

A CYCLICAL APPEARANCE OF ANTIBODY-PRODUCING CELLS
AFTER A SINGLE INJECTION OF SERUM PROTEIN ANTIGEN*

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The role of antibody in the regulation of the immune response has become a major issue only in the past few years, even though it has been known for over half a century that mixing antigen with excess antibody can suppress the antibody response (1). A regulatory role of antibody in the immune response was first suggested by Uhr and Baumann (2) in 1961, based on experiments showing that passive antibody inhibits the immune response. Although it has clearly been demonstrated by numerous workers (cited in reference 1) that passive antibody can interfere with the expression of an immune response, little information has been obtained which demonstrates the role of antibody produced during an antibody response on the control of that same response. The best evidence supporting the latter point is the enhancement of the immune response after removal of circulating antibody (3, 4). Such information strongly suggests that circulating antibody plays an important role in regulation of the immune response once it has been initiated. In the case of antigens which persist in the body tissues, antibody may also regulate the immune response over longer periods of time. In fact, it has been demonstrated that the immune response to lipopolysaccharide from *Escherichia coli* shows a cycling phenomenon which appears to be controlled by the level of circulating antibody (5). Thus, while both normal catabolism of antigen and inhibition by antibody may terminate the immune response to antigens that are readily catabolized, it appears that antibody alone is probably responsible for the regulation of the immune response to antigens such as lipopolysaccharides which are poorly catabolized and persist in tissues for long periods of time.

The present paper shows that injection of the aggregated form of serum protein antigens in rabbits results in a cyclic appearance of antibody-producing cells which appears to be synchronized. Experiments will be presented which both implicate locally produced antibody in regulation and delineate some of the possible cellular events that may be involved.

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Materials and Methods

Animals.—New Zealand White rabbits weighing 2.5–3.0 kg were used in all experiments.

Antigens.—Cohn fraction II from human sera (HGG)¹ was obtained through the courtesy of the American Red Cross National Fractionation Center. The IgG fraction (HuIgG) was isolated by diethylaminoethyl (DEAE)-cellulose chromatography on columns equilibrated with 0.01 M phosphate buffer, pH 8.0. Pooled turkey sera was precipitated (twice) with ammonium sulfate [(NH₄)₂SO₄] at a final concentration of 35% and the IgG fraction (TuIgG) was isolated by filtration on G-200 Sephadex essentially according to the method described by Benedict (6). Both immunoglobulins were heat aggregated at 63°C for 25 min (7).

Radioisotope Studies.—Proteins were trace labeled by the chloramine-T method as modified by McConahey and Dixon (8). Radioactivity was measured in a gamma scintillation counter with a sodium iodide crystal.

X Radiation.—Two opposing 220 kV Picker machines, 160 cm apart, were used to deliver 500 R whole body irradiation (50–55 R/min) to rabbits placed in a leucite container midway between the sources. The machines were operated at 210 kV, 15 mA, with an inherent filter of 0.25 mm Cu and 1 mm Al.

Hemolytic Plaque Assay.—Spleens and mesenteric lymph nodes were removed from rabbits immediately after exsanguination, trimmed of fat, and teased apart in balanced salt solution (BSS) buffered to pH 7.0 with ethylenediaminetetraacetic acid (EDTA) at a final concentration of 0.02 M. Spleen fragments were teased through 90-mesh wire screens (Newark Wire Cloth Co., Newark, N. J.) and filtered through nylon monofilament cloth (Tobler, Ernst, Traber, Inc., New York). Cells were washed one time in EDTA-BSS and three times in BSS (all centrifugations at 250 g for 10 min at 4°C) before suspending to a concentration of $2-4 \times 10^7$ cells/ml.

Blood was collected sterily by cardiac puncture into tubes containing EDTA, penicillin, and streptomycin at final concentrations of 0.02 M, 100 U/ml and 100 µg/ml, respectively. The blood was mixed in a graduated cylinder with an equal volume of 3% gelatin (Matheson, Coleman and Bell, Los Angeles, Calif.) dissolved in EDTA-BSS. The cells were allowed to settle 30 min at 37°C, and the supernate was aspirated as closely as possible to the red blood cell interface. The supernate was centrifuged at 250 g for 10 min at 37°C, washed one time in EDTA-BSS also at 37°C, and treated with 10 ml 0.83% ammonium chloride for 5 min at room temperature (to lyse contaminating red blood cells) before centrifugation at 4°C. Cells were washed three times in BSS and suspended to a concentration of $2-4 \times 10^7$ cells/ml. All procedures for isolating cells from blood were carried out with siliconized glassware.

