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Derralynn A. Hughes and Siamon Gordon

Macrophage (Mφ) expression of the leukocyte integrins has been implicated in their adhesion and migration in the adult. Little is known, however, of the expression or function of these molecules during development. This study defines the spatial and temporal sequences of expression of the type 3 complement receptor (CR3) in the developing mouse; establishes the functional efficacy of this molecule in spreading, adhesion, and phagocytosis; and investigates its role in inflammatory and constitutive migration. Expression of CR3 on monocytes occurred early compared to Mφ-restricted glycoprotein F4/80, but expression on stellate tissue Mφ appeared later than F4/80 and was transient. Expression of CR3 on resident tissue Mφ is more widespread during development, being retained on only very specific Mφ populations in the adult. Neutrophil polymorphs expressed CR3 from day 17 of gestation onward. The anti-CR3 mAb 5C6 was used to investigate the role of CR3 in adhesion, spreading, and phagocytosis by neonatal Mφ. Neonatal macrophages were found to adhere, spread, and phagocytose by CR3-dependent mechanisms, and a CR3-independent system was implicated in the spreading of neonatal Mφ. The role of CR3 in migration during development was then investigated. 5C6 had potent effects on the early stages of the migration of myelomonocytic cells to an inflammatory stimulus in vivo. Despite efficient transplacental transfer of the Ab from pregnant mother to fetus, the process by which monocytes generate populations of resident tissue Mφ was undisturbed, indicating the existence of CR3-independent mechanisms of monocyte migration during development.


In the adult mouse, constitutive migration of monocytes from the bone marrow maintains most resident tissue Mφ populations (7) and continues alongside inflammatory and immunologically driven recruitment processes in which a family of structurally and functionally related surface glycoproteins, the leukocyte β2 integrins (LFA-1, CR3, and p150,95) (8), have been implicated (9–11). Each of these molecules consists of an antigenically distinct α-chain (150–190 kDa) noncovalently associated with a β-chain (95 kDa) as an αβ dimer (reviewed in Ref. 12). Congenital inability to express the β2 chain results in an impairment of surface expression of all three heterodimers, severe defects in leukocyte accumulation in vivo and adherence in vitro, and an increase in life-threatening infections (13). However, the role of these molecules in the constitutive or inflammatory migration of monocytes during development is unknown.

Similarly, while the distribution of leukocyte integrins in the adult is well documented (14, 15), little is known of their expression during ontogeny. In the adult, CR3 is expressed on monocytes, neutrophil polymorphonuclear leukocytes (PMN), and NK cells in blood and hemopoietic tissues, but not on populations of stellate tissue Mφ, except microglia in the brain, marginal zone Mφ in the spleen, and Mφ in the subcapsular sinus of the lymph node (16). This suggests that CR3 expression is widely downregulated as a consequence of constitutive entry of monocytes into tissues and differentiation into resident Mφ such as Kupffer cells and implies a possible role for CR3 in development. In contrast, high levels of CR3 expression are retained on Mφ that have been recruited to tissues in response to inflammatory signals.

The aim of this study, therefore, was to analyze the spatial and temporal sequences of CR3 expression in fetal and neonatal tissues as a first step to define its role in constitutive and inflammatory monocyte recruitment during development. Subsequently, mAb 5C6, which blocks CR3-dependent adhesion of Mφ to bacteriologic plastic (BP) and has potent effects on some forms of myelomonocytic migration in vivo (17–20), also provided a useful tool to determine the ability of CR3 on isolated neonatal Mφ to...
mediate adhesion, spreading, and phagocytosis. Having then established the efficacy of neonatal CR3 in vitro, its role in the processes of constitutive and inflammatory myelomonocytic migration during development was also investigated.

Materials and Methods

Animals

Embryos and newborn mice (Pathology Oxford (PO)) were bred at the Sir William Dunn School of Pathology (Oxford, U.K.) and maintained under conventional laboratory conditions with free access to food and water. Females were inspected daily for the presence of a vaginal plug, which was designated day 0 of pregnancy. The day of birth (usually day 19 of pregnancy) was designated day 0 of neonatal life.

Antibodies

The following rat mAbs were used as hybridoma supernatants for immunohistochemistry. F4/80 (21) recognizes a 160-kDa Ag of unknown function. M1/70 (22) and 5C6 (18) were used in combination at a final dilution of 1:10 in PBS to recognize the CR3. Biotinylated rabbit anti-rat IgG were obtained from Vector Laboratories (Peterborough, U.K.). Polyclonal Abs recognizing F4/80 and sialoadhesin were prepared in our laboratory by Drs. P. Dri and P. Crocker and were routinely used at a dilution of 1/500 for immunohistochemistry.

Immunocytochemical techniques

Fixation and sectioning of murine organs. Embryos from day 8 to birth and neonatal animals up to 2 wk after birth were examined. Visceral yolk sac, liver, spleen, bone marrow, thymus, lung, kidney, and gut were dissected from conceptuses as they became readily identifiable. Organs were washed in PBS before freezing in OCT embedding medium (Miles, Elkhart, IN) cooled in isopentane (BDH-Merck, Poole, U.K.) over liquid nitrogen. Frozen sections were cut on a cryostat (Leica, Wetzlar, Germany) at 5 µm, air-dried for 1 h, and frozen at −20°C until further use. Fresh organs were fixed for 10 min in 2% paraformaldehyde in HEPES-buffered isotonic saline before staining; 2 mM calcium chloride was added to the fixative to maintain the structural conformation of the integrin.

