The Arthus Reaction in Rodents: Species-Specific Requirement of Complement

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The Arthus Reaction in Rodents: Species-Specific Requirement of Complement


We induced reverse passive Arthus (RPA) reactions in the skin of rodents and found that the contribution of complement to immune complex-mediated inflammation is species specific. Complement was found to be necessary in rats and guinea pigs but not in C57BL/6J mice. In rats, within 4 h after initiation of an RPA reaction, serum alternative pathway hemolytic titers decreased significantly below basal levels, whereas classical pathway titers were unchanged. Thus the dermal reaction proceeds coincident with systemic activation of complement. The serine protease inhibitor BCX 1470, which blocks the esterolytic and hemolytic activities of the complement enzymes C1s and factor D in vitro, also blocked development of RPA-induced edema in the rat. These data support the proposal that complement-mediated processes are of major importance in the Arthus reaction in rats and guinea pigs, and suggest that BCX 1470 will be useful as an anti-inflammatory agent in diseases where complement activation is known to be detrimental. The Journal of Immunology, 2000, 164: 463–468.

The Arthus reaction is the classic in vivo model for immune complex (IC)-mediated acute inflammatory tissue injury. In the reverse passive Arthus (RPA) reaction, an excess of Ab is injected into the skin of animals previously infused i.v. with the corresponding Ag. This ensures perivascular deposition of ICs in the dermis and reproducibly elicits a rapid inflammatory response characterized, in order, by edema, neutrophilia, hemorrhage, and finally tissue necrosis (reviewed in Ref. 1). The events that initiate and propagate inflammatory tissue injury during the RPA reaction have been the subject of many studies using rabbits (2), guinea pigs (3), and rats (4). These early animal studies led to general agreement that complement activation by ICs is the key initiating step and demonstrated a requirement for neutrophils and mast cells for propagation of the inflammatory cascade. However, more recent studies have suggested that IC-mediated inflammation in the RPA reaction is initiated via Fcγ receptor-triggered pathways. For example, treatment of rats with recombinant soluble human FcγRIIa was shown to lead to a dose-dependent inhibition of the RPA response (5), and targeted deletion of the FcγR γ-chain resulted in markedly attenuated RPA reactions in mice (6, 7). In addition, it was reported that in mice deficient in complement proteins C3, C4, or C5 the RPA reaction develops normally (8). Thus, it was proposed that IC-initiated inflammation in the RPA response relies on activation of cellular responses triggered by FcγRs and does not require complement activation (8). Subsequent experiments utilizing mice deficient only in FcγRIIa indicated that this receptor is involved in mediation of the RPA reaction (9). However, in contrast to the studies using mice deficient in the γ-chain of FcγR, experiments using FcγRIII-deficient animals indicated that in addition to FcγR-triggered pathways, complement activation also contributes significantly to the mediation of the RPA reaction. Hörken et al. (10) who used mice deficient in the C5a receptor also demonstrated that complement contributes to the RPA reaction.

Obviously, the exact roles of FcγRs and complement in the RPA reaction remain equivocal. Nevertheless, the combined data suggested to us that the degree of dependence on complement- and FcγR-mediated pathways in IC-induced injury is species specific. We tested this hypothesis by directly comparing the RPA reactions elicited in the skin of normocomplementemic and decomplemented rats, guinea pigs, and mice and found that the contribution of complement in the early edematous phase of inflammation indeed exhibits species specificity; in rats and guinea pigs complement plays a major role, whereas in C57BL/6J mice no requirement for complement could be demonstrated. In addition, using the rat model, we show that complement is activated systemically during the dermal RPA reaction and that treatment with the serine protease inhibitor BCX 1470 blocks the inflammatory response. This therapeutic effect of BCX 1470, and perhaps of other similar inhibitors, may prove useful for the treatment of human diseases where tissue injury is initiated by IC deposition and is mediated by complement activation.