Antibody-forming cells were measured by a modification (9) of the Jerne plaque assay (10). Goat red blood cells (GRBC) (Colorado Serum Co., Denver, Colo.) were used as indicator cells. GRBC-adsorbed HGG or GRBC-adsorbed turkey gamma globulin, prepared by 2× precipitation of turkey sera with 35% (NH₄)₂SO₄, were covalently bound to the GRBC with 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide HCl (Ott Chemical Co., Muskegon, Mich.). Conjugated GRBC and appropriate dilutions of either spleen, lymph node, or blood cells were added to 0.5 ml of 0.5% agarose (L'Industrie Biologique Francaise, Gennevilliers, France) at 45°C, and the suspension was immediately plated on microscope slides which were then incubated in a humidifying chamber for 1 h at 37°C. Plaque-forming cells (PFC) were developed by inverting the slides for 2½ h in complement (1:10 dilution of guinea pig sera in BSS), either with amplifying antibody (sheep anti-rabbit IgG at an optimal dilution of 1:100) (indirect PFC), or without amplification (direct PFC). For differentiation of IgM- and IgG-PFC, slides were first immersed in 0.005 M dithiothreitol (DTT) (Calbiochem, San Diego,

¹ Abbreviations used in this paper: BSS, balanced salt solution; DTT, dithiothreitol; GRBC, goat red blood cells; HGG, Cohn fraction II from human sera; IA, iodoacetamide; PFC, plaque-forming cells.

Calif.) diluted in pH 8.6 Tris-NaCl buffer (0.15 M), followed by immersion in 0.011 M iodoacetamide (IA) (three times recrystallized) (K & K Laboratories, Plainview, N. Y.) diluted in pH 7.3 Tris-NaCl, and three washes in BSS at 4°C (11).

Hyperimmune Cells.—Hyperimmune cells were obtained by giving rabbits five daily subcutaneous injections of antigen (8 mg soluble HGG and 2 mg aggregated HuIgG). After 2 wk, the rabbits were given a second series of injections of antigen (13 mg soluble HGG and 2 mg aggregated HuIgG) subcutaneously daily for 4 days, with 30 mg/kg body weight given intravenously on the 5th day. Additional courses of 15 mg antigen were given to some animals 1 mo or more after the last course. Animals were bled 7 days after the last injection and individual sera were tested for precipitating antibody. The average antibody titer of donors used in each experiment ranged from 170 to 850 μ g antibody N/ml. Cells were used in transfer experiments 3–7 wk after the last injection. Spleen cells and mesenteric lymph nodes were removed immediately after exsanguination, trimmed of fat, and teased apart in Auto-Pow minimal essential media (Flow Laboratories, Inglewood, Calif.) containing 1.7 mg sodium bicarbonate, 100 μ g streptomycin, and 100 U penicillin per ml. Cell suspensions were filtered as described previously, centrifuged at 250 g, and resuspended to the desired concentration.

Measurement of [¹²⁵I]HuIgG in Tissues.—HuIgG was radiolabeled with ¹²⁵I and aggregated, and 2 mg (52 μ Ci/mg) was injected intravenously into rabbits. The animals were exsanguinated 2 or 10 days later, and sections of various tissues were immediately removed and dissected free of gross fat and blood clots. Thin sections were prepared for autoradiography and processed with NTB-2 Nuclear Track Emulsion (Eastman Kodak, Rochester, N. Y.). Slides were developed after 14 days and stained with hematoxylin. The remaining tissue sections were weighed, teased apart in saline, and counted in a gamma scintillation counter with a NaI crystal. Tissues were then sonicated in a 250 W, 10 kc cycle Raytheon Sonic Oscillator and counted for ¹²⁵I activity before and after precipitation with an equal volume of 20% trichloroacetic acid (TCA). All procedures up to TCA precipitation were carried out at 4°C. The TCA precipitates were centrifuged at 1100 g for 15 min and washed three times in 10% TCA before counting.

Antibody Analyses.—The levels of precipitating antibody to HuIgG were measured by a quantitative precipitation technique (12) and calculated on the basis of a ratio of antibody to antigen nitrogen of 2.5.

RESULTS

Cyclical Appearance of PFC after a Single Injection of Antigen.—The splenic PFC response to a single intravenous injection of 2 mg aggregated HuIgG is shown in Fig. 1, which gives the average PFC from spleens of 5–20 rabbits on each day. PFC were first detected 3 days after injection and reached a peak on day 5. The number of PFC then decreased rapidly, with less than 20% of the peak number remaining on day 6. The number of PFC remained at a low level until day 12, at which time there was a significant increase. A second peak of PFC occurred on day 13, 8 days after the first peak, and was comparable to the level of the day 5 peak. 8 days later (21 days after injection) a third peak of PFC was detected, although not as high as the preceding peaks. Both direct and indirect PFC followed this pattern.