Ag detection. Fixed sections were washed in PBS containing 0.1% (v/v) Triton X-100 (BDH-Merck) and treated with 2% normal rabbit serum for 30 min. Sections were incubated for 90 min in hybridoma supernatants. Endogenous peroxidase activity was inactivated by incubation of sections with 10−2 M glucose, 10−3 M NaNO3, and 40 U glucose oxidase in 100 ml 0.1 M phosphate buffer for 15 min at 37°C (23). Affinity-purified, mouse-adsorbed, biotinylated second Ab (Vector) was used at 1% for 45 min, and avidin-biotin-peroxidase complex (ABC elite, Vector) (24) was used according to the supplier’s recommendation. The presence of Ag was revealed by incubation with 0.5 mg/ml 3,3′-diaminobenzidine, HCl (Polysciences, Northampton, U.K.) and 0.024% H2O2 in 10 mM imidazole in PBS, pH 7.4.

Double labeling. To detect a second Ag, polyclonal Abs were incubated on sections for 90 min followed by 1% alkaline phosphatase-conjugated goat anti-rabbit second Ab for 60 min (Vector). The Ag was revealed by incubation with a 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium alkaline phosphatase substrate kit (Vector), and sections were counterstained in cresyl fast violet acetate (BDH-Merck) and mounted in DPX (BDH-Merck). Sections in which primary Ab, secondary Ab, or avidin-biotin-peroxidase complex reagent were omitted and sections treated with nonimmune IgG were stained in cresyl fast violet acetate (BDH-Merck) and mounted in DPX.

In vivo detection of Ags. Pregnant mice were injected i.v. with 0.5 mg of 5C6 mAb or isotype-matched control mAb, 1C5 (which does not react with the surface of murine leukocytes) (25), on days 14 and 17 of pregnancy. Newborn PO mice were injected i.p. with 0.05 mg of purified rat mAb. Offspring of the pregnant mice or the newborn mice injected directly were subsequently fixed by perfusion with 2% periodate-lysine-paraformaldehyde solution (PLP) (26). After postfixing in the same solution for 4 h, tissues were impregnated with 20% sucrose in 0.1 M phosphate overnight before freezing and sectioning, as described. No further fixation of these sections was required. Affinity-purified, mouse-adsorbed, biotinylated anti-rabbit Ab (Vector) was used to locate injected rat Abs, and polyclonal rabbit anti-F4/80 antisera was used to define the distribution of F4/80+ monocytes or Mφ in these sections.

Photography. Representative black and white photographs were taken using a blue filter (Watten Gelatin Filter no. 47, Kodak, Rochester, NY) that intensifies the brown precipitate. Color photographs were taken using a 15-cc magenta filter.

Neonatal peritoneal Mφ. Resident peritoneal Mφ (RPM) were obtained from the cavities of 3-day-old PO mice by lavage with 1 ml of PBS. Recruited cells were similarly harvested from 3-day-old PO mice that had been injected i.p. with 25 µl of Brewar’s complete thioglycollate broth on the day of birth.

Adhesion to artificial substrate (18). Cells to be assayed for adhesion were suspended in RPMI 1640 with 10% PBS and plated at a density of 3  × 106 Mφ/well in flat-bottom BP or tissue culture plastic (TCP) 96-well plates (Flow Laboratories (Rickmansworth, U.K.) and Nunc/Life Technologies (Paisley, U.K.)). mAb (5 µg/ml) alone or in combination with divalent cation chelator (5 mM EDTA) were added to test wells and incubated for 30 min at 4°C. After incubation for 90 min at 37°C, plates were washed three times in PBS, and adherent cells were fixed in methanol and stained with 40% Giemsa solution for 1 h. Plates were washed in tap water, and the retained dye was solubilized in methanol and quantified by measuring absorbance at 450 nm in an automatic plate reader (Anthos III, Denley Instruments, Billinghamurst, U.K.). In some assays cell viability was assessed at each stage using trypan blue dye exclusion (Sigma, Poole, U.K.).

Spreading assays

Cells were resuspended in RPMI 1640 plus 10% FBS and plated at a density of 103 Mφ/chamber in multichamber slides. mAb (2 µg/ml 5C6 or control 1C5) or chelator (5 mM EDTA) was added to test chambers and retained throughout the experiment. Slides were washed in PBS, and adherent cells were fixed in 0.2% glutaraldehyde at 30 min or 1, 3, 6, 10.5, or 24 h. The number of cells with a spread morphology was assessed using phase contrast microscopy and expressed as a percentage of the total adherent cells.

