MODULATION OF REGULATORY MECHANISMS OPERATIVE IN THE CYCLICAL PRODUCTION OF ANTIBODY*

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Cyclical fluctuations in both the humoral antibody response (1-6) and in cell-mediated immunity (7-11) have been observed in several species after a single injection of persisting antigens. The possibility that these fluctuations reflect regulatory mechanisms operating within the immune system has been previously reviewed (12). The response in rabbits to a single intravenous injection of aggregated human gamma globulin (AHuIgG)1 has been characterized by the successive appearance of peaks of plaque-forming cells (PFC) in the spleen and peripheral blood, separated by well-defined 8-day periods of decreased responsiveness (4). Although there is evidence in other systems for regulation of the immune response by circulating antibody, resulting in cyclical variations of antibody and PFC (2), the 8-day latent period in rabbit spleens is observed regardless of the dose of antigen injected or the amount of circulating antibody formed. Thus, it was postulated that if antibody played a role in this system, the antibody produced locally, at the site of PFC production, was the best candidate for exerting such precise control of the response (4).

The amount of antibody produced to several antigens has been shown to be enhanced in rabbits by simultaneous injection of antigen and lipopolysaccharide (LPS) (13, 14), phytohemagglutinin (PHA) (14, 15), or concanavalin A (Con A) (14) and by sublethal irradiation after injection of antigen (14, 16). Therefore, the effect of these treatments on the cyclical nature of the PFC response in rabbits to AHuIgG was examined to determine if their ability to exert an adjuvant effect was regulated in the same manner as the normal PFC response to AHuIgG. In addition, different injection regimens have been used in an attempt to define the conditions essential for cycling.

Materials and Methods

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

Antigens. AHuIgG 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 isolated by chromatography on DEAE-

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1 Abbreviations used in this paper: AHuIgG, aggregated human gamma globulin; Con A, concanavalin A; LPS, lipopolysaccharide; PFC, plaque-forming cells; PHA, phytohemagglutinin.
cellulose by elution with 0.01 M phosphate buffer, pH 8.0. Ammonium sulfate precipitation (35%) was used in purifying the IgG fraction from pooled turkey sera. The precipitate was dialyzed against borate-buffered saline, pH 8.2, and the IgG fraction was isolated by filtration on G-200 Sephadex, essentially according to the method described by Benedict for purification of chicken immunoglobulin (17). Both immunoglobulins were aggregated by heating at 63°C for 25 min followed by precipitation with 0.62 M sodium sulfate as previously described (18).

Mitogens. LPS from Escherichia coli K235 was generously supplied by Abbott Laboratories, North Chicago, Ill., through Dr. Floyd C. McIntire, now at the School of Dentistry, University of Colorado Medical School, Denver, Colo. Con A (lots 88 and 96), three times crystallized, was purchased from Miles Laboratories, Inc., Kankakee, Ill. PHA-P (lots 59855 and 59875) was purchased from Difco Laboratories, Detroit, Mich.

Irradiation. Irradiated rabbits were given a whole body dose of 500 R. They were restrained in a Lucite chamber and placed in a Gamma Cell 40 small animal irradiator (Atomic Energy of Canada, Ltd., Ottawa, Canada) which contained a cesium-137 source emitting a central dose of 115 R/min.

Hemolytic Plaque Assay. PFC were measured by a modification (19) of the Jerne and Nordin hemolytic plaque assay (20). Cohn Fraction II from human sera or the ammonium sulfate-precipitated fraction of turkey sera were coupled to goat erythrocytes (Colorado Serum Co., Denver, Colo.) by 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide HCl (Story Chemical Corp., Muskegon, Mich.) for enumeration of PFC to HuIgG or turkey IgG, respectively. Indirect PFC were developed with an antiserum to rabbit IgG prepared in sheep.

Antibody Analysis. Circulating antibody levels were determined by a quantitative precipitin test which measures the amount of 125I-labeled antigen precipitated at equivalence by 1 ml of serum (21). Amounts of antibody protein were calculated on the basis of an antibody to antigen (HuIgG) weight ratio at equivalence of 2.5.

