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IgG and Complement-Mediated Tissue Damage in the Absence of C2: Evidence of a Functionally Active C2-Bypass Pathway in a Guinea Pig Model

Eric Wagner,*‡ Jeffrey L. Platt,¶ David N. Howell,‡‖ Henry C. Marsh, Jr.,** and Michael M. Frank‡‡§

In vitro complement-mediated lysis of heavily sensitized sheep erythrocytes by C4-deficient (C4D) guinea pig and C2-deficient (C2D) human sera was demonstrated some years ago. It was postulated that these “complement-bypass” pathways resulted from activation of C1 and components of the alternative pathway. We used normal, C2D, and C4D guinea pigs in a Forssman shock model to test the in vivo relevance of the C2- and C4-bypass pathways of complement activation. High concentrations of both anti-Forssman Ab and C2D or C4D guinea pig serum induced efficient lysis of sheep erythrocytes in vitro. The most efficient lysis was observed when IgG Ab and C2D guinea pig serum were used. Blocking either the classical pathway (treatments with EGTA-Mg²⁺ or soluble recombinant complement receptor type 1 (sCR1)) or the alternative pathway (treatment with heating at 50°C, sCR1, or soluble recombinant CR1 lacking the first of the four long homologous repeat sequences (sCR1[desLHR-A])) inhibited lysis; both pathways were required for lysis of sheep erythrocytes by C2D and C4D guinea pig sera. i.v. injection of anti-Forssman Ab in normal guinea pigs resulted in rapid death from pulmonary shock, whereas C4D guinea pigs had no adverse effect. Surprisingly, C2D guinea pigs either died in a delayed fashion or had a sublethal reaction. sCR1 treatment prevented Forssman shock in both normal and C2D guinea pigs, whereas sCR1[desLHR-A] prevented Forssman shock only in C2D animals. Our results suggest that the C2-bypass pathway occurs in vivo to produce tissue damage. Activation of complement in the absence of C2 appears to be far more efficient than in the absence of C4. The Journal of Immunology, 1999, 163: 3549–3558.

Genetic deficiency of the second component of the complement system (C2) is one of the most common deficiencies of a complement protein in Caucasians (1). Curiously, the clinical consequences of this complement deficiency, particularly with respect to an increased susceptibility to infection, are usually less severe than in patients with other early component deficiencies (C1 or C4). At least 25% of C2-deficient patients appear completely healthy (1). Whereas the total hemolytic activity of sera from C2-deficient patients is undetectable by standard methods, we and others have shown that, under special circumstances, complement-mediated lysis of Ab-sensitized sheep erythrocytes occurs in vitro (2–4). Heavy sensitization of cells is required for cell lysis to occur. This lysis appears to require the action of proteins of both the classical and alternative complement pathways.

To this point, several pathways for lysis of cells involving C1 and components of the alternative pathway have been described.

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pulmonary shock occurred with pathologic findings that resembled those in normal guinea pigs. We have analyzed the difference in this immunopathologic reaction between C2-deficient and C4-deficient animals in detail in both in vivo and in vitro studies. This appears to represent the first example of in vivo involvement of the C2-bypass pathway of complement activation in the induction of tissue damage.

Materials and Methods

Complement buffers, cells, and sera

Isotonic Veronal-buffered saline (VBS) containing either 0.5 mM MgCl₂, 0.15 mM CaCl₂, and 0.1% gelatin (GVBS³⁻) or 10 mM EDTA and 0.1% gelatin (EDTA-GVBS) as well as low ionic strength dextrose-GVBS³⁻ (DGVBs³⁻) were prepared as described (13). Isotonic GVBS²⁺ containing 8 mM EGTA and 5 mM MgCl₂ (EGTA-GVBS) for alternative pathway activation assays was prepared as described (14). In some experiments, EGTA-GVBS contained 20 mM EDTA to ensure maximal calcium ion chelation in highly concentrated serum.

Sheep blood was collected aseptically in Asever’s solution. After removal of the buffy coat and several washes in EDTA-GVBS, GVBS³⁻, and 60% DGVBs²⁺, the erythrocytes were standardized to 1 × 10⁸/ml and stored at 4°C in DGVBs³⁻. For alternative pathway assays, blood was collected from normal New Zealand white rabbits into disodium EDTA and processed as sheep blood.

Blood samples were collected from normal Hartley, C2-deficient, and C4-deficient guinea pigs via cardiac or cranial vena cava puncture under anesthesia with isoflurane. Serum was prepared from these blood samples using standard methods and immediately frozen at ~80°C. Sera were adsorbed twice with packed fresh sheep erythrocytes or fresh rabbit erythrocytes on ice for 30 min to remove normal Abs.