Phagocytosis of opsonized red cells

Sheep erythrocytes (Becton Dickinson, Oxford, U.K.; stored in Alsever’s solution at 4°C until used) were incubated with 30% mouse IgM anti-sheep erythrocyte in PBS (Nordic Immunological Laboratories, Tilburg, The Netherlands) for 45 min at 4°C, followed by fresh mouse serum for 30 min at 37°C to fix iC3b. Opsonized sheep erythrocytes (EAiC3b) were used at a 5% (v/v) suspension for 60 min to assay for rosetting at 4°C or phagocytosis at 37°C (27, 28). Binding was quantified by counting the number of erythrocytes attached to 100 Mφ. Noninternalized erythrocytes were lysed in 0.147 M NH4Cl before counting the number of erythrocytes ingested per 100 Mφ.

LPS-induced myelomonocytic recruitment

Newborn PO mice were injected intradermally with 10 µl of PBS or LPS (0.2 µg) containing 1% Monensal blue to mark the site of the lesion and at the same time i.p. with 0.05 mg of purified 5C6 or isotype-matched control mAb (1C5). Animals were perfusion fixed with PLP at 1, 4, 8, 10, 5, 24, and 56 h after injection and processed as above to reveal injected mAb and F4/80 on recruited cells.

Results

Expression of the leukocyte integrin, CR3, during murine development

To establish a basis for manipulation of myelomonocytic cell migration during the ontogeny of the mononuclear phagocyte system, the expression of CR3 on monocytes, Mφ, and PMN during murine development was investigated. Tissue samples were taken throughout fetal and neonatal life and stained using a combination of two mAbs, M1/70 and 5C6, which show differential sensitivity to digestion of the cell surface with pronase and are therefore believed to recognize different epitopes of CR3 (18). No staining was observed with the irrelevant isotype-matched mAbs CAMPATH-1G or 1C5. Organs from at least two mice from each of three independent litters were examined at each developmental stage, and in each case a comparison was made with consecutive sections stained with the F4/80 polyclonal Ab.

Lymphohemopoietic organs

Liver (Fig. 1). The liver primordium develops relatively earlier in the mouse than in man and is visible at only 9 days of gestation, when it is composed of broad hepatic cords separated by large sinusoids containing nucleated erythroblasts (Eh). CR3+ monocytes were found in the fetal liver of whole embryos from this time.
cells (Kc) are only occasionally in contact with hemopoietic cells. f, Day 17 fetal liver: CR3 monocytes are associated with clusters of erythroid and CR3 myeloid cells (both monocytes and PMN); c, e, Day 1 newborn liver: hemopoietic activity continues, and CR3 is now expressed at low levels on stellate Mφ as well as on clustered monocytes. d, Day 17 fetal liver: CR3 stellate Mφ are associated with clusters of erythroid and CR3 myeloid cells (both monocytes and PMN); c, e, Day 1 newborn liver: hemopoietic activity continues, and CR3 Mφ plasma membrane processes are still associated with hemopoietic cell clusters. f, Day 3 newborn liver: hemopoietic activity is now much reduced and CR3 plasma membrane processes of the immature Kupffer cells (Kc) are only occasionally in contact with hemopoietic cells. g, Day 8 newborn liver: there are many highly membranous CR3 Kc. No hemopoietic clusters. h, Adult liver: CR3 is expressed on spindle shaped sinus-lining Mφ. Membrane staining of the cells is much reduced compared with that in the neonate. Bar = 50 μm.

FIGURE 1. CR3 is expressed on hepatic monocytes and stellate Mφ throughout murine development. a, Day 11 fetal liver: monocytes (arrows) are scattered among endodermal cells. b, Day 13 fetal liver: the number of CR3 monocytes has increased and are located around the margins of the liver sinususes (s). c, Day 15 fetal liver: hemopoietic activity has increased and CR3 is now expressed at low levels on stellate Mφ as well as on clustered monocytes. d, Day 17 fetal liver: CR3 stellate Mφ are associated with clusters of erythroid and CR3 myeloid cells (both monocytes and PMN); c, e, Day 1 newborn liver: hemopoietic activity continues, and CR3 Mφ plasma membrane processes are still associated with hemopoietic cell clusters. f, Day 3 newborn liver: hemopoietic activity is now much reduced and CR3 plasma membrane processes of the immature Kupffer cells (Kc) are only occasionally in contact with hemopoietic cells. g, Day 8 newborn liver: there are many highly membranous CR3 Kc. No hemopoietic clusters. h, Adult liver: CR3 is expressed on spindle shaped sinus-lining Mφ. Membrane staining of the cells is much reduced compared with that in the neonate. Bar = 50 μm.

FIGURE 2. Expression of CR3 cells in the developing spleen occurs before the onset of local hemopoiesis. a, Day 12 fetal spleen: occasional CR3 monocytes appear in the mesenchymal mass (arrow). b, Day 13 fetal spleen: CR3 monocytes are distributed throughout the spleen. c, Day 15 fetal spleen is now an active site of hemopoiesis. The tissue is homogeneous in architecture and contains many CR3 monocytes. d and e, Day 16 and 18 fetal spleen: some areas destined to become white pulp (w) are free of CR3 cells. f, Day 1 newborn spleen: small areas of white pulp (wp) are already distinct; CR3 monocytes, PMN, and Mφ are present only in the red pulp. g, Day 4 newborn spleen: hemopoiesis is reduced, but continues throughout adult life. h, Day 8 newborn spleen: the spleen now resembles that of a normal adult. i, Adult spleen: stellate CR3 marginal zone Mφ (mz) delineate the white pulp (wp). The red pulp (rp) contains few CR3 mature Mφ but many monocytes and PMN. Bar = 50 μm.