Results

Effect of LPS on Cycling. A cyclical production of indirect PFC was observed in spleens of rabbits injected simultaneously with 2 mg AHuIgG and 10 μg LPS intravenously (Fig. 1). The kinetic pattern of the response, as assayed 5, 8, and 13 days after injection, was similar to that in rabbits injected with antigen only, in which peak PFC were obtained on day 5 and several succeeding peaks were observed at 8-day intervals (4). However, injection of antigen and LPS resulted in an average sixfold enhancement of the first peak of PFC (day 5) compared to rabbits receiving antigen only. LPS had no apparent effect on the second peak (day 13) since it was of the same magnitude regardless of whether rabbits were injected with AHuIgG alone or with AHuIgG and LPS. Both groups of rabbits had minimal splenic PFC on day 8. Precipitating antibody levels on day 8, the day of maximal titer, were sixfold higher in rabbits injected with AHuIgG and LPS compared to rabbits injected with antigen alone (565 μg antibody/ml and 87 μg antibody/ml, respectively).

Effect of PHA on Cycling. Simultaneous injection of 2 mg AHuIgG and 10 mg PHA also resulted in a cyclical pattern of splenic PFC production to HuIgG (Fig. 2). Injection of PHA with antigen resulted in a threefold enhancement of PFC on day 5 compared to rabbits injected with AHuIgG only. Precipitating antibody levels on day 8 reflected the differences observed in PFC on day 5, with fourfold higher antibody levels in rabbits injected with PHA and antigen compared to those injected with antigen alone (302 μg antibody/ml and 81 μg antibody/ml, respectively). PFC in rabbits injected with AHuIgG and PHA did not decrease to levels as low as on day 8, as in rabbits injected with antigen only, although the percentage decrease from day 5 levels was similar in the two
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"On day 13, in contrast to results obtained with LPS, PFC levels were again markedly enhanced in rabbits injected with PHA and antigen as compared to rabbits injected with antigen only."

Effect of Con A on Cycling. In contrast to the lack of effect of either LPS or PHA injected simultaneously with antigen on the kinetic pattern of PFC produc-
tion to HuIgG in spleens of rabbits, Con A injected under the same conditions disrupted the cyclical appearance of PFC (Fig. 3). Injection of Con A with AHuIgG also caused an increase (approximately 1.5 times) in the total number of lymphocytes in the spleen on all days tested compared to the number of splenic lymphocytes in rabbits injected with AHuIgG only. Indirect PFC on day 5 in rabbits injected with 10 mg Con A and 2 mg AHuIgG were depressed, compared to PFC in rabbits injected with antigen only. However, PFC (per $10^6$ spleen cells) showed an approximately linear increase on days 8 and 13 in rabbits injected with Con A and simultaneously with antigen, so that by day 13, PFC (per $10^6$ spleen cells) were five times greater than in spleens from rabbits injected with antigen alone. On day 8, a time when minimal PFC were detected in rabbits injected with antigen only (less than 30 PFC per $10^6$ spleen cells), an average of 160 PFC per $10^6$ spleen cells was detected in rabbits injected with Con A and 2 mg AHuIgG. Despite the marked difference in the kinetics of the PFC response in the spleens of rabbits injected with Con A and antigen and those injected with antigen only, precipitating antibody levels in both groups peaked on day 8 (557 μg antibody/ml and 62 μg antibody/ml, respectively) and declined in both groups by day 13 (462 μg antibody/ml and 38 μg antibody/ml, respectively).