A similar type of response was seen in the spleen after a single intravenous injection of 2 mg aggregated TuIgG (Fig. 2). Indirect PFC reached a peak 4½ days after injection, decreased, and peaked a second time on day 12. In this case, the interval between peaks was again approximately 8 days. Direct PFC

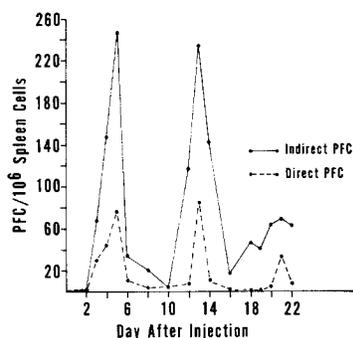


FIG. 1. Kinetics of the appearance of PFC in spleens of rabbits after a single intravenous injection of 2 mg aggregated HuIgG.

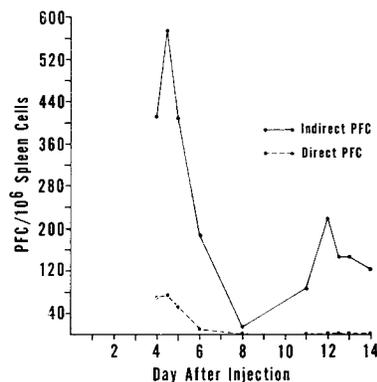


FIG. 2. Kinetics of the appearance of PFC in spleens of rabbits after a single intravenous injection of 2 mg aggregated TuIgG.

were only 13% of the indirect PFC on day 4½ and were not detected on day 12, possibly because the plaque technique was not sensitive enough to detect the small number that would have been expected. On day 12, the indirect PFC were only 40% of the initial peak. The rapid disappearance of PFC after the day 5 peak was specific since the response to an injection of 2 mg of TuIgG was not affected by an injection of 2 mg of HuIgG given 4½ days previously.

Correlation of Direct and Indirect PFC with 19S and 7S Antibody-Producing Cells.—Reduction and alkylation of antibody produced by PFC (with DTT and IA, respectively) before addition of complement was used to determine the class of antibody to HuIgG secreted from spleen cells of rabbits injected with 2 mg of aggregated HuIgG. Direct PFC were inhibited at least 95% in both primary (day 5) and secondary responses (day 6), whereas indirect PFC were inhibited only 7% (Table I). Therefore, the sheep antirabbit IgG used as amplifying antibody would appear to be capable of both enhancing IgG PFC

and inhibiting IgM PFC. Treatment with IA alone had no significant effect on PFC.

Secondary PFC Response.—The splenic PFC response in primed rabbits (injected with 2 mg aggregated HuIgG 40–85 days before the second injection) represented a typical secondary response (Fig. 3). On day 2, the 1st day tested, there were already significant numbers of PFC, whereas PFC could not be detected until day 3 of the primary response. The peak PFC occurred on day 3 instead of day 5 (as shown in an experiment where indirect PFC in the peripheral blood on days 2, 3, and 4 after a second injection of antigen averaged 249, 3,923, and 452 PFC/10⁶ cells, respectively, whereas the primary response

TABLE I
*Effect of Reduction and Alkylation on PFC to HuIgG**

Treatment	Primary response		Secondary response	
	Direct	Indirect	Direct	Indirect
None	26	301	546	3083
DTT + IA	0 (100%)‡	298 (0%)	30 (95%)	2862 (7%)
IA	22 (14%)	303 (0%)	561 (0%)	3054 (0%)

DTT = dithiothreitol; IA = iodoacetamide.

* Per 10⁶ spleen cells.

‡ The figures in parentheses represent the percent reduction in PFC of treated cells as compared with cells receiving no treatment.

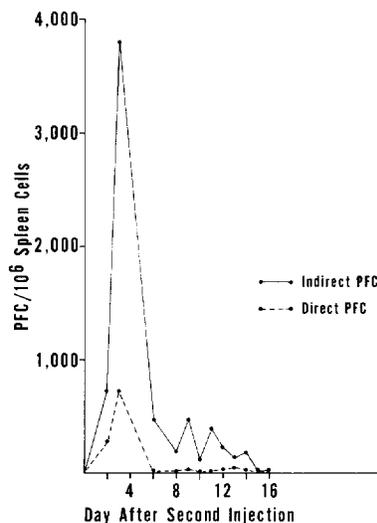


FIG. 3. Kinetics of the appearance of PFC in spleens of rabbits after a second injection of 2 mg aggregated HuIgG. All rabbits were injected intravenously with a primary dose of 2 mg aggregated HuIgG 40–85 days before the second injection.

occurred on day 5 in both the spleen and blood). Also, the magnitude of the peak secondary response was approximately 10-fold greater than that of the primary response. No additional significant peaks of direct or indirect PFC were detected even though PFC were measured on day 6 and daily from days 8 to 16. Again, both indirect and direct PFC followed the same kinetics, although direct PFC were less than 20% of the indirect PFC. As each point represents the average PFC from only three rabbits, the minor variations in numbers of PFC that occurred were probably the result of animal variability.

