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ROLE OF C3 IN THE REGULATION OF A SPLENIC PFC RESPONSE IN RABBITS

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The effects of in vivo C3 depletion on the immune response were examined in rabbits by assaying for splenic PFC after immunizing normal or cobra venom factor-treated animals with aggregated human γ-globulin. The response to this T-dependent antigen has previously been shown to be regulated such that several cycles of PFC appear following a single intravenous injection of antigen. C3 depletion had no effect on the first peak of PFC (appearing 5 days after injection), but resulted in depression of the second peak of PFC (day 13). In rabbits depleted of C3, antigen localization in splenic germinal centers was markedly decreased. Delaying C3 depletion until after antigen localization had occurred resulted in no depression of the second peak of PFC. These results suggest that one mechanism by which C3 affects immune responses in vivo is via its role in influencing the persistence of antigen. In the absence of C3, no significant localization of antigen occurs, resulting in interference with the cyclical production of antibody.

As important as the events involved in the initiation of antibody production are those events responsible for the regulation of the immune response after it has been initiated. Availability of antigen persisting in the host tissue plays an important role in such regulation. With some antigens, the cessation of antibody production is, in part, due to the catabolism of the stimulating antigen. However, with antigens that persist in the host for even short periods of time, the regulation of antibody production is more complex and other regulatory mechanisms such as suppressor cell activity, antibody feedback, and possibly anti-idiotypic reactivity may be more important.

A system lending itself to examination of these controlling mechanisms has been described in the rabbit, where a single intravenous injection of aggregated human gamma globulin (AHGG) has been shown to result in the cyclical appearance of plaque-forming cells (PFC) to human γ-globulin (HGG) (1). A single intravenous injection of 0.02 to 20.0 mg of AHGG results in a peak of both IgM and IgG PFC on day 5, followed by a rapid decline in the PFC response. At least two additional peaks and declines, separated by 8 day intervals have been observed. In addition, the splenic response is capable of regulating the PFC response of distal lymph nodes and appears to involve suppressor cells generated in the spleen (2).

After intravenous injection, AHGG is localized in the follicles of the spleen and persists there in an antigenically active form throughout the period in which cycling is observed (1). An essential role for C3 in the localization of antigen in lymphoid follicles has been suggested by others (3, 4), since in vivo decomplementation has been shown to result in impaired localization of AHGG in splenic germinal centers. Furthermore, this ability to localize in germinal centers has been correlated with the complement-fixing ability of the various classes and subclasses of immunoglobulins (5). In addition to the role C3 plays in the follicular localization of antigen, a role for complement in the initiation of immune responses has been proposed. In vivo experiments using cobra venom factor (CoF) to deplete C3 have indicated that C3 depletion results in a preferential suppression of T-dependent responses over T-independent responses (6-8). In addition, B lymphocytes possessing receptors for C3 (CR+) have been implicated as functioning in T-dependent responses, whereas B lymphocytes lacking C3 receptors (CR−) appear to function in T-independent responses (3, 9, 10). Thus, the proposal was made that C3 plays a role in facilitating T-B cell cooperation (6).

In the present study, it was of interest to investigate the effect of C3 depletion on the cyclical PFC response to AHGG because AHGG is a thymus-dependent antigen (11) and the cyclical nature of the response appears to require follicular localization of antigen (1). The results demonstrate that C3 does not appear to be required in the initiation of this response, but C3 does appear to be involved in regulating the appearance of PFC. The ability of C3 to influence the cyclical PFC response appears to be mediated by its effect on antigen localization.

MATERIALS AND METHODS

Animals. Male New Zealand White rabbits 3 to 4 months old were used throughout these studies.

