

MONOCLONAL ANTIBODY TO THE MURINE TYPE 3
COMPLEMENT RECEPTOR INHIBITS ADHESION OF
MYELOMONOCYTIC CELLS IN VITRO AND
INFLAMMATORY CELL RECRUITMENT IN VIVO

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Macrophage ($M\phi$) adhesion plays an important role in a variety of processes including phagocytosis, inflammation, immunity, and atherosclerosis. Selective interactions with other cells and with extracellular matrix depend on specific plasma membrane receptors, for example, for Fc and complement, fibronectin and laminin (1). Unique adhesion and spreading of $M\phi$ on artificial substrata such as glass or plastic often form the basis of cell isolation, but the surface molecules involved in these immunologically nonspecific interactions have not been identified. In early morphologic studies, Rabinovitch (2) described a role for Mg^{2+} in spreading of murine peritoneal $M\phi$ on glass, and Bianco et al. (3) and Gotze et al. (4) implicated proteolytic enzymes generated by activation of the coagulation and complement cascades as modulators of human monocyte spreading in vitro. However, the relationship between adhesion and spreading and the relevance of these in vitro phenomena to adhesive interactions in vivo, such as endothelial cell binding and migration into tissues, remain obscure.

Some structural insights have recently been gained into molecules responsible for the recruitment of myelomonocytic cells to inflammatory sites. Human patients lacking the leukocyte function-associated antigen (LFA)¹ family of surface receptors present with recurrent infection characterized by the failure of circulating myelomonocytic cells to adhere to endothelium and to leave the vascular compartment for tissue spaces (5), a disease known as leukocyte adhesion deficiency. The LFA family of noncovalently linked heterodimers consists of LFA-1, p150/95, and the Mac-1 cell surface receptor for the complement cleavage product iC3b, the type 3 complement receptor (CR3) (6). All three proteins share a common β chain of 95 kD while having specific α chains of 150–

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¹ Abbreviations used in this paper: BCG, Bacille Calmette-Guerin; BP, bacteriologic plastic; CB, cytochalasin B; DOC, sodium deoxycholate; EAC142, sheep erythrocytes opsonized with rabbit anti-sheep erythrocyte IgM and C142 complement components; EAiC3b, sheep erythrocytes opsonized with IgM iC3b complement cleavage product; EIgG, sheep erythrocytes opsonized with rabbit anti-sheep erythrocyte IgG; LFA, leukocyte function-associated antigen; $M\phi$, macrophage; PMN, polymorphonuclear leukocyte; RPM, resident peritoneal macrophage; TCP, tissue culture-treated plastic; TPM, thioglycollate-elicited peritoneal macrophage.

170 kD. The recent cloning of the β chain (7) has revealed an unusual repeated cysteine-rich motif in the extracellular domain of the molecule and considerable homology to the Integrin family, which includes fibroblast and platelet surface receptors for fibronectin and other adhesive proteins. Myelomonocytic cells, especially polymorphonuclear leukocytes (PMN) isolated from patients with leukocyte adhesion deficiency also display defective adhesion to various substrata in vitro (8), but since all three members of the LFA family are absent at the cell surface, it is not clear which component is normally responsible for particular adhesive functions, nor has the role of these glycoproteins in M ϕ adhesion been adequately defined.

In this study we describe the characteristics of adhesion of murine peritoneal M ϕ and PMN in vitro and report the isolation of an anti-M ϕ mAb that prevents Mg $^{2+}$ -dependent adhesion of M ϕ to bacteriologic plastic. This mAb, which is directed against an epitope on CR3 that is distinct from the iC3b binding site, inhibits myelomonocytic cell recruitment into the peritoneal cavity after injection in vivo. We have therefore mimicked aspects of the leukocyte adhesion deficiency in the mouse with a reagent restricted to only one member of the LFA family and provide a strategy to isolate new potent inhibitors of cell adhesion acting in vitro and in vivo.

Materials and Methods

Cells. Murine elicited macrophages (TPM) and PMN were harvested from the peritoneal cavity by lavage 4 and 1 d, respectively, after the intraperitoneal injection of 1 ml Brewer's complete thioglycollate broth. Cytospin preparations stained with Giemsa revealed that ~80% of the 4-d exudate (3×10^7 cells on average) were M ϕ and >95% of the 1-d exudate (4×10^7 cells) were PMN. Bone marrow cells, ~45% PMN, were obtained by flushing the femoral cavity with PBS. Bacille Calmette-Guerin (BCG)-activated and resident peritoneal M ϕ and Kupffer cells were isolated as described (9).

Animals. Mice (C57/BL or Pathology Oxford, PO), of either sex, were bred at the Sir William Dunn School of Pathology. AO rats (specific pathogen-free) were from the MRC Cellular Immunology Unit in our department.

Adhesion Assays. Cells were suspended in Iscove's modification of Dulbecco's Medium with 20% FCS and plated at a density of 10^5 cells/well in 96-well plates that were either flat-bottomed bacteriologic plastic (BP) (Flow Laboratories, Rickmansworth, United Kingdom) or tissue culture-treated plastic (TCP) (Sterilin, Ashford, United Kingdom). After incubation for 30 min at 37°C, plates were washed three times in PBS and adherent cells were fixed with methanol. After staining with 10% Giemsa solution for 10 min, plates were washed in tap water, dried, and the retained dye was solubilized in methanol. Stain was quantified by measuring absorbance at 460 nm in an automatic plate reader (Dynatech Laboratories, Inc., Alexandria, VA). This assay was linear between 5×10^3 and 10^5 adherent cells/well. In antibody inhibition experiments, hybridoma-conditioned media were added to microtiter plates and cooled to 4°C before addition of cells in the same medium. After preincubation at 4°C for 30 min, neither antibody-treated nor control cells had adhered and the plates were then warmed to 37°C for 30 min before washing and processing. In detachment assays, 10^5 M ϕ were plated in serum containing medium and allowed to adhere for 60 min at 37°C. After washing to remove nonadherent cells, antibody was added in medium and cells incubated at 37°C for 30 min. Thereafter, plates were washed and processed as above and the percentage of detached cells was calculated. The effects of various inhibitors on adhesion were studied by preincubating cells in one or more of 5 μ M cytochalasin B (CB), 10 μ g/ml colcemid, or 5 mM 2-deoxyglucose for 30 min at 4°C before performing the adhesion assay in medium with 10% FCS as described above. Experiments assessing the cation requirements of adhesion were carried

out in Iscove's medium and 10% FCS with either 5 mM EDTA or 5 mM EGTA to restrict Ca²⁺ and Mg²⁺, or Ca²⁺ alone. The absolute cation requirements were determined by performing adhesion assays in Ca²⁺- and Mg²⁺-free HBSS, with 10% FCS dialyzed to remove divalent cations and defined quantities of added CaCl₂ or MgCl₂. The effect of surface proteolysis on adhesion was assessed by treating cells in suspension with 0.5 mg/ml Pronase (Boehringer Mannheim Biochemicals, Lewes, United Kingdom) or 0.1 mg/ml crystalline trypsin in Iscove's medium without added protein for 30 min at 37°C. After washing the cells in 10% FCS, they were plated in serum-containing medium on the appropriate substratum and assayed in the standard adhesion assay.