Comparison of Spleen and Mesenteric Nodes.—Indirect PFC in the mesenteric lymph nodes were measured on days 5, 8, and 13 after a single intravenous injection of 2 mg aggregated HuIgG and compared with indirect PFC in the spleens of the same rabbits (Table II). The results, representing the average PFC per 10^6 node or spleen cells of five rabbits for each day, show that PFC per 10^6 cells in the nodes were approximately 50% of those in the spleen on

TABLE II
Appearance of PFC in Spleens and Mesenteric Lymph Nodes of Rabbits after a Single Intravenous Injection of 2 mg Aggregated HuIgG

Organ	Indirect PFC/ 10^6 Cells*		
	Day 5	Day 8	Day 13
Spleen	160.8	4.1	119.1
Nodes	75.5	9.0	11.5

*Values represent average of five rabbits for each day.

day 5. By day 8, PFC in the nodes had decreased almost to background levels and, in contrast to splenic PFC, did not peak again on day 13.

Tissue Localization of HuIgG.—Rabbits were injected intravenously with 2 mg aggregated ^{125}I HuIgG and exsanguinated on either day 2 or day 10. Lung, kidney, liver, spleen, mesenteric lymph node, and thymus (day 10 only) sections were taken for histological examination and for radioactivity measurements. Less than 5% of the injected radioactivity could be detected in the blood within 10 min after injection of the antigen. 2 days after injection, counts of sonicated tissues showed that lungs retained the greatest amount of TCA-precipitable radioactivity per gram of tissue ($9.8 \mu\text{g } ^{125}\text{I}$ HuIgG/g tissue) and spleens retained the next highest amount ($1.14 \mu\text{g } ^{125}\text{I}$ HuIgG/g tissue) (Table III). By day 10, the amount of antigen retained had decreased by approximately 90% in all tissues (giving a half-life of approximately 2.5 days) except the spleen. In contrast, the half-life of ^{125}I HuIgG in the spleen was 12.8 days. Autoradiographs of spleen and lymph node sections on day 2 showed diffuse label throughout the follicles, whereas by day 10, label was concentrated in the germinal centers of the spleen, and no label could be detected in similar areas of the nodes (Fig. 4).

Persistence of Immunogenicity of Injected HuIgG.—Irradiated rabbits injected with 2 mg aggregated HuIgG 24 h after irradiation (500 R) were injected with $1.8\text{--}2.1 \times 10^9$ sensitized cells at various times after receiving the antigen. Cell suspensions were prepared from spleens and mesenteric lymph nodes of hyperimmune animals and injected (without antigen) in approximately equal proportions, spleen cells intravenously and node cells intravenously and intraperitoneally. Spleens of the recipients were plaqued $3\frac{1}{2}$ days after injection of hyperimmune cells. The number of indirect PFC was calculated as a percent

TABLE III
Organ Distribution of Aggregated [125 I]HuIgG (2 mg) in Rabbits after Intravenous Injection on Day 0

Organ	$\mu\text{g}[^{125}\text{I}]\text{-HuIgG/g tissue}$				$t_{1/2}$ (days)†
	Day 2		Day 10		
	Total	TCA-ppt*	Total	TCA-ppt	
Lung	10.62	9.8	0.91	0.86	2.3
Kidney	0.63	0.26	0.03	0.02	2.5
Liver	0.25	0.15	0.02	0.02	2.7
Spleen	1.36	1.14	0.79	0.74	12.8
Mesenteric lymph nodes	0.17	0.09	0.01	0.01	2.5
Thymus	—	—	0.01	0.01	—

Values represent averages of three animals for each day.

* Trichloroacetic acid precipitable.

† The $t_{1/2}$ value was calculated between day 2 and day 10 and was based on TCA-precipitable activity.

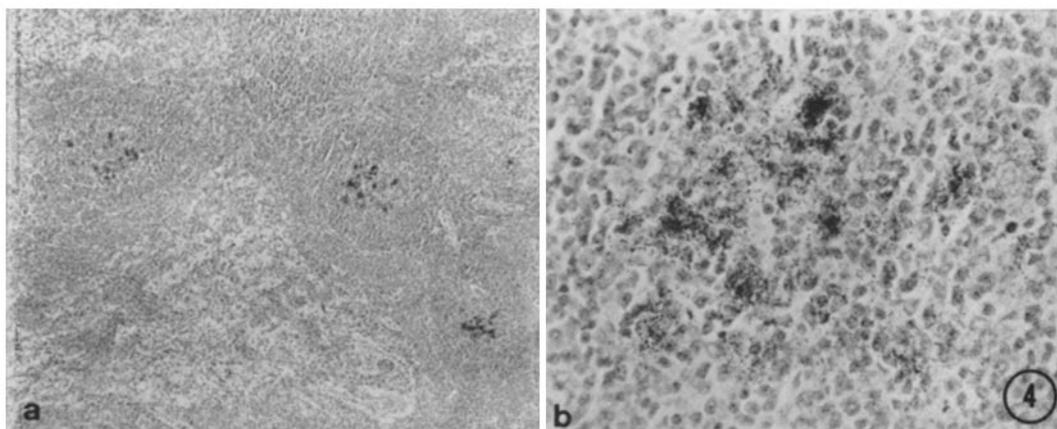


FIG. 4. Autoradiographic evidence of follicular localization of [125 I]HuIgG in the spleens of rabbits stained with hematoxylin. (a) $\times 50$; (b) $\times 320$.

