecipitation (22).

Because of two previous failures to demonstrate anti-p150,95 inhibitable EC3bi rosettes (2, 22), two different approaches were adopted in the current study. First, with neutrophils and monocytes, a mixture of three MAbs to CR_3 was combined with polyclonal anti-CR_1 to block all CR_1- and CR_3-dependent rosette formation with EC3bi. This was essential with neutrophils that expressed 10 times more of both CR_1 and CR_3 than p150,95. Second, culture-derived macrophages were examined because previous studies had demonstrated that these cells expressed very avid receptors for EC3bi that were only poorly inhibited by mixtures of anti-CR_1 and anti-CR_3 (26). With both neutrophils and macrophages, CR_4-dependent rosetting was blocked by anti-p150,95. The data suggested that p150,95 might be a major iC3b-receptor on macrophages because modulation of p150,95 with a single MAb produced significant inhibition of EC3bi rosette formation without additional blockade of CR_1 and CR_3.

It should be noted that the p150,95/CR_4 molecule may be different from the neutrophil/platelet receptor for fluid-phase C3dg-dimers that has recently also been called CR_4 (40). Although the p150,95 molecule may bind to fixed C3dg in addi-

tion to fixed iC3b (23), our current data with rosetting as well as published data with the isolated molecule indicate that p150,95 binding to iC3b is completely inhibited by EDTA. In contrast, the binding of ^{125}I-C3dg-dimers to neutrophils was unaffected by EDTA (41). Moreover, p150,95 antigens and CR_4-dependent EC3bi rosetting activity are expressed on both monocytes and neutrophils, but are undetectable on platelets (14, 42). In contrast, the C3dg-dimer receptor is present on platelets and absent from monocytes (40).

The possible specificity of CR_4 for fixed C3dg was not investigated in the present study because of the difficulty in obtaining EC3dg that were totally free of fixed iC3b. EC3dg usually bear 5–10% contamination with fixed iC3b detectable with ^{125}I-monoclonal anti-C3c (17). If such EC3dg form rosettes that are not inhibitable by mixtures of anti-CR_1 and anti-CR_3 (23), then it can not be excluded that such CR_4-dependent rosettes might be due to this small amount of fixed iC3b. Quantitatively, however, such 10% contamination with iC3b would amount to < 6,000 iC3b molecules on EC3dg bearing 60,000 total fixed iC3b/C3dg molecules, and EC3bi specifically prepared with this amount of fixed iC3b did not form neutrophil CR_4-dependent rosettes (23). Although C3dg can be obtained free of iC3b in the fluid-phase, available data suggests that the C3dg-dimers or C3dg-coated microspheres generated from such pure C3dg probably do not bind to CR_4, as their uptake by neutrophils is unaffected by EDTA (17, 41). It is thus likely that the analysis of CR_4 binding of C3dg will require indicator particles bearing only C3dg fixed via its physiologic ester bond site.

The data presented here and in previous reports (14, 31, 43, 44) support the concept that monocytes undergo a switch in C3-receptor types from CR_1 and CR_3 (Mac-1) to CR_4 (p150,95) during differentiation into macrophages. Quantitation of surface antigens with ^{125}I-MAbs showed that culture-derived macrophages expressed eight times more CR_4 than freshly isolated monocytes. Although this greatly facilitated tests of the p150,95 relationship to CR_4, the data suggests that the differentiation of C receptor phenotypes produced in vitro is different from that of normal resting macrophages because CR_1 and CR_3 were also increased on the culture-derived macrophages. When tissue macrophages from several organs were examined by immunoperoxidase staining, the majority of macrophages stained for CR_4 (p150,95) and failed to stain for CR_1 and CR_3. Normally there was little CR_3 on tissue macrophages in combination with strong expression of CR_4. Much larger amounts of CR_3 have been observed in inflammatory tissues containing granulocyte and monocyte infiltrates, such as those frequently seen in synovial tissues from patients with rheumatoid arthritis (45).

Preliminary findings further suggest that CR_4 can acquire the ability to induce ingestion of EC3bi in a manner similar to CR_3. Adherent culture-derived macrophages ingested EC3bi after blockade of CR_1 and CR_3, and then this CR_4 dependent ingestion was inhibited by anti-p150,95. Others have also reported that p150,95 (as well as CR_3 and LFA-1) could mediate phagocytosis of unopsonized bacteria and yeast (46, 47).

If CR_4 is primarily expressed and functional on tissue macrophages rather than on blood phagocytes, then characterization of its activity will be more difficult than previous functional studies of CR_1 and CR_3. It will be particularly important to determine whether CR_4 mediates clearance of iC3b-coated complexes in the liver. Studies in both humans and monkeys infused with C3-coated red blood cells or immune complexes have demonstrated a major role for fixed C3 in clearance by the liver (48, 49). Fixed iC3b, rather than fixed C3b, is probably the most prevalent opsonic C3 fragment on immune complexes and bacteria in the blood. Thus, the current data showing the function of CR_4 as an iC3b-receptor and its preferential expression on tissue macrophages suggest that CR_4 may be an important receptor involved in C3-mediated clearance. Preliminary studies of liver sections by immunoperoxidase staining and of isolated Kupffer cells by assay for uptake of ^{125}I-labeled MAbs have indicated that Kupffer cells express more CR_4 than either CR_1 or CR_3 (Ross, G. D., M. J. Walport, and N. Hogg, unpublished observation).

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