Reagents. AHGG was prepared from Cohn Fraction II of human sera provided by the American Red Cross National Fractionation Center, with the partial support of National Institutes of Health Grant No. 13881 (HEM). The IgG fraction was purified by elution from diethyliaminoethy cellulose columns with 0.01 M phosphate buffer, pH 8.0. Aggregation of the IgG fraction was performed essentially as described by Gamble (12), by heating at 63°C for 25 min, followed by precipitation with a final concentration of 0.62 M sodium sulfate.
Purification of CoF. CoF was routinely purified by ion exchange chromatography (DEAE-Sephadex) and assayed as described by Ballow and Cochrane (13). One unit of CoF is defined as that amount in 0.1 ml of CoF required to cause 50% inhibition of lysis of sensitized erythrocytes (13). In some experiments CoF was further purified by preparative polyacrylamide gel electrophoresis as previously described and was determined to be free of phospholipase A₂ enzymatic activity (14).

Assay for C3. C3 levels were measured in samples of serum by radial immunodiffusion, as described previously (15). Antisera to C3 was obtained by immunizing guinea pigs with zymosan treated with normal rabbit serum. The zymosan was extensively washed before immunization. Antisera monospecific for C3 were obtained by selecting those bleedings obtained early after immunization that showed no reaction with other serum components, as assayed by immunoelectrophoresis.

Hemolytic plaque assay. PFC were enumerated by a modification (16) of the Jerne and Nordin plaque assay (17), in which spleen cells and indicator erythrocytes were added to 0.5 ml of 0.5% agarose (Indubiose A-37, Accurate Chemical and Scientific Corp., Hicksville, N. Y.) at 45°C and poured onto microscope slides. Spleens were excised from rabbits immediately after sacrifice and teased in balanced salt solution (BSS) (18) buffered to pH 7.0 with ethylenediaminetetraacetic acid (EDTA) at a final concentration of 0.02 M. Fragments of spleen were then teased through 90-mesh wire screens (Newark Wire Cloth Co., Newark, N. J.) and then filtered through nylon monofilament cloth (Kressilk Products, Inc., Monterey Park, Calif.). Cells were washed once in EDTA-BSS, followed by three washes in BSS (250 X G, 10 min, 4°C) and suspended to a final concentration of 2 to 4 X 10⁶ cells/ml. Indicator erythrocytes were prepared by coupling Cohn Fraction I with human sera to goat erythrocytes (Colorado Serum Co., Denver, Colo.) with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCI (Storv Chemical Co., Muskegon, Mich.). After an initial incubation at 37°C, slides prepared for enumeration of direct PFC were bathed in a 1:10 dilution of guinea pig sera (Pel Freeze, Rogers, Ark.) in BSS. Indirect PFC were bathed in the same solution containing, in addition, sheep anti-rabbit IgG at an optimum dilution.

Measurement of ¹²⁵I in tissues. HGG was first iodinated with ¹²⁵I according to the method of McConahey and Dixon (19) and then aggregated as described above. Rabbits were injected intravenously with 2 mg of the iodinated protein (65 μCl/mg) and exsanguinated 10 days later. The kidneys and spleens were excised and dissected free of gross fat and blood clots. The tissue was weighed, teased in saline, and counted in a gamma scintillation counter with a NaI crystal. The tissues were sonicated at 4°C in a Sonifier Cell Disrupter (Heat Systems-Ultronics, Inc., Plainview, N. Y.). The sonicated material was then centrifuged at 4°C in a Sonifier Cell Disrupter (Heat Systems-Ultronics, Inc., Plainview, N. Y.). The sonicated material was then counted for ¹²⁵I activity before and after precipitation with an equal volume of 20% trichloroacetic acid (TCA). All procedures were carried out at 4°C. The TCA precipitates were centrifuged at 110 x G for 15 min and washed three times in 10% TCA before counting.