onward, whereas F4/80 was only detected on monocytes from day 10 of gestation (day 10e; not shown). The density of CR3 monocytes in liver increased as hemopoiesis progressed from day 11e (Fig. 1a) and by day 13e, when the liver is well developed, labeled monocytes could be seen in clusters around liver sinusoids (Fig. 1b). Hemopoietic foci were found intermingled within the hepatic cords, but unlike the stellate Mφ detected by F4/80, CR3 monocytes were not associated with developing Eb. The F4/80 stellate Mφ first exhibited CR3 expression on day 15 (Fig. 1c). By day 17e, CR3 PMN were apparent and located in clusters of erythroid and myeloid cells around a CR3 stromal Mφ (Fig. 1d). CR3 PMN and monocytes increased in number until shortly after birth (Fig. 1, e and f) and continued to be distributed unevenly throughout the liver, with high numbers of cells adjacent to the sinusoids. CR3 staining of stellate Mφ was maximal around 8 days after birth (Fig. 1g) even though at this stage the Mφ were no longer associated with remaining erythroid cells. Hemopoietic clusters containing CR3 monocytes were rare by this stage, and mature Mφ accounted for most of the CR3 staining. After birth, declining hemopoietic activity in the liver was associated with a decreased number of CR3-staining monocytes and PMN, while CR3 staining of mature Mφ progressively decreased in intensity. In the adult, CR3 expression on spindle-shaped sinus-lining Mφ (Kupffer cells) was much reduced or absent (Fig. 1h), in contrast to F4/80, which is highly expressed on mature Kupffer cells in the liver (not shown).

Spleen (Fig. 2). By 11 days of gestation, the epithelial lining of the dorsal mesentery of the stomach is thickened and represents the anlage of the spleen. CR3 monocytes first appeared among the cells of this rudimentary spleen on day 12e (Fig. 2, a and b), at the same time as F4/80 expression was first detected on splenic monocytes. Ramified F4/80 Mφ appear on day 15e and are surrounded by developing red cells, but at this stage these cells do not
express CR3 (Fig. 2c). From day 16e (Fig. 2d) the splenic architecture becomes more complex, with distinct arteries and veins and regions destined to become lymphoid areas, containing none of the monocytes or numerous CR3+ granulocytic cells that are then apparent in the developing red pulp (Fig. 2e). CR3+ monocytes and PMN continue to be detected in the red pulp immediately after birth and during adult life (Fig. 2, f and g). In contrast, CR3+ stellate Mϕ were most evident in the expanding marginal zone of the white pulp 1 wk after birth (Fig. 2h), and in the adult could be detected both in the marginal zone and occasionally around the central arteriole in the white pulp (Fig. 2i). The network of stromal Mϕ expressing F4/80 in the red pulp never expressed CR3.

Yolk sac. CR3 was detected on stellate Mϕ and monocytes within and along vessels of the vitelline yolk sac from the ninth day of gestation (day 9e; Fig. 3a), which is 1 day earlier than F4/80 expression has been observed on cells seen mainly in the yolk sac mesoderm; CR3 is also expressed on more stellate Mϕ within vessels than F4/80. The density of CR3 staining peaked on day 10e, when nucleated Eb were also seen within the blood spaces. At no stage were Mϕ associated with clusters of Eb.

Bone marrow. Hemopoietic stem cells and F4/80+ Mϕ invade the cartilaginous rudiment of bone on day 16e, resulting in the formation of the marrow cavity. CR3+ monocytes and PMN were evident within the stroma of the bone marrow from day 17e, when hemopoietic activity is initiated (Fig. 3b). Clusters of erythroid and CR3+ myeloid cells surround central F4/80+ Mϕ during development and in the adult mouse, but CR3 staining was only observed on the stromal Mϕ of the bone marrow from day 17e until shortly after birth and, unlike F4/80, was absent from these Mϕ in the adult as previously reported (29).

Nonhemopoietic tissues

F4/80+ and CR3+ monocytes were seen throughout the undifferentiated tissue within which organs develop. As organogenesis proceeds, F4/80+ Mϕ persist to become components of loose connective tissue of differentiated organs, whereas CR3 expression is variable depending on the organ.

Gut. CR3 was first detected on spindle-shaped Mϕ around the capsule of the gut on day 11e. One day later, when the lumen became visible, CR3+ Mϕ were still confined to the capsule, whereas F4/80+ Mϕ appeared in the lamina propria at this stage. From day 13e CR3+ monocytes appeared in the lamina propria circumferential to the developing villi, and on day 15 mature Mϕ were visible within the villi. The pattern of moderate CR3 expression on the stellate Mϕ within the villi and strong expression on monocytes of the lamina propria continued throughout development and in the adult (Fig. 3, c and d).