Materials and Methods

Animals

All animals were fed and watered ad libitum and maintained according to protocols established by the Animal Resources Program at the University of Alabama at Birmingham. Eight-week-old C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) weighing 30 ± 2 g, 12-wk-old Sprague Dawley rats (Charles River Laboratories, Boston, MA) weighing 343 ± 15 g, and 18-wk-old HsdPoc:DH guinea pigs (Harlan Sprague-Dawley, Indianapolis, IN) weighing 278 ± 4 g were used.
Materials

Polyclonal rabbit anti-sheep RBC serum, chicken egg albumin (grade VII), Evans blue dye, 5,5′-dithiobis (2-nitrobenzoic acid), Na-benzoyl-L-Arg-nitroanilide, propidium, and DMSO were from Sigma (St. Louis, MO). Benzoyloxy carbonyl-Lys-thiobenzyl was from Nova Biochem (La Jolla, CA), bovine trypsin was from Worthington Biochemical (Freehold, NJ), and eosin Y was from Eastman (Rochester, NY). Bovine trypsin was from Worthington Biochemical (Freehold, NJ), bovine trypsin, and the rabbit polyvalent polyclonal rabbit anti-chicken egg albumin IgG was from Cappel (West Chester, PA), and sheep and guinea pig erythrocytes were from Colorado Serum (Denver, CO). Polyclonal rabbit anti-chicken egg albumin IgG was from Cappel (West Chester, PA), and sheep and guinea pig erythrocytes were from Colorado Serum (Denver, CO). Polyclonal rabbit anti-chicken egg albumin IgG was from Cappel (West Chester, PA), and sheep and guinea pig erythrocytes were from Colorado Serum (Denver, CO).

Determination of the esterolytic activity of factor D, C1s, and trypsin

The esterolytic activity of factor D, C1s, and trypsin was determined by measurement of their ability to hydrolyze appropriate synthetic substrates (15, 16). All esterolytic assays utilized 1.29 mM of substrate dissolved in 0.1 mM HEPES, 0.5 M NaCl, and 10% DMSO and were performed in 96-well microtiter plates (200 μl per reaction). In these assays, hydrolysis of the substrate benzoyloxycarbonyl-Lys-thiobenzyl by factor D (104 nM) or by C1s (3 × 105 nM) liberates an active group which reacts with the chromogen 5,5′-dithiobis (2-nitrobenzoic acid), whereas trypsin hydrolyzes the chromogenic substrate Na-benzoyl-L-Arg-nitroanilide. In both cases the change in absorbance of the colored end-product was monitored at 405 nm (15, 16). All esterolytic assays utilized 1.29 mM of substrate dissolved in 0.1 mM HEPES, 0.5 M NaCl, and 10% DMSO and were performed in 96-well microtiter plates (200 μl per reaction). In these assays, hydrolysis of the substrate benzoyloxycarbonyl-Lys-thiobenzyl by factor D (104 nM) or by C1s (3 × 105 nM) liberates an active group which reacts with the chromogen 5,5′-dithiobis (2-nitrobenzoic acid), whereas trypsin hydrolyzes the chromogenic substrate Na-benzoyl-L-Arg-nitroanilide. In both cases the change in absorbance of the colored end-product was monitored at 405 nm (15, 16).

Hemolytic assays

Total classical pathway hemolytic activity of human and rat serum was measured using 100 μl rabbit anti-sheep erythrocytes (1 × 107/m in) with 50–150 μl of a 1/10 dilution of serum in GVB-EDTA (half-strength veronal-buffered saline (pH 7.3) containing 1 mM MgCl2, 0.15 mM CaCl2, and 0.1% gelatin). The reaction volume was adjusted to 1.5 ml with GVB and the mixture was incubated for 60 min at 37°C. The absorbance of the supernatants at 413 nm was then used to calculate CH50 U/ml. Total hemolytic activity defined as: [(rate of esterolysis in absence of inhibitor) – (rate of esterolysis in absence of inhibitor)] × 100.

Hypocomplementemia was induced via i.p. injection of CoVF (30 μg) (19) 18 h before initiation of RPA reactions. We have shown (20) using ELISA (21) that this treatment reduces mouse serum antigenic C3 to less than 3% of initial levels within 4 h, and that the hypocomplementemic condition persists for at least 48 h. We verified that after injection of CoVF, antigenic C3 was also not detected in rat and guinea pig sera collected up to 24 h after CoVF treatment (data not shown). To test whether administration of BCX 1470 inhibits the RPA reaction, the compound was mixed with the OVA/Ab solution and administered i.v. to rats (10 mg/kg) via the right femoral vein immediately before initiation of dermal RPA reactions. Alternatively, BCX 1470 was infused during the initial hour of the RPA reaction (0.25 mg/kg final dose), beginning immediately after injection of OVA and just before dermal injection of Ab. Preliminary tests confirmed that 24-h survival of rats treated with 10 mg/kg of BCX 1470 was 100%. Control rats (no inhibitor) received a bolus or an infusion of 5% dextrose.