RESULTS

Effect of C3 depletion on the in vivo PFC response to AHGG. Rabbits were immunized with 2 mg AHGG intravenously either with or without prior injection of 800 units of CoF. In order to obtain maximal depletion of C3, CoF was administered i.p. in three injections spaced 4 hr apart beginning 24 hr before injection of antigen (20). Rabbits were bled before and at various times after injection of CoF, and their sera assayed for C3 by radial immunodiffusion with guineas pig anti rabbit C3. Figure 1 shows the level of C3 in the serum of rabbits injected with CoF 24 hr before injection of AHGG, calculated as a percent of the prebleeding level. C3 levels fell to 10% of pretreatment levels at the time of injection of antigen and did not increase during the next 3 days. By 5 days after injection of antigen, C3 levels had increased, presumably because of the formation of an antibody response to CoF, as observed by others (20). Injections of AHGG alone resulted in no significant change in serum C3 levels. PFC values were assayed 5 days after injection of antigen, a time previously shown to elicit peak PFC values in rabbits injected only with AHGG. As shown in Table I, the day 5 direct PFC response of rabbits injected with 2 mg AHGG only, was not affected by injection of rabbits with CoF before injection of antigen. The indirect PFC response of rabbits injected with AHGG only, although in each experiment slightly higher than the response in CoF-treated rabbits, was also not significantly different from that of CoF-treated rabbits (p > 0.5). Direct PFC in this system enumerate IgM antibody-producing cells, as previously shown by their sensitivity to reduc-
tion and alkylation with dithiothreitol and iodoacetamide, respectively (1). In contrast, indirect PFC represent IgG-producing cells as evidenced by their insensitivity to this treatment.

**Effect of C3 depletion on the cyclical PFC response to AHGG.** It has previously been reported that rabbits injected intravenously with 2 mg AHGG exhibit periodic fluctuations in both direct and indirect PFC, with peaks appearing 5, 13, and 21 days after injection of antigen (1). PFC assayed during the intervening times were at markedly depressed levels. Although injection of CoF prior to antigen injection did not alter the early phase of the response (day 5 to day 8), PFC on day 13 were markedly reduced in CoF-treated rabbits (174 indirect PFC/10^6 cells after antigen injection alone vs 50 indirect PFC/10^6 cells after antigen injection in CoF-treated rabbits) (Fig. 2). Thus, immunization of C3-depleted rabbits results in the initial formation of IgG-producing PFC on day 5 that is comparable to that observed in rabbits containing normal levels of C3. However, IgG-producing PFC that appear subsequently (day 13) and that seem to be dependent on follicular antigen retention (1) are shown to be markedly decreased in C3-depleted rabbits. The reduction in PFC at this time occurs despite the reappearance of C3 in the circulation, reaching normal levels by day 8.

**Effect of delayed C3 depletion on the late PFC response to AHGG.** To further analyze the role of complement in the immune response, treatment with CoF was delayed until after localization of the injected antigen was essentially complete (day 3). As shown in Figure 3, delaying injection of CoF until 3 days after antigen injection resulted in abrogation of the inhibitory effect of CoF treatment on day 13 PFC, with even some enhancement of the response. As observed above, the same preparation of CoF administered 1 day before injection of antigen resulted in marked depression of day 13 PFC assayed at the same time.

**Effect of C3 depletion on the splenic localization of AHGG.** Previous studies have indicated that the appearance of the late peak of PFC (day 13) in spleens of rabbits immunized with AHGG appears to be correlated with the prolonged retention of antigen in the spleen (1). Therefore, the localization of AHGG in the spleen was examined after injection of antigen in either untreated rabbits or rabbits treated with CoF prior to injection of radiolabeled antigen. Rabbits were exsanguinated 10 days after antigen injection; their spleens and kidneys were homogenized and the TCA-precipitable material was counted. Those rabbits pretreated with CoF showed a markedly reduced retention of protein-bound radiolabel in their spleens when compared to rabbits injected with antigen only (Table II). The retained antigen has previously been shown to be present in a functional form as assayed by its ability to stimulate primed lymphocytes transferred without injection of antigen (1). Table II also shows that the decreased retention of antigen in CoF-treated rabbits is accompanied by a decrease in day 13 PFC in these rabbits, compared to those injected with antigen alone. No difference was observed in the amount of radioactivity detected in the kidneys of rabbits injected with antigen only and those injected with CoF and antigen.

