Immunologic "Amnesia" of Antibody-Forming Cells after RES Blockade* (34096)

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The immunologic response to a specific antigen may be characterized both by the time of appearance of antibody and the amount produced. In general, upon two or more exposures to an antigen an individual usually responds more rapidly and develops higher levels of serum antibody than during the primary response (1–3). Often such antibody is predominantly 7S IgG, whereas during the primary response much of the detectable antibody is 19S IgM (4–6). Appearance of these different molecular forms of antibody depends, in part, upon the nature of the antigen used initially, the route of inoculation, and the time–dose relationship between immunization and serologic testing. The characteristic anamnestic response after a second exposure to an antigen thus appears to depend upon development of "immunologic memory" during primary stimulation.

Methods have been recently developed for rapid detection and enumeration of antibody-forming cells after either primary or secondary immunization with sheep erythrocytes (7–9). These methods, based on localized hemolysis in semisolid agar containing target erythrocytes and complement, differentiate 7S IgG from 19S IgM antibody-forming cells (8–10).

In previous studies it was observed that blockade of the reticuloendothelial system (RES) with carbon particles markedly suppressed the immune response of mice to a primary immunizing injection of sheep erythrocytes (11, 12). In the present study experiments were performed to determine further whether suppression of the initial immune response to erythrocytes by RES blockade could affect development of "immunologic memory," as determined on both the cellular and humoral level.

Methods and Materials. Animals. Young adult male NIH albino A mice were used. They were maintained as a closed colony by a local breeder and were generally 6–8 weeks of age at the start of an experiment (11–13).

Immunization and serology. For challenge immunization, individual mice were inoculated intraperitoneally (ip) with 0.2 ml of a 10% suspension of washed sheep red blood cells (RBC). Blood samples were obtained prior to and at various time intervals after immunization of individual mice by retro-orbital venous puncture. Serum hemagglutinins and hemolysins were determined by the microtiter procedure, using 0.025 ml of physiological saline solution and twofold serial dilutions. Samples of sera were also treated with an equal quantity of 0.2 *M* 2-mercaptoethenol (2-ME) prior to titration to distinguish between 7S IgG and 19S IgM hemolysins. Selected serum specimens obtained late in the primary and during the secondary response were analyzed by sucrose gradient centrifugation to differentiate the two molecular forms of antibody. Immunoelectrophoresis with specific antiserum was performed also to confirm the presence of either IgG or IgM globulins in the serum fractions.

RES blockade. Experimental mice were inoculated ip with 10 mg Pelikan carbon, as

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described previously (11, 12), in order to suppress phagocytic activity of macrophages (13–16). The charcoal suspension was designated C11-1143a and was manufactured by Gunther-Wagner, Hanover, Germany. Control mice were inoculated with Hanks' balanced salt solution only.

Determination of high- and low-efficiency antibody-forming cells. The number of high-efficiency antibody plaque-forming cells (PFC), considered to be producing 19S IgM hemolysins, was determined in spleens of control mice by the direct localized antibody plaque procedure in agar gel essentially as described previously (7, 13). Spleens obtained at autopsy were "teased" individually into a small amount of cold Hanks' solution. Resulting cell suspensions were washed several times and the number of nucleated cells per milliliter determined with a hemocytometer. Cell viability was estimated by trypan blue stain technique. The number of direct PFC's was determined for 0.1-ml aliquots of each cell suspension, using 0.7% Noble agar containing DEAE-dextran and target sheep erythrocytes. The plates were treated once with guinea pig complement for 1 hr at 37°. Clear zones of hemolysis which appeared on these "direct" plates were considered to be due to individual 19S hemolysin-producing cells (7, 10). The number of PFC per plate was counted and used to calculate the number of PFC per million spleen cells tested and per whole spleen.

The numbers of low-efficiency PFC, presumably due to 7S IgG hemolysins, were determined by an indirect antibody-plaque technique whereby duplicate samples of the cell suspensions were plated in agar exactly as for the direct procedure. The plates were then treated with hyperimmune rabbit polyvalent anti-mouse gamma globulin serum, diluted 1:50 (8). After further incubation with guinea pig complement, additional plaques often appeared on the plates and could be correlated with appearance of 7S IgG antibody (8).

Experimental design. Development of immunologic "memory," both on the serum and cellular level, was determined in groups of mice which were either untreated or had been previously injected with carbon. The numbers of 19S and 7S PFC, as well as the level of 2-ME–sensitive or resistant serum antibody, were determined after a second injection of sheep RBC. Representative mice from each of the groups were also tested for PFC after the initial injection of red blood cells only or red cells and carbon.

Results. Large numbers of antibody-forming cells appeared in the spleens of control mice after a single inoculation of sheep RBC (Fig. 1, Table I). A peak of 40,000 to 80,000 PFC per spleen generally appeared about Day 4 after immunization. There was a rapid decline thereafter, with a low level of approximately 1000 PFC per spleen by Day 10 to 17 after immunization. The first 7S PFC appeared in spleens of control mice by Day 5 after RBC injection, and generally reached a peak level by Day 8 to 10. At this time usually two-thirds or more of the PFC in spleens of the normal immunized mice could be detected only by the indirect procedure with anti-globulin serum (Fig. 1).

When mice were treated with 10 mg of colloidal carbon at the same time or 1–3 days before initial immunization, there was a marked suppression of appearance of both 19S and 7S PFC throughout an observation period of 30 days. Although the peak number of 19S PFC appeared in spleens of carbon-treated mice Day 4 after immunization, the total number was only about 5–10% of that observed in spleens of control animals (Fig. 1, Table I). There were few if any detectable 7S PFC in spleens of carbon treated mice during the first 2 weeks after immunization.