of indirect PFC in irradiated recipients receiving 2 mg aggregated HuIgG at the time of injection of sensitized cells. Rabbits receiving antigen 24 h after irradiation and not receiving sensitized cells showed essentially no PFC when plaqued at the same time. Based on the above criteria, 52% of the injected aggregated HuIgG remained in an immunogenic form in rabbits injected with antigen 10 days earlier (Fig. 5). By days 20 and 30, the effective concentration decreased to 12% and 3%, respectively. At days 60 and 90, essentially no "immunogenicity" remained.

Effect of Dose of Antigen on Kinetics of PFC.—Rabbits were injected with 0.02, 0.2, 2.0, or 20 mg aggregated HuIgG intravenously and spleens from each group were plaqued 4, 5, 6, 8, 12, 13, or 14 days after injection. With each dose of antigen, two peaks of PFC were detected (Fig. 6). In this single experiment,

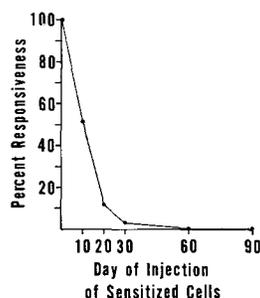


FIG. 5. Persistence of HuIgG in irradiated rabbits as measured by its *in vivo* ability to stimulate sensitized donor cells. Recipient rabbits received 500 R whole-body irradiation 24 h before an intravenous injection of 2 mg aggregated HuIgG. For all days except day 10, an additional 500 R was given 24 h before injections of sensitized cells on the days indicated. Splenic PFC were assayed 4½ days after injection of sensitized cells and expressed as a percentage of the response of irradiated rabbits injected with both sensitized cells and 2 mg aggregated HuIgG.

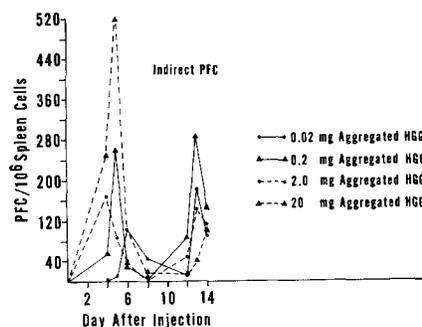


FIG. 6. Kinetics of the appearance of indirect PFC in spleens of rabbits after a single intravenous injection of various doses of aggregated HuIgG.

the PFC to 2.0 mg aggregated HuIgG peaked on day 4 and the PFC to 0.02 mg aggregated HuIgG peaked day 6, while the PFC in rabbits injected with the other two doses peaked at the usual time, on day 5. Also, animals injected with 20 mg aggregated HuIgG showed a second peak later than day 13. The variability in time of appearance of peak PFC may reflect a true variability in the kinetic pattern among different rabbits. More likely, the variability is the result of the small number of animals used and the quantitative differences in PFC among different rabbits. In any event, with all doses, two peaks of PFC could be detected and were separated by approximately 8 days of markedly decreased responsiveness. The height of the second peak obtained with the various doses was inversely proportional to the height of the first peak and to the dose of antigen injected.

Kinetics of PFC in Spleen vs. Peripheral Blood.—The kinetics of the PFC response in the peripheral blood were essentially identical with that in the spleen as can be seen in Fig. 7, which shows the average PFC responses of four rabbits

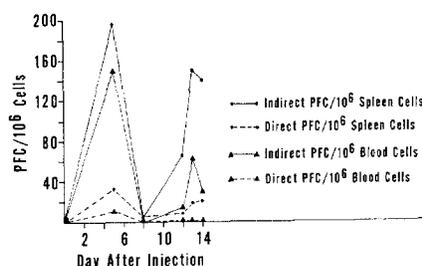


FIG. 7. Kinetics of the appearance of PFC in spleens and peripheral blood of rabbits after a single intravenous injection of 0.2 mg aggregated HuIgG.

after injection of a single dose of 0.2 mg aggregated HuIgG. If there is a lag between appearance of PFC in the spleen and in the blood, it is less than 24 h as can be seen by comparing the PFC responses on days 12, 13, and 14.