Hemolytic assays

The ability of sera from C2-deficient, C4-deficient, and normal guinea pigs to lyse Ab-coated sheep erythrocytes was assayed as follows. Sheep erythrocytes, containing antiserum or a rabbit anti-boiled sheep erythrocyte stroma IgG fraction, were prepared from these blood samples using standard methods and immediately frozen at ~80°C. Sera were diluted with 0.1% gelatin, 0.15 mM CaCl₂, and 1 mM MgCl₂ in VBS³⁻. 1 × 10⁵ cells/ml were sensitized with various dilutions of either a rabbit anti-boiled sheep erythrocyte stroma IgM-containing antiserum or a rabbit anti-boiled sheep erythrocyte stroma IgG fraction. It is known that virtually all of the hemolytic Ab in these preparations reacts with purified Forssman glycosphingolipid. The antiserum used in our experiments contained anti-sheep red blood cell stroma IgM Abs with undetectable IgG hemolytic reactivity (data not shown). Sensitized cells (25 μl) containing antiserum or a rabbit anti-boiled sheep erythrocyte stroma IgG fraction were incubated at 37°C for 1 h in the presence of various dilutions of guinea pig serum in a total volume of 75 μl. Where indicated, inhibitors of complement were added to the reaction mixture. The reaction was stopped by adding 2 ml of ice-cold EDTA-GVBS, the tubes were spun, and the extent of hemolysis in the supernatant was read at 412 nm. Total hemolytic complement activity (13) and alternative pathway activity (14) assays were performed as described.

Complement inhibitors

To assess the role of both the classical and alternative pathways of complement activation in our in vitro and in vivo assays, soluble forms of complement receptor type 1 (CR1) were used. Soluble CR1 (sCR1), lacking the cytoplasmic and transmembrane domain of CR1, and a soluble recombinant form of sCR1, lacking the first of the four long homologous repeat sequences (LHR-A), which contains one of the three moderate-affinity binding sites for C4b (sCR1[desLHR-A]), were provided by Avant Immunotherapeutics (Needham, MA). sCR1 blocks C3 and C5 convertases of both the classical and the alternative complement pathways (15). Recently, sCR1[desLHR-A] was produced and shown to block preferentially the alternative pathway (16). LHR-A contains one of the three moderate-affinity C4b binding sites. Absence of LHR-A, within the engineered molecule, abrogates control of the classical pathway C3 convertase. Thus, the classical pathway remains intact, whereas alternative pathway function is blocked. In in vivo experiments, both of these agents were injected i.v. at a dose of 15 mg/kg body weight (17).

Abbreviations used in this paper: VBS, Veronal-buffered saline; GVBS³⁻, VBS with 0.1% gelatin, 0.15 mM CaCl₂, and 1 mM MgCl₂; EDTA-GVBS, VBS with 0.1% gelatin and 10 mM EDTA; EGTA-GVBS, GVBS³⁻ with 8 mM EGTA and 5 mM MgCl₂; CR1, complement receptor type 1; sCR1, soluble form of CR1 lacking the transmembrane and cytoplasmic domains; sCR1[desLHR-A]; sCR1 lacking the first long homologous repeat (LHR-A); CVF, cobra venom factor; MBL, mannamin-binding lectin.

Animals

Normal Hartley guinea pigs (400–800 g) were purchased from Charles River Laboratories (Wilmington, MA), and guinea pigs deficient in the second (C2) and fourth (C4) components of complement (500–800 g) were from colonies housed at the Duke University Vivarium (Durham, NC). Both C2-deficient (18) and C4-deficient (19) guinea pigs were derived from the same original colonies. All animals used in our experiments were devoid of either functional C2 or functional C4 activity according to well-established and highly sensitive hemolytic assays (20, 21).