Effect of Irradiation on Cycling. Irradiation (500 R) of rabbits 24 h after injection of 2 mg AHuIgG resulted in greatly enhanced PFC levels compared to rabbits injected with antigen only (Fig. 4). PFC per $10^6$ spleen cells in irradiated rabbits averaged 1,355 and 1,781 on days 5 and 13, respectively, compared to PFC values of 149 and 66 on days 5 and 13, respectively, for rabbits injected with antigen only. Antibody titers on day 8 were markedly enhanced in irradiated rabbits injected with AHuIgG (494 μg antibody/ml) when compared to rabbits only injected with AHuIgG (61 μg antibody/ml). A cyclical pattern of PFC production occurred, although there was an average of 606 indirect PFC per $10^6$ spleen cells on day 8, a time when only minimal PFC are detected after injection of antigen alone. Total splenic PFC were also enhanced by irradiation and showed a similar pattern of cycling although only approximately half as many lymphocytes were recovered from spleens of irradiated rabbits on all days tested.

Kinetics of Splenic PFC Response after Intraperitoneal Injection of Antigen. A cyclical pattern of splenic PFC production was found after intraperitoneal injection of 2 mg AHuIgG (Fig. 5). Although in this experiment no comparison was made with a similar group of animals injected intravenously, PFC numbers were comparable to those observed in other experiments after intravenous injection of the same dose of AHuIgG.

Kinetics of Secondary Splenic PFC Response after Low-Dose Priming. A cyclical appearance of splenic PFC was observed in rabbits primed with an intravenous injection of 0.002 mg AHuIgG and injected with 2 mg AHuIgG intravenously approximately 1 mo later (Fig. 6). PFC were assayed 3, 5, 8, 11, and 13 days after the secondary injection and showed peak levels at both days 3 and 11. Thus, although PFC were not assayed daily, it would appear that an 8-day cyclical PFC response occurred. The primary PFC response to 0.002 mg AHuIgG was similar, although lower than, the response to 2 mg AHuIgG.

Kinetics of PFC Response in the Draining Lymph Node after Subcutaneous
Fig. 3. Comparison of PFC kinetics in spleens of rabbits after a single intravenous injection of 2 mg AHuIgG with, or without, a simultaneous injection of 10 mg Con A. Each point represents an average of PFC values from 9 to 10 rabbits.

Fig. 4. Comparison of PFC kinetics in spleens of rabbits after a single intravenous injection of 2 mg AHuIgG with, or without, irradiation (500 R) 24 h after injection of antigen. Each point represents an average of PFC values from five rabbits.

Injection of Antigen. A linear increase in PFC to HuIgG was found on days 5, 8, and 13 in the draining popliteal lymph node after subcutaneous injection of 0.2 mg AHuIgG into the hind foot of rabbits (Fig. 7). Little or no precipitating antibody was detected in the sera of the rabbits on any of these days. Rabbits injected with 0.2 mg AHuIgG subcutaneously in the hind foot and simultaneously injected intravenously with 2 mg AHuIgG had levels of PFC in the popliteal node on day 5 comparable to rabbits injected only subcutaneously. However, PFC in the nodes 8 days after both subcutaneous and intravenous injection were suppressed compared to PFC in the nodes of rabbits injected only subcutaneously. Between days 8 and 13, PFC in the nodes appeared at relatively the same rate in both groups; however, day 13 PFC in the nodes of rabbits in-
Discussion

Various mechanisms have been proposed for the regulation of physiological responses in which the controlling factors are generated during the response. In some immunological systems, regulation has been reported to occur via a feedback mechanism involving circulating antibody (22). In other systems, activation of a regulatory population of T cells has been proposed (23). Self-regulation by the immune system is apparently involved in the cyclical antibody response to persisting antigens. In the present system, the precision of the regulation is such that successive peaks of PFC appear in the spleens of rabbits...
at exact intervals of 8 days after the initial peak of PFC, after a single intravenous injection of AHuIgG.