Antibody Production. mAb 5C6 was the product of a fusion between spleen cells from an AO rat, immunized four times with TPM, and the Y3 rat myeloma line. The fusion was performed as described by Galfre et al. (10). Hybridoma supernatants were screened for inhibition of attachment and for detachment of TPM. One positive culture out of 1,056 wells was isolated and cloned twice by limiting dilution. Antibody was purified from ascites by sodium sulphate precipitation, anion-exchange chromatography on DEAE-Sephacel (Pharmacia Fine Chemicals, Hounslow, United Kingdom) and gel filtration chromatography on S-300 Sephadex (Pharmacia Fine Chemicals). Purity was assessed by SDS-PAGE in 10% acrylamide gels using the buffer system of Wyckoff et al. (11) and staining with Coomassie Blue. The IgG was free of any other protein bands when run either reduced or nonreduced. F(ab')₂ fragments were prepared by pepsin digestion of IgG at pH 4.0 in 0.1 M acetate after Rousseaux et al. (12) and purified by gel filtration chromatography. Fab fragments were produced by papain digestion of IgG in 75 mM sodium phosphate, pH 7.0/75 mM NaCl and purified by anion-exchange chromatography on DEAE-Sephacel. 5C6 is a rat IgG2b as typed by Ouchterlony gel diffusion using antisera directed against rat IgG subclasses supplied by Dr. H. Bazin (Catholic University, Louvain, Belgium). 5C6 was fluoresceinated by the method of Mason (13).

Labeling, Immunoprecipitation, and SDS-PAGE. 4-d TPM were plated at 10⁷ cells/10-cm tissue culture dish and cultivated overnight in methionine-free medium with 200 μCi [³⁵S]methionine. After a chase incubation with unlabeled methionine, the cells were washed in PBS and lysed in 1% vol/vol Triton X-100 in PBS, 10 mM EDTA, 3 mM PMSF and 3 mM iodoacetamide. After preclearing the lysate with protein A-Sepharose, mAb in detergent was added for 60 min at 4°C. Thereafter, the mAb-antigen complex was precipitated with rabbit IgG anti-rat IgG-protein A-Sepharose. The beads were washed by standard methods and boiled in sample buffer containing 2% SDS. The eluates were analyzed by electrophoresis on 10% polyacrylamide gels and autoradiography. Control preparations contained an mAb to determinants not found in mouse Mφ. In peptide mapping experiments, 5C6 and M1/70 immunoprecipitates on protein A-Sepharose beads were digested for varying times at 37°C with 100 μg/ml crystalline trypsin. The reaction was stopped by boiling the beads in SDS sample buffer and the resultant cleavage patterns were analyzed by SDS-PAGE.

Preparation of Derivatized Substrata. Fibronectin-coated surfaces were prepared by coating tissue-culture plastic surfaces with gelatin followed by fresh mouse serum for 1 h at room temperature. The wells were then extensively washed with PBS. Poly-L-lysine-coated, glutaraldehyde-activated surfaces were derivatized with DNP/BSA anti-DNP immune complexes as described (14).

Chemotaxis Assay. The directional migration of myelomonocytic cells in response to the chemotactic tripeptide FMLP was assayed under agarose as described (15).

Recruitment Assays. PO mice were injected intravenously with PBS, 5C6 IgG, 5C6 F(ab')₂, or the anti-L3T4 subclass-matched (IgG2b) YTA mAb, 4 h before injection of 1 ml thioglycollate broth intraperitoneally. All antibody injections were 0.5 mg unless stated otherwise. At 18 or 96 h after the first injection, mice were killed and peritoneal exudate cells, blood, livers, spleens and bone marrows were removed. Total and differential counts of exudate cells, bone marrow cells, and blood leukocytes (after sedimentation of erythrocytes with 1% Dextran T500) were obtained using May-Grunwald-Giemsa stain. Inhibition of recruitment by mAb was calculated as: Inhibition = [(total exudate cells) - (resident cells in antibody-treated mice)]/[recruited cells from control mice]. Cells were

then tested for adhesion to BP or TCP. Livers and spleens were examined histologically as follows. In some experiments tissues were fixed in glutaraldehyde and stained with haematoxylin and eosin. In experiments where FITC-5C6 had been injected *in vivo*, organs were examined for the presence of mAb by direct immunofluorescence of frozen sections (5 µm) embedded in Tissuetek (Miles Laboratories Inc., Naperville, IL). Alternatively, animals were perfusion-fixed as described (16) and frozen sections of tissues were analyzed by the immunoperoxidase technique without adding exogenous first antibody to the sections.

Rosetting Assays. Sheep erythrocytes (E) were opsonised with IgM antibody and iC3b or C142 (17), or rabbit anti-sheep E IgG and used as a 5% vol/vol suspension for rosetting at 4°C or phagocytosis at 37°C by adherent TPM (18). Binding was quantified by counting the number of attached erythrocytes to 100 TPM by phase-contrast microscopy after washing and glutaraldehyde fixation.

Microscopy. Fluorescence microscopy was performed using a Zeiss Axiophot epifluorescence microscope. Scanning electron microscopy was performed as described (18).

Reagents. M1/70, a rat anti-CR3 mAb, was used either as IgG or hybridoma supernatant from laboratory stocks. The cell line was the gift of Dr. T. Springer (Dept. of Pathology, Harvard Medical School, Boston, MA). YTA mAb was a gift of Dr. H. Waldmann (Dept. of Immunology, University of Cambridge, Cambridge, United Kingdom). Rabbit anti-E IgM (EA) and EA iC3b were provided by Dr. R. Sim (MRC Immunoochemistry, University of Oxford, Oxford, United Kingdom). Reagents were obtained from the following sources: Iscove's medium (Flow Laboratories, Inc.); FCS (Gibco Laboratories, Paisley, Scotland); Colcemid (Dr. E. P. Evans, Sir William Dunn School of Pathology); Deoxyglucose, cytochalasin B, EDTA, and EGTA (Sigma Chemical Co., Poole, United Kingdom); Protein A-Sepharose (Pharmacia Fine Chemicals).

Protein Assays. Protein concentrations of IgG were assayed according to Lowry et al. (19) using BSA as the standard.

