

Neutrophil and Monocyte Cell Surface p150,95 Has iC3b-Receptor (CR₄) Activity Resembling CR₃

Barry L. Myones,* Juanita G. Dalzell,* Nancy Hogg,† and Gordon D. Ross*

*Division of Rheumatology-Immunology, Department of Medicine, and The Department of Microbiology-Immunology, University of North Carolina, Chapel Hill, North Carolina 27599; and †The Macrophage Laboratory, Imperial Cancer Research Fund, London WC2A 3PX, United Kingdom

Abstract

Previous investigations of p150,95 (CD11c), the third member of the CD18 membrane glycoprotein family that includes CR₃ (Mac-1 or CD11b) and LFA-1 (CD11a), had demonstrated that solubilized p150,95 bound to iC3b-agarose in a manner similar to isolated CR₃. 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 CR₄. Normal neutrophil and macrophage CR₄-dependent EC3bi rosettes were inhibited by monoclonal anti-p150,95, and cells from a patient with CD18 deficiency did not form CR₄-dependent EC3bi rosettes. With neutrophils that bore large amounts of CR₁ and CR₃ and little p150,95, EC3bi were bound primarily via CR₁ and CR₃, and demonstration of p150,95-dependent rosettes required large amounts of fixed iC3b, low-ionic strength buffer, and antibody blockade of CR₁ and CR₃. By contrast, culture-derived macrophages expressed eight times more p150,95 than did monocytes and EC3bi were bound to both p150,95 and CR₃ when EC3bi bore small amounts of fixed iC3b and assays were carried out in isotonic buffer. Comparison of the amounts of CR₁, CR₃, and CR₄ in various tissues by immunoperoxidase staining revealed that CR₄ was the most abundant C3 receptor molecule on tissue macrophages, and suggested that CR₄ 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 CR₃ (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, CR₃ 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 CR₃ 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 CR₃ and small amounts of p150,95 and LFA-1, CR₃ 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 CR₃ from iC3b-agarose affinity columns (21), or to be purified on iC3b-agarose after selective removal of CR₃ by immunoprecipitation (22), suggesting that it might function as an iC3b-receptor analogous to CR₃. Up until this time, however, attempts to demonstrate

Address reprint requests to Dr. Ross, Division of Rheumatology-Immunology, CB# 7280 Faculty Laboratory Office Building 231-H, University of North Carolina, Chapel Hill, NC 27599.

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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 so-

dium 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), CR₃ (complement receptor type three, same as Mac-1 or CD11b, specific for iC3b), and CR₄ (complement receptor type four, same as p150,95, Leu M5, or CD11c, specific for iC3b); CR₁, 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-CR₁ and anti-CR₃ antibodies. This receptor activity on neutrophils appears to be the same as the receptor for EC3bi and EC3dg rosettes that had been previously designated CR₄ (23). CR₄ 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 CR₄ rosetting activity and large amounts of p150,95. With both cell types, CR₄ rosetting activity was inhibited by antibodies to the α -chain of p150,95, suggesting that cell surface p150,95 is probably identical to CR₄.

Methods

Neutrophils and monocytes. Blood was obtained from normal volunteers or a patient with inherited deficiency of LFA-1/CR₃/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 μ l of Hanks' balanced salt solution containing 1% bovine serum albumin; HBSS/BSA).

Antibodies. Rabbit F(ab')₂ anti-CR₁ was used to block CR₁ rosettes (27), and E11 MAb anti-CR₁ was used to quantitate CR₁ expression (28). MAbs to CR₃ 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 IgG₁ 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 IgG₁ mAb 3.9 (31). L29 is an IgG₁ 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/CR₃/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 ¹²⁵I 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 IgG₁ 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 IgG₁ 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 ¹²⁵I-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 μ l/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 mM at 22°C). With macrophages in 4-well chambers, 225 μ l of isotonic HBSS/BSA was used instead of BDVA. Next, 100 μ l 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 \times 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 μ l (8-well chambers) or 225 μ l (4-well chambers) with buffer, and 100 μ l 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-CR₁ used was sufficient to block all monocyte and neutrophil rosettes with EC3b (17). To determine the appropriate amount of each anti-CR₃ 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 CR₄ rosetting activity, amounts of anti-CR₃ 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-CR₁. 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-CR₃, anti-MHC class I, or myeloma IgG served as controls. MAbs to CR₃ and/or p150,95 were then added in the fluid phase prior to EC3bi addition.