Appearance of Precipitating Antibody in Relation to Appearance of PFC.—Spleens of rabbits were plaqued after intravenous injection of 2 mg aggregated HuIgG and sera from these rabbits analyzed for precipitating antibody. Precipitating antibody was too low to be detected until day 5 (the peak day for PFC) but rose to a peak on day 8 (Fig. 8). The antibody level decreased rapidly until after the second peak, when it rose again. When individual rabbits were analyzed (on days 5, 8, 13, 16, and 21 after injection of antigen) for PFC in their peripheral blood and precipitating antibody in their sera, the dependence of antibody on PFC can be seen clearly (Fig. 9). After the first peak of PFC on day 5 for rabbit 1, antibody peaked on day 8, then started to decrease until the second peak of PFC appeared on day 13. The serum antibody level then remained at a high level. In rabbit 2, no second peak of PFC occurred and antibody levels in the serum showed a continued decrease after peaking on day 8.

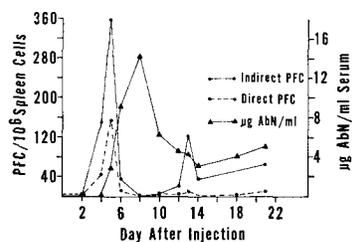


FIG. 8. Relationship between PFC in spleens of rabbits and precipitating antibody in peripheral blood after a single injection of 2 mg aggregated HuIgG.

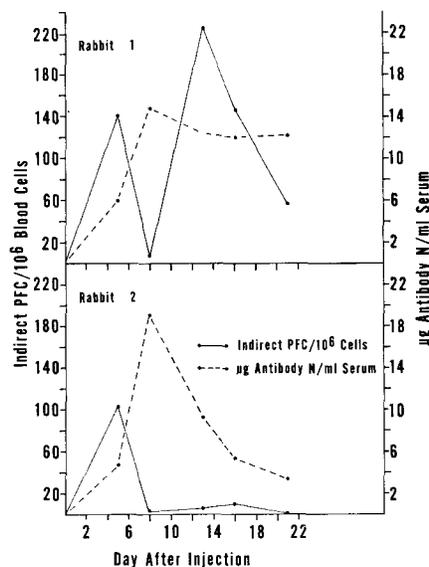


FIG. 9. Relationship between PFC and precipitating antibody in peripheral blood after a single intravenous injection of 2 mg aggregated HuIgG. The data are from representative rabbits which were bled 25 ml of blood (cardiac) on the respective days.

DISCUSSION

The above data suggest that a specific control mechanism regulates the formation of antibody-producing cells to a persisting antigen, resulting in the cyclical production of PFC. PFC in the spleens of rabbits were shown to appear, disappear, and reappear after a single intravenous injection of either aggregated HuIgG or aggregated TuIgG. After an initial peak of PFC on day 5, two subsequent peaks of PFC were observed at 8-day intervals, with a marked decrease in the number of PFC detected during these intervals. The levels of precipitating antibody in the serum reflected events occurring at the single cell level with a delay of approximately 3 days, accounting for the period of time needed for

secretion and equilibration of antibody into the fluid spaces. Such fluctuations in PFC levels after a single injection of antigen have been described previously. Britton and Möller (5) showed cyclical peaks of PFC in mice injected intraperitoneally with heat-killed *Escherichia coli*. Sell et al. (13) demonstrated two peaks of IgA-, $\gamma 1$ -, $\gamma 2a$ -, and $\gamma 2b$ -producing PFC in the spleens of mice after a single intravenous injection of SRBC. However, only a single peak of IgM-producing PFC was detected.

The identical kinetics of IgM and IgG synthesis in rabbits immunized with aggregated HuIgG indicates that if a switch from IgM to IgG production occurs, it takes place at a stage in differentiation before the secretion of immunoglobulins. Evidence for a switch has been observed by several groups of workers. Lymphocytes in rabbits have been shown to have cell surface IgM² while synthesizing IgG, suggesting that after triggering of the cell by antigen there is a point when the cell switches from the synthesis of IgM to the synthesis of IgG (14). In the rabbit, this switch may explain why, for many antigens, the dominant molecular class for B-cell receptors is IgM, while in the plasma cell and serum IgG predominates (15). Pierce et al. (16) provided further evidence of a switch mechanism by inhibiting in vitro the IgG and IgA responses to SRBC with incorporation of anti- μ -chain into the cultures. Furthermore, the first appearance of IgG-PFC to SRBC in spleens of recipient mice injected with SRBC and normal lymphocytes was associated with clones of cells previously producing IgM (17-19). Other data are also available which indirectly support either an in vivo or in vitro switch in immunoglobulin class during differentiation (20-23). Direct evidence for double producers of IgG and IgM has been reported by Nossal and coworkers (24, 25), who were able to isolate cells containing both IgM and IgG. However, since only a few (1.5%) double producers were detected, it has been suggested that the majority of IgG-synthesizing cells could not have arisen from IgM producers (26-28).