Results

Adhesion Assays. We characterized Mφ and PMN adhesion in a variety of simple, reproducible, short-term adhesion assays to develop a screening method for mAbs directed against functional epitopes of phagocyte surface molecules. Adherence of various primary murine Mφ to TCP or BP (polystyrene) was examined because selective adherence of Mφ to these substrata forms the basis of their rapid purification from other blood cells and fibroblasts (20). All assays were carried out in the presence of serum. The comparative adhesive qualities of different cells to the plastic substrata are shown in Table I. The adhesion of Mφ and exudate PMN to BP was Mg²⁺-dependent, pronase-sensitive, and required elevated temperature as well as cytoskeletal stabilization for efficient binding. Exposure of TPM to 0.5 mg/ml pronase at 37°C for 30 min completely abolished their ability to adhere to BP. This effect persisted for at least 6 h and cells recovered fully by 24 h in culture. Resting PMN and tissue Mφ such as Kupffer cells were unable to adhere to BP. Adhesion to BP required at least 100 µM extracellular Mg²⁺, and Ca²⁺ alone was unable to substitute for this requirement. The adhesion of PMN (whether resting or exudate) to TCP had the same qualities as Mφ adhesion to BP. All cell types tested adhered well to TCP and showed varying degrees of spreading. As in BP adhesion, Mφ adhesion to TCP required elevated temperature and an intact cytoskeleton, but differed in being resistant to proteolysis and was partially cation-independent.

The adherence of Mφ to BP appeared to be a useful means of identifying a pronase-sensitive, cation-dependent surface component involved in cell adhesion.

TABLE I
Adhesion of Cells In Vitro After 30 Min at 37°C in 10% FBS. Absorbance of Giemsa at 460 nm was Used to Determine Adherent Cell Numbers

Cell type and treatment	Tissue culture plastic		Bacterial plastic	
	Absorbance	Cell number	Absorbance	Cell number
		$\times 10^{-3}$		$\times 10^{-3}$
Resident peritoneal Mφ	0.174	96 ± 4	0.135	74 ± 3
Thioglycollate PMφ	0.189	104 ± 5	0.147	81 ± 5
Kupffer cells	0.181	99 ± 3	0.002	1 ± 3
Bone marrow PMN	0.180	99 ± 3	0.008	4 ± 3
Exudate PMN	0.186	102 ± 4	0.151	83 ± 5
Cation requirement (TPM)				
Mg ²⁺ present (no Ca ²⁺)	0.161	88 ± 4	0.118	65 ± 3
Ca ²⁺ present (no Mg ²⁺)	0.104	57 ± 5	0.004	2 ± 2
Neither present	0.104	57 ± 5	0.004	2 ± 2
Protease sensitivity (TPM)				
Trypsin	0.178	98 ± 3	0.152	84 ± 4
Pronase	0.182	100 ± 2	0.005	3 ± 2
Metabolic and cytoskeletal factors (TPM)				
Controls (37°C, no inhibitors)	0.152	84 ± 4	0.135	74 ± 3
Temperature <4°C	0.008	4 ± 3	0.004	2 ± 2
Cytchalasin B 5 μM	0.072	40 ± 3	0.058	32 ± 3
Colcemid (10 μg/ml)	0.063	35 ± 5	0.049	27 ± 4
Cytchalasin B + colcemid	0.006	3 ± 2	0.002	1 ± 2
2-Deoxyglucose	0.151	83 ± 3	0.051	28 ± 1

Results of quadruplicates in three separate experiments are expressed as the mean absorbance and the mean ± SD of adherent cell number calculated from an internal standard. ~10⁵ myelomonocytic cells were plated/well.

We thus used this assay to screen for functional mAbs capable of, first, inhibiting attachment of TPM to BP and, second, detaching adherent TPM from BP.

mAb 5C6 Inhibits Adhesion to BP and Detaches Adherent TPM. In each of two consecutive fusions, one hybridoma secreting a mAb capable of inhibiting attachment of TPM to BP was isolated. Both identify the same antigen and this report is confined to the first of these mAbs, designated 5C6. The effects of mAb 5C6 on adhesion of TPM to bacterial and TCP are illustrated in phase micrographs in Fig. 1. Fig. 1A shows that untreated 4-d TPM adhered and spread on BP after 30 min at 37°C in serum-containing medium. Preincubation of these cells in 5C6 hybridoma supernatant for 30 min at 4°C before warming to 37°C to allow adhesion, led to the complete abolition of TPM attachment to BP (Fig. 1B). While untreated TPM adhered and spread on TCP after 30 min at 37°C (Fig. 1C), cells preincubated with 5C6 showed unimpaired adhesion to TCP, but did not spread at all (Fig. 1D). The adhesion of untreated TPM to glass was similar to that observed on TCP. Control TPM adherent to glass as shown in the scanning electron micrograph in Fig. 2A, were flat and well spread. After these cells had been preincubated in 5C6 mAb (Fig. 2B), the cells adhered via a relatively small, snail-like foot process while the bulk of the plasma membrane remained in a highly ruffled dome over the cell body. Adhesion of Mφ to glass

