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Immunopharmacology 46 (2000) 39–54

Immunopharmacology

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Critical role of Kupffer cell CR3 (CD11b/CD18) in the clearance of IgM-opsonized erythrocytes or soluble β-glucan

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Accepted 28 June 1999

Abstract

Liver macrophages (Kupffer cells) play a major role in blood clearance of both C3-opsonized immune complexes and therapeutic β-glucan polysaccharides. Human Kupffer cells express three types of C3-receptors: CR1 (C3b-receptor; CD35), CR3 (iC3b- and β-glucan-receptor), and CR4 (iC3b-receptor; CD11c/CD18). Studies of isolated macrophages have suggested that CR3 is the major receptor mediating capture of either C3-opsonized erythrocytes (E) or β-glucans. In this investigation, the organ distribution and function of CR3 in the clearance of IgM-opsonized E and soluble CR3-binding polysaccharides were explored in normal vs. CR3-knockout (CR3-KO) mice. Analysis of intravenously (i.v.) injected ^{125}I-anti-CR3 showed that the major vascular reservoir of CR3 was the liver, followed by spleen and lungs. By contrast, clearance of ^{125}I-anti-CR1 appeared to be mediated predominantly by splenic B lymphocytes, as only subsets of splenic macrophages or Kupffer cells were found to express CR1. Clearance of IgM-opsonized ^{51}Cr-E occurred rapidly to the livers of normal mice but was nearly absent in CR3-KO mice. Soluble ^{125}I-β-glucan exhibited rapid clearance to the liver in normal mice, whereas clearance in CR3-KO mice was significantly reduced. In conclusion, Kupffer cell CR3 plays a crucial role in the clearance of both IgM-opsonized E and β-glucans.

Keywords: Complement; Biological response modifier; Clearance; Macrophage; C3-receptor; Liver

Abbreviations: Ab, antibody; Ag, antigen; BRM, biological response modifier; C, complement; C3b, iC3b (or C3bi), C3dg, and C3d represent fragments of the third component of C; CR1, C receptor type 1 or CD35, the C3b/C4b-receptor; CR2, C receptor type 2 or CD21, a receptor for iC3b, C3dg, and C3d; CR3, C receptor type 3, a receptor for iC3b and β-glucan known also as CD11b/CD18, Mac-1, or $\alpha_M\beta_2$-integrin; CR3-KO mice, CR3-knockout mice; CR4, C receptor type 4, a receptor for iC3b known also as CD11c/CD18, p150,95, or $\alpha_x\beta_2$-integrin; EAC, E opsonized with Ab and C; EC3bi and EC3dg, E opsonized with iC3b or C3dg, respectively; EDTA, ethylenediamine tetraacetate; FCS, fetal calf serum; fMLP, formylmethione-leucine-phenylalanine tripeptide; GFN, grifolan, a soluble β-glucan derived from *Grifola frondosa*; HBSS, Hank's balanced salt solution; i.v., intravenous; ICAM-1, intercellular adhesion molecule 1 or CD54; mAb, monoclonal Ab; NK cell, natural killer cell; PBS, phosphate buffered saline; E, red blood cell; SZP_g and SZP_m, soluble zymosan-derived polysaccharides consisting primarily of mannose (β-mannan) or glucose (β-glucan), respectively

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0162-3109/00/$ - see front matter
PII: S0162-3109(99)00157-5

1. Introduction

The liver plays a major role in the removal of pathogenic substances from the blood. Hepatocytes function to metabolize drugs, ethanol, and various toxic compounds, while Kupffer cells have a predominant role in the clearance of microbial organisms and immune complexes (Benacerraf, 1964; Lepay et al., 1985; Loegering, 1986). The removal of IgM/C3-opsonized cells and immune complexes by Kupffer cells is thought to be mediated by the same types of C3-receptors expressed by macrophages in other tissues (Brown et al., 1970; Smedsrød et al., 1985; Hinglais et al., 1989). However, splenic macrophages do not exhibit a similar efficiency in the blood clearance of C3-opsonized immune complexes or E, unless there is simultaneous opsonization with both IgG and C3 (Schreiber and Frank, 1972a,b; Davies et al., 1993).

Complement (C) activation by immune complexes or Ab-opsonized cells results in the covalent attachment of C3b molecules to surface hydroxy or amino groups. On E in blood, surface-bound C3b has a half-life of ~ 90 s because the plasma protease factor I rapidly cleaves surface-bound C3b into iC3b. By contrast, iC3b has a much longer ~ 45 min half-life in blood, and tests in vitro have shown that iC3b is the major C3-fragment mediating attachment of microbial pathogens to neutrophils, monocytes, or macrophages (Ross, 1986; Cain et al., 1987; Rosenthal et al., 1996).

Human leukocytes express three types of C3-receptors (Ross, 1998). CR1 binds immune complexes or particles bearing C3b, but exhibits reduced avidity for particles bearing iC3b. CR3 (Mac-1 or $\alpha_M\beta_2$-integrin) promotes avid leukocyte attachment to iC3b-opsonized particles or immune complexes, but has reduced avidity for particles bearing C3dg or C3d. CR4 (p150,95 or $\alpha_x\beta_2$-integrin) mediates adhesion to iC3b-opsonized particles. In comparison to monocytes, tissue macrophages have reduced expression of CR1 and more CR4 than CR3 (Hogg et al., 1986; Myones et al., 1988). Nevertheless, in vitro studies have shown that CR4 has less avidity for iC3b than does CR3. Moreover, in mice and rats, bone marrow and peritoneal macrophages are devoid of CR1 (Martin and Weis, 1993; Quigg and Holers, 1995) and the putative CR4 (CD11c/CD18) molecule is a dendritic cell-specific marker that is undetectable on macrophages (Metlay et al., 1990). Thus, CR3 may be the most important receptor mediating clearance of iC3b-opsonized immune complexes by the macrophage phagocytic system.

CR3 functions both as a receptor for phagocytosis or cytotoxicity and as an adhesion molecule responsible for leukocyte diapedesis (Ross et al., 1999; Xia and Ross, 1999). Regulation of these seemingly disparate functions occurs through a lectin site located within the CD11b portion of CR3 (Thornton et al., 1996; Ross et al., 1999; Xia and Ross, 1999). Serum-opsonized yeast particles simultaneously present to CR3 both cell wall-bound β-glucans and iC3b. The cell wall β-glucan binds to a lectin site in CR3 and primes the receptor for later cytotoxic degranulation in response to the opsonic iC3b that engages a separate binding site within CR3 (Thornton et al., 1996; Větvička et al., 1996; Xia et al., 1999). Recent reports have shown that lectin site regulation of CR3-dependent cytotoxicity also explains the action of several soluble therapeutic polysaccharide biological response modifiers (BRM). Soluble fungal polysaccharides bind with high affinity to the CR3 of circulating leukocytes, and this primes the receptor for cytotoxic recognition of iC3b-opsonized tumor cells that lack endogenous CR3-binding polysaccharides and are otherwise inert in stimulating leukocyte CR3-dependent cytotoxicity (Větvička et al., 1996, 1997; Ross et al., 1999; Xia et al., 1999). Many fungal β-glucans stimulate potent cell-mediated tumoricidal activity in animals, and several of these polysaccharide BRM such as lentinan and schizophyllan have been administered to patients with cancer (Fujimoto, 1989; Shimizu et al., 1991; Chihara, 1992; Kimura et al., 1994; Matsuoka et al., 1997; Yoshida et al., 1997). Previous reports that have examined the clearance of these β-glucan BRM have shown rapid clearance to liver Kupffer cells (Smedsrød and Seljelid, 1991; Miura et al., 1996; Suda et al., 1996; Yoshida et al., 1996; Adachi et al., 1998; Sveinbjornsson et al., 1998). Because of the high affinity of CR3 for soluble β-glucans (Thornton et al., 1996; Xia et al., 1999), it is reasonable to propose that Kupffer CR3 has a primary role in the clearance of β-glucan BRM, as well as IgM/C3-opsonized cells.