An interesting aspect of the present study is that the appearance of PFC after a single intravenous injection of the antigen seems to be synchronous. Although it appears most likely that the apparent "synchrony" can be attributed at least indirectly to antibody, the precise mechanism is difficult to understand. It has often been demonstrated that passive antibody can interfere with the development of an immune response (cited in reference 1). Britton and Möller (5) have suggested that antibody controls fluctuations in the immune response to persisting antigens. In their experiments, a cyclical appearance of both antibody and PFC occurred after injection of the lipopolysaccharide of *Escherichia coli*, although synchrony was not

² Monomeric IgM has been isolated and identified as the predominant immunoglobulin on the lymphocytes of mice (Vitetta, E. S., S. Baur, and J. W. Uhr. 1971. Cell surface immunoglobulin. II. Isolation and characterization of immunoglobulin from mouse splenic lymphocytes. *J. Exp. Med.* **134**:242; Kennel, S. J., and R. A. Lerner. 1973. Isolation and characterization of plasma membrane associated immunoglobulin from cultured human diploid lymphocytes. *J. Mol. Biol.* **76**:485).

apparent. It has been proposed that the regulation of antibody production to both persisting and readily catabolizable antigens is controlled by antibody via a dynamic equilibrium among circulating antibody, antigen, and antigen-antibody complexes throughout the extracellular compartments (29). It would be difficult to explain the synchrony observed in the present experiments by such a mechanism since the interval between the peaks was not affected by the serum level of the antibody either produced or present at the time of appearance of the second PFC peak. However, the precise timing of peaks of PFC could be explained in part by antibody control if the regulating antibody was effective locally at the sites of production. The local concentration of antibody, in any given foci, would be the same in all animals, and it would be independent of the total amount in either the extra- or intravascular compartments. The latter possibility would explain the rapid decrease in PFC numbers which occurs regardless of the total antibody present in the serum. In addition, another event must occur to account for both the reappearance of PFC at a precise time and the longer induction period. Such synchrony could be explained by control mechanisms that have been described in other systems. The available data suggest that the regulation of cell renewal can be achieved by extending or contracting the length of the G_1 period (30, 31), and it appears that such regulation is tissue specific. In addition, cells from a variety of organs can be stimulated to begin dividing again after a period in which DNA synthesis had been arrested. From incorporation studies of cells blocked at different phases of the mitotic cycle, it has been determined that cells can enter mitosis upon an appropriate stimulus from G_1 , or less likely, directly from G_2 (32). Furthermore, a latent period has been observed when cells can not respond to a stimulus by dividing (33). In spleens of rabbits producing PFC to aggregated HuIgG, inactivation of antigen by antibody produced locally could result in cells being arrested in some phase of the cell cycle. Only cells developed beyond a certain point would continue to differentiate to PFC, while cells developed to a lesser degree would be blocked temporarily from further division and differentiation. Upon termination of this latent period, cells would again be stimulated in the spleen by antigen uncovered when antibody diffused into the circulation. Thus, due to a transient curtailment of antigenic stimulation, cells that had not completed enough differentiation to become PFC without further antigenic stimulation may be arrested temporarily. When they are again capable of responding, antigen would be available in the spleen to restimulate them and synchronization of PFC could occur. In any event, it appears likely that the control of the latent periods and the synchrony of the appearance of PFC in the present system are exercised through some event(s) in the G_1 period. In this case, the effect on G_1 is probably the result of an indirect effect of antibody present at or near the site of its production.

The above reasoning would imply that although the initial peak of PFC results from the stimulation of virgin precursor cells, succeeding peaks must be the result of stimulation of memory cells. This same reasoning makes it difficult to imagine a mechanism for stimulation of virgin precursor cells over different periods of time that would result in such precise timing of peak PFC over a 1,000-fold range in the dose of antigen administered. Nor is it easy to visualize a mechanism that would result in the synchronized reappearance of

PFC arising directly from virgin B cells. Also, although the magnitude of the first peak of PFC varied directly with the dose of antigen administered, the second peak was inversely related to dose, suggesting that with large amounts of antigen, more cells were stimulated directly to PFC, leaving fewer memory cells available for restimulation. In contrast, with small amounts of antigen, cells were stimulated to differentiate, yet with most cells not enough hits per cell occurred to complete the pathway from precursor to PFC. This would explain why, after injection of extremely small amounts of antigen (0.02 mg), the second peak of PFC was greater than the first peak.