Lung. The lung anlage develops on day 9e as rudiments of larynx-trachea-branchi. CR3+ Mϕ, with extensive processes, were evident around the outer margins of the mesenchyme of the developing lung from day 11e, at the same stage that F4/80+ cells were observed randomly distributed within the mesenchyme. By day 13 the lungs were clearly subdivided into lobes, and by day 14 the disappearance of mesenchyme from the lung was accompanied by the formation of airways and vessels with which some CR3+ monocytes and more spindle-shaped Mϕ were associated (Fig. 3e). CR3+ cells, often in pairs, and F4/80+ Mϕ were distributed throughout the intraalveolar septa of the lung from just before birth (Fig. 3f), whereas the alveolar Mϕ populations of the adult failed to express CR3 or F4/80.

Mϕ of the kidney failed to express CR3 at any point during development, whereas F4/80 was expressed on Mϕ between the tubules from day 12e.

Thymus. The thymus becomes visible as epithelial lobes surrounded by a thin outer capsule of mesenchyme at 13 days of gestation. Flattened F4/80+ cells can be seen in the thymus from this time. As the organ grows, the F4/80+ Mϕ increase in number and become more stellate, the majority of Mϕ were found in the cortical regions among developing thymocytes. By contrast, CR3+ monocytes could only be detected in the vascularized corticomediullary region of the thymus from day 16e onward when the thymus increases strikingly in volume and the flattened F4/80+ Mϕ do not express detectable levels of CR3 at any point during development or in the adult (Fig. 3, g and h).

Peritoneal cells

Resident and elicited neonatal peritoneal Mϕ were immunoreactive for CR3 (not shown).

The immunohistochemistry outlined above established that CR3 was expressed on monocytes and tissue Mϕ during development, but was gradually lost after birth from mature tissue Mϕ in many regions. The pattern of CR3 expression differed from that of F4/80.
both spatially and temporally (summarized in Table I). CR3 was only retained on the marginal zone M\(\phi\) of the spleen and the subcapsular M\(\phi\) of the lymph nodes (not shown), which may be involved in trafficking of leukocytes into these organs in the adult. This was suggestive of a specific role for CR3 during the developmental period and was consistent with a possible role for CR3 in the adhesion or migration of monocytes into and within organs before they differentiate into resident tissue M\(\phi\). It was impossible to obtain neonatal peripheral blood monocytes in sufficient numbers to assess their use of CR3 in vitro; however, in the adult RPM have been successfully used as surrogate monocytes in adoptive transfer assays of migration (25). Isolated neonatal peritoneal M\(\phi\) were therefore used to establish the functional competence of the CR3 expressed during murine development. The spreading, adhesion, and phagocytosis of neonatal peritoneal M\(\phi\) were assessed at a stage when there was still significant expression of CR3 on the stromal M\(\phi\) of spleen and liver.

**CR3-dependent adhesion of neonatal M\(\phi\)**

mAb 5C6 was initially selected for its ability to inhibit the adhesion of adult M\(\phi\) to BP by blocking the interaction between CR3 and an undefined component of FBS that coats the plastic surface (18). This interaction is also inhibited by EDTA, since the receptor function of integrins depends on the presence of divalent cations (30, 31). In this study, short term adhesion assays to BP were performed with neonatal and adult peritoneal cells lavaged from the resting peritoneal cavity or after its stimulation 3 days previously with thioglycolate broth. The comparative adhesion qualities

### Table I. Expression of F4/80 and CR3 during murine development

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<th>CR3 Monocytes</th>
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![FIGURE 4. CR3-dependent adhesion and spreading of neonatal and adult peritoneal M\(\phi\).](http://www.jimmunol.org/)

A and B. Adhesion of neonatal and adult M\(\phi\) to bacteriologic plastic: preincubation of the cells with 5C6 mAb or chelation of divalent cations with EDTA inhibited the adhesion of both RPM and TPM to BP. Each data point represents eight replicate wells; results were confirmed in four independent experiments. C. Spreading of neonatal and adult peritoneal M\(\phi\) on glass: 100% neonatal cells exhibited ramified morphology 21 h before the adult cells. Each data point represents three replicate wells; results were confirmed in three independent experiments. D. The spreading of neonatal and adult peritoneal M\(\phi\) at 6 h was only partially inhibited by incubation with 5C6 mAb and was more fully inhibited by chelation of divalent cations using EDTA. Each data point represents three replicate wells; results were confirmed in three independent experiments.
of the cells and the effects of chelation of divalent cations or pre-incubation of the cells with mAb 5C6 are shown in Figure 4, A and B. The adhesion of neonatal peritoneal Mφ to BP, like that of adult Mφ, was divalent cation dependent and almost completely inhibited by 5C6. The CR3 of neonatal cells is therefore functional in adhesion to BP. However, in contrast to the adult where thioglycolate-elicited peritoneal Mφ (TPM) were more adherent than RPM, neonatal RPM adhered more readily to BP than neonatal TPM.