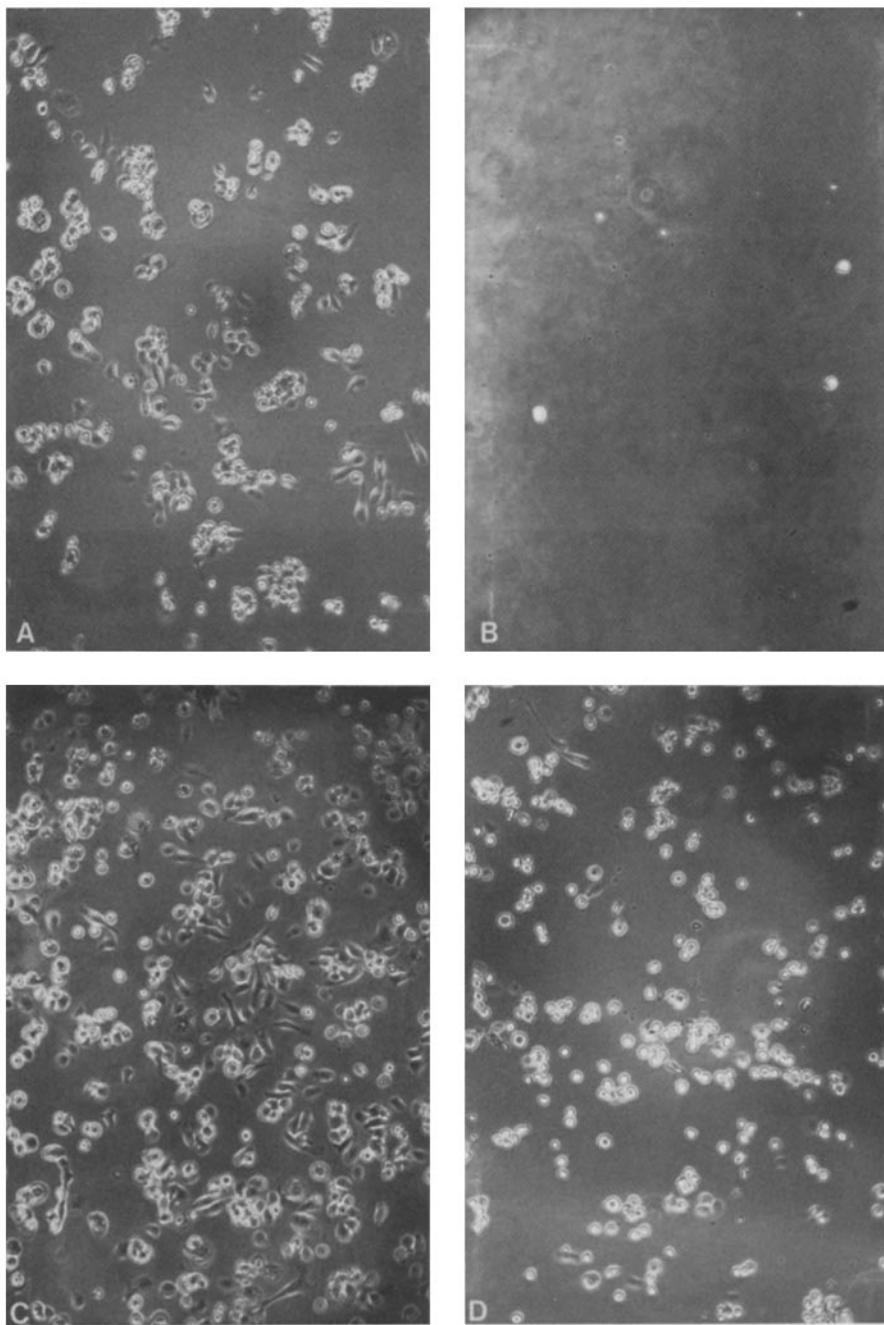


FIGURE 1. mAb 5C6 inhibited adhesion of TPM to BP and spreading of TPM on TCP. TPM were preincubated in medium alone (*A* and *C*) or 5C6 hybridoma supernatant (*B* and *D*) and 10^6 TPM were plated in 1 ml of medium with 10% FCS on a 35-mm petri (*A* and *B*) or tissue culture dish (*C* and *D*) and allowed to adhere for 30 min at 37°C. After washing three times in PBS, adherent cells were fixed in 0.5% glutaraldehyde and examined by phase-contrast microscopy. 5C6-treated cells failed to adhere to BP (*B*) and failed to spread on TCP (*D*), while control cells adhered and spread on both BP (*A*) and TCP (*C*).

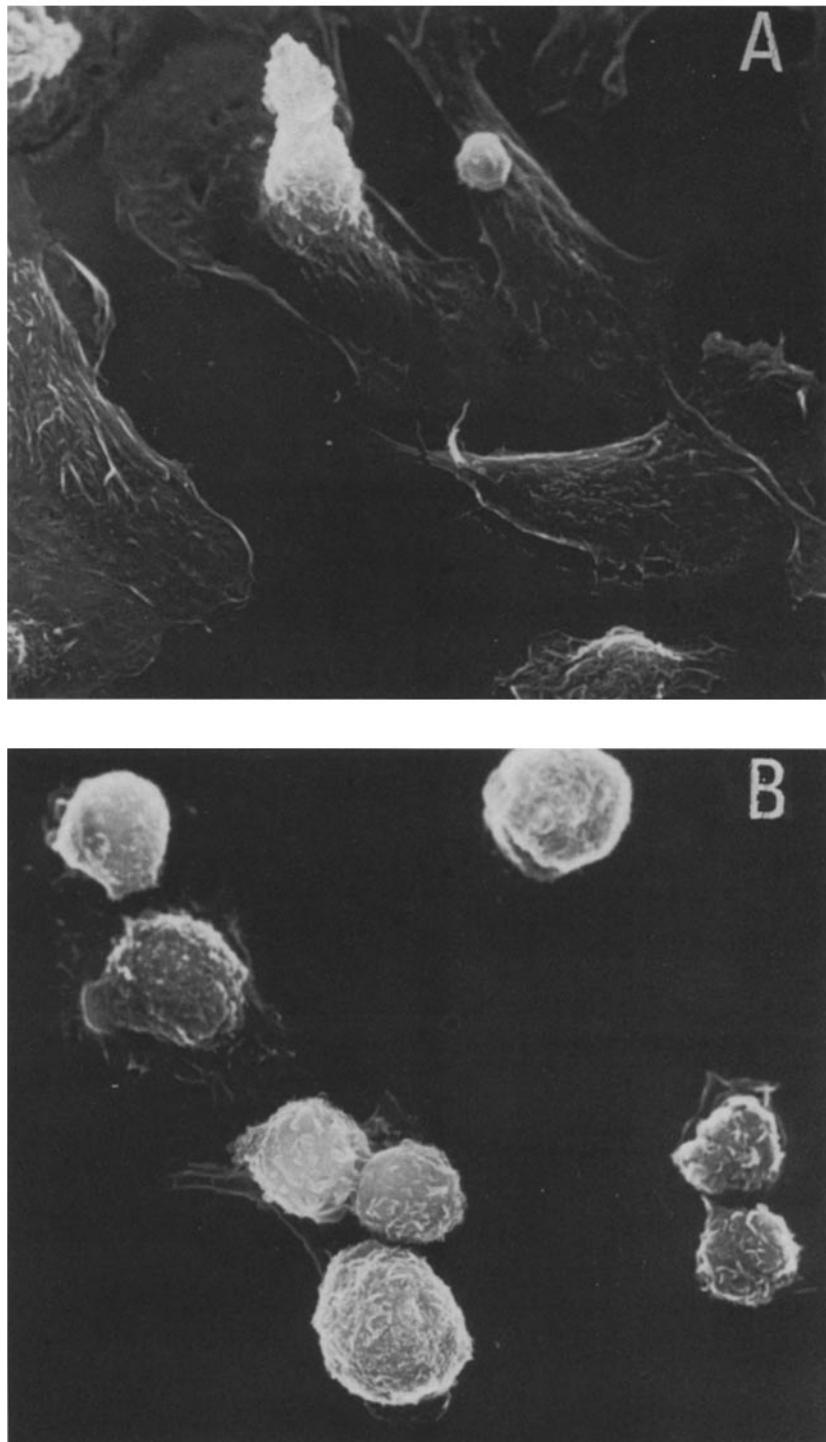


FIGURE 2. Scanning electron micrographs of control and 5C6-treated TPM adherent to glass. Treatment and preparation as in Fig. 1. After fixation in glutaraldehyde and cacodylate buffer, preparations were critical point-dried and shadowed with gold. Control preparations (A) were well spread while 5C6-treated preparations (B) adhered through a snail-like foot process and remained rounded with plasma membrane ruffles over the cell body.