Histology

Skin biopsies were taken from saline-injected and Ab-injected sites, embedded in OCT compound (Sakura Fineteck USA, Torrance, CA), flash frozen in liquid nitrogen, and stored frozen (at −70°C) until processed. Tissue sections (4 μm) were cut from each lesion on a cryostat microtome set at −20°C, beginning at the periphery of each lesion and ending proximal to but not including the point of injection. Sections were mounted on glass slides, air dried, acetone fixed, and stained with hematoxylin and eosin to visualize overall tissue pathology. To reveal ICs and the extent of deposition of C3, sections were blocked with normal horse serum and double stained with tetrathymid rhodamine isothiocyanate (TRITC)-labeled (1:100) rabbit anti-chicken egg albumin (ICN Pharmaceuticals, Aurora, OH). To reveal the injected Ag, tissue sections were stained with FITC-labeled rabbit anti-chicken egg albumin (ICN Pharmaceuticals). Sections were washed and mounted in Fluormount G (Southern Biotechnology Associates, Birmingham, AL) and were viewed with a Leica/Leitz DMRB fluorescence microscope equipped with appropriate filter cubes (Chroma Technology, Brattleboro, VT). Images were acquired with a C5810 series digital color camera (Hamamatsu Photonics, Bridgewater, NJ) and processed with Adobe Photoshop and IP LAB Spectrum software (Signal Analytics Software, Vienna, VA). Images of TRITC

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To determine whether the role of complement in IC-initiated inflammatory reaction is species specific in rodents and to identify an appropriate animal model for testing complement inhibitors in vivo, we compared the RPA reaction in the skin of rats, guinea pigs, and mice. Under identical experimental conditions, intradermal injection of IgG anti-chicken egg albumin (50 μg) into animals receiving i.v. 10 mg/kg of chicken egg albumin, intradermally with saline or 20 μg IgG, and typically they attain comparable intensity in rats and mice but was much more pronounced in guinea pigs (Fig. 1). As judged by similar lesion size, the inflammatory response was of comparable intensity in rats and mice but was much more pronounced in guinea pigs (Fig. 1). The intensity of the RPA response was reduced significantly (6-fold) in rats made hypocomplementemic by injection of CoVF and was also reduced significantly (2-fold) in hypocomplementemic guinea pigs (Fig. 1). In contrast, the size of RPA lesions elicited in mice was not reduced by CoVF-induced hypocomplementemia (Fig. 1). Thus, in rodents, sensitivity to RPA-induced extravasation is species specific, and in rats and guinea pigs, but not in mice, complement appears to be a major mediator of the inflammatory response.

Kinetics and dose response of the rat RPA reaction

Because the net effect of hypocomplementemia on the RPA response was greatest in the rat, we studied in more detail the kinetics and IgG dose responsiveness of the reaction in this species. As shown in Fig. 2, dermal lesions are already of substantial size by 2 h after injection of 20 μg IgG Ab, and typically they attain maximum size within 3–4 h. Beyond 4 h there is gradual regression of the size of the lesions, although they are still obvious even at 18 h. The intensity of the dermal edema increased with the amount of injected IgG (Fig. 3). A strict requirement for complement was confirmed by the fact that hypocomplementemic rats showed no significant edema development even when 100 μg of IgG was used (Fig. 3). The dermal edema elicited by this dose of Ab in hypocomplementemic rats was not significantly greater than that elicited in control rats by injection of saline (Fig. 3).

Microscopic examination of skin biopsies from normocomplementemic rats revealed extensive edema of the hypodermis at the sites of Ab injection (Fig. 4B), whereas saline-injected skin showed normal histology (Fig. 4A). Immunofluorescence confirmed the presence of ICs in Ab-injected sites (Fig. 5). These contained rabbit IgG (Fig. 5B), chicken egg albumin (Fig. 5C), and extensive deposits of C3 (Fig. 5B). By comparison, in the dermis of hypocomplementemic rats, IgG was deposited (Fig. 6C) but C3 was not (Fig. 6D). These combined data indicate that in the rat during the first 4 h of the RPA reaction, the dermal inflammatory response requires the deposition of ICs and an intact serum complement system.