Forssman shock experiments

Details relative to the preparation and characterization of rabbit anti-boiled sheep erythrocyte stroma Abs are as described (12). An IgG fraction from rabbit antisera collected several weeks after primary immunization was prepared according to standard methods (22). Heat inactivation (56°C, 30 min) of all sera before Ab purification was performed to ensure absence of hemolytically active complement. Hemolytic titrations of Ab preparations in the presence of excess complement was conducted as described (23). Both the IgM-containing antiserum and the IgG fraction were used in in vitro assays, whereas only the IgG fraction was used in in vivo experiments, because only anti-Forssman IgG Abs are capable of inducing the Forssman shock reaction (24). A total of 0.5 ml IgG anti-Forssman Ab injected i.v. proved to be sufficient to cause the classical symptoms associated with Forssman shock (13) and uniformly led to pulmonary shock and death within 10 min after injection in normal Hartley guinea pigs of 350–500 g body weight. A volume of 1 ml induced a similar reaction in 800-g guinea pigs. Briefly, animals were injected via either the lateral saphenous vein or one of the branches of the cephalic vein and observed until reactions occurred. In experiments in which complement inhibitors were used, a catheter was inserted into a branch of one of the cephalic veins while the animal was under isoflurane anesthesia. scR1 or sCR1[desLHR-A] was injected to achieve a final dose of 15 mg/kg body weight. Endotoxin-free sterile PBS (Life Technologies, Grand Island, NY) served as a control (vehicle). Fifteen minutes later, anti-Forssman IgG was injected. Blood samples were collected before inhibitor or Ab administration and when severe respiratory distress, which preceded death, was observed. In cases in which no distress was observed, animals were bled and killed 90–90 min after Ab injection.

Tissue sampling and processing

At the animal’s death, the lungs were rapidly removed. Small sections were embedded in OCT medium, quick-frozen in liquid nitrogen precooled isopentane, and stored at ~80°C until further analysis. Sections (5 μm) of the frozen tissues were cut and tested by immunofluorescence for the presence of rabbit IgG, guinea pig C3, and guinea pig C4. FITC-labeled goat anti-rabbit IgG (IgG fraction) and FITC-labeled goat anti-guinea pig C3 (IgG fraction) were purchased from ICN Biomedicals (Costa Mesa, CA). A FITC-labeled nonimmune goat IgG fraction (ICN Biomedicals) was used as a negative control. A polyclonal Ab directed against guinea pig C4 was prepared in our laboratory by immunizing C4-deficient guinea pigs with normal guinea pig serum. An IgG fraction was prepared that reacted with guinea pig C4 on the basis of immunodiffusion. Because of its weak reactivity toward guinea pig C4, this Ab preparation was used in immunofluorescence studies using a two-step amplification system. The IgG fraction was labeled with biotin (Sigma, St. Louis, MO), and its binding to guinea pig lung tissues was evidenced using streptavidin-FITC (Jackson ImmunoResearch, West Grove, PA). An IgG fraction from a nonimmune C4-deficient guinea pig serum was prepared and labeled with biotin and used as a negative control. Abs to guinea pig C4 and C3 were adsorbed with normal rabbit serum, whereas the anti-rabbit IgG was adsorbed with normal guinea pig serum to avoid nonspecific reactivity.

Results

In vitro lysis of sensitized erythrocytes by complement-deficient sera

As expected from earlier studies (4, 5), lysis of Ab-sensitized sheep erythrocytes was noted when high concentrations of both C2-deficient and C4-deficient guinea pig sera were used (Fig. 1). Ab isotype and the density of Ab molecules on the surface of the target cell also played a crucial role in the complement-mediated lysis of these cells. Dose-response curves were established for lysis of IgG and IgM Ab-sensitized sheep erythrocytes in the presence of excess C4-deficient and C2-deficient guinea pig serum. Whereas
sera from C2-deficient guinea pigs lysed both IgM- and IgG-sensitized sheep erythrocytes (Fig. 1, A and B), in general, more efficient lysis was observed when IgG was used as a sensitizing agent (Fig. 1B). C4-deficient guinea pig serum proved much less efficient than C2-deficient guinea pig serum at lysis of sheep erythrocytes at all concentrations of Ab. Sera from C4-deficient guinea pigs induced lysis of IgG-sensitized cells, whereas the effect on IgM-sensitized cells was negligible (Fig. 1, C and D). Maximal lysis of cells was seen with high concentrations of sensitizing Ab and high concentrations of C2- and C4-deficient sera. The observed lysis of erythrocytes was complement dependent, as incubation in the presence of EDTA in all cases decreased specific lysis to <5% in cases in which lysis in the absence of EDTA was maximal (data not shown). Heat inactivation of serum samples at 56°C for 30 min abolished their lytic activity.