**Effect of treatment with a phospholipase-free preparation of**

![Figure 2](http://www.jimmunol.org/)

**Figure 2.** Rabbits were injected with a single i.v. injection of 2 mg AHGG on day 0. One group of rabbits also received a total of 800 units of CoF i.p. given in three injections starting 24 hr before injection of antigen. Each point represents the mean indirect PFC of 12 rabbits, with brackets indicating 1 S.E.M.

<table>
<thead>
<tr>
<th>Table II Effect of C3 depletion on antigen retention and PFC responses</th>
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<tbody>
<tr>
<td>Treatment</td>
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<tr>
<td>---</td>
</tr>
<tr>
<td>Day 1</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>CoF</td>
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* AHGG, 2 mg AHGG injected i.v. day 0. For antigen localization studies, the antigen was radiolabeled with ^125I. Indirect PFC were assayed on rabbits immunized with either the labeled or unlabeled form of antigen.

**Assayed 10 days after injection of antigen.**

**Assayed 13 days after injection of antigen.**
CoF on splenic localization of AHGG. A preparation of CoF free of contamination with phospholipase A₁ was used to determine if folliculipases were involved in the decreased retention of follicular antigen observed in rabbits pretreated with standard preparations of CoF. The results obtained in Table III show that pretreating rabbits with a phospholipase-free preparation of CoF resulted in decreased localization of AHGG compared to rabbits injected only with AHGG, similar to that observed with preparations of CoF prepared by ion-exchange chromatography and Sephadex gel filtration.

**DISCUSSION**

These studies demonstrate that the C₃ component of complement plays a role in regulating the cyclical appearance of PFC to AHGG in rabbit spleens. The ability to detect a cyclical response in rabbits after immunization with AHGG allows an investigation of the effect of C₃ on the regulation of different phases of the immune response. At least three successive peaks of PFC to HGG appear after a single intravenous injection of rabbits with the aggregated form of the antigen (1). Each peak is separated from the previous peak by an 8-day period in which minimal PFC responses are observed. Although no difference is observed between normal and CoF-treated rabbits in the number of PFC to HGG at the time of the first peak of PFC (day 5), PFC arising subsequently are markedly reduced (day 13). Depletion of C₃ also results in the inability of the antigen to localize in the splenic follicles. It is proposed that C₃ plays a regulatory role in this response primarily by its effect on the persistence of antigen in the splenic follicles.

Previous studies have shown a relationship between localization of antigen in germinal centers and the humoral immune response (1, 21, 22). In the cyclical PFC response to AHGG, HGG has been shown to be localized in splenic germinal centers throughout the period in which at least three successive peaks of PFC appear (1). In contrast, a single early peak of PFC was observed in the mesenteric nodes of the same animals, with no later cycles of PFC. Correspondingly, no follicular localization of antigen could be detected in the nodes. The above results show that complement is involved in the process of antigen localization since treatment with CoF prior to injection of antigen results in the absence of localized antigen in splenic follicles. In addition, delaying injection of CoF in rabbits until after AHGG has had a chance to localize (day 3), abrogates the ability of CoF treatment to decrease the PFC response on day 13. Failure of aggregated immunoglobulins to localize in splenic follicles has previously been observed in mice treated with CoF (3, 4). As observed above with rabbits, only a minimal amount of radiolabeled AHGG was found in the mesenteric nodes of mice, and this was unaffected by C₃ depletion (4). Although with most antigens both antibody and C₃ appear to be necessary for antigen localization to occur (23), it is the activation of C₃ that appears to play a critical role since antigens capable of activating C₃ can localize without the presence of antibody.

These effects of C₃ depletion do not appear to result from a disruption in early events involved in T-B cell collaboration. Despite the T dependency of this antigen (11), there was a marked decrease in the magnitude of only the second peak of PFC, with no significant alteration in the appearance of the first peak of PFC, as assayed by the appearance of either IgM or IgG PFC. The involvement of C₃ in T-B cell collaboration was initially proposed as the result of studies showing that the ability of C₃ depletion to affect antibody responses was correlated with the thymic dependency of the antigen (6–8). C₃ involvement in T-B cell collaboration was further implicated by data showing that IgM antibody responses were found to be more sensitive to C₃ depletion than IgG (6, 7), IgA (24, 25), or IgE (25) responses, which are relatively more T dependent (26–28). These observations contrast with the failure to show depression of the early PFC response after depletion of C₃ in rabbits immunized with AHGG. Others have found that some T-independent responses can be decreased by in vivo complementation (29, 30), which also indicates that C₃ may affect at least some immune responses by mechanisms other than facilitating T-B cell collaboration.