Statistical analysis

Significant differences were determined by Student’s t tests. Statistical significance was defined as p < 0.05. All averaged values are expressed as the mean ± SEM.

Results

Requirement of complement in the RPA reaction is species specific

FIGURE 1. Species-specific requirement of complement in the RPA reaction. Rats, guinea pigs, and mice were injected i.v. with 10 mg/kg of chicken egg albumin in 2% Evans blue dye, after which they immediately received an intradermal injection of saline or 50 μg of rabbit IgG anti-chicken egg albumin (25 μl per site). The Arthus reaction was allowed to proceed for 4 h, and the net area of each IgG-induced dermal lesion was determined as described in Materials and Methods. To deplete serum complement, animals were injected i.p. with 30 μg CoVF 18 h before initiation of the RPA reaction. Control animals received no CoVF treatment. The data are presented as the mean ± SEM from three experiments, n = 3–4 animals (6–8 lesions) per group. *, p < 0.009 vs untreated group (Student’s t test). ns, not significant.

FIGURE 2. Time course of development of the RPA reaction. Rats were injected i.v. with chicken egg albumin, intradermally with saline or 20 μg IgG, and killed at the times indicated. The net area of IgG-induced dermal lesions was determined. Data are presented as the mean ± SEM from three experiments, n = 1–5 animals (2–10 lesions) per point.

FIGURE 3. The rat RPA reaction is Ab- and complement-dependent. RPA reactions were initiated in the dermis of control rats (○) and CoVF-treated rats (●) using increasing doses of IgG Ab. The area of dermal lesions was measured at 4 h. The data are presented as the mean ± SEM from two experiments, n = 2–4 rats (4–8 lesions) per point. *, p < 0.005 vs CoVF-injected rats receiving an equivalent amount of IgG (Student’s t test).
BCX 1470 inhibits complement activation in vitro and blocks the Arthus reaction in vivo

The serine protease inhibitor BCX 1470 inhibits the esterolytic activity of factor D and C1s 3.4- and 200-fold better, respectively, than that of trypsin (Table I). The ability of BCX 1470 to inhibit esterolytic activity translates into potent inhibition of the proteolytic activity of C1s, factor D, and consequently of the classical and alternative pathway-mediated hemolysis of target RBC (Table I). As expected from its more effective inhibition of C1s esterolytic activity, classical pathway hemolytic activity is inhibited 7-fold more effectively than that of the alternative pathway. Inhibition of the alternative pathway is apparently due to inhibition of factor D and not factor B (Table I).

We tested the ability of BCX 1470 to inhibit development of the rat dermal RPA reaction. BCX 1470 administered i.v. as either a single bolus or a 1-h infusion significantly reduced edema (Fig. 7). Remarkably, the inhibition of edema development achieved by 1-h infusion of BCX 1470 was equivalent to that achieved by induction of hypocomplementemia with CoVF (Fig. 7). Evidence that the mode of action of BCX 1470 in vivo involves inhibition of complement activity was obtained by serial measurements of serum hemolytic activity during development of RPA lesions (Fig. 8). During the RPA reaction in control rats, no significant change in serum classical pathway hemolytic activity was detected, but alternative pathway hemolytic activity decreased by 50% (Fig. 8A). By comparison, in rats infused for 1 h with BCX 1470 the...
decrease in alternative pathway activity was delayed by at least 2.5 h (Fig. 8B). Based on the in vitro data, we suggest that BCX 1470 abrogates the RPA reaction in rats via blockade of complement activation, most likely via inactivation of C1s and factor D enzymatic activity.

Discussion

Two major conclusions can be drawn from these data. First, in rodents the contribution of complement to RPA-induced dermal edema is species specific. Complement is necessary in rats and guinea pigs but not in mice. Second, the serine protease inhibitor BCX 1470 blocks development of RPA-induced edema in the rat, apparently by inhibiting complement activation.