Quantitation of CR₁, CR₃, and p150,95. Neutrophils were tested in suspension for uptake of ¹²⁵I-MAbs (34). Serial dilutions of ¹²⁵I-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 ¹²⁵I-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 ¹²⁵I-MAb. The amount of ¹²⁵I-MAb bound in the presence of a 100-fold molar excess of homologous unlabeled MAb was subtracted from the total amount of bound ¹²⁵I-MAB to determine the amount of specifically bound ¹²⁵I-MAB. With each mAb (E11, MN-41, and 3.9) titration studies showed that 400 ng of ¹²⁵I-MAB in 300 μ l of HBSS/BSA was saturating. Cells were first incubated with either unlabeled MAB or

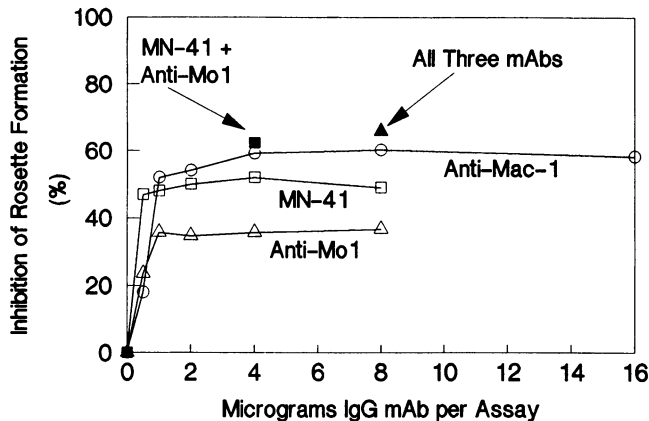


Figure 1. Titration of anti- CR_3 MABs for inhibition of adherent neutrophil EC3bi rosette formation. The results plotted are the mean of three assays of adherent neutrophils with EC3bi bearing 5.7×10^4 iC3b/E in BDVA low-ionic strength buffer. Neutrophils adhered to eight-well slide chambers ($\sim 4 \times 10^4$ adherent neutrophils per chamber) were treated with $F(ab')_2$ anti- CR_1 (140 μ g) plus the amount of each MAB listed in a total volume of 150 μ l for 20 min before addition of 100 μ l of EC3bi for assay of rosette formation. A mixture of 4 μ g each of MN-41 and anti-Mo1 (solid square) was compared to the same mixture containing 8 μ g 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 μ m 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 μ l of various IgG₁ MAB-containing supernatants, followed, after appropriate washing by 40 μ l 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'-diaminobenzidine (0.6 mg/ml). Controls for the IgG₁ MABs E11, 44 and 3.9 were a nonspecific IgG₁ 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-Mo1 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-Mo1). When tested in this way, neutrophils from 6 of 14 donors formed $\leq 15\%$ 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 $\geq 23\%$ 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 IgG₁ anti-p150,95 MABs individually. By contrast, substitution of an IgG₁ anti-class I MAB for one of the IgG₁ 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; <i>P</i> < 0.004	85	63
Anti-CR ₁ /CR ₃ + 3.9	12±2.9; <i>P</i> < 0.004	87	68
Anti-CR ₁ /CR ₃ + L29	16±5.6; <i>P</i> < 0.004	83	58
Anti-CR ₁ /CR ₃ + anti-Leu M5/3.9/L29	6±6.1; <i>P</i> < 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; <i>P</i> > 0.5	59	11
Anti-CR ₁ /CR ₃ + anti-Leu M5/3.9	35±10; <i>P</i> < 0.5	64	22
Anti-CR ₁ /CR ₃ + anti-Leu M5/3.9/L29	14±6.2; <i>P</i> < 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-CR₁/CR₃ versus anti-CR₁/CR₃ plus anti-p150,95 were calculated by the Student's *t* test. * The following amounts of antibodies were used: 140 µg rabbit anti-CR₁, 10 µg anti-Mac-1, and 5 µg each of MN-41, anti-Mol, anti-Leu M5, 3.9, L29, and HB-120. ‡ Anti-CR₁/CR₃: mixture of rabbit anti-CR₁, MN-41, anti-Mac-1, and anti-Mo-1. § This is the value for CR₄-dependent rosette formation used to calculate the values for inhibition of CR₄ rosettes given in the third column.