Serum antibody titers were also depressed in carbon-treated animals, as compared to controls (Table I). Both hemagglutinins and hemolysins appeared in the sera of control mice by Day 3 to 4 after immunization, reached a peak by Day 7 to 10, and remained either constant or declined during the next 2–3 weeks. Most of the antibody activity was susceptible to 2-ME treatment during the first week after immunization. The serum antibody then became resistant to 2-ME and sedimented slowly during sucrose gradient centrifugation. Nearly all of the carbon-treated mice had a delayed appearance of

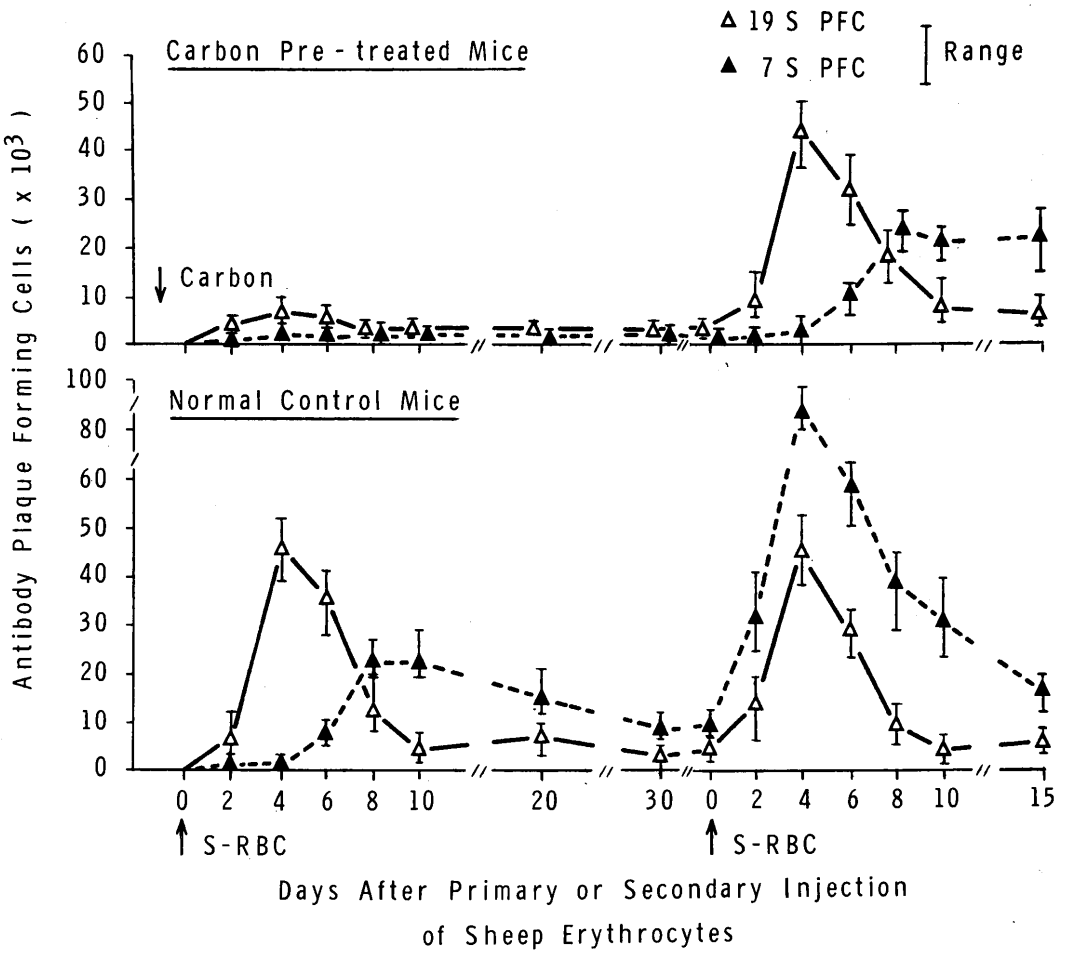

FIG. 1. Comparison of the cytokinetics of appearance of antibody plaque-forming cells, both 19S and 7S, in spleens of normal control mice immunized with sheep erythrocytes and experimental mice pretreated with 10 mg of carbon 1 day prior to initial immunization (first arrow). All mice immunized with sheep erythrocytes on Day 0 and Day 35 (arrows). Each point represents the average antibody-plaque count of spleens from four to six mice.

serum antibody. Titers in all treated animals were usually lower than that observed in controls. Most of the antibody detected in carbon-treated mice was susceptible to 2-ME inactivation throughout the observation period of a month.

When control mice were injected with erythrocytes for a second time 1 month after primary immunization, there was a vigorous anamnestic response, both on the cellular and humoral level (Fig. 1). Large numbers of both 19S and 7S PFC appeared in the spleens of the control mice by Day 3 to 4 after RBC challenge. There were nearly twice times as many 7S as 19S PFC. In contrast, very few 7S PFC appeared until about Day 5 to 6 after secondary immunization of mice treated prior to the initial injection of red cells with carbon. The number of 7S PFC remained relatively low as compared to the control group of mice not previously treated with carbon (Fig. 1).

Control mice responded to the second injection of red blood cells with relatively high hemolysin and hemagglutinin titers, most of which was resistant to 2-ME treatment (Table I), and sedimented as 7S globulins. Antibody to sheep red blood cells in the sera of

TABLE I. Effect of Carbon Injection on Appearance of Circulating Antibody and 19S and 7S Antibody Plaque-Forming Cells in Spleens of Mice Receiving a Primary and