The PFC responses in the two phases (day 5 vs day 13) differ in that the first peak of PFC appears to be the result of virgin precursor stimulation, whereas the second peak appears to be dependent on the stimulation of memory cells (1). Thus, the differential effects on the two peaks of PFC could be explained by the involvement of different precursor cells, with differing requirements for antigen presentation in the two phases of the response. Localized antigen has been implicated in the generation of memory cells in other systems, and generation of these memory cells also appears to be dependent on the presence of C₃ (22, 23, 31). The present results would be compatible with a role for C₃ in memory-cell generation. Thus, in vivo complementation prior to antigen injection decreases the second peak of PFC (day 13), which appears to be generated from memory cells, but not the primary response (day 5).

Since depletion of C₃ was accomplished by administration of CoF, both the effect on antigen localization and the PFC response could be due to contaminating proteins isolated with the de-complementing protein or to depletion of complement components other than C₃. Although it is possible that other complement components may be involved in facilitating antigen localization, this would be limited to the terminal components since it has been shown (in subhuman primates at least) that CoF treatment does not alter Clq, C₄, or Factor B levels as measured by Mancini techniques, but does reduce C₃ and C₅ levels significantly (32). Experiments utilizing C₆-deficient rabbits also appear to rule out a regulatory role for components C₆ and beyond. The cyclical response to AHGG was examined in genetically C₆-deficient rabbits that were bred into the colony of New Zealand White rabbits. Both C₆-sufficient littermates as well as the C₆-deficient rabbits were relatively hyporesponsive to injection of AHGG, when compared to rabbits from the New Zealand White closed colony. However, a cyclical response was observed, but no difference in the day 13 PFC response was seen between C₆-deficient rabbits and littermates. CoF treatment has also been shown to result in depletion of the IgG response in mice to SRBC, regardless of whether the strains of mice tested have normal or deficient levels of C₅ (33). Thus, it seems reasonable to attribute the effects observed after C.F. administration as being due to depletion of C₃.

Preparations of CoF prepared by ion-exchange chromatog-

### Table III

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Antigen Localization (cpm/gm) ± S.E.*</th>
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<tbody>
<tr>
<td>Day 1</td>
<td>Day 0</td>
</tr>
<tr>
<td>None</td>
<td>125I-AHGG</td>
</tr>
<tr>
<td>pCoF</td>
<td>125I-AHGG</td>
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* AHGG, 2 mg 125I AHGG injected i.v. pCoF, 800 units phospholipase-free cobra venom factor injected i.p. in three doses spaced 4 hr apart.

* Assayed 10 days after injection of antigen. Data are expressed as the mean values from four rabbits ± S.E.
raphy and Sephadex gel filtration have been shown to contain contaminating phospholipases that can suppress immune responses (34). In the present study, however, a phospholipase A₂-free preparation of CoF produced a loss of antigen retention in the spleen comparable to that observed with preparations purified by ion-exchange chromatography and Sephadex gel filtration only. Thus, it seems most likely that the effects observed by in vivo decompartmentalization with CoF are the result of depletion of complement, and, in particular, the C₃ component of complement.

Although CoF acts as an immunogen in rabbits, which apparently accounts for the rapid reappearance of C₃ due to antibody clearance of CoF from the circulation (20), antigenic competition does not appear to be a factor in explaining these results since the noncross-reacting antigen, aggregated turkey γ-globulin, which is strongly immunogenic, does not interfere with the PFC response to HGG when injected simultaneously with AHGG (2).

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REFERENCES