Role of CR3 in the spreading of neonatal Mφ

The interaction of Mφ with more complex substrata such as serum coated glass or TCP can be described in the two phases: adhesion and spreading. 5C6 and EDTA, which inhibit Mφ adhesion to BP, reduce the spreading of adult cells on glass and TCP, but fail to inhibit their adhesion. The kinetics of spreading of adult and neonatal peritoneal Mφ on glass and its dependence on CR3 were assessed using a method previously described by Haynes et al. (32). Neonatal RPM spread more rapidly than adult RPM, such that 100% adherent neonatal cells had an extensively arborized morphology after only 3 h of incubation compared with 24 h for the adult RPM (Fig. 4C). Chelation of divalent cations inhibited the spreading of neonatal RPM on TCP (p < 0.01), whereas blocking CR3 with 5C6 mAb up to the 6 h point did not significantly inhibit their spreading on this surface. This indicates that the spreading of neonatal Mφ involves divalent cation-dependent receptors other than CR3 or a conformation of CR3 not recognized by 5C6 (Fig. 4D). Since the proportion of adult Mφ fully spread at the 6 h point was small (only 18.7%), the inhibitory effect of 5C6 and EDTA (20 and 58%) did not reach statistical significance. This time point was chosen to fall on the up-slope of spreading for both cell populations, thus potentially allowing both positive and negative effects of the Ab to be observed. The inhibitory effect of 5C6 and EDTA on adult cells at time points that allow full spreading has been well documented (18).

Role of CR3 in phagocytosis by neonatal Mφ

In addition to adhesion and spreading, CR3 is known to mediate binding and ingestion of complement-opsonized particles by adult peritoneal Mφ (33, 34). Figure 5 summarizes the binding and phagocytosis of iC3b EAiC3b by neonatal and adult resident and recruited Mφ. As previously documented (34), adult TPM were able to both bind and ingest EAiC3b, whereas RPM bound opsonized particles well, but ingested relatively few. Similarly, in the neonate both RPM and TPM bound EAiC3b, but ingestion of EAiC3b was greater by TPM than by RPM. In each case EAiC3b rosetting was inhibited by 5C6, indicating that the CR3 on both the resident and elicited neonatal peritoneal Mφ is capable of binding iC3b and to some extent mediates ingestion of EAiC3b.

LPS-induced myelomonocytic cell recruitment to neonatal skin

Since the epitope of CR3 recognized by 5C6 and implicated in adult myelomonocytic cell recruitment is present and active in adhesion and phagocytosis in the neonate, the ability of 5C6 to block inflammatory recruitment of cells in the neonate was examined. It is well documented that thioglycolate broth elicits a 10- to 20-fold influx of myelomonocytic cells into the adult peritoneal cavity 2 to 4 days after injection. Recruitment of myelomonocytic cells to the neonatal peritoneal cavity was examined by i.p. injection of sterile thioglycolate broth. However, in contrast to the adult, there was no detectable increase in the total number of cells within the peritoneal cavity over the 7 days after thioglycolate injection. Furthermore, the developmental, weight-related increase in the number of cells in the cavity was suppressed by thioglycolate (not shown).

Since injection of control Ab, PBS, or 5C6 also prevented any developmental increase in cell number in the cavity, it is likely that any injection results in a sufficient inflammatory stimulus to induce the resident cells to leave or die within the cavity. For this reason an alternative model of inflammatory recruitment in neonatal mice was employed. It has been established that an acute inflammatory response in adult murine skin may be elicited by LPS. After intradermal injection, myelomonocytic recruitment into adult skin occurs within 2 h (35). This model was therefore adapted for the neonate.

One-day-old mice were injected i.p. with control Ab or 5C6 and intradermally with PBS or LPS, including monestral blue to mark the site of injection. Animals were fixed by PLP perfusion at various times, and the lesions were analyzed immunohistochemically.

One hour after injection, many CR3-labeled cells were found at the lesion, but few could be identified as monocytes using polyclonal F4/80. Cell recruitment was increased at 4 h; it was maximal 8 h after injection and consisted mainly of PMN (Fig. 6, a and b). By 24 h the total numbers of recruited cells had started to decline,
and the infiltrate became predominantly monocytic in character (Fig. 6, c and d). The early, largely neutrophil, response was inhibited by the i.p. injection of 5C6 mAb. Figure 6, E and F, shows the absence of F4/80<sup>+</sup> monocytes/MΦ by means of a polyclonal Ab (b, d, f, and h: blue). Results were confirmed in three independent experiments. a and b, Skin 8 h after injection of LPS intradermally and control mAb i.p.: CR3<sup>+</sup> PMN (p), but no F4/80-stained monocytes are visible at the site of the lesion marked with monestral blue (mb). c and d, Skin 24 h after injection of LPS intradermally and control mAb i.p.: CR3<sup>+</sup> PMN (brown) and CR3<sup>+</sup> F4/80<sup>+</sup> monocytes (m, blue) are now both present at the site of LPS injection. e and f, Skin 24 h after injection of LPS intradermally and 5C6 mAb i.p.: the recruitment of both PMN and monocytes was blocked by 5C6. g and h, Skin 56 h after injection of LPS intradermally and 5C6 mAb i.p.: CR3<sup>+</sup> F4/80<sup>+</sup> monocytes (mo) are again visible at the site of LPS injection.