inhibition 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-CR₃. This suggested that p150,95 could mediate EC3bi rosette formation independently of CR₃. 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; <i>P</i> < 0.03	80	50
Anti-CR ₃ + 3.9	21±8.6; <i>P</i> < 0.05	76	42
Anti-CR ₃ + L29	19±9.1; <i>P</i> < 0.03	79	47
Anti-CR ₃ + Anti-Leu M5/3.9	18±9.1; <i>P</i> < 0.03	80	50
Anti-CR ₃ + Anti-Leu M5/3.9/L29	13±5.2; <i>P</i> < 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 CR₁. The results given are the mean values±1 standard deviation for five preparations of monocyte-derived macrophages. In comparisons of rosette formation with anti-CR₃ versus anti-CR₃ 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-CR₃ 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 CR₄-dependent rosette formation used to calculate the values for inhibition of CR₄ rosettes given in the third column.

§ Anti-CR₃: mixture of MN-41, anti-Mac-1, and anti-Mol.

Table III. Blockade of Macrophage CR₄-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; <i>P</i> < 0.002	83	60
Mab 3.9 anti-p150,95	None	59±15; <i>P</i> < 0.02	31	—
	Anti-CR ₃	2.8±1.6; <i>P</i> < 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-CR₃ (*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-CR₃ 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 CR₄-dependent rosette formation; i.e., the level of EC3bi rosettes in the presence of anti-CR₃. The inhibition of CR₄-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-CR₃. Another important finding was that modulation on a surface coated with anti-CR₃ MAb produced reciprocal results to those obtained with MAb anti-p150,95 coated surfaces. Surface modulation with a single MAb anti-CR₃ was as effective in blocking EC3bi rosettes as was the addition of three fluid phase anti-CR₃ MAbs to cells on myeloma IgG or anti-class I coated surfaces. The majority of the remaining CR₄-dependent rosettes expressed after CR₃ 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 ¹²⁵I-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 CR₄-dependent rosettes with neutrophils and monocyte-derived macrophages from a patient with partial deficiency of the LFA-1/CR₃/p150,95 membrane antigen family. Normal neutrophils, tested in parallel with the patient's neutrophils, formed 23% CR₄-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 CR₄ activity, because all of the EC3bi rosettes formed in low-ionic strength BDVA buffer were blocked by the anti-CR₁/CR₃ mixture. Tests of this patient's neutrophils revealed 6,300 CR₃ per neutrophil (~ 10% of normal) by assays for uptake of ¹²⁵I-anti-CR₃, 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 CR₄ 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 CR₁, CR₃, and p150,95 on neutrophils, monocytes, and macrophages. The relative CR₄ dependence of EC3bi rosette formation appeared to correlate with the level of surface p150,95 versus the levels of CR₁ and CR₃ (Table V). Neutrophils and monocytes expressed 3-12 times more CR₁ and CR₃ than p150,95, whereas monocyte-derived macrophages expressed increased amounts of p150,95 that approached levels of CR₁ and CR₃.

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 CR₁ and CR₃ (Table VI). Figs. 2-4 illustrate the indirect immunohistochemical staining patterns obtained on a variety of tissues using MAbs E11 (CR₁), 44 (CR₃), 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 CR₁ and CR₃ on the same cells (Fig. 2 b, c, e, f). Strong expression of CR₁ was evident in lymphoid tissues, but it was restricted to lymphocytes (probably B cells, Fig. 2 f) and dendritic reticulum cells. CR₃ 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 CR₃-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 CR₃-expressing granulocytes were seen in blood vessels (Fig. 3, d: see arrow), or in situations of inflammation

Table IV. Absence of CR₄ Rosette Formation with Neutrophils and Monocyte-derived Macrophages from a Patient with Partial Deficiency of LFA-1/CR₃/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)	None	99	0
	Tested on myeloma IgG surface	52	0
Tested on MAb 3.9 surface	None	47	0
	Anti-CR ₃	4	0