The cyclic appearance of peaks of PFC in the spleens of rabbits after an intravenous injection of aggregated HuIgG apparently depends on the persistence of antigen in this organ. The preferential persistence of this antigen in the spleen was demonstrated both functionally and directly by determining organ distribution of TCA-precipitable [125 I]HuIgG and by autoradiography. First, it was shown that sensitized cells could be specifically stimulated in irradiated recipients injected with aggregated HuIgG at least 30 days before transfer of the cells. Using similar cell transfer procedures, Britton et al. (34) have shown the persistence of SRBC for at least 14 days and *Escherichia coli* lipopolysaccharide for at least 45 days. Second, the injection of [125 I]HuIgG resulted in localization of some antigen in most of the tissues tested; however, the major localization was in the lung and spleen as would be expected after an intravenous injection. The labeled HuIgG was rapidly catabolized in the lungs and other tissues, but persisted in the spleen with a half-life of approximately 13 days. Autoradiography showed dense localization of the [125 I]HuIgG in the germinal centers of the spleen 10 days after injection, while no such localization was observed at this time in the lymph nodes. The failure to observe such localization in lymph nodes corresponds with the failure to observe a second peak of PFC in lymph nodes on day 13. Localization of antigen after various routes of injections has been extensively studied and was reviewed by Nossal and coworkers (35, 36). In the present studies, dense deposits of [125 I]HuIgG were localized in the central areas of germinal centers of spleens, but not in lymph nodes, 10 days after injection. It is not clear whether the dense localization in the germinal centers of the spleen was induced by antibody, as has been demonstrated with soluble antigens (35, 37), or whether after development of germinal centers and organ redistribution of antigen the aggregated antigen localized directly through the Fc fragment. Brown et al. (38) previously demonstrated that aggregated human gamma globulin, but not the monomeric form, rapidly localized in the germinal centers of the lymph nodes in a dendritic pattern after intradermal injection into guinea pigs. The failure of succeeding peaks in the secondary response may be related to the more rapid disappearance of antigen (39).

The PFC to HuIgG which appear in the peripheral blood apparently originate from cells stimulated in the spleen, as was proposed by Sorokin and coworkers (40, 41). The

identical kinetic pattern observed in cells from the blood and spleen are consistent with an exit of cells from the spleen into the blood. Strober (42) has obtained evidence for a qualitative change in the B cells after stimulation which allows them to migrate. He observed that thoracic duct cells from primed donors were capable of responding to antigen in an irradiated recipient after passage through an intermediate host, while similarly passed normal thoracic duct cells were not able to respond, even though capable of responding if not passed. The differences in the ability of the normal and primed passed cells to respond were attributed to a change at the B-cell level, in which the primed B₂ cell became capable of recirculating, while the virgin B₁ cell could not. In the present situation, the ability of the stimulated B₂ cell to enter the circulation may be dependent on the loss of receptor sites during the process of differentiation, thus freeing it from the antigenic stimulus. Depending on the degree of stimulation, the B₂ cell may either differentiate further into a PFC, or regenerate receptors and return to the antigen deposits in the spleen (or other organs) where, after a latent period, it can be stimulated to differentiate further. Retention of specifically sensitized cells from the circulation in lymphoid tissue containing the specific antigen has been shown by Ford (43) and Durkin and Thorbecke (44). Migration of stimulated cells has also been observed by Cannon and Wissler (45), who demonstrated [³H]thymidine-labeled cells in the blood of spleen-shielded, irradiated rats after antigen stimulation. Unfortunately, the specificity of the cell in the blood was not determined. Of course, not all cells stimulated by antigen and developing into B₂ cells leave the spleen, since more PFC are usually found in the spleen than in the peripheral blood. Other factors obviously play a role in whether a B₂ cell leaves the spleen or develops into either plasma or memory cells within the spleen, e.g., logistics of the cell, effectiveness of antigenic stimulus, available space in the spleen, and possibly the inherent ability of each B₂ cell to migrate.

A similar phenomenon has been observed in the cellular responsiveness of rabbits to rabbit thyroglobulin after immunization with aqueous preparations of bovine thyroglobulin (46). A major peak of PFC appeared in the spleen 25 days after a series of injections of bovine thyroglobulin was initiated and was directed mainly against those determinants specific for bovine thyroglobulin. 7-8 days later, PFC in the thyroid gland reached a peak, and these, in contrast to the early splenic PFC, were specific for only those determinants on bovine thyroglobulin shared with rabbit thyroglobulin. Thus, it would appear that rabbit thyroglobulin deposited in the injured gland was able to sequester B₂ cells sensitized to determinants for rabbit thyroglobulin, and after an appropriate latent period possibly regulated by G₁, stimulated the cells to produce antibody to rabbit thyroglobulin.

SUMMARY

After a single intravenous injection of rabbits with aggregated HuIgG, IgM- and IgG-plaque-forming cells (PFC) in both the spleens and peripheral blood of rabbits peaked 5, 13, and 21 days after injection, while almost no PFC could be detected on days 8 and 16. The available data suggest that the secondary peaks of PFC (days 13 and 21) resulted from stimulation of memory

cells by persisting antigen that was localized in the germinal centers in the spleen. No such persistence of antigen occurred in the lymph nodes, and these lymphoid tissues did not exhibit secondary peaks of PFC.

The identical kinetic patterns for IgM- and IgG-PFC indicate that the major portion of IgG-PFC did not result from IgM-secreting cells switching to IgG synthesis and secretion.

The present data suggest that the antibody produced and present at the site of interaction between committed cells and antigen is responsible for the regulation of antibody synthesis to persisting antigens. Possible cellular events involved in both the regulation and an apparent synchronous appearance of antibody producing cells in the spleens of rabbits were presented.

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