Involvement of the classical and alternative complement pathways in vitro

To assess the relative role of the classical and alternative pathways of complement activation in the lysis of heavily sensitized sheep erythrocytes by C2-deficient and C4-deficient guinea pig sera, a buffer containing 20 mM EGTA and 5 mM MgCl₂ was used to allow only the activation of the alternative complement pathway. Cells were sensitized with a 1:125 dilution of either IgM or IgG anti-Forssman Ab. These were the highest concentrations of Ab that could be used without agglutination of cells. As shown in Fig. 2, EGTA treatment, which blocks C1 function, markedly impaired the lysis of both IgM- (Fig. 2A) and IgG-sensitized (Fig. 2B) erythrocytes by C2-deficient, C4-deficient, and normal guinea pig sera, indicating that the classical pathway was utilized in these sera. Treatment of the various sera at 50°C for 30 min, which destroys factor B activity, impaired the ability of C2-deficient and C4-deficient guinea pig sera to lyse sensitized sheep erythrocytes, whereas normal guinea pig serum was unaffected by such treatment (Fig. 2, A and B), indicating that the alternative pathway contributed to lysis in C2-deficient and C4-deficient sera.

The contribution of the classical and the alternative pathways of complement activation in the observed phenomenon was further analyzed using specific inhibitors of each pathway. sCR1, which interferes with C3 and C5 convertases of both the classical and alternative pathways, and sCR1[desLHR-A], which specifically blocks the alternative pathway C3 and C5 convertases, were used in our assays. As expected from previous studies on human complement activation, when normal guinea pig serum was incubated with sheep erythrocytes sensitized with either an optimal or a high concentration of anti-Forssman IgG, sCR1 inhibited the lysis of target cells in a dose-dependent fashion. sCR1[desLHR-A] had no effect on the lytic activity of normal guinea pig serum (Fig. 3A). In sharp contrast, both sCR1 and sCR1[desLHR-A] inhibited the lysis of heavily sensitized sheep erythrocytes by C2-deficient and C4-deficient guinea pig sera in a dose-dependent fashion. On a molar basis, a higher concentration of sCR1[desLHR-A] than sCR1 was required to achieve the same extent of inhibition of lysis (Figs. 3, B and C). In all cases, substitution of complement inhibitors by a control protein (human serum albumin) induced no significant change in the percentage of lysis induced by serum alone (data not shown). These results confirm that the alternative pathway is necessary for lysis of sensitized sheep erythrocytes by C2-deficient and C4-deficient guinea pig sera. In addition, our data show for the first time that sCR1 and sCR1[desLHR-A] derived from human CR1 have the ability to interfere with guinea pig complement activation, as they do with human complement. However, there are

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** Lysis of Ab-sensitized sheep erythrocytes in C2-deficient and C4-deficient guinea pig sera. Standardized cells sensitized with various dilutions of either a rabbit anti-Forssman IgM (A and C) or IgG Ab (B and D) were incubated in the presence of C2-deficient guinea pig serum (A and B) or C4-deficient guinea pig serum (C and D) diluted as follows: 1:3 ( ), 1:9 ( ), and 1:18 ( ).
no published data to indicate that human CR1 has all of the functional activities in guinea pig serum that exist in human serum.

**In vivo complement activation as assessed by the Forssman shock reaction**

Normal, C2-deficient, and C4-deficient guinea pigs were subjected to Forssman shock according to well-established methods. Observations are depicted in Table I. All normal guinea pigs died of pulmonary shock within 2–10 min after injection of anti-Forssman IgG Ab, as expected. Three C4-deficient guinea pigs were subjected to the same treatment, but no adverse effects were seen at up to 70 min of observation after Ab administration. Macroscopic observation of the lungs after the animals were sacrificed showed no signs of pulmonary damage or hyperinflation, which is the hallmark of Forssman shock. In striking contrast, C2-deficient guinea pigs demonstrated sensitivity to the i.v. injection of rabbit anti-Forssman Ab with reactions appearing in a delayed fashion as compared with normal guinea pigs. Three animals died of pulmonary shock within 10–30 min after challenge with the Ab. Three other guinea pigs experienced a sublethal reaction observed as respiratory distress without death. In the latter case, the lungs were markedly hyperinflated, as were those of normal guinea pigs with Forssman shock. In cases in which the shock was lethal, blood-tinged sputum obstructing the airways was observed, as is observed in complement-sufficient guinea pigs. Control experiments in two normal guinea pigs injected with a similar amount of preimmune rabbit IgG fraction failed to demonstrate any pulmonary reaction. The dependence on complement of the reaction observed in C2-deficient guinea pigs was tested by treatment of two animals with cobra venom factor (CVF). As observed in normal guinea pigs, treatment with CVF completely eliminated any reaction to the i.v. injection of rabbit anti-Forssman IgG (data not shown). Two C2-deficient animals were also injected i.p. with normal guinea pig citrate plasma as a source of C2 and were subjected to Ab challenge 18 h later. Even though the functional C2 levels in the blood of each animal roughly approximated 5% of the activity found in the injected material, a normal pulmonary reaction followed by death within 5 min after Ab injection was observed in C2-reconstituted C2-deficient guinea pigs (not shown).