* Anti-CR₁/CR₃: mixture of rabbit anti-CR₁, 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-CR₁ was not required and only the anti-CR₃ mixture of three MAbs was used. [‡] By definition, this is the control level of CR₄-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 CR₃ 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 CR₃ (Fig. 3 g). Various tissues that contained p150,95-expressing macrophages are illustrated in Fig. 4. Both alveolar macrophages and skin macrophages expressed CR₃ at much lower levels than p150,95. Although macrophages from bronchus stained predominantly for CR₃ 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 CR₄ (23). Because neutrophils and monocytes express the iC3b-receptors CR₁ and CR₃ in addition to CR₄, demonstration of p150,95 (CR₄) dependent EC3bi rosette formation required prior blockade of CR₁ and CR₃ with a polyclonal mixture of antibodies to CR₁ and CR₃. 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 CR₄-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 CR₄-dependent rosette formation that was readily detectable with small amounts of fixed iC3b and isotonic buffer. Normal neutrophil and macrophage CR₄-dependent rosettes were inhibited by anti-p150,95, whereas neutrophils and macrophages from a patient with inherited deficiency of the entire LFA-1/CR₃/p150,95 membrane antigen family failed to form CR₄-dependent rosettes.

Table V. Quantitation of CR₁, CR₃, and CR₄ on Neutrophils, Freshly Isolated Monocytes, and Monocyte-derived Macrophages (M ϕ)

	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 M ϕ [§]	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 M ϕ 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 CR₁, CR₃, 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