The effects of mitogens on the cyclical antibody response to AHuIgG, either in enhancing the initial and/or succeeding peaks of PFC or in disrupting the periodic variations in the response, have been studied in an attempt to correlate these effects with the known cellular sites of action of the mitogens. In this regard, the effect of LPS in enhancing the splenic PFC response to AHuIgG in rabbits is consistent with the possibility that enhancement of the response is due to the ability of LPS to act as a mitogen for B cells. LPS has been shown to be a mitogen for B cells in the mouse (24) and, although less potent, for B cells in the rabbit (25, 26). Furthermore, a correlation has been demonstrated between the mitogenic and adjuvant activities of LPS (27). Thus, LPS may act to enhance the initial peak of PFC by providing a "second signal" to B cells (28), thereby allowing low avidity precursors to receive sufficient triggering to become PFC. Injection of LPS with AHuIgG, however, does not result in enhancement of the second peak of PFC. Although LPS persists for prolonged periods of time (29), its failure to enhance the latter PFC response may be due to the differential localization of AHuIgG and LPS, resulting in the inability of LPS to function effectively with antigen in the restimulation of cells. Thus, only those cells capable of being restimulated by persisting levels of antigen alone would participate in the succeeding peaks of PFC. In support of this, a strict time relationship between injection of LPS and the antigen has previously been shown to be necessary in order for LPS to exert its adjuvant effect (30). It is unlikely that the failure of enhanced PFC in the second peak is due to suppression by high levels of antibody since either PHA or irradiation likewise enhance antibody synthesis while also enhancing the second peak. Although the above model is compatible with the experimental results, LPS does affect a variety of physiological re-
sponses, any of which could also be responsible, at least in part, for these effects. Indeed, effects of LPS on other cells directly involved in the immune response, i.e. T cells and macrophages, have also been reported (31–33).

In contrast to LPS, activation of a T-cell helper population could explain the enhancement of both the initial and succeeding peaks of PFC observed when PHA is injected with AHuIgG. Once the T-cell population has been expanded, these T cells would be available for cooperation with competent B cells, resulting in triggering of a larger population of specific B cells. A similar stimulation has been observed in vivo in mice (34). However, PHA has been shown to suppress, not enhance the in vitro response of mouse spleen cells (35). Since a strict comparison has not been made in the mouse between in vivo and in vitro effects of PHA on the PFC response to a particular antigen, it is possible that PHA acts via different mechanisms under in vivo and in vitro conditions. Furthermore, although PHA has been shown to be a mitogen for a subpopulation of T cells in both mice (36) and rabbits (25, 37), and there appears to be a similarity in the function of different cell types in the mouse and rabbit (38), subtle differences have become apparent (39) which might result in PHA acting via different mechanisms in the mouse and rabbit. In addition, an in vivo effect of PHA in stimulating B cells directly cannot be ruled out since PHA also binds to B cells in the mouse (40) and is capable of stimulating B cells in vitro under conditions in which it is locally concentrated (41). However, if PHA enhances the response by stimulating B cells, it does so differently than LPS, since only the initial peak of PFC is enhanced by LPS, but both the initial and succeeding peaks are enhanced by PHA. Neither mitogen, however, has an effect on the precise timing of the cyclical appearance of PFC.