**FIGURE 2.** Contribution of the alternative pathway in the lysis of Ab-sensitized sheep erythrocytes by normal, C2-deficient, and C4-deficient guinea pig sera. Sheep erythrocytes were sensitized with a 1:125 dilution of rabbit anti-Forssman IgM (A) or IgG (B) and mixed with guinea pig sera diluted 1:9. Sera were used untreated (open bars) or in a buffer containing 20 mM EGTA and 5 mM MgCl₂ (filled bars) or were heated at 50°C for 30 min (hatched bars).

**FIGURE 3.** Effect of sCR1 and sCR1[desLHR-A] on the lysis of heavily sensitized sheep erythrocytes. Sheep erythrocytes were sensitized with a 1:125 dilution of rabbit anti-Forssman IgG and incubated with either sCR1 (●) or sCR1[desLHR-A] (○) in the presence of a 1:120 dilution of normal guinea pig serum (A), a 1:9 dilution of C2-deficient guinea pig serum (B), or a 1:9 dilution of C4-deficient guinea pig serum (C).
Role of the classical and alternative pathways in vivo

Because the pulmonary reaction we observed in C2-deficient guinea pigs was most unexpected, given that the Forssman reaction is strictly dependent on the classical pathway of complement activation (as proven by the lack of a reaction in C4-deficient guinea pigs) and C2-deficient animals have total absence of C2, we conducted experiments to assess the role of both pathways of complement activation in this reaction. We used sCR1 to block both the classical and the alternative complement pathways and sCR1[desLHR-A] to block the alternative complement pathway. In standard hemolytic assays, administration of sCR1 completely inhibited complement activity via the classical and alternative pathways. Administration of sCR1[desLHR-A] resulted in complete inhibition of the alternative pathway but not of the classical pathway. Representative examples are given in Fig. 4. It is noteworthy that blood samples were taken after Forssman shock. Some depression of serum complement titers is noted compared with preshock samples in animals in which there was no complement inhibition. Reactions observed in each animal are described in Table II. As expected, sCR1 protected both normal guinea pigs and C2-deficient guinea pigs from Forssman shock (Table II). In contrast, normal guinea pigs injected with sCR1[desLHR-A] before injection of the Ab underwent pulmonary shock, as did control animals injected with PBS before anti-Forssman Ab challenge. However, C2-deficient guinea pigs were protected from Forssman shock when treated with sCR1[desLHR-A] before Ab challenge. The lungs in these animals appeared normal with no sign of pulmonary damage.

Lung sections from normal, C2-deficient, and C4-deficient guinea pigs were frozen upon the animals’ deaths. Immunofluorescent staining for the presence of rabbit IgG, guinea pig C4, and guinea pig C3 was performed. In all animals tested, rabbit IgG deposition was observed in alveolar septa, primarily in association with alveolar capillaries of the lung (Fig. 5). C4 deposition was observed within the lungs of both normal (Fig. 6A) and C2-deficient (Fig. 6B) guinea pigs subjected to Forssman shock. Anti-guinea pig C4 staining occurred in alveolar septa, mainly in association with alveolar capillaries. Anti-guinea pig C4 staining, although relatively weak in intensity, was clearly above that observed with a matched control Ab (Fig. 6, C and D). However, we were not able to find differences in the intensity of anti-guinea pig C4 staining in lung tissues from normal and C2-deficient guinea pigs treated with PBS, sCR1, or sCR1[desLHR-A] before Forssman shock induction (data not shown). Massive C3 deposition was observed in the alveolar septa, mostly associated with alveolar capillaries of lung tissues from normal and C2-deficient guinea pigs either untreated or treated with PBS before anti-Forssman Ab injection (Fig. 7, A and B). When both normal and C2-deficient guinea pigs were treated with sCR1 15 min before Forssman shock induction, the intensity of anti-guinea pig C3 staining in the lung tissues decreased dramatically (Fig. 7, C and D) to levels comparable with the fluorescence intensity observed with a matched control Ab (not shown). When sCR1[desLHR-A] was used as an anti-complement agent, the intensity of anti-guinea pig C3 staining decreased dramatically only in C2-deficient guinea pigs (Fig. 7F), whereas it was intense in normal animals treated with the same agent at the same dose (Fig. 7E).