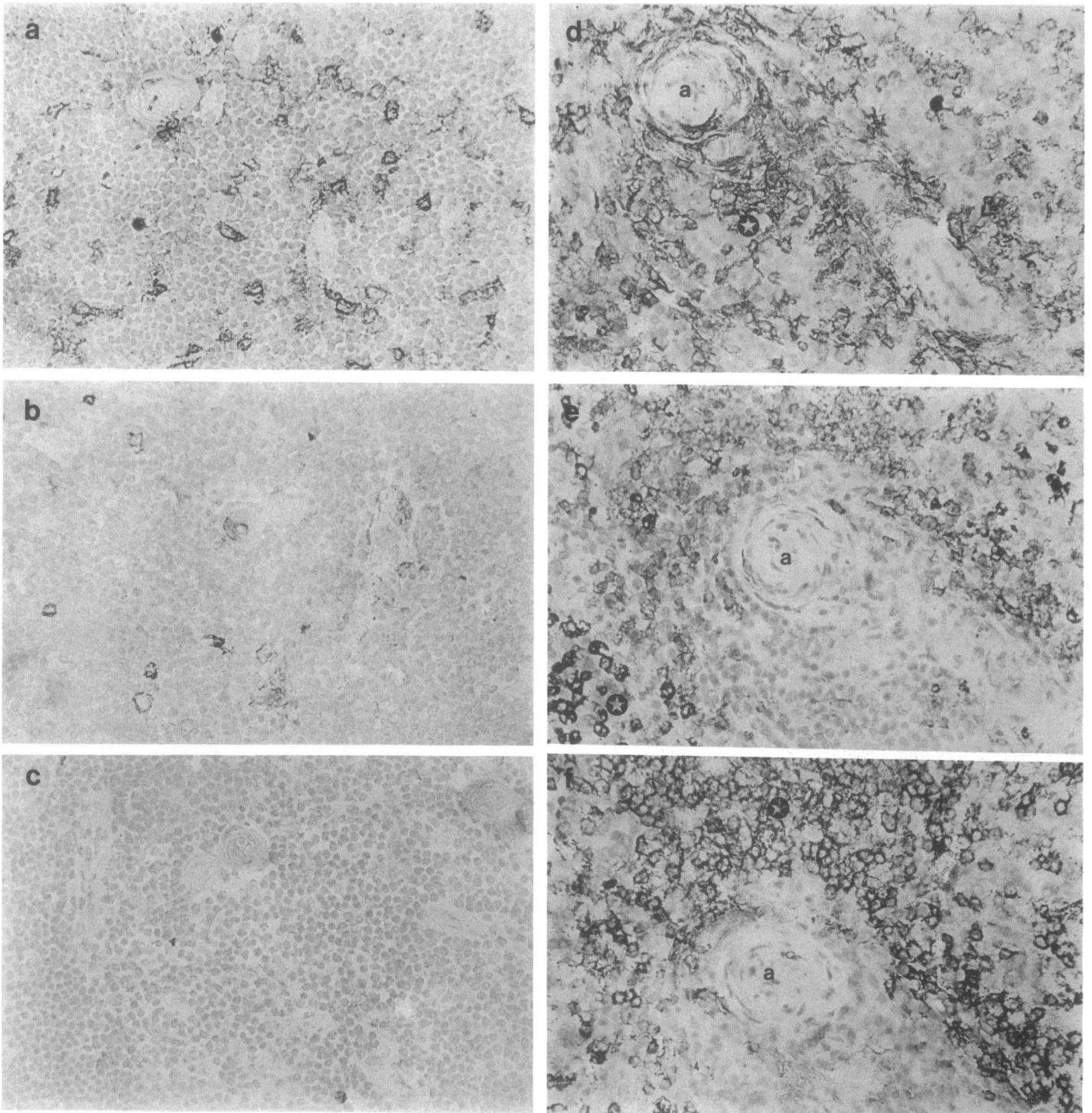


Figure 2. Immunoperoxidase labeling of thymus and spleen with mAbs specific for CR₁, CR₃, and p150,95. Sections of thymus medulla labeled with: (a) MAb 3.9 (p150,95) showing many reactive macrophages, versus sections (b) MAb 44 (CR₃), (c) MAb E11 (CR₁) showing little positive staining; magnification, 680. Consecutive sections of spleen white pulp labeled with: (d) MAb 3.9 illustrating ag-

gregations 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 CR₃ on the same cell. Evidence that p150,95 functions along with CR₃ 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