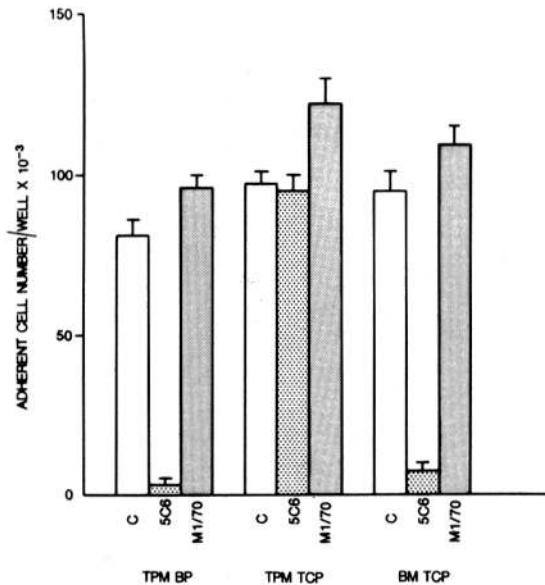


FIGURE 3. Quantitative adhesion assays after preincubation of cells in medium alone (C), 5C6, or M1/70 mAb. 10^5 TPM or marrow PMN (BM) were plated per well in 96-well plates on BP TCP. Results show the mean \pm SD of quadruplicates in three separate experiments after incubation for 30 min at 37°C, washing, and Giemsa staining. Cell number was calculated from an internal standard of 10^5 adherent cells/well.

TABLE II
A Comparison of the Functional Characteristics of 5C6 mAb [Intact IgG, F(ab')₂, and Fab Fragments] and M1/70 IgG

	5C6 IgG	5C6 F(ab') ₂	5C6 Fab	M1/70 IgG
Inhibition of TPM adhesion to BP	92 \pm 1%	51 \pm 3%	0%	-22%
Detachment of adherent TPM (BP)	65 \pm 4%	8 \pm 3%	5 \pm 2%	6 \pm 3%
Inhibition of EiC3b rosetting	>95%	>95%	0%	>95%
Pronase* resistance of epitope	85 \pm 1%	ND	ND	26 \pm 1%

5C6 IgG, F(ab')₂ and Fab were used at 1, 10, and 100 μ g/ml respectively while M1/70 was used at 10 μ g/ml. Rosetting was quantified as described in Materials and Methods. Results reflect the mean \pm SD of at least three separate experiments.

* TPM were digested in suspension (30 min, 37°C) with 0.5 mg/ml pronase, fixed in 0.25% glutaraldehyde, quenched in FCS, and 10^6 cells were assayed in quadruplicate by trace indirect binding assay (two experiments). Untreated cells bound 3,903 \pm 47 cpm of second antibody after binding of 5C6 and 3,336 \pm 51 cpm after pronase digestion. Untreated cells bound 3,292 \pm 35 cpm using M1/70 as first antibody and only 866 \pm 12 cpm after pronase treatment. The Mφ-specific F4/80 antigen known to be pronase-sensitive was abolished by this treatment.

and TCP had two phases, mAb 5C6-resistant adherence followed by mAb 5C6-sensitive spreading.

Fig. 3 and Table II summarize quantitative assays of adhesion in the presence or absence of mAb and show that 5C6 IgG at 0.5 μ g/ml inhibited attachment of TPM to BP by 92 \pm 1%, while untreated cells or cells treated with saturating concentrations of the anti-CR3 mAb M1/70, used for comparison, displayed unaltered adhesive capacities. The increased adherence of M1/70-treated cells as compared with untreated cells reflected aggregation of cells by this antibody and adhesion of these clusters. In addition to its effects on Mφ adhesion, 5C6 also abolished the adhesion of PMN to TCP, which was unimpaired in control or M1/70-treated PMN. While M1/70 treatment led to aggregation of myelomonocytic cells in suspension, 5C6-treated PMN or Mφ remained discrete.

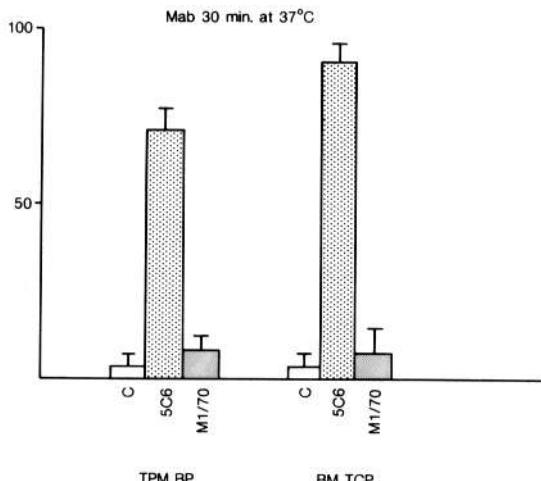


FIGURE 4. Detachment by mAb 5C6 of TPM adherent to BP, or of bone marrow PMN (BM) adherent to TCP. Assay as described in Materials and Methods. Medium alone (C) or M1/70 supernatant (M1/70) failed to detach either TPM from BP or PMN from TCP. Results expressed as mean \pm SD (triplicates in two separate experiments).

The CSAT antigen or fibronectin receptor of avian fibroblasts was identified by the ability of antibody to detach cells adherent to a fibronectin-coated substratum (21). After this precedent, we examined the ability of 5C6 to detach TPM from BP or bone marrow PMN from TCP. After allowing cells to attach and adhere for 60 min at 37°C, plates were washed and medium containing saturating concentrations (5 μ g/ml) of 5C6 or M1/70 or no antibody was added. Within 5 min at 37°C, spread cells treated with 5C6 had become rounded while M1/70-treated and control cells were unaltered. By 30 min at 37°C (Fig. 4), 65% of 5C6-treated TPM and >85% of 5C6-treated PMN were detached. This differed substantially from the 3% of M1/70-treated TPM or PMN detached in the same period. Detachment by lower concentrations of mAb (0.05 μ g/ml) was slower, with 30 min needed for rounding of adherent cells and 90 min for detachment. The attachment of TPM to defined substrata such as fibronectin or immune complexes was unaffected by 5C6 mAb (not shown).

mAb 5C6 Binds to CR3 and Inhibits EAiC3b Binding. The antigen recognized by 5C6 was characterized by the cell binding profile of the mAb and the molecular species identified by immunoprecipitation. Indirect binding RIAs with fixed cells and direct immunofluorescence studies of live cells revealed that 5C6 bound to a methanol- and glutaraldehyde-stable epitope on TPM, RPM, BCG-activated M ϕ , and PMN that was absent from the surface of Kupffer cells (as gauged by immunoperoxidase staining) and from resting or BCG-activated lymphocytes.