In this study, the clearance of intravenously (i.v.) injected IgM-opsonized E and two soluble CR3-binding polysaccharides were examined in normal vs. CR3-KO mice (CR3-deficient mice generated through knockout of the CD11b gene). The data indicated that Kupffer cell CR3 has a primary role in the clearance of both IgM/C3-opsonized E and CR3-binding polysaccharides such as β-glucan.

2. Materials and methods

2.1. Reagents

RPMI-1640 medium was obtained from Atlanta Biologicals (Norcross, GA); tyramine (4-hydroxyphenethylamine) and Protein A, insoluble lyophilized cell powder from Sigma (St. Louis, MO); fetal calf serum (FCS) from Hyclone Laboratories (Logan, UT); Hanks balanced salt solution (HBSS) from Life Technologies (Grand Island, NY).

2.2. Mice

Female, 6–8 weeks old, BALB/c mice were purchased from the Jackson Laboratory, Bar Harbor, ME. Generation and characterization of BALB/c/129Sv mice deficient in CD11b (CR3-KO) and their normal littermates were previously described (Coxon et al., 1996). Age and sex-matched normal and CR3-KO littermates were used in parallel assays of clearance.

2.3. MAbs and non-specific rat IgG

Rat 8C12, an IgG_{2a},κ mAb to murine CR1 (Kinoshita et al., 1988), was purchased from Pharmingen (San Diego, CA) as pure IgG and as a biotin-conjugate. The M1/70 rat IgG_{2b},κ anti-mouse CR3 mAb (specific for CD11b) was isolated from ascites fluid (Xia et al., 1999). Pure rat IgG_{2a},κ R35–95 with unknown specificity (Pharmingen) was obtained as an isotype control for 8C12 anti-CR1. All three rat IgG preparations were labeled with ^{125}I using Iodogen (Fraker and Speck, 1978) with resulting ^{125}I uptake of 0.34 μCi/μg 8C12, 0.30 μCi/μg M1/70, and 0.44 μCi/μg R35–95. The proportion of the ^{125}I-anti-CR3 representing specific Ab was estimated by an in vitro assay for the fraction of ^{125}I-labeled IgG able to bind to macrophage CR3. This assay was carried out as previously described (Myones et al., 1988; Ross et al., 1992; Xia et al., 1999) using the mouse monocytoid cell line P388D1 obtained from the American Type Culture Collection (ATCC, Manassas, VA). Briefly, aliquots of 5×10^7 P388D1 cells in 12×75 mm^2 tubes immersed in an ice bath were incubated with 1.4 μg/ml of ^{125}I-M1/70 anti-CR3 mAb (~100,000 cpm) in the presence or absence of 1 mg/ml of unlabeled M1/70 mAb in 100 μl RPMI-1640 medium/10% FCS. After 30 min, cells with bound ^{125}I-labeled mAb were separated from unbound ^{125}I-labeled IgG by layering 80 μl of the cell suspension onto 200 μl of a 6:4 mixture of *n*-butyl phthalate and dioctylphthalate in 300-μl polypropylene microprecipitin tubes and centrifugation at $14{,}000 \times g$ for 1 min. After freezing the tube contents at −140°C for 20 min, the frozen tips of the tubes were cut off and the radioactivity associated with both the tip containing the cells with bound mAb and the top of the tube containing unbound IgG were measured.

2.4. Preparation of mouse E opsonized with rabbit IgM Ab (EA)

E suspensions prepared from EDTA–citrate blood were washed three times with ice-cold PBS, labeled with [^{51}Cr]sodium chromate (Větvička et al., 1996), and suspended at $\sim 3.3 \times 10^8$ cells/ml in PBS. Portions of the ^{51}Cr-E was opsonized with IgM Ab forming EA by dropwise addition with constant mixing of equal volumes of rabbit IgM anti-mouse E Ab (Accurate Chemical and Scientific, Westburg, NY) diluted 1:25, 1:50, 1:100 or 1:200 in PBS, incubation at 37°C for 30 min, and three washes with ice-cold PBS. Assay of this Ab for hemagglutination indicated a titer of 1:100, and a dilution of 1:50 produced optimal fixation of C with whole mouse serum (Ross and Winchester, 1980). A portion of the EA prepared with a 1:50 dilution of IgM Ab were also incubated further with fresh C5-deficient mouse serum to form EAC (Ross, 1981) for in vitro tests of phagocytosis by unactivated peritoneal macrophages (Kruisbeek, 1996; Xia et al., 1999). Because these EAC were ingested by macrophages, it was likely

that the IgM contained minor contamination with IgG (Mantovani et al., 1972; Ehlenberger and Nussenzweig, 1977). Accordingly, for some experiments as indicated, this trace IgG contamination was removed from the IgM Ab by adsorption to insoluble Protein A. Briefly, 300 μl of IgM was mixed with 25 mg of Protein A for 30 min at 4°C, and then the Protein A was removed by centrifugation. EAC generated with this IgG-depleted IgM were not ingested, indicating successful removal of IgG.

2.5. Soluble zymosan-derived polysaccharide (SZP)

Methods for preparation of SZP were previously described (Thornton et al., 1996). As reported recently (Xia et al., 1999), the size and sugar composition of such SZP preparations is somewhat dependent on the starting batch of yeast. SZP preparations used in this investigation either consisted almost entirely of mannose, and are referred as SZP_m (Mr ~ 20 kDa), or consisted primarily of β-(1,3)-glucan and are referred as SZP_g (Mr ~ 6 kDa). For labeling with ^{125}I, reductive amination was used to couple tyramine to the reducing end of SZP_m (Cheong and Hahn, 1991) or SZP_g (Cosio et al., 1988) prior to Iodogen-catalyzed iodination (Thornton et al., 1996). The affinity of ^{125}I-SZP_m preparations for CR3 was 5- to 10-fold higher than current preparations of ^{125}I-SZP_g (Xia et al., 1999).