**FIGURE 6.** CR3 is active in the initial stages of LPS-induced recruitment of myelomonocytic cells to the skin of neonatal mice. Newborn PO mice were injected intradermally with PBS or LPS containing Monestral blue to mark the site of the lesion and i.p. with purified 5C6 or isotype-matched control mAb (1C5). Animals were fixed by perfusion with PLP at various times after injection and stained to reveal CR3 on PMN and monocytes (a, c, e, and g; brown) and F4/80<sup>+</sup> monocytes and MΦ by means of a polyclonal Ab (b, d, f, and h: blue). Results were confirmed in three independent experiments. a and b, Skin 8 h after injection of LPS intradermally and control mAb i.p.: CR3<sup>+</sup> PMN (p), but no F4/80-stained monocytes are visible at the site of the lesion marked with monestral blue (mb). c and d, Skin 24 h after injection of LPS intradermally and control mAb i.p.: CR3<sup>+</sup> PMN (brown) and CR3<sup>+</sup> F4/80<sup>+</sup> monocytes (m, blue) are now both present at the site of LPS injection. e and f, Skin 24 h after injection of LPS intradermally and 5C6 mAb i.p.: the recruitment of both PMN and monocytes was blocked by 5C6. g and h, Skin 56 h after injection of LPS intradermally and 5C6 mAb i.p.: CR3<sup>+</sup> F4/80<sup>+</sup> monocytes (mo) are again visible at the site of LPS injection.

and the infiltrate became predominantly monocytic in character (Fig. 6, c and d). The early, largely neutrophil, response was inhibited by the i.p. injection of 5C6 mAb. Figure 6, E and F, shows the absence of F4/80<sup>+</sup> monocytes/MΦ or CR3<sup>+</sup> monocytes and PMN at the 24 h lesion. The later phase of monocytic recruitment appeared to be at least partially 5C6 resistant (Fig. 6, e–h), with both CR3<sup>-</sup> and F4/80-staining cells detectable in the vicinity of the LPS injection after 56 h. This result confirms the activity of CR3 in the inflammatory recruitment of neonatal PMN and monocytes and also suggests that, as in some models in the adult, other adhesion molecules are also involved.

**Role of CR3 in the constitutive migration of myelomonocytic cells during murine development**

We next investigated whether the migration of cells expressing CR3 during prenatal development could be affected by 5C6. It was essential to know that the Ab could reach sites of expression if its effects on monocyte migration were to be assessed. Animals were injected with mAb i.v. on days 14 and 17 of pregnancy, and their offspring were fixed by perfusion 1 day after birth. 5C6 could be detected on MΦ in the neonatal spleen (Fig. 7a), lamina propria of the gut (Fig. 7b), and Kupffer cells in the liver (Fig. 7c) and had therefore crossed the placenta. When Ab was injected i.p. into newborn mice, followed by PLP perfusion-fixation of the whole animal, 5C6 could be detected in the liver, thymus, and spleen 1, 4, 6, and 9 days after injection using a rat monoclonal detection system. In control animals that received PBS or isotype-matched control Ab, no in vivo labeling of cells could be detected (not shown). In animals injected with 5C6, PMN, monocytes, and some tissue MΦ were strongly labeled in the developing hematolymphoid organs. Monocytes and marginal zone MΦ were labeled in the spleen (Fig. 7d), monocytes were labeled in the corticomedullary region of the thymus (Fig. 7e), and Kupffer cells and monocytes were labeled in the liver (Fig. 7f). In the nonhematolymphoid organs of gut, brain, and skin, CR3<sup>+</sup> cells were detected in architectural arrangements similar to those of conventionally stained specimens (not shown).
gestation: the injected 5C6 stains monocytes and M₄ neonatal gut after injection of 5C6 to the pregnant mother on days 14 and the placenta to label monocytes and PMN in the developing spleen.

b.
aclonal F4/80.

stellate M₄ Ab.

marks myelomonocytic cells in the corticomedullary junction (cmj).
f.
cells show mitotic figures.

d.
liver. Figure 7 shows Kupffer cells in the liver 9 days after SC6 administration, illustrating the morphologic integrity of the organs after Ab injection. This pattern of staining was identical with that detected in animals preinjected with control Ab and with that detected in native animals by Morris et al. (5). Therefore, while 5C6 exhibits the capacity to cross the placenta and be retained on the surface of myelomonocytic cells for several days after birth, such intervention does not interfere with the ability of the cells to find their organ-specific locations.