Immunoprecipitation of 5C6 antigen from [35 S]methionine-labeled TPM revealed a heterodimer of 165 kD and 95 kD which comigrated with CR3 as precipitated by M1/70 (Fig. 5). Further proof of the identity of these molecules was that the 165-kD chain precipitated by 5C6 peptide mapped identically to the α chain of CR3 after limited tryptic cleavage (not shown). In addition, like intact M1/70, both 5C6 IgG and its F(ab')₂ fragment, but not Fab, inhibited rosetting of EAiC3b to TPM (Table II) whereas no inhibition of rosetting of EAC142 or EIgG (FcR) was seen (not shown). Maximal inhibition of EAiC3b rosetting was obtained with 0.35 μ g/ml of 5C6 IgG.

The 5C6 Epitope Is Distinct from the M1/70 Epitope. The disparity between

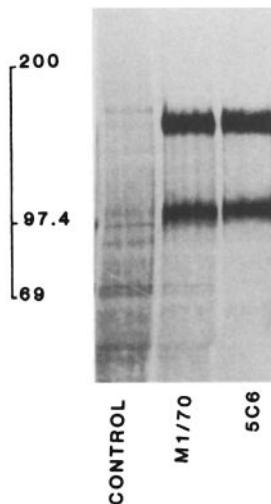


FIGURE 5. SDS-PAGE (10% gel) and autoradiography of [35 S]-methionine-labeled immunoprecipitate of cultured TPM. Control preparation was precipitated with the rat anti-guinea pig PMN mAb 1A10.4, while both M1/70 and 5C6 precipitated a heterodimer of 165 and 95 kD that comigrated.

5C6 and M1/70 in their ability to inhibit adhesion of TPM to BP led us to explore the possibility that the two antibodies identify distinct epitopes on the CR3 molecule. We therefore compared the sensitivity of the binding of these antibodies to proteolytic digestion of the TPM cell surface. Digestion of the cell surface with pronase (0.5 mg/ml for 30 min at 37°C) destroyed most of the M1/70 binding sites and abolished the M ϕ -specific F4/80 epitope, whereas the 5C6 sites were more resistant to proteolysis (Table II). The 5C6 antigen, like that of M1/70, is probably an α chain epitope, as it was restricted to myelomonocytic cells and was absent on lymphocytes that are rich in LFA-1, which shares a common β chain with CR3. The 5C6-epitope might require α chain together with the common β chain since after dissociation of chains by pH 11.5 in solution, 5C6 failed to precipitate any antigen. The 5C6 epitope was also dissociated from the adhesive domain of CR3 because pronase digestion abolished adhesion of TPM to BP while binding of 5C6 was largely intact (Table I).

Optimal Inhibition of Adhesion Requires Intact IgG. We examined the ability of defined Ig fragments to alter M ϕ adhesion to BP in vitro. Intact 5C6 IgG inhibited adhesion of TPM by 92% compared with untreated cells (Table II). The F(ab') $_2$ fragment of 5C6 inhibited ~50% adhesion despite binding comparably to the intact IgG in indirect binding assays. The Fab fragment also bound well showing 50% saturation at 0.1 μ g/ml of protein, but did not inhibit TPM adhesion. Interestingly, in experiments using purified 5C6 IgG at concentrations of 0.05 mg/ml where monovalent binding becomes significant, functional activity of the IgG diminished in a prozone-like phenomenon. It was clear that divalent binding was thus a prerequisite but alone was insufficient to inhibit adhesion. Only the intact 5C6 IgG was able to reverse adhesion, but detachment under these conditions was partial.

The Effect of mAb 5C6 on Recruitment of Myelomonocytic Cells to an Inflammatory Site In Vivo. We next examined the possible role of CR3 in the recruitment of myelomonocytic cells to the peritoneal cavity. Intravenous injection of mice with

TABLE III

Injection of 5C6 IgG In Vivo Inhibited Recruitment of Myelomonocytic Cells to a Peritoneal Exudate: The Effect of Treatments on 18-h Peritoneal Exudates and Blood Leukocytes

Pretreatment	Mice	Peritoneal exudate		Blood leukocytes	
		Number of cells	Differential	Number of cells/ml	Differential
	<i>n</i>	$\times 10^{-6}$		$\times 10^{-6}$	
PBS i.v. or nothing	16	36 ± 4	54P 40M 6L	16 ± 0.2	80P 12M 8L
0.5 mg i.v. 5C6 IgG	18	9.0 ± 2	35P 51M 14L	17 ± 0.2	77P 13M 10L
0.5 mg i.v. 5C6 F(ab') ₂	2	26	71P 29M	15	72P 13M 15L
0.5 mg i.v. YTA IgG	2	22	65P 33M 2L	14	75P 10M 15L

The treatment preceded intraperitoneal thioglycollate injection by 4 h in all cases. Controls, animals not exposed to thioglycollate broth, with or without 5C6 IgG pretreatment, yielded $5-6 \times 10^6$ cells, 35% M, 65% L. Blood and bone marrow cell counts were also unchanged from controls.

TABLE IV

The Effects of 5C6 IgG Treatment on 4-d Peritoneal Exudates and Blood Leukocytes

Treatment	Mice	Peritoneal exudate		Blood leukocytes	
		Number of cells	Differential	Number of cells/ml	Differential
	<i>n</i>	$\times 10^{-6}$		$\times 10^{-6}$	
PBS i.v.	4	27 ± 4	P25 M66 L9	9.1	55P 12M 33L
0.5 mg 5C6 IgG	4	9.4 ± 2	P8 M38 L54	8.6	52P 10M 38L

See footnote in Table III regarding controls.

purified 5C6 IgG was followed 4 h later by an intraperitoneal challenge of sterile thioglycollate broth. A total of 22 5C6 IgG-injected mice and 20 control mice were examined in this series of experiments (Tables III and IV).