Activation of a helper population of T cells by Con A is also compatible with the enhancement of the immune response to AHuIgG by this mitogen. However, additional events must also occur after injection of Con A since a marked disruption of the cyclical response occurs, resulting in a linear increase in PFC over the 13-day period assayed. Con A has been shown to activate both helper and suppressor populations of mouse lymphocytes in vitro depending on the conditions of culture (35). Similarly, activation of T cells by Con A could result in an alteration of the regulatory balance between helper and suppressor populations of T cells normally present in vivo after antigen activation. The decreased response observed five days after injection of AHuIgG and Con A as compared to injection of AHuIgG alone might be due to the preferential activation of a nonspecific suppressor cell population by Con A. A similar early suppression followed by subsequent enhancement was observed in the response of rabbits to sheep erythrocytes (14). Such early activation of suppressor cells could then exhaust this population so that specific activation of suppressor cells by antigen would not occur later in the response. Expression of helper or suppressor function after injection of AHuIgG alone may be, in part, mediated by the amount of functional antigen available (42). In any event, both of the putative suppressor effects observed in this system are short-lived, a characteristic of the suppressive effects found in other systems (43, 44). However, again, alternative mechanisms are possible for the regulation of the AHuIgG response by Con A. As with PHA, Con A also binds to B cells (40) and is capable of stimulating them
When locally concentrated (45). Secondly, Con A has been shown to alter the migration of cells, in that mouse lymphocytes treated in vitro with Con A preferentially migrate to the spleens of normal syngeneic recipients (46). However, differential migration of cells would not appear to explain the effect of Con A on this system since the kinetics of appearance of PFC in the peripheral blood after injection of Con A with antigen were altered in a similar manner as the kinetics of appearance of PFC in the spleen. Also, PFC in the mesenteric nodes showed an increase in numbers between days 5 and 8 when Con A was injected simultaneously with AHuIgG. PFC in the mesenteric nodes showed no further increase on day 13, but it has previously been shown that antigen is only retained in the node for a short period of time and cannot be observed in autoradiographs of sections of nodes on day 10, at a time when labeled antigen can be seen in the spleen (4). That PFC do not increase in the mesenteric nodes between days 8 and 13 can therefore be attributed to the lack of antigen in this organ. Thus all lymphoid organs examined—spleen, mesenteric node, and peripheral blood—showed an alteration of the appearance of PFC after injection of Con A with AHuIgG as compared to injection of AHuIgG alone. However, despite the continued increase in antibody-forming cells in these organs, the kinetics of appearance of circulating antibody did not differ in animals injected only with antigen or with Con A and antigen, although antibody levels were markedly enhanced in rabbits injected with Con A. Thus, although PFC levels maintained a constant increase between days 5 and 13 in the spleen and peripheral blood of rabbits injected with Con A and AHuIgG, the levels of circulating antibody declined slightly by day 13, at a rate similar to that in rabbits injected only with AHuIgG, in which PFC on day 8 were negligible. It remains to be determined whether this difference reflects a decrease in the amount of antibody secreted by cells late in the response after injection of Con A with AHuIgG, early production of more avid antibody, or a relatively greater contribution by mesenteric node PFC to the circulating antibody pool. Regardless, it would be difficult to explain the cyclical appearance of PFC on the basis of regulation by circulating antibody since PFC increase during a period when circulating antibody levels are high.

The enhancing effect of irradiation 24 h after injection of AHuIgG could be explained by elimination of suppressor cells, a subpopulation of T cells reported to be relatively radiosensitive (47). Thus, the enhanced levels of PFC could be due to elimination of cells which, in the normal animal, are capable of limiting the immune response. This effect has been observed with many antigens and has previously been considered a result of more space made available in lymphoid organs for division of specifically stimulated cells (16). However, although markedly greater numbers of PFC are observed in the interval between days 5 and 13 in rabbits injected with antigen and irradiated, compared to those only injected with antigen, a definite cyclical response is still apparent after irradiation. Thus, it would be attractive to consider two suppressor populations of cells, one regulating the quantity of antibody produced, the second regulating the turn-on or shutoff of antibody production.

The capacity of splenic lymphocytes to exhibit a cyclical response to AHuIgG is not limited to an intravenous injection, nor is it limited to a primary response.
Intraperitoneal injection of antigen resulted in a cyclical appearance of PFC practically identical to that observed after intravenous injection of antigen. Cycling was also observed in the secondary response to 2 mg AHuIgG when the priming dose was 0.002 mg. Previously, only a single peak of PFC was observed in the secondary response to an intravenous injection of 2 mg AHuIgG in animals primed with 2 mg AHuIgG (4). Since PFC were observed in the primary response after injection of both 0.002 mg and 2 mg AHuIgG, the absence of a cyclical response with the latter dose cannot be attributed to the presence of primed, rather than virgin B cells, unless the effect is due to quantitative differences.