2.6. Analysis of clearance kinetics and organ distribution of i.v. ^{125}I-IgG, ^{125}I-SZP, or ^{51}Cr-E

Mice were injected in a tail vein with 100–200 μl of PBS containing 1–10 μg of ^{125}I-IgG, 1 μg hexose of ^{125}I-SZP, or 2.5%–25% ^{51}Cr-E. Drops of blood were collected from a small cut on the lower side of the tail at timed intervals, and 25 μl samples from each blood drop were analyzed for radioactivity with a gamma counter. At the end of each clearance experiment, the mice were sacrificed, and the radioactivity associated with major organs was measured. It should be noted that uncleared radioactive IgG, SZP, or E remained in the blood that was trapped in the various organs analyzed for associated radioactivity at the end of clearance experiments. Thus, the amount of radioactivity associated with an individual organ in which there was no specific clearance (e.g., livers of CR3-KO mice following clearance of ^{125}I-anti-CR3) represented the unbound radioactivity contained within the blood that was trapped in that organ. Because the liver contains 40%–50% of the trapped blood recovered in the six organs removed for analysis of radioactivity, the liver should contain 40%–50% of the unbound radioactivity recovered among these six organs.

In experiments that measured the organ distribution of i.v. ^{125}I-IgG–anti-CR1 vs. ^{125}I-non-specific rat IgG, the entire vasculature was perfused with 20 ml of PBS/heparin to remove most of the blood containing unbound ^{125}I-IgG prior to removal of organs for analysis of bound radioactivity. The method was derived from an investigation of adhesion molecules exposed on the vascular endothelium (McHale et al., 1999). Briefly, 1 h after injection of 5 μg of ^{125}I-IgG, mice were euthanized by CO_2 asphyxiation and the heart was exposed surgically. A syringe with 23 gauge needle was used to inject PBS containing 10 U/ml of sodium heparin (Sigma) into the left ventricle while simultaneously the perfusate was aspirated from an incision made in the right atrium.

2.7. Immunofluorescence staining and flow cytometry

Two-color flow cytometry of cell suspensions was carried out to identify cell types expressing CR1 using standard techniques (Shevach, 1992). Before removing organs, the majority of blood leukocytes were removed by perfusing the vasculature with PBS/heparin as before. Single cell suspensions were prepared by gently mincing small pieces of organs in a tissue homogenizer. Splenic mononuclear cells and granulocytes were isolated from spleen cell suspensions by two-step ($d = 1.08$ and 1.105 g/ml) Ficoll–Hypaque density gradient centrifugation ($1000 \times g$, 20 min, 4°C). Alveolar macrophages or Kupffer cells in lung or liver cell homogenates, respectively, were enriched by flotation on a single layer of Ficoll–Hypaque ($d = 1.08$ g/ml) using centrifugation at $400 \times g$ for 20 min at 4°C. Peritoneal exudate macrophages were harvested by standard techniques 4 days after peritoneal injection of

brewer's thioglycollate media (Kruisbeek, 1996; Xia et al., 1999). CR1 was measured on surface IgM^+ B lymphocytes in splenic mononuclear cell fractions using 8C12 anti-CR1–biotin followed by streptavidin–phycoerythrin (PE) in combination with goat $F(ab')_2$ anti-IgM–FITC (Cappel Laboratories, Cochranville, PA). The same 8C12 anti-CR1–biotin followed by streptavidin–FITC (Pharmingen) was used to assess CR1 in combination with anti-CD80–PE (Pharmingen) to identify monocytes/macrophages, or with anti-Gr1–PE (Pharmingen) to identify neutrophils. Cells expressing CR3 were identified by use of M1/70 anti-CR3–FITC (Winchester and Ross, 1986) instead of anti-CR1–biotin plus streptavidin–FITC. Flow cytometry was carried out with a Coulter Profile II instrument (Coulter, Miami Lakes, FL), and list mode data were analyzed using WinList 3.0 (Verity Software House, Topsham, ME). Non-specific IgG isotype-matched controls were used for each fluorochrome type to define background staining, and dead cells were excluded by addition of propidium iodide (Shevach, 1992).

2.8. Pharmacokinetics data and statistical analysis

Blood clearance and organ distribution data were analyzed using GraphPad PRISM (GraphPad Software, San Diego, CA). Clearance curves for pharmacokinetic parameters were derived using non-linear regression methods that are part of this software package. The first phase half-lives ($T1/2\alpha$) were calculated from standard pharmacokinetics equations using first-order rate constants (Iznaga-Escobar et al., 1998). Statistical significance of differences was determined using Student's *t*-test.

3. Results

3.1. Clearance kinetics and organ distribution of i.v. IgG anti-CR3 mAb

The kinetics of blood clearance and subsequent organ distribution of i.v. injected ^{125}I-labeled M1/70 anti-CR3 was determined in normal vs. CR3-KO mice (Fig. 1). As expected, clearance of the mAb was rapid in normal mice but insignificant in mice lacking the target Ag. Incomplete clearance in normal mice was probably due to the parallel in vitro

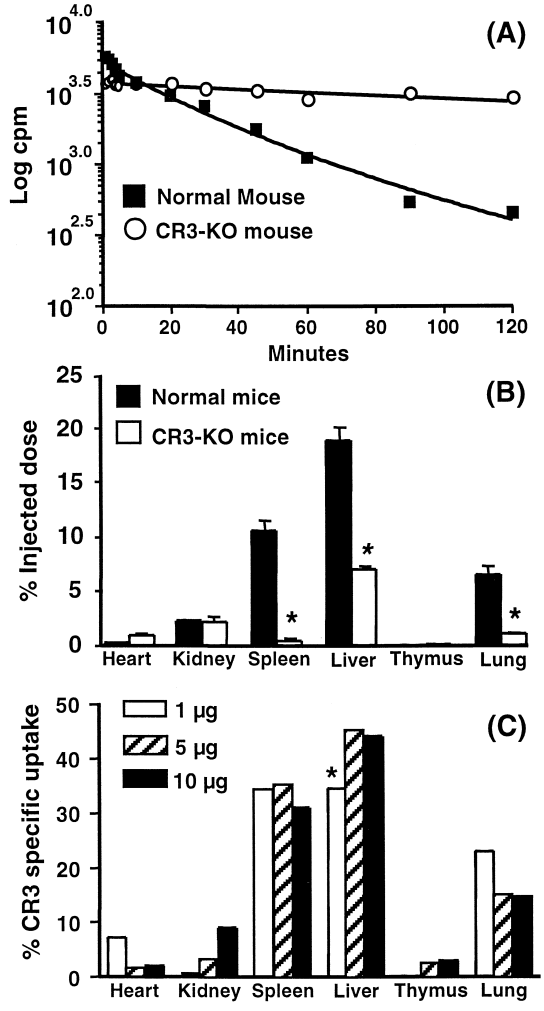

Fig. 1. Clearance kinetics and organ distribution of i.v. injected ^{125}I-M1/70 anti-CR3 mAb in normal vs. CR3-KO mice. Normal mice were injected with 1 µg ^{125}I-anti-CR3 and blood samples were analyzed for radioactivity at timed intervals (A). After 120 min, the percent of injected radioactivity remaining in major organs was determined (B). Asterisks indicate that significant ($p < 0.001$) differences were detected in the deposition of ^{125}I-anti-CR3 in the spleen, liver, and lungs of normal vs. CR3-KO mice. The data represent the mean ± SD from three experiments. (C) shows the CR3-specific organ distribution of radioactivity 2 h after i.v. doses of 1, 5, or 10 µg of ^{125}I-anti-CR3. This experiment was done twice with each mAb dose in pairs of normal and CR3-KO mice. The asterisk indicates that more CR3 ($p < 0.05$) was detected in the liver vs. the spleen with the 5 and 10 µg doses of ^{125}I-anti-CR3.