In control animals, which received nothing or PBS alone, recruitment of cells to the peritoneal cavity 18 h after thioglycollate injection was unimpaired. Cell yields were enhanced three- to fourfold over untreated controls and consisted predominantly of PMN and Mφ. By 4 d the recruited peritoneal cell yields had dropped by 25%, with a higher proportion of Mφ and fewer PMN. In two mice injected intravenously with 0.5 mg of YTA, an isotype-matched mAb directed against CD4 antigen, 22×10^6 peritoneal cells were recovered at 18 h compared with 26×10^6 cells recovered from PBS injected animals in the same experiment, showing a small diminution of recruitment. In striking contrast, peritoneal recruitment in the 5C6 IgG-injected animals was reduced by $84 \pm 3\%$ at 18 h, whereas peritoneal yields in animals that received the mAb, but no thioglycollate broth, were unaffected. Inhibition of recruitment persisted for at least 4 d in 5C6 IgG-treated animals. From differential counts we calculated that 3.1×10^6 PMN were recovered at 18 h in antibody-treated mice compared with 2.0×10^7

in controls and 3.5×10^6 M ϕ after 4 d, compared with 1.8×10^7 in controls. The ability of 5C6-intact IgG to inhibit recruitment of myelomonocytic cells correlated with its inhibition of adhesion in vitro, whereas the F(ab') $_2$ fragments, which showed only partial inhibition of adhesion in vitro, failed to impair recruitment in vivo. The inhibition of recruitment by 5C6 IgG was dose-dependent: 0.5 mg i.v. IgG was the smallest dose of IgG able to inhibit peritoneal recruitment >80% at 18 h and inhibition of recruitment by 0.1, 0.2, and 0.35 mg was 40, 51, and 68%, respectively (one experiment, two mice per data point).

Peritoneal cells recovered after intravenous treatment with mAb adhered well to both BP and TCP in vitro, suggesting that their exposure to 5C6 had been subsaturating. Experiments in which 5C6 mAb was injected directly intraperitoneally, resulted in binding of antibody to all peritoneal M ϕ which, when harvested, had lost their ability to adhere to BP or spread on TCP, like M ϕ treated with mAb in vitro.

The blood, bone marrow, liver, and spleen of control and mAb-treated mice were examined to identify its site of action. There was no difference in number or differential counts of femoral marrow cells between control and 5C6-treated mice (six experiments, not shown). Marrow plugs were lightly fixed in paraformaldehyde, embedded, and stained by the immunoperoxidase method after frozen section to detect mAb that had bound in vivo. Marrows from 5C6-treated mice showed heavy labeling of most mature myelomonocytic cells (not shown). Blast cells, lymphocytes, and resident bone marrow M ϕ remained unlabeled, as expected. There was no evidence of cell death.

Table III shows the analysis of blood leukocytes in control and experimental animals. Thioglycollate broth elicited a PMN leukocytosis at 18 h, which was similar in antibody-treated and untreated mice. In mice that had not been exposed to thioglycollate, with or without 5C6 IgG pretreatment, there were $7-10 \times 10^6$ cells/ml, 60–67% PMN. These data showed that the egress of myelomonocytic cells from marrow into blood had not been impaired. Furthermore, blood leukocytes in both control and 5C6 IgG-treated mice were not aggregated. Blood PMN from control animals adhered well to BP and TCP in vitro, whereas blood leukocytes from 5C6-IgG-treated mice failed to adhere to either substratum (not shown).

Histological analysis of sections of liver and spleen showed no evidence of leukocyte aggregation, cell death, or removal by phagocytosis. Particular attention was paid to the examination of liver sinusoids and the splenic red pulp and no differences were discerned between control and mAb-treated animals. These observations made it unlikely that excessive PMN margination or phagocytic clearance was responsible for the failure to recruit myelomonocytic cells to the peritoneal cavity.

The observed inhibition of recruitment by antibody could be due to inhibition of adhesion of circulating cells to endothelium and/or failure of marginated cells to undergo directional migration into the tissue spaces. We therefore examined the ability of bone marrow leukocytes from 5C6-treated and control mice to undergo chemotaxis in response to FMLP in an under-agarose assay (six animals, three experiments). There was no difference in the distance migrated by bone marrow PMN from 5C6 IgG-treated or control mice (not shown), indicating

that the mAb treatment *in vivo* had not impaired the ability of leukocytes to respond to a defined chemotactic stimulus.

Discussion

Our main conclusion is that the CR3 molecule contributes to a particular type of adhesion of myelomonocytic cells *in vitro* and *in vivo*. Our strategy of screening for mAbs that could inhibit or reverse the adhesion of TPM to BP yielded an inhibitor of inflammatory cell recruitment *in vivo*, most likely by interfering with adhesion to endothelium. In the light of findings in the leukocyte adhesion deficiency syndrome, it is perhaps not surprising that the 5C6 mAb was directed against the CR3 molecule, to an epitope distinct from the iC3b binding site. Our studies show that the CR3 is necessary for certain adhesive and migratory functions of leukocytes and suggest a strategy to isolate novel inhibitors of inflammation that interfere with recruitment of myelomonocytic cells.

Adhesion of Myelomonocytic Cells In Vitro. The differences between BP and TCP are not well documented, but it is clear that leukocytes are unusual in their ability to adhere to both substrata, in the presence of serum. Our studies with various inhibitors have defined at least two modes of adhesion, firstly CR3-dependent, pronase-sensitive adhesion of myelomonocytic cells to BP. This depends on Mg²⁺, like some other LFA-adhesive interactions (22) and is readily reversible by chelation or selected mAb such as 5C6. The requirement of Mg²⁺ for CR3-dependent adhesion correlates with the cation requirements for the rosetting of complement-opsonised erythrocytes to murine myelomonocytic cells (23), but differs from human monocyte CR3, which requires both calcium and magnesium for binding and phagocytosis (24). This adhesive mechanism provides a useful method for isolation of certain Mφ populations. Only Mφ that express surface CR3 can bind to BP, unlike Kupffer cells (9) and certain tissue Mφ (25), suggesting that other adhesive molecules on Mφ cannot replace this particular function. Immature PMN do not adhere to BP under these conditions, but this deficiency can be overcome by inflammatory stimuli, which are known to increase surface expression of CR3 in PMN by exocytosis (26).

Secondly, there is CR-independent, pronase-resistant, and Mφ-specific adhesion to TCP or glass. Studies with mAb 5C6 show that the CR3 can contribute to spreading of elicited Mφ on glass, but adhesion depends on an undefined mechanism, which at present can only be reversed by local anaesthetics (2). Further studies, perhaps with specific mAbs that inhibit this function, are needed to establish whether this is due to a single or multiple Mφ-adhesive molecule(s).