Discussion

Some years ago, it was demonstrated that high concentrations of serum from guinea pigs totally deficient in the fourth component of
Complement had the ability to induce lysis of heavily sensitized sheep erythrocytes via a new complement activation pathway that we termed the C1-bypass activation pathway (2). The pathway was shown to require the activity of C1 and components of the alternative pathway for its action. It was inefficient and required high concentrations of complement-activating Ab to induce lysis. At that time, one name for the alternative complement pathway was the bypass pathway. Because C1 was required for function, we termed the pathway “the C1-bypass pathway” to indicate that it required C1 and proteins of the “bypass” pathway for function. The term bypass pathway has fallen out of favor for the alternative complement activation pathways, although different IgM preparations have differing activities on the observed pattern of lysis (6). In isolated reports, the C4-bypass pathway of complement activation was proposed as being involved in human pathologic situations, such as chronic urticaria and angioedema (9), and hemolytic uremic syndrome (10). Using *Giardia lamblia* trophozoites as a target, Deguchi et al. (8) demonstrated a role of the C4-bypass activation pathway in the lysis of this protozoan parasite. IgM Abs isolated from patients with high lytic activity toward the parasite were shown to activate the bypass pathway in C2-deficient human serum and C4-deficient guinea pig serum. The lytic activity was lost on depletion of C1 or factor B from the serum samples, indicating an involvement of both the classical and alternative pathways of complement activation with no requirement for C4 and C2.

Lysis of sensitized sheep erythrocytes via the C2-bypass pathway depends on alternative pathway function (3). In addition, activation of the alternative pathway leading to erythrocyte lysis in the C2-bypass activation pathway was shown to require the presence of C4b on the sensitized cell rather than C1q (3). By depleting specific complement components, Steuer et al. (4) demonstrated that the lysis of heavily sensitized sheep erythrocytes by C2-deficient human serum depends on activation of C1, C4, and components of the alternative pathway. In binding studies, this group further demonstrated that C4b could bind the alternative pathway C3 convertase via a weak interaction with C3b (7) instead of directly forming a C3 convertase by binding factor B, as was previously proposed (3). Both of the above-cited reports were studies performed with human serum as a source of complement. Our in vitro studies, using C2- and C4-deficient guinea pig sera, are in agreement with the above observations and show for the first time the activity of a C2-bypass pathway in C2-deficient guinea pig serum.

Abs of IgG isotype are more effective than IgM in inducing the lysis of sheep erythrocytes by complement-deficient sera (Fig. 1), as was reported by Matsushita and Okada (3) for C2-deficient human serum and by us (5) for C4-deficient guinea pig serum. It is interesting that IgG Ab causes more lytic damage in the in vitro complementary pathway. This pathway was termed the C2-bypass pathway. Both IgM and IgG Abs have the ability to activate these unusual complement activation pathways, although different IgM preparations have differing activities on the observed pattern of lysis (6). In isolated reports, the C4-bypass pathway of complement activation was proposed as being involved in human pathologic situations, such as chronic urticaria and angioedema (9), and hemolytic uremic syndrome (10). Using *Giardia lamblia* trophozoites as a target, Deguchi et al. (8) demonstrated a role of the C4-bypass activation pathway in the lysis of this protozoan parasite. IgM Abs isolated from patients with high lytic activity toward the parasite were shown to activate the bypass pathway in C2-deficient human serum and C4-deficient guinea pig serum. The lytic activity was lost on depletion of C1 or factor B from the serum samples, indicating an involvement of both the classical and alternative pathways of complement activation with no requirement for C4 and C2.

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bypass pathways and is the only Ab isotype capable of causing Forssman shock (24). The reason why IgG Abs induce more extensive tissue damage is not clear. However, it should be noted that rabbit anti-Forssman IgG was shown to induce far more pore-forming lytic lesions than IgM when guinea pig complement is used as a complement source (25). The presence of IgG rather than IgM could favor alternative pathway activation by its ability to amplify C3b deposition on a target surface (26).

In the studies reported here, C2-deficient guinea pig serum proved to be more potent than C4-deficient guinea pig serum in the lysis of heavily sensitized sheep erythrocytes. As shown in Fig. 1, higher specific erythrocyte lysis was achieved with C2-deficient serum whether an IgM antiserum or an IgG fraction was used as a complement source (25). The presence of IgG rather than IgM could favor alternative pathway activation by its ability to amplify C3b deposition on a target surface (26).

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In our in vitro studies depended on both the classical and the alternative pathways. As demonstrated in Fig. 2, incubation of cells and sera (both from C2-deficient and C4-deficient guinea pigs) in a calcium ion-chelating buffer containing EGTA and magnesium ions, which eliminates classical pathway activation, substantially decreased the ability of the serum to induce lysis of sensitized cells. Furthermore, heat inactivation of serum for 30 min at 50°C, which is known to destroy factor B activity, also impaired markedly the lytic activity of the serum samples from C2- and C4-deficient animals, suggesting a need for components of the alternative pathway as well in the lysis of sensitized erythrocytes.