finding that only 60% of the ^{125}I-labeled mAb preparation exhibited CR3-binding activity (this is a typical finding for IgG mAbs isolated from ascites fluid that contains variable amounts of serum-derived IgG). Major differences in the organ distribution of the infused anti-CR3 were observed in the liver and spleen where vascular sinusoids are lined with CR3-bearing macrophages. The lungs of normal mice also represented a significant reservoir of CR3 available for capturing the i.v. ^{125}I-labeled mAb. Because there was some concern that the relative organ distribution of CR3 might not be accurately determined with the 1 μg dose of ^{125}I-anti-CR3 mAb used to assess clearance kinetics, additional tests of CR3 organ distribution were carried out in normal vs. CR3-KO mice using 5 or 10 μg of i.v. ^{125}I-anti-CR3. After 2 h, mice were exsanguinated to remove as much blood as possible before removal of organs to assess uptake of ^{125}I-anti-CR3. The CR3-specific uptake of mAb by normal mice was determined by subtraction of the organ-associated radioactivity measured in CR3-KO mice. An increase in the liver-associated radioactivity from 34.5% to 45% was observed when the 5 μg dose of mAb was compared to the 1 μg dose, but there was no further increase in the proportion of recovered radioactivity in the liver with a 10 μg dose of ^{125}I-anti-CR3 (Fig. 1C).

3.2. Clearance and organ distribution of i.v. anti-CR1 mAb

In humans, CR1 is expressed along with CR3 on peripheral blood monocytes and neutrophils, and has been thought to play a role in clearance via the macrophage phagocytic system. However, studies of mice have shown that CR1 is absent on bone marrow and peritoneal exudate macrophages (Martin and Weis, 1993), although its expression on neutrophils can be induced by stimulation with fMLP (Kinoshita et al., 1988; Quigg et al., 1997). To determine the organ distribution of exposed CR1 in the mouse vasculature, the deposition of i.v. injected ^{125}I-8C12 rat IgG_{2a} anti-CR1 was compared to ^{125}I-R35–95 rat IgG_{2a} that has no known specificity (Fig. 2). Normal mice were compared to CR3-KO mice using the same 5 μg i.v. dose of ^{125}I-mAb or non-specific IgG

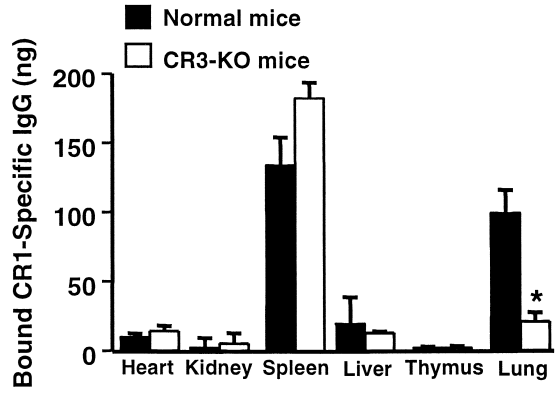

Fig. 2. Organ distribution of specifically bound ^{125}I-8C12 anti-CR1 following i.v. injection. Three pairs of normal and CR3-KO mice were injected i.v. with 5 μg of either ^{125}I-8C12 rat IgG_{2a} anti-CR1 or ^{125}I-non-specific rat IgG_{2a}. After 60 min, the mice were asphyxiated by CO_2 inhalation and their vasculature was perfused with PBS/heparin to remove the majority of blood containing unbound ^{125}I-IgG. The nanograms of CR1-specific IgG bound to major organs was determined by subtracting the nanograms of non-specific IgG from the nanograms of anti-CR1 IgG measured in the same organs. The bars represent the mean ± SD for three normal or three CR3-KO mice. The only significant difference between normal and CR3-KO mice (indicated by asterisk) was that more CR1 was detected in the lungs of normal mice.

found to be optimal with ^{125}I-anti-CR3. CR1 was detected predominantly in the spleen followed by the lungs, and much less fixed CR1 was detected in the liver. CR3-KO mice appeared to have similar amounts of exposed CR1 in the spleen and liver, but less in the lungs ($p < 0.01$) than did normal mice.

Flow cytometry of isolated splenocytes showed CR1 on 80% of IgM^+ B lymphocytes and 79% of $CD80^+$ macrophages, but no CR1 on $Gr1^+$ neutrophils. In Kupffer cell-enriched liver cell suspensions, CR1 was detected on only 60% of $CD80^+$ Kupffer cells. By contrast, nearly all $CD80^+$ splenic macrophages and Kupffer cells exhibited staining for CR3. The CR1 staining intensity of splenic B cells greatly exceeded the CR1 staining of either splenic macrophages or Kupffer cells, indicating that splenic B cells were the major vascular reservoir of CR1. However, it was not possible to use these data to assess the relative amounts of CR1 vs. CR3 expressed by these macrophages. No $CD80^+$ macrophages were recovered in lung cell suspensions, preventing tests of alveolar macrophages. Analysis

of thioglycollate-elicited peritoneal macrophages confirmed that these cells do not express CR1 (Martin and Weis, 1993), and showed also that the CR3 staining intensity of these activated macrophages was far greater than the CR3 staining measured on splenic macrophages and Kupffer cells.

3.3. Accelerated CR3-dependent clearance of IgM-opsonized E

The availability of CR3-KO mice provided the opportunity to determine the contribution of CR3 to the clearance of a model IgM-bearing immune complex. When mice were injected i.v. with ^{51}Cr-labeled syngeneic E, with or without IgM opsonization, rapid ($T1/2\alpha = 1.5$ min) IgM-dependent clearance of E occurred only with normal (Fig. 3A) and not with CR3-KO mice (Fig. 3C). There was a slight enhancement ($p < 0.05$) of clearance of IgM-opsonized E vs. unopsonized E in CR3-KO mice after 1–2 h (Fig. 3C). Examination of major organs after 5 min or 2 h showed that the liver was the primary site responsible for clearance of IgM-opsonized vs. unopsonized E in normal mice ($p < 0.001$, Fig. 3B), whereas there was no specific organ deposition of IgM-opsonized E in CR3-deficient mice (Fig. 3D). There was little clearance of the IgM-opsonized E to