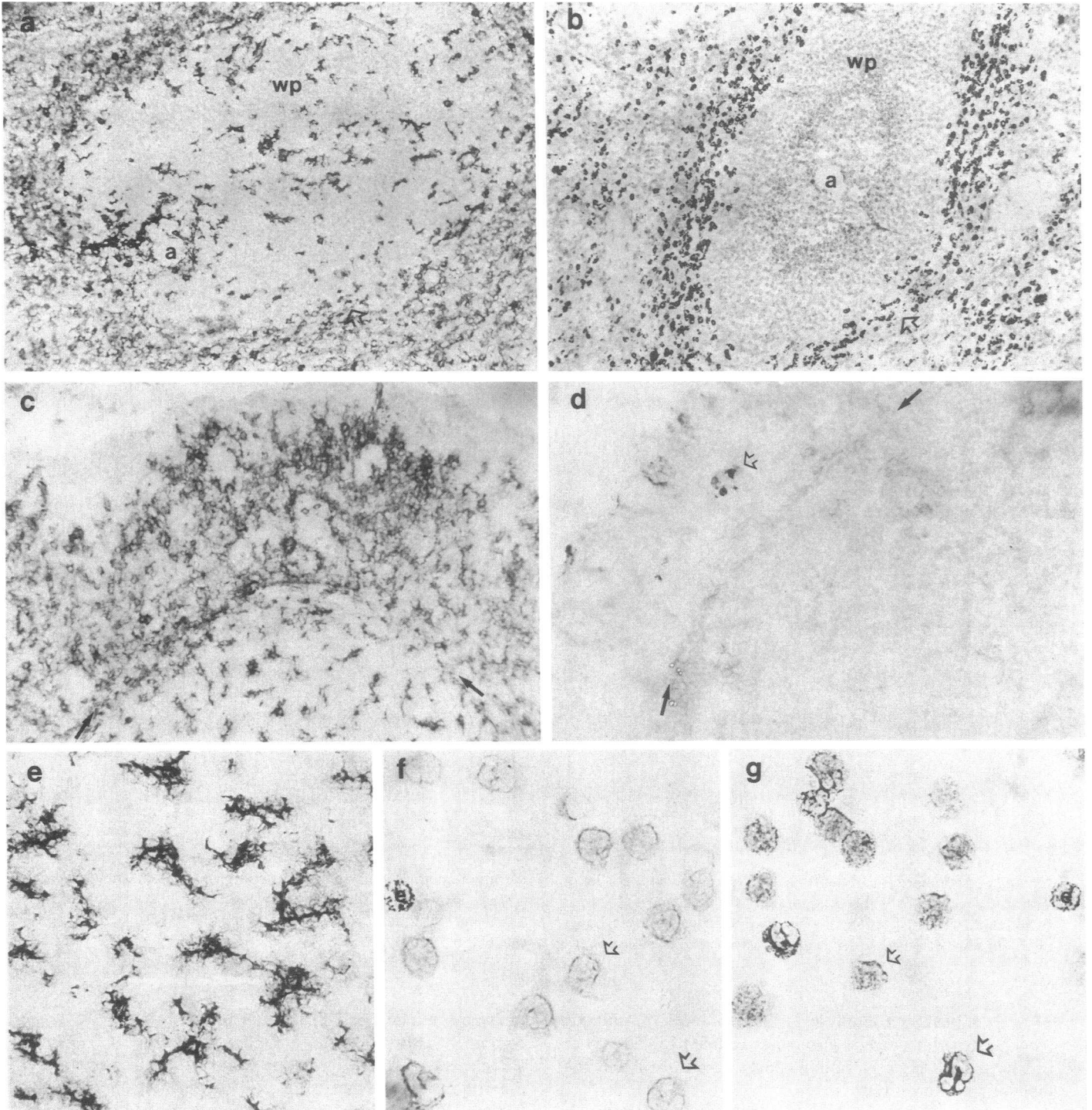


Figure 3. Immunoperoxidase labeling of monocytes, spleen, and tonsil with MAbs specific for CR₃ and p150,95. Sections of spleen labeled with: (a) MAb 3.9 (p150,95) showing macrophage staining in the white pulp (wp) area and in the surrounding marginal zone (*open arrow*) and red pulp; (b) MAb 44 (CR₃) 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.