An additional demonstration of the role of the alternative pathway on the lysis of heavily sensitized sheep erythrocytes by C2-deficient and C4-deficient guinea pig sera in our in vitro studies depended on both the classical and the alternative pathways. As demonstrated in Fig. 2, incubation of cells and sera (both from C2-deficient and C4-deficient guinea pigs) in a calcium ion-chelating buffer containing EGTA and magnesium ions, which eliminates classical pathway activation, substantially decreased the ability of the serum to induce lysis of sensitized cells. Furthermore, heat inactivation of serum for 30 min at 50°C, which is known to destroy factor B activity, also impaired markedly the lytic activity of the serum samples from C2- and C4-deficient animals, suggesting a need for components of the alternative pathway as well in the lysis of sensitized erythrocytes.

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is therefore a preferential inhibitor of the alternative pathway. In our hands, sCR1[desLHR-A] did not block the lysis of Ab-sensitized sheep erythrocytes by the classical pathway. The fact that it inhibits lysis in C2-deficient and C4-deficient guinea pigs argues strongly that components of the alternative pathway are required for lysis.

We tested the ability of the C2-bypass and the C4-bypass pathways to mediate immunologic damage in the Forssman shock model. Using C2-deficient and C4-deficient guinea pigs, we were able to show for the first time the involvement of the C2-bypass pathway in vivo. As reported in our earlier studies (12), C4-deficient guinea pigs had no adverse reaction to the i.v. injection of rabbit IgG anti-Forssman Abs (Table I). To our surprise, C2-deficient animals underwent either sublethal or lethal reactions that appeared in a delayed fashion as compared with normal guinea pigs treated the same way (Table I). This phenomenon was complement dependent, as animals treated with CVF to deplete C3 and late-acting components behaved as C4-deficient guinea pigs. Reintroduction of guinea pig C2 by i.p. injection of normal guinea pig plasma accelerated the symptoms observed after i.v. injection of

FIGURE 7. Detection of anti-guinea pig C3 binding to lung sections from normal (A, C, and E) and C2-deficient (B, D, and F) guinea pigs by immunofluorescence. Massive staining of the alveolar septa, in association with alveolar capillaries, is observed in both normal (A) and C2-deficient (B) guinea pigs treated with PBS before Forssman shock induction. A striking decrease in C3 binding, comparable with that using a matched control Ab, is noted when both normal (C) and C2-deficient (D) guinea pigs are treated with sCR1. After treatment with sCR1[desLHR-A], C3 deposition remains massive in the lungs of normal guinea pigs undergoing Forssman shock (E), whereas it is inhibited in the lungs of C2-deficient guinea pigs that survived Forssman shock after anti-Forssman Ab injection (F) (magnification, ×185).
the anti-Forssman Ab, and the animals died in a time course similar to that observed in normal guinea pigs.

To investigate the role of the classical and alternative pathways in the development of the Forssman reaction in C2-deficient animals, we used sCR1 and sCR1[desLHR-A] injected i.v. 15 min before Ab administration. In normal animals, only sCR1 was potent at blocking the Forssman reaction. In sharp contrast, both sCR1 and sCR1[desLHR-A] protected C2-deficient guinea pigs from Forssman shock (Table II), suggesting that the alternative pathway of complement activation was recruited in C2-deficient guinea pigs but was not required in normal animals. The requirement for the classical pathway function can be deduced by the absence of shock in C4-deficient guinea pigs. Therefore, components of the classical and alternative pathways appear to be required in Forssman shock in C2-deficient animals. Our previous studies have shown that there is no blocking factor in C4-deficient guinea pigs that might interfere with potential complement-related activity and purified C4 can restore function to the deficient pathway in these animals (29).

Our immunopathologic studies using lungs from animals subjected to Forssman shock revealed similar C4 deposition patterns in normal and C2-deficient guinea pigs (Fig. 6). The same pattern of C4 deposition was observed in all animals, irrespective of the treatment regimen administered before anti-Forssman Ab injection (data not shown). In contrast, no reaction was observed when this Ab was used on lung sections from normal and C2-deficient guinea pigs that were not injected with anti-Forssman IgG or when a matched control Ab was used. This observation therefore suggests that activation of early components of the classical pathway up to the missing component (C2) occurs on IgG-coated cellular targets in vivo in C2-deficient guinea pigs, ruling out the sole activation of components of the alternative pathway in the observed phenomenon.