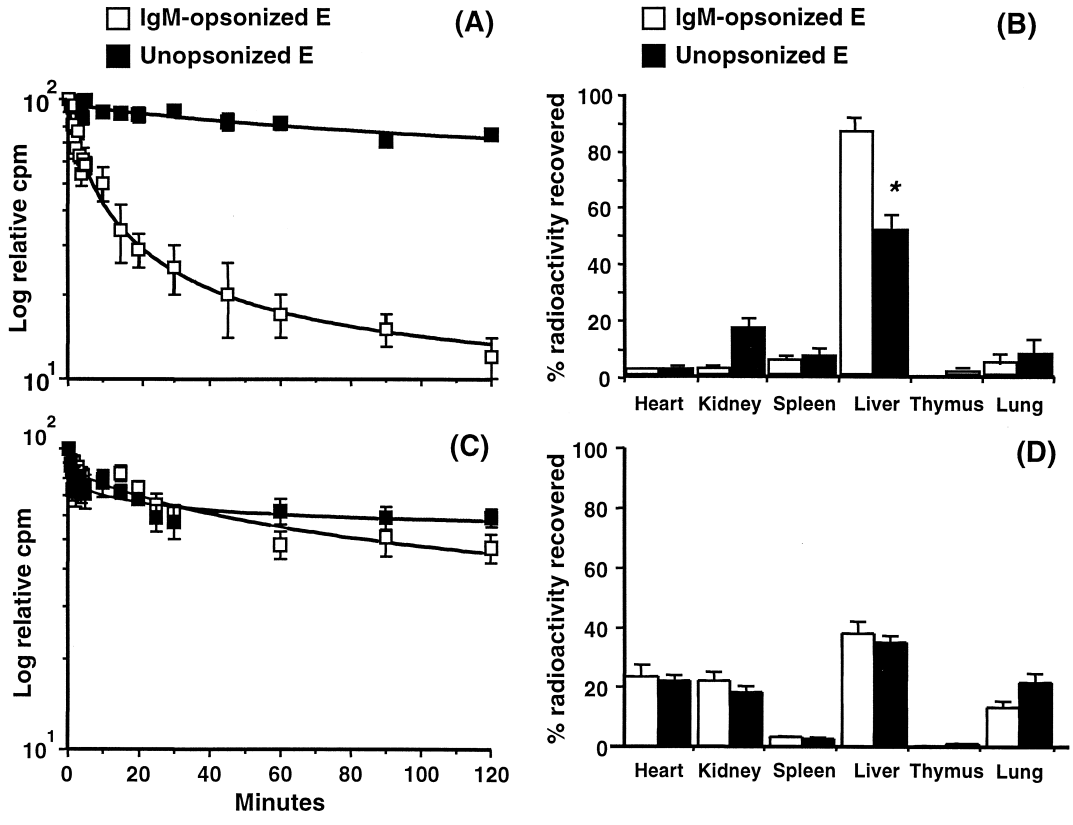

Fig. 3. Clearance and organ distribution of i.v. injected ^{51}Cr-labeled E, with or without opsonization with IgM, in normal vs. CR3-KO mice. The distribution of radioactivity in blood and organs were determined as in Fig. 1 during a 2-h interval after i.v. injection. (A) and (B) show the clearance and organ distribution in normal mice; (C) and (D) show the results with CR3-KO mice. The cpm value measured at 0.5 min was designated as 100 relative cpm units to allow comparisons between experiments with different preparations of radiolabeled E. The asterisk in (B) indicates that the liver was the only organ where there was a significant difference in recovery of radioactivity when unopsonized vs. IgM-opsonized E were compared ($p < 0.001$). Each experiment was done three times with pairs of normal and CR3-KO mice, and each bar in (B) and (D) represents mean values from three mice ± SD.

the spleen or lungs, despite their content of exposed CR3 (Fig. 1B).

In reports of similar studies with guinea pigs or human volunteers, most of the IgM-opsonized E were sequestered rapidly by the liver but later released back into the circulation over a 2-h period (Schreiber and Frank, 1972b; Atkinson and Frank, 1974a). By contrast, IgG-opsonized E were sequestered and not released (Schreiber and Frank, 1972b), presumably because IgG- and C3-opsonized E that are bound to macrophages are phagocytosed (Huber and Fudenberg, 1970; Mantovani et al., 1972; Ehlenberger and Nussenzweig, 1977). Thus, the absence of such release of sequestered IgM-opsonized E during a similar 2 h period could have been due to a demonstrated minor contamination of the commercial rabbit IgM Ab preparation with amounts of IgG Ab that were shown to be sufficient to mediate phagocytosis in vitro by unactivated peritoneal macrophages following treatment of the IgM-opsonized E with normal mouse serum to generate opsonic C3 (data not shown). Accordingly, these clearance experiments were repeated after removal of contaminating IgG from the rabbit IgM anti-E Ab. In addition, because other reports had shown that liver release of sequestered IgM-opsonized E also depended on both the amount of IgM Ab on the E (Frank et al., 1977) and the total liver burden of bound E (Nilsson Ekdahl et al., 1991), clearance was also investigated with varying doses of E opsonized with the same amount of IgM (Fig. 4) or with a constant amount of E opsonized with different amounts of IgM Ab (not shown). Within a 2-h period, a 100-μl injection of 2.5% IgM-opsonized E (Fig. 4) showed the same clearance pattern as observed earlier with this amount of E opsonized with the IgM prior to removal of contaminating IgG (Fig. 3). As greater amounts of opsonized E were infused, the rate of clearance was reduced. Some release of sequestered E back into the circulation (denoted by a small increase in blood cpm) appeared to occur briefly between 10 and 60 min time points after infusion of 5% or 25% IgM-opsonized E, but clearance (i.e., sequestration, denoted by a decrease in blood cpm) predominated after 1 h. When E clearance was measured with varying dilutions of the opsonizing IgM Ab (1:25, 1:50, 1:100, or 1:200) and a constant 100 μl dose of 2.5% E, the rate of

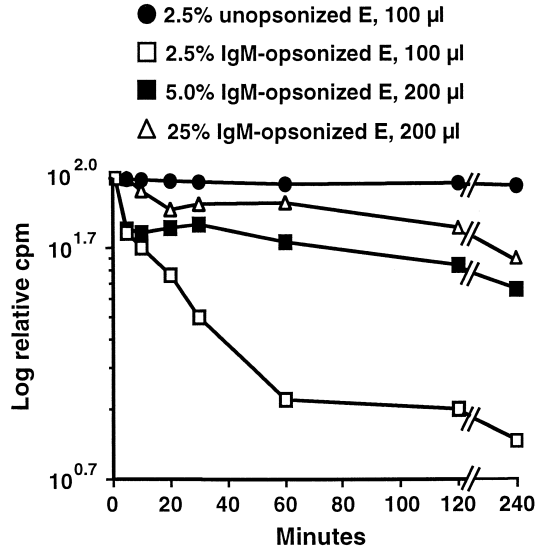

Fig. 4. Sequestration without subsequent release of IgM-opsonized ^{51}Cr-labeled E. After removal of detectable IgG contamination from the rabbit IgM anti-mouse E Ab, the same protocol as in Fig. 3 was used except that the dilution of IgM employed for E opsonization was decreased from 1:50 to 1:25 to compensate for the loss in E agglutinating activity that was noted after IgG removal. The cpm value measured at 1 min was designated as 100 relative cpm units. The data represent mean values obtained from two identical experiments with each injected amount of unopsonized or IgM-opsonized E.

sequestration was proportional to the amount of Ab but no release of sequestered E was observed (data not shown).