Our data support the proposal that complement-mediated processes are of major importance in the RPA reaction in rats (22). The weak dermal response we observed in hypocomplementemic rats after administration of a high dose of Ab and the weak RPA reaction seen by others in rats treated with the complement inhibitor soluble complement receptor type I, sCR1 (22), suggest that complement-independent processes do not make a significant contribution to the initial increased vascular permeability of the RPA reaction. Our data show that guinea pigs are more sensitive to IC-mediated inflammation than rats, but the contribution of complement is likely similar in these two species. In contrast, complement has little or no effector role in RPA reaction in C57BL/6J mice. Thus, our data do not contradict the proposal (23) that in mice, complement is not required for IC-mediated inflammation. However, because only C57BL/6J mice were used in our work, we cannot exclude the possibility of complement participation in the dermal RPA reaction in other strains of mice. In fact, soon after the initial submission of this paper, it was reported that the major mechanism promoting immune complex-triggered peritonitis in C57BL/6J mice was activation of macrophages via FcγRI, whereas in BALB/c mice the response was complement dependant (24). The rat RPA reaction has often been used as an in vivo model to test the efficacy of anti-inflammatory drugs (25–31). Yet, despite

Table I. Inhibitory activity of the serine protease inhibitor BCX 1470

<table>
<thead>
<tr>
<th>Activity</th>
<th>IC_{50} (μM)</th>
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<tbody>
<tr>
<td>Esterolytic</td>
<td></td>
</tr>
<tr>
<td>Factor D</td>
<td>0.096</td>
</tr>
<tr>
<td>Cls</td>
<td>0.0016</td>
</tr>
<tr>
<td>Trypsin</td>
<td>0.326</td>
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<tr>
<td>Hemolytic</td>
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<tr>
<td>Classical pathway</td>
<td>0.046 ± 0.011</td>
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<tr>
<td>Alternative pathway</td>
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<tr>
<td>Factor D</td>
<td>0.075 ± 0.013</td>
</tr>
<tr>
<td>Factor B</td>
<td>NI*</td>
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* NI, no inhibition when tested at up to 20 μM.

FIGURE 7. BCX 1470 blocks the RPA reaction in rats. Immediately after i.v. injection of chicken egg albumin and just before intradermal injection of 25 μg rabbit IgG, BCX 1470 was injected as a single i.v. bolus (10 mg/kg), or a 1-h i.v. infusion was started (0.25 mg/kg). Net area of the resultant dermal lesions was quantitated at 4 h after injection of IgG. Rats pretreated with CoVF served as controls. * p < 0.05 vs untreated rats (Student’s t tests). The data are presented as the mean ± SEM from six experiments. The numbers above the columns indicate the number of lesions measured.
these efforts, an extensive literature search suggests that ours is the first study to quantify the effects of an RPA reaction on the activity of serum complement. Despite evidence that ICs activate the classical pathway in vitro (32), we detected no change in rat serum classical pathway hemolytic titers during the dermal RPA reaction. In contrast, the serum alternative pathway hemolytic activity decreased significantly during the rat RPA response. We do not know the reason for this discrepancy. The results of both hemolytic assays depend on the levels of C3 to C9, although not necessarily to the same extent. Therefore, the observed difference could be due either to a greater degree of activation/consumption of factor B than C2 and/or C4, or to different sensitivities of the two assays to decreased levels of C3 to C9.

We have shown that the synthetic serine protease inhibitor BCX 1470 is a potent inhibitor of C1s and factor D in vitro and that it prevents the initial phase of IC-mediated inflammation in rats and the associated systemic complement activation. We did not exhaustively investigate the effects of the inhibitor on tissue deposition of the various complement components during the Arthus reaction, but presumably deposition of C1q is not affected, whereas deposition of later components (e.g., C3, C4, and C5b-9) is reduced. Also we cannot exclude the possibility that the BCX 1470 effects on RPA were at least in part due to inhibition of some other serine protease. However, taken together with the results of the CoVF decomplementation experiments, these results support the conclusion that complement is necessary for the development of RPA lesions in rat skin. Although the role of complement in IC-mediated diseases may differ in rats and humans, BCX 1470 or other similar protease inhibitors have potential medical applications in the pharmacologic control of complement activation in human diseases.

References