There was massive C3 deposition within the lungs of normal and C2-deficient guinea pigs that experienced Forssman shock (Fig. 7, A and B). Treatment of both normal and C2-deficient guinea pigs with sCR1 before induction of Forssman shock decreased the binding of C3 into the lungs (Fig. 7, C and D) to levels comparable with those observed with a matched control Ab. CR1 allows for a faster decay of both the classical and alternative pathway C3 and C5 convertases and is a cofactor for factor I-mediated cleavage of C3b to iC3b in humans (30). These functions of CR1 might explain our data and further support the strict dependency of Forssman shock on complement activation. When normal guinea pigs and C2-deficient guinea pigs were treated with sCR1[desLHR-A], an inhibitor of the alternative pathway and not of the classical pathway (16), only C2-deficient guinea pigs survived the shock and showed almost complete absence of C3 deposition within their lungs (Fig. 7F). In contrast, normal animals treated with the same agent failed to show any decrease in C3 binding in the lung (Fig. 7E) and died of Forssman shock as untreated animals did. This result demonstrates that C3 deposition and Forssman shock in C2-deficient guinea pigs are dependent on the triggering of the alternative pathway. Although this pathway is clearly not required for Forssman shock to occur in normal guinea pigs, the possible contribution of this pathway to the reaction in such animals cannot be ruled out.

As we observed C4 deposition within the lungs of both normal and C2-deficient guinea pigs, we can postulate that in animals lacking functional C2, a C2-bypass pathway can be triggered in vivo to induce tissue damage. As was demonstrated in in vitro experiments, this pathway requires Ab, early components of the classical pathway (C1, C4), and components of the alternative pathway. No obvious quantitative difference in IgG, C4, and C3 binding within the lungs of normal and C2-deficient guinea pigs was noted. Thus, our in vitro findings correlate with our in vivo observations. The C2-bypass pathway can lead to complement-mediated tissue damage but is relatively inefficient. High concentrations of Ab and complement are required to induce lysis of sheep erythrocytes in vitro. In vivo, the clinical signs of Forssman shock occur in C2-deficient guinea pigs in a delayed fashion as compared with normal guinea pigs. Therefore, more complement activation via the alternative pathway is required, which may explain the delay in the occurrence of the symptoms of Forssman shock.

One might postulate that the newly discovered mannan-binding lectin (MBL) pathway plays a role in the C2-bypass pathway, as observed in C2-deficient guinea pigs. MBL can substitute for Clq in activating the classical pathway on binding to microbial carbohydrates. MBL associates with two serine proteases, termed MASP-1 and MASP-2 (31). This pathway was shown to be activated in the presence of sensitizing Ab. However, MBL appears to require IgG that lacks terminal galactose residues to trigger complement activation (32). Normal mammalian IgG has terminal galactose on carbohydrate residues, and the Abs used in this study lacked terminal galactose residues. MBL is reported to activate the alternative pathway on sheep erythrocytes coated with mannan (33). Activation of the alternative pathway in this system is reported to require C4 and C2 of the classical pathway. If these early reports prove correct, it is unlikely that MBL plays a role in the C2-bypass pathway. Nevertheless, as more is learned about the MBL pathway of complement activation, it may be found to play a role in this phenomenon.

The presence of bypass pathways of complement activation may well give insights on the evolution of the complement system, as proposed by Farries et al. (34). It may be that C1 first evolved as a way of allowing Ab to activate the alternative pathway via the C4-bypass pathway. The appearance of C4 would have improved the ability to induce complement activation by Abs bound to a target surface, although with much less efficiency than the classical pathway as currently known. The further addition of C2 to the cascade may have allowed efficient complement activation through the classical pathway.

In summary, we confirm the presence of C1-dependent C4- and C2-bypass pathways in the guinea pig, demonstrate that the C2-bypass pathway is far more damaging immunopathologically, and demonstrate for the first time the ability of the C2-bypass pathway to cause an immunopathologic effect in vivo. The human pathologic relevance of our findings is still not clear, but it is noteworthy that Traustadottir et al. (11) recently observed that a similar activation pathway depending on C1, C4, and components of the alternative pathway is involved in a model of immune complex binding to red blood cells mediated via CR1. Our results may explain why few C2-deficient patients have recurrent infectious problems. It is known that the classical complement pathway contributes to the immune response in guinea pigs (35–37). It has been shown that C2-deficient guinea pigs differ from C4-deficient guinea pigs in that they do respond with an adequate IgG response to high antigenic loads of T-dependent Ags (36). The presence of the C2-bypass pathway may explain these differences between C4-deficient and C2-deficient animals.

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