3.4. Clearance and organ distribution of therapeutic CR3-binding soluble polysaccharides

The clearance of two different preparations of ^{125}I-labeled SZP was examined following i.v. administration in the same way as with the anti-CR3 mAb and IgM-opsonized E (Figs. 5 and 6). SZP_m, a mannan-rich 20 kDa yeast-derived soluble polysaccharide that exhibits a binding affinity for CR3 of $\sim 5 \times 10^{-8}$ M (Thornton et al., 1996), exhibited rapid clearance with nearly 50% of the injected dose being taken out of the circulation by the liver within the first 5 min after i.v. injection (Fig. 5). Notably, only very minor proportions of SZP_m localized to the spleen or lung, the other two organs shown to express CR3 detectable with anti-CR3 mAb (Fig. 1).

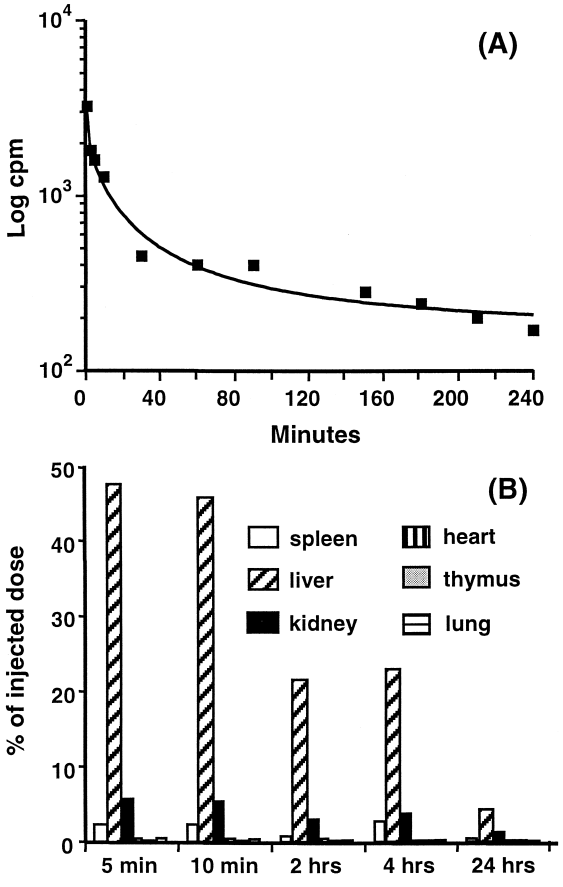

Fig. 5. Blood clearance of i.v. injected ^{125}I-SZP$_m$. BALB/c mice were injected i.v. with 1 μg of ^{125}I-SZP$_m$, and at timed intervals, 25 μl blood samples were analyzed for radioactivity (A) and the distribution of radioactivity in major organs was determined (B). The clearance results shown for one mouse (A) are representative of similar results obtained with two other mice. The organ distribution results (B) were derived from similar tests of five mice sacrificed for analysis of organ-associated radioactivity at each of the indicated time intervals.

SZP$_g$, a yeast-derived soluble β-glucan of ~6 kDa that exhibits 5- to 10-fold lower binding affinity for CR3 than does SZP$_m$ (Xia et al., 1999), was examined in the same way for clearance and organ localization following i.v. administration, except that CR3-KO mice were available for comparison to normal mice (Fig. 6). The CR3-deficient mice exhibited delayed clearance as compared to normal mice, with a calculated $T1/2\alpha$ of 10 vs. 3 min in normal mice. Analysis of major organs 2 h after i.v. injection of normal mice indicated that most of the deposited ^{125}I-SZP$_g$ had been removed by the kidneys, as only 24.8% of the SZP$_g$ was found in the liver (Fig. 6B). By contrast, the organ distribution in CR3-KO mice showed that the reduced blood clear-

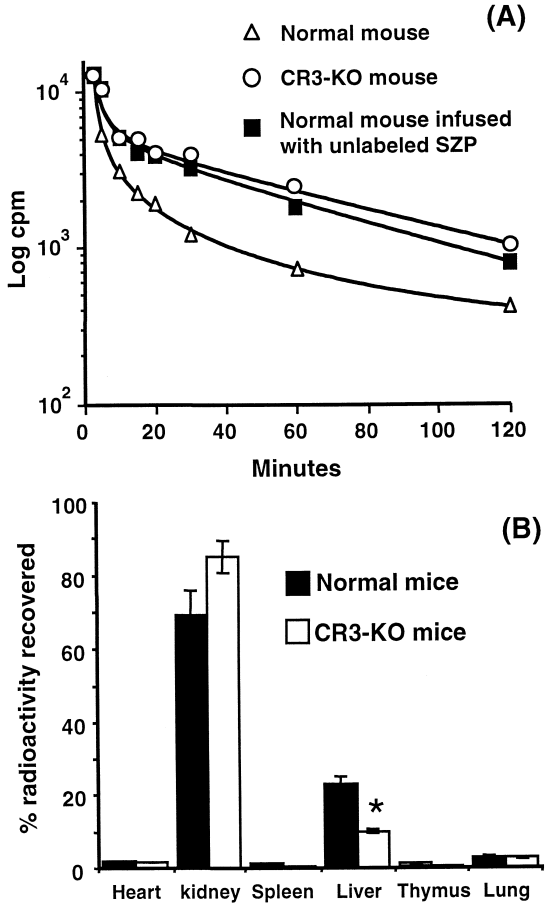

Fig. 6. Differences in the clearance of ^{125}I-SZP$_g$ in normal vs. CR3-KO mice, and inhibition of ^{125}I-SZP$_g$ clearance in normal mice by mixture of the ^{125}I-SZP$_g$ with excess unlabeled SZP$_g$. Normal mice were injected i.v. with 1 μg of ^{125}I-SZP$_g$ either alone or in mixture with 2 mg of unlabeled SZP$_g$. Clearance was measured simultaneously with a CR3-KO mouse given only 1 μg of ^{125}I-SZP$_g$. (A) shows radioactivity remaining in the blood at timed intervals and (B) shows the tissue distribution of radioactivity at the end of the experiment. The percent radioactivity recovered in the liver was significantly greater in normal mice vs. CR3-KO mice ($p < 0.01$). The blood clearance results shown from one experiment with a pair of normal and CR3-KO mice are representative of similar results obtained in three experiments. Each bar in (B) represents the mean ± SD for three mice.

ance resulted from removal of significantly less ^{125}I-SZP$_g$ by the livers of CR3-deficient mice as compared to normal mice ($p < 0.001$). A similar organ distribution was also observed when mice were sacrificed 30 min after i.v. infusion of ^{125}I-SZP$_g$, with ≥ 2 times more ^{125}I-SZP$_g$ in the livers of normal vs. CR3-KO mice ($p < 0.01$; data not shown).