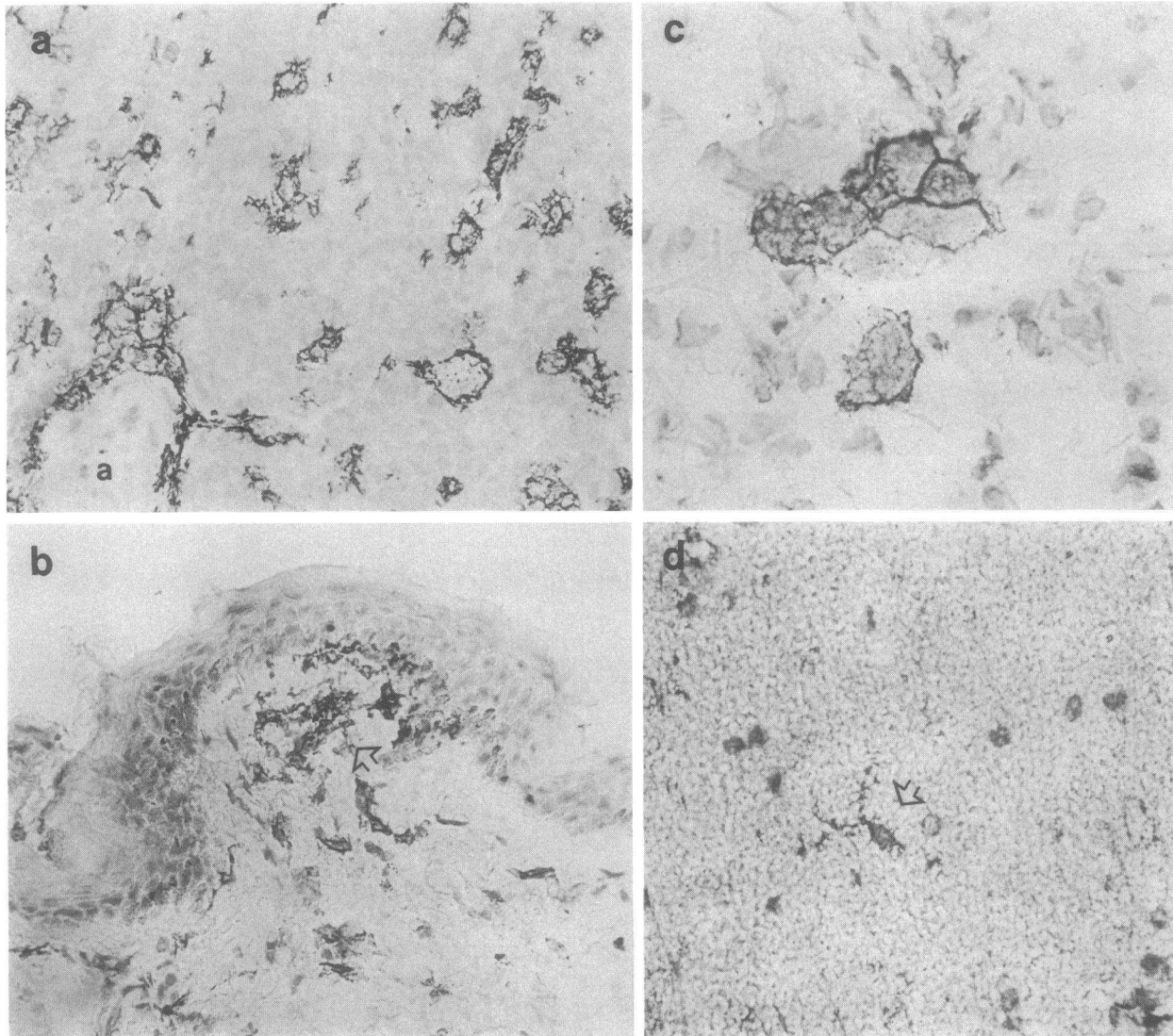


Figure 4. Immunoperoxidase labeling of spleen, lung, skin, and brain with MAb 3.9 (p150,95). Tissue section of (a) spleen, showing macrophages adjacent to arteriole in white pulp at higher magnification (680); (b) skin, showing positively stained macrophages within

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/CR₃/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-CR₃ or anti-LFA-1 (39). Second, an iC3b/C3dg receptor activity (CR₄) was demonstrated on neutrophils that was not inhibited by anti-CR₁ and anti-CR₃, and which was absent on neutrophils from patients with inherited deficiency of the LFA-1/CR₃/p150,95 (23). Third, detergent-solubilized p150,95 from U937 cells was shown to bind and elute from iC3b-Sepharose after selective removal of CR₃ 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 neutro-

phils and monocytes, a mixture of three MAbs to CR₃ was combined with polyclonal anti-CR₁ to block all CR₁- and CR₃-dependent rosette formation with EC3bi. This was essential with neutrophils that expressed 10 times more of both CR₁ and CR₃ 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-CR₁ and anti-CR₃ (26). With both neutrophils and macrophages, CR₄-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 CR₁ and CR₃.

It should be noted that the p150,95/CR₄ molecule may be different from the neutrophil/platelet receptor for fluid-phase C3dg-dimers that has recently also been called CR₄ (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 ¹²⁵I-C3dg-dimers to neutrophils was unaffected by EDTA (41). Moreover, p150,95 antigens and CR₄-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 CR₄ 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 ¹²⁵I-monoclonal anti-C3c (17). If such EC3dg form rosettes that are not inhibitable by mixtures of anti-CR₁ and anti-CR₃ (23), then it can not be excluded that such CR₄-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 CR₄-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 CR₄, as their uptake by neutrophils is unaffected by EDTA (17, 41). It is thus likely that the analysis of CR₄ 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 CR₁ and CR₃ (Mac-1) to CR₄ (p150,95) during differentiation into macrophages. Quantitation of surface antigens with ¹²⁵I-MAbs showed that culture-derived macrophages expressed eight times more CR₄ than freshly isolated monocytes. Although this greatly facilitated tests of the p150,95 relationship to CR₄, the data suggests that the differentiation of C receptor phenotypes produced in vitro is different from that of normal resting macrophages because CR₁ and CR₃ 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 CR₄ (p150,95) and failed to stain for CR₁ and CR₃. Normally there was little CR₃ on tissue macrophages in combination with strong expression of CR₄. Much larger amounts of CR₃ 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 CR₄ can acquire the ability to induce ingestion of EC3bi in a manner similar to CR₃. Adherent culture-derived macrophages ingested EC3bi after blockade of CR₁ and CR₃, and then this CR₄ dependent ingestion was inhibited by anti-p150,95. Others have also reported that p150,95 (as well as CR₃ and LFA-1) could mediate phagocytosis of unopsonized bacteria and yeast (46, 47).

If CR₄ 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 CR₁ and CR₃. It will be particularly important to determine whether CR₄ 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 CR₄ as an iC3b-receptor and its preferential expression on tissue macrophages suggest that CR₄ 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 ¹²⁵I-labeled MAbs have indicated that Kupffer cells express more CR₄ than either CR₁ or CR₃ (Ross, G. D., M. J. Walport, and N. Hogg, unpublished observation).

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