The exquisiteness of the regulation of the immune response by the lymphoid system is illustrated by the regulation of the response in the popliteal lymph node as the result of an injection which stimulates the spleen. After subcutaneous injection of AHuIgG in the hind foot, the PFC response in the draining popliteal node increased linearly throughout a 13-day time period. However, if, in addition to subcutaneous injection, the antigen was also given intravenously, a normal cyclical PFC response was observed in the spleen. Furthermore, the PFC in the node on day 8 were depressed in rabbits injected with antigen subcutaneously and intravenously compared to the response in rabbits receiving only the subcutaneous injection. Recovery of the PFC response in the node was apparent on day 13 in rabbits injected subcutaneously and intravenously. Thus, the suppression of the popliteal node response in rabbits by simultaneous intravenous injection of AHuIgG could be the result of suppressor cells activated in the spleen which then circulate to the node. Since measurable levels of circulating antibody were produced after intravenous, but not subcutaneous injection, this shutoff could also be mediated by circulating antibody produced by PFC stimulated in the spleen and mesenteric nodes as a result of intravenous injection. However, this possibility seems unlikely since, after an intravenous injection of AHuIgG, not only is there no correlation between the amount of antibody formed and the time of appearance of peak PFC, but the time of appearance and disappearance of antibody is the same whether a cyclical pattern of PFC production exists (AHuIgG injection) or does not exist (Con A and AHuIgG injection). Furthermore, circulating antibody levels after injection of Con A with AHuIgG are extraordinarily high during a time when PFC are increasing linearly.

It is tempting to postulate that lymphocytes in the node are being regulated by suppressor T cells activated in the spleen as the result of intravenous injection of AHuIgG. A similar regulatory role of spleen-localizing parental thymocytes on the DNA synthetic response of lymphocytes in the nodes of F₁ recipients has been described by Gershon et al. (48). Although these regulatory T cells were shown to preferentially localize in the spleen, antigenic stimulation may result in changes in the spleen and nodes, allowing these cells to exit from the spleen and migrate to the node. Indeed, antigenic stimulation of the lymph node has been shown to result in expansion of postcapillary venules in the nodes, making them permeable to normally nonmigratory cells (C. Metzler and R. Gershon, personal communication), as has been shown for sites involved in delayed hypersensitivity reactions (49).
The biological role for such precisely controlled regulation is as yet unclear. However, striking examples exist as in the case of patients immunized with Rh 1 by transfusion or pregnancy who have exhibited 7-day periodical variations in anti-Rh 1 production for as long as 40 yr after immunization (50). Whatever the mechanism involved in these regulatory phenomena, the spleen appears to play a major role.

Summary

Modulation of the cyclical response in rabbits to aggregated human gamma globulin (AHuIgG) was investigated in order to study some of the parameters involved in self-regulation of the immune response. Several mitogens (lipopolysaccharide [LPS], phytohemagglutinin [PHA], and concanavalin A [Con A]), when injected simultaneously with antigen, have been shown to modulate the normal splenic plaque-forming cell (PFC) response in rabbits to a single intravenous injection of AHuIgG. This response to AHuIgG has previously been characterized by the initial appearance of PFC in the spleen 3 days later, with a peak of PFC at 5 days after injection. The number of PFC in the spleen then decreases and remains at a low level until a second increase begins on day 10, peaking on day 13. The 8-day cycle between peak PFC repeats, with a third peak appearing on day 21. In the present studies, injection of LPS with AHuIgG was shown to affect the PFC response by enhancing only the initial peak of PFC, PHA was shown to enhance both the initial and secondary peaks of PFC, while injection of Con A with AHuIgG resulted in a prolonged increase in PFC with no apparent cycling. Irradiation 24 h after injection of antigen resulted in PFC kinetics similar to those observed with PHA, although the increase in PFC was more marked with irradiation. Thus, although LPS, PHA, Con A, and irradiation markedly affected the immune response to AHuIgG, Con A was the only substance which altered the cyclical appearance of PFC to HuIgG. The cyclical nature of the PFC kinetics was shown to occur with either intravenous or intraperitoneal injection of antigen and in both primary and secondary responses, provided that the rabbits were primed with a low dose of antigen.

Data were obtained that suggest that the response in distal lymph nodes may be regulated by immunological events occurring in the spleen. Cycling of PFC was not observed in the draining node after subcutaneous injection of AHuIgG in the hind foot. However, if the antigen was also injected intravenously at the same time as the subcutaneous injection, the response in the node became cyclical.

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