Discussion

In this study we have used a combination of mAbs that recognize different epitopes of CR3 (18, 36), a cell surface component involved in monocyte and neutrophil migration in the adult, to analyze its expression in the developing mouse. CR3⁺ monocytes and M₆ were distinguished on account of their round or stellate morphology and frequent presence of phagocytic material in M₆. Neutrophils, which only became evident on day 16 or 17 of gestation, were small with a multilobed nucleus. Flow cytometric analysis has previously demonstrated large populations of CR3-positive myeloid cells in the fetal liver from day 11 of gestation, increasing up to birth and decreasing from the first to third weeks after birth (37). However, the study in question does not detect heterogeneity of expression within individual organs or distinguish between monocytes produced as a result of hemopoietic activity at the height of the CR3 expression and mature resident tissue M₆. Here we have compared the immunohistochemical distribution of CR3 during development with that of F4/80 (Table I) to analyze the expression of a known adhesion molecule on a population of developing cells identified by an independent marker. The expression of CR3 on monocytes within most developing organs preceded the appearance of F4/80⁺ monocytes or M₆, but the expression of CR3 on stellate tissue M₆ appeared later than F4/80 and was transient, often being lost shortly after birth. The expression of CR3 on resident tissue M₆ is therefore more widespread in the fetus and neonate than in the adult, except on very specific populations of adult M₆ (Table II). LFA-1 was detected on monocytes and M₆ at the same stage as CR3 in most organs, but was retained during development and in the adult (not shown). This raises interesting questions about the regulation of CR3 expression during development, its function on monocytes and M₆ in the embryo and neonate, and the significance of its down-regulation in the adult. It may be that its function is specific to an early window of monocyte/M₆ life cycle and is lost as a consequence of monocyte differentiation within tissues. For example, CR3 expression on monocytes may be related to their capacity for migration and may no longer be required once a M₆ is fixed within a tissue. CR3 expression on mature M₆ in the lymph node and spleen occurs in areas of leukocyte entry into these organs (38); elimination of marginal zone M₆ in the spleen reduces the accumulation of lymphocytes in this area (39), suggesting that these M₆ have an active role in the trafficking of other cells. Furthermore, adoptively transferred RPM are first evident in the marginal zone of the spleen and subcapsular sinus of the lymph node (25), so that CR3 expressed in these regions might be related to newly extravasated cells that have not yet assumed a tissue phenotype.

The CR3 of M₆ isolated from the peritoneal cavities of neonatal mice was functional in in vitro assays of adhesion to BP and could be completely inhibited by mAb 5C6 or EDTA. However, the spreading of neonatal RPM, which was more rapid and extensive than that of adult RPM, could not be completely inhibited by 5C6. Integrins on leukocytes are normally inactive, in that they bind with only very low avidity to their ligands but can be triggered
period of these animals have not been reported (46). The development of mice deficient in CR3 is also grossly are found in CR3-deficient human patients to the extent examined

administration. This is in keeping with studies indicating that migra-

distribution in tissues by 5C6 up to 9 days after its original ad-

enhanced spreading may be a reflection of recent extravasation from
the blood to form the resident population or may be due to the presence of mediators within the newborn cavity, such as IFN-γ (42) and TNF (43) or macrophage CSF (44), which are known to enhance the spreading of murine adult RPM in vitro.

To investigate the role of CR3 in inflammatory migration in
neonates, LPS was introduced into the skin of neonatal mice. The initial phase of LPS-induced myelomonocytic recruitment in the neonate was blocked by mAb 5C6. However, despite the continued presence of 5C6-labeled Mφ at the lesion, a later phase of my-
elomonocytic recruitment was still evident and therefore was independent of CR3. Analogous studies in the adult indicate that T cell-dependent inflammation induced by specific antigenic chal-

ence in sensitized mice is also biphasic and is inhibited by 5C6 only in its initial phase even though the Ab is present and func-
tional for 4 or more days (20) and recruitment of cells to pulmo-

granulomata following Calmette-Guérin bacillus infection or to hepatic sinusoids in murine malaria are mostly independent of CR3-mediated mechanisms (9). The present study shows that in-
duced monocyte migration in the neonate also has CR3-dependent and -independent components.

Administration of 5C6 to pregnant or newborn mice resulted in
labeling of the CR3 Ag in vivo and demonstrated that mAb 5C6 was able to cross the placenta. This is of interest since 5C6 is also able to cross the blood-brain barrier (45), whereupon it induces mitoses and apoptosis of microglia. Low levels of mitoses were also observed in neonatal spleens and thymi after injection of this Ab, indicating that ligation of CR3 in the periphery may also stimu-
late cell division, but further quantitative studies are needed to establish whether this was enhanced relative to the background in
the present model. 5C6 failed to disrupt constitutive monocyte migration to nonhematolymphoid organs or to affect myelomonocy-
cyte distribution in hematolymphoid organs. The expression of other differentiation markers, including F4/80 and sialoadhesin,
was unaffected by the administration of 5C6 at or before birth. A variety of Abs did not detect any significant disruption of Mφ distribution in tissues by 5C6 up to 9 days after its original admin-
istration. This is in keeping with studies indicating that migra-
tion of fluorescently labeled Mφ from the blood to normal tissue in
the adult is CR3 independent (25) and that normal Mφ populations are found in CR3-deficient human patients to the extent examined (13). The development of mice deficient in CR3 is also grossly normal; however, details of Mφ distribution during the embryonic period of these animals have not been reported (46).

This study has demonstrated high levels of CR3 expression in
hematolymphoid and nonhematolymphoid organs during develop-
ment that coincide with high levels of hemopoiesis and decline after birth. Prominent expression during the developmental period is compatible with a role for CR3 in hemopoietic cell migration. CR3 expression on isolated neonatal peritoneal Mφ is able to mediate adhesion and phagocytosis, and it contributes to the initial phase of myeloid cell recruitment to an inflammatory stimulus in vivo. However, mAb 5C6 was unable to inhibit the largely mono-
cytic, delayed phase of recruitment to LPS or the constitutive mi-
gregation of monocytes during development. Mφ migration in the normal developing animal is therefore largely independent of CR3, which may perform additional, undiscovered functions in tissue modelling and myelomonocytic cell ontogeny.

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References