The reduced clearance of the ^{125}I-SZP$_g$ in CR3-KO vs. normal mice was presumably due to the absent β-glucan-receptor activity provided by leukocyte CR3. Since the uptake of ^{125}I-SZP$_g$ by normal neutrophils or macrophages in vitro is saturable, reversible, and blocked by excess unlabeled β-glucan (Xia et al., 1999), the clearance of 1 μg of ^{125}I-SZP$_g$ was examined in normal mice given simultaneously either 0.2, 1.0 or 2.0 mg of unlabeled SZP$_g$. The results indicated that ~2.0 mg of unlabeled SZP$_g$ were required to retard the rate of clearance of 1 μg of ^{125}I-SZP$_g$ to a rate similar to that observed in CR3-KO mice (Fig. 6A).

4. Discussion

Kupffer cells play a major role in host defense through their ability to remove specific substances from the blood via high avidity membrane binding sites that include C3-receptors, Fc-receptors, mannose receptors, and scavenger receptors (Benacerraf, 1964; Crofton et al., 1978; Lepay et al., 1985; Lee et al., 1986; Loegering, 1986). The availability of mice deficient only in CR3 provided the opportunity to investigate the specific function of CR3 in the clearance of two types of materials known to be removed by Kupffer cells. The data indicated a critical role for Kupffer cell CR3 in the clearance of IgM/C3-opsonized E and soluble CR3-binding polysaccharides.

Analysis of the exposed reservoirs of fixed CR3 in the vasculature showed that the liver was the most abundant source of CR3, followed next by spleen and lungs. All CR3-specific clearance of E or soluble polysaccharides occurred in the liver and there was no detectable clearance in the spleen or lungs. This finding probably relates to the mechanisms of blood filtration available in the liver vs. the spleen or lungs (Benacerraf, 1964; Crofton et al., 1978). The liver also has the greatest blood volume of the organs examined, and this explained why ~50% of the total unbound ^{125}I-IgG or unopsonized E were also recovered in the liver. Although some investigators have estimated that Kupffer cells represented 80%–90% of the entire macrophage phagocytic system throughout the vasculature (Crofton et al., 1978; Bouwens et al., 1986), the current investigation detected only 45% of the exposed CR3 (Mac-1) macrophage surface marker in the liver.

IgM-opsonized syngeneic E are a well characterized type of immune complex. IgM efficiently activates C via the classical pathway resulting in abundant deposition of C3b. However, plasma factor I quickly mediates the proteolysis of this C3b into iC3b through recognition by cofactors present in both plasma (factor H) and the red cell membrane; i.e., MCP or CD46 in humans (Atkinson et al., 1991) or the homologous Crry/p65 molecule in mice (Kim et al., 1995). Thus, iC3b predominates on syngeneic E in plasma, and little fixed C3b is available as a potential ligand for CR1. Other types of target cells, particularly microorganisms, can offer a protected environment for C3b deposition in which a high proportion of the fixed C3b is not broken down into iC3b (Newman and Mikus, 1985). Early investigations that tried to define the type of C3 fragment present on serum-opsonized E that was functional in clearance concluded that it was probably C3b because of positive immune adherence tests (Brown et al., 1970; Atkinson and Frank, 1974a). However, later research showed that E bearing large amounts of iC3b could form CR1-dependent rosettes with human E (immune adherence reaction), and that only E bearing relatively small amounts of fixed iC3b were bound exclusively to CR3 (Ross et al., 1983). In addition to this reaction of fixed iC3b with CR1 and CR3, both CR2 and CR4 have also been shown to mediate leukocyte adhesion to iC3b-opsonized E (Ross et al., 1983; Myones et al., 1988). Although CR2 is undetectable on macrophages, follicular dendritic cells use CR2 for capture of iC3b- or C3dg-opsonized protein Ags as part of the primary immune response (Fang et al., 1998). Thus, even if iC3b were the predominant C3 fragment bound to a particle, it could not be predicted with certainty which type of C3-receptor would be functional in the clearance of that particle. Finally, the true half-life of fixed iC3b in vivo is unknown and is likely to be

much shorter than the 45 min measured in serum (Ross et al., 1985) because of blood cell-associated CR1 that functions as a cofactor for factor I proteolysis of fixed iC3b into fixed C3dg (Ross et al., 1982; Kinoshita et al., 1985). E bearing only C3dg exhibit a greatly reduced avidity for CR3 and CR4 (Frade et al., 1985; Ross and Reed, 1992).

Rodent C3-receptors have similarities and important differences from their human counterparts. CR1 is more closely related to CR2 and is derived from an alternate transcript of the Cr2 gene rather than from a separate gene. Moreover, CR1 is found primarily on follicular dendritic cells and B lymphocytes, and little is detectable on myeloid series cells. However, CR1 expression by murine myeloid cells appears to be variable and dependent upon cell differentiation or activation state. Highly activated thioglycollate-elicited peritoneal exudate macrophages were convincingly shown to lack detectable CR1 protein or mRNA (Martin and Weis, 1993), whereas this study demonstrated subsets of splenic macrophages and Kupffer cells that expressed CR1. Notably, this investigation used the same biotin-labeled 8C12 anti-CR1 mAb followed by streptavidin–FITC that had been used by Martin and Weis, and confirmed their finding of absent CR1 expression by thioglycollate-elicited peritoneal exudate macrophages. Although peripheral blood neutrophils do not express CR1, they can be induced to express CR1 by stimulation with fMLP (Kinoshita et al., 1988; Quigg et al., 1997). However, a C3b-binding protein that was isolated by affinity chromatography from murine neutrophils was shown to be distinct from CR1 (Quigg et al., 1997), and the Crry/p65 molecule that shares epitopes with human CR1 does not function as a C3b-receptor capable of binding C3b-opsonized E (Kim et al., 1995). It was predictable that the spleen would be the major site for capture of i.v. ^{125}I-anti-CR1, since B cells had previously been shown to be the major mouse leukocyte type expressing CR1 (Kinoshita et al., 1988; Takahashi et al., 1997) and the spleen contains the only lymphoid tissue directly exposed to the blood.

The major difference in clearance of IgM-opsonized E in normal vs. CR3-KO mice shows that CR3 plays a major role in clearance of C3-opsonized particles. Moreover, the near absence of clearance in CR3-deficient mice suggests that macrophage CR1 does not participate in E clearance. If CR1 were to have any role in E capture by liver Kupffer cells, then E capture by CR1 would have been expected at the earliest time points when some fixed C3b might still exist on the cell surface. Only after 1–2 h was there a small but significant IgM-dependent clearance of E in CR3-deficient mice. It is unknown whether this low level of clearance was due to CR2- or CR4-dependent adhesion to fixed iC3b or C3dg. Arguing also against a role of C3dg in clearance of E are previous reports that E from patients with chronic cold agglutinin disease frequently bear $1–2 \times 10^4$ C3dg per cell (Ross et al., 1985) without evidence of reduced survival (Jaffe et al., 1976; Kirschfink et al., 1994). Moreover, CR4 is undetectable on murine splenic macrophages and Kupffer cells (Metlay et al., 1990), even though it is the major β_2-integrin of human tissue macrophages (Hogg et al., 1986; Myones et al., 1988; Ross et al., 1992).