The relationship between Mφ adhesion and spreading is complex, but specific inhibitors such as 5C6 make it possible to dissect the contribution of the CR3 molecule to these processes. The mechanism of 5C6 IgG-mediated inhibition of adhesion is unclear. Inhibition of CR3 adhesion by an antibody or antibody fragment was not conferred by the ability of an antibody to prevent the binding of iC3b to its receptor. This is evident from the failure of M1/70 and the 5C6 F(ab')₂ adequately to inhibit adhesion while efficiently blocking iC3b binding. The epitope recognized directly by 5C6 is also dissociated from both the adhesion and iC3b binding sites of CR3 because the 5C6 Fab fragment bound excellently in direct binding assays (not shown), and in fact, competed with the intact IgG

while having no functional inhibition of either of those two functions. The 5C6 antigen, unlike adhesion function, was relatively resistant to pronase digestion, again suggesting that this epitope is distinct from both adhesion and iC3b binding domains. The requirement for divalent binding for functional inhibition by antibody is consistent with a mechanism that involves steric hindrance. The requirement for intact IgG to inhibit adhesion efficiently could be ascribed to the following mechanisms: (a) the Fc gives added length to the intact molecule, which is necessary for efficient steric hindrance; (b) the Fc portion might alter the relative mobility of the two antigen binding sites, altering their functional activity; and (c) the Fc might bind to the surface of the same cell as the antigen binding site (?) via FcR, and in some indirect way further impair adhesion. In any event, further experiments are needed to elucidate the role of the Fc fragment of intact 5C6 IgG.

The reversible nature of CR3-mediated adhesion is shown in the detachment assays. Such detachment could result from limiting available receptor numbers below a threshold critical for adhesion, by competing with substratum for receptor binding or by uncoupling receptors from cytoskeleton. The inhibitory effect of cytoskeletal inhibitors in the *in vitro* assay system highlights the role of cytoskeleton in stabilizing CR3-mediated attachment.

Inhibition of Inflammatory Cell Recruitment In Vivo. Intravenous injection of relatively small amounts of 5C6 mAb, 0.5 mg/animal, resulted in a profound and relatively prolonged (>4 d) inhibition of recruitment of PMN (1 d) or M ϕ (4 d) into a thioglycollate-challenged peritoneal cavity. The effect was highly reproducible, antibody-specific, dose-dependent, and not observed with the F(ab')₂ fragment, a poor inhibitor of adhesion *in vitro*. Further studies confirmed that the 5C6 antibody bound to bone marrow and circulating PMN *in vivo* and had no detectable effect on leukocytosis or egress from bone marrow. Circulating PMN tested after antibody treatment could not adhere *in vitro*, suggesting functional activity of antibody *in vivo*. The failure to recruit cells was not due to cytotoxicity, opsonization and clearance by phagocytosis, or aggregation within the circulation. Furthermore, treated PMN had unimpaired adhesion to other substrata *in vitro*, such as fibronectin-coated surfaces (our unpublished observations) and were fully able to respond to chemotactic stimuli such as FMLP. Taken together, these studies suggest that 5C6 impaired the interaction of myelomonocytic cells with endothelium, but further studies are needed to substantiate this hypothesis. Studies by others have implicated CR3 and LFA-1 molecules in leukocyte adhesion to endothelial cells *in vitro* (27). In an *in vivo* study similar to ours, Arfors et al. (28) have shown that a murine mAb directed against the CD18 complex, i.e., all members of the LFA family, inhibited both neutrophil accumulation and plasma leakage in rabbits, presumably by impairing interactions with the vascular endothelium. Our study now shows that an equally striking inhibition can be achieved with an antibody directed to CR3 alone.

The cells obtained from the peritoneal cavity after thioglycollate broth injection under antibody cover showed no impaired adhesion. These could be either original resident cells or recruited cells which had been insufficiently coated with antibody, since injection of antibody intraperitoneally confirmed that direct access to antibody *in vivo* inhibited subsequent adhesion *in vitro*.

These studies shed further light on the leukocyte adhesion deficiency syndrome in that it appears, at least in mice, that CR3 is necessary for migration of myelomonocytic cells to an inflammatory focus. However, indirect effects of CR3 ligation on expression of other members of the LFA family cannot be entirely ruled out by the present experiments. The failure of M1/70, unlike 5C6, to inhibit adhesion or detach adherent cells in vitro supports the notion of multiple functional domains on CR3 (29). The differences between 5C6 and M1/70 underline the dissociation between binding to a particular antigen and consequent functional effects. Strategies that are directly functional, as pursued in this study, and in which in vitro observations can be correlated with effects in vivo, are more likely to yield reagents that clarify complex physiologic processes, regardless of the antigen to which they bind. The definition of discrete adhesion-promoting domains on receptors such as CR3 can lead to development of novel, specific inhibitors of inflammation that act at the point of myelomonocytic cell exit from the circulation. Although the present studies implicate CR3 as a suitable target for such therapeutic agents, other molecules may be involved in constitutive entry of monocytes into tissues and enhanced recruitment of inflammatory cells during T cell-dependent recruitment and activation of macrophages.

Summary

Macrophage interactions with extracellular matrix and other cells are important in phagocytosis, inflammation, and immunity. To learn more about the surface molecules involved in adhesion we compared the binding of murine macrophages and polymorphonuclear leukocytes (PMN) with artificial substrate in vitro. A distinctive type of adhesion of thioglycollate-elicited peritoneal macrophages (TPM) to bacteriologic plastic (BP) was defined, which was pronase-sensitive, Mg²⁺-dependent, and required cytoskeletal stabilization. A rat mAb designated 5C6 was isolated because it inhibited TPM attachment to BP, as well as mediating detachment of TPM adherent to that substratum. In addition, it inhibited the attachment of PMN to tissue culture plastic. This antiadhesive property of 5C6 mAb required intact IgG; the F(ab')₂ fragment was partially effective and Fab was ineffective. 5C6 recognized the type 3 complement receptor, inhibiting rosetting of EAC3bi to TPM and immunoprecipitating a heterodimer of 160 and 95 kD that comigrated with the M1/70 immunoprecipitate. 5C6 recognized a pronase-stable epitope distinct from that of M1/70. Other mAbs, including M1/70 (CR3) and 2.4G2 (FcR), failed to have any antiadhesive effect in vitro.

The inhibitory activity of 5C6 in short-term adhesion assays correlated with its inhibition of recruitment of myelomonocytic cells to a thioglycollate-elicited peritoneal exudate in vivo, after intravenous injection of mAb. 5C6 IgG inhibited recruitment of myelomonocytic cells by 84 ± 3% at 1 d compared with saline-injected controls. The F(ab')₂ fragment and a class-matched control IgG had little effect. Recruitment of TPM at 4 d was also efficiently inhibited by 5C6 IgG. 5C6 IgG was not cytotoxic, had no effect on marrow egress, did not cause increased phagocytic clearance of circulating neutrophils, and had no adverse effect on chemotaxis in vitro. We show that CR3 alone of the LFA-family is necessary for the recruitment of myelomonocytic cells to inflammatory stimuli

such as thioglycollate broth. This strategy may be of general use in isolating reagents that inhibit the adhesive function of CR3 and provides a novel approach to antiinflammatory therapy.

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