Several previous reports on the clearance of IgM- and/or C3-opsonized E have shown that E were first sequestered rapidly in the liver and then a portion of the E were released slowly back into the circulation where they survived normally (Brown et al., 1970; Frank et al., 1977; Nilsson Ekdahl et al., 1991). The release of sequestered E has been shown to be dependent upon both the amount of IgM (Atkinson and Frank, 1974a,b), as well as the quantity of infused E (Nilsson Ekdahl et al., 1991). The release of sequestered C3-opsonized E from the liver is likely to be due both to a lack of phagocytosis of the E after they are bound to Kupffer cell CR3, followed by the release of the bound E by proteolytic cleavage of the fixed iC3b by factor I using adjacent Kupffer cell CR1 as a cofactor. A similar mechanism was proposed 25 years ago when the Kupffer cell C3b-receptor was thought to be responsible for sequestration of IgM-opsonized E and factor I cleavage of C3b was thought to form C3d (Atkinson and Frank, 1974a).

In the current investigation, little release of sequestered IgM-opsonized E was observed. Since phagocytosis of sequestered IgG-opsonized E prevents their later release (Frank et al., 1977), the trace amounts of IgG detected in the IgM preparation were removed. Although this was effective in vitro in preventing macrophage phagocytosis of bound EAC generated with the IgM and mouse serum C, the

IgM-opsonized E generated with the IgG-depleted IgM were not significantly released following sequestration in the liver. Numerous additional tests with varying amounts of IgM Ab or varying numbers of infused E also did not show significant release of sequestered E. The reason why the mouse may be different from other animals that have been examined with this same E clearance model is unknown, but may be due to the subset of Kupffer cells detected in this study that express only CR3 and no detectable CR1. Factor I-dependent cleavage of iC3b into C3dg requires CR1 as an essential cofactor (Ross et al., 1982; Kinoshita et al., 1985). A relatively small amount of CR1 as compared to CR3 on murine Kupffer cells, particularly the $CR1^{-}CR3^{+}$ subset shown to represent ~40% of Kupffer cells, might prevent the release of CR3-bound iC3b-E by not providing adequate cofactor activity for factor I cleavage of the iC3b into C3dg.

Clearance of β-glucan BRM by Kupffer cell CR3 was expected both because Kupffer cells had been shown to be the major site of uptake of i.v. infused β-glucans (Hamuro et al., 1970; Suda et al., 1996; Yoshida et al., 1996; Adachi et al., 1998; Sveinbjornsson et al., 1998) and because macrophage CR3 had been shown to serve as a major receptor for β-glucans (Xia et al., 1999). In particular, in vitro studies had shown that there was virtually no uptake of ^{125}I-labeled SZP_g by peritoneal macrophages from CR3-KO mice as compared to normal macrophages, and the uptake by normal macrophages was blocked by mAbs to CR3 (Xia et al., 1999).

Two CR3-binding soluble polysaccharides were examined for clearance. SZP_m, a ~20 kDa β-mannan exhibiting a high binding-affinity (5×10^{-8} M) for human (Thornton et al., 1996) or mouse (Xia et al., 1999) CR3, was cleared rapidly with little glomerular filtration. By contrast, SZP_g, a smaller (~6 kDa) β-glucan exhibiting 10-fold less affinity for CR3 (Xia et al., 1999), was also cleared rapidly, but most was removed by the kidneys before it reached Kupffer cell CR3. Others have also reported that large β-glucans were cleared by the liver while small glucans were removed by glomerular filtration (Smedsrød and Seljelid, 1991). Because the binding of β-glucans to CR3 is saturable and reversible (Thornton et al., 1996; Xia et al., 1999), experiments were carried out to determine the amount of unlabeled competing SZP_g required to block the CR3-dependent clearance of 1 μg of ^{125}I-SZP_g. The relatively large quantity of SZP_g required (2 mg) was probably a function of both its rapid removal by the kidneys as well as its lower affinity for CR3. With the large β-glucan ''Grifolan'' (GRN; $> 5 \times 10^5$ kDa) a much smaller injected dose (250 μg) inhibited Kupffer cell clearance (Miura et al., 1996).

The pharmacokinetic parameters of β-glucan BRM are important for calculating therapeutic dose levels. Although larger β-glucan BRM appear to be better because of their resistance to glomerular filtration and saturation of liver clearance, large β-glucan BRM have a greater potential for producing undesired therapeutic side effects. β-Glucans that are large enough (2×10^5) to cross-link membrane surface CR3 can activate inflammatory cells nonspecifically in the absence of a target pathogen. Lentinan, schizophyllan, GRN, and the large glucan from *Sclerotinia sclerotiorum* (SSG) have been shown to trigger neutrophil degranulation and a respiratory burst, and to stimulate macrophage and NK cell secretion of TNFα, IFNγ, and IL-6 (Sakagami et al., 1988; Adachi et al., 1994; Sakurai et al., 1994; Větvička et al., 1996; Ljungman et al., 1998; Ross et al., 1999). By contrast, low m.w. soluble β-glucans bind only to individual CR3 molecules and induce a primed state that is capable of stimulating cytotoxic degranulation if, and only if, the primed CR3 is later cross-linked by the clustered iC3b found on a target pathogen (Větvička et al., 1996; Xia et al., 1999). This requirement for cell-bound iC3b to activate β-glucan-primed CR3 makes the cytotoxic function of low m.w. β-glucans specific for iC3b-opsonized microbial pathogens or tumor cells. A polysaccharide BRM with the size and CR3-binding affinity of SZP_m appears to be ideal because its size is large enough to prevent glomerular filtration and its high affinity for CR3 results in most of the injected dose being taken up rapidly by the liver.

The preferential clearance of β-glucans to the liver is one explanation for the frequent effectiveness of β-glucan BRM against hepatomas (Abe et al., 1984; Williams et al., 1985; Sherwood et al., 1988; Sveinbjornsson et al., 1998). Moreover, the ability of β-glucans to saturate Kupffer cells facilitates the maintenance of therapeutic blood levels (Miura et al., 1996; Suda et al., 1996), allowing β-glucans to

prime the CR3 of circulating leukocytes for cytotoxic destruction of tumors in other anatomic locations. In addition, the primed state of macrophage and NK cell CR3 resulting from a brief exposure to β-glucan remained detectable for up to 24 h (Xia et al., 1999). Thus, daily i.v. therapy for 2 weeks with 200 μg of SZP_g, that remains only briefly in the blood because of rapid clearance, was adequate to promote a 70%–90% reduction in the weight of established mammary tumors (Yan et al., 1999).

Acknowledgements

The authors gratefully acknowledge the technical assistance provided by Jana Větvičková. The authors particularly thank Dr. Brian P. Thornton, University of Louisville, for assistance with early preparations and clearance studies of SZP_m labeled with ^{125}I. This work was supported by research grants from the United States Department of Public Health, National Institutes of Health, AI27771 [G.D.R.] and DK51643 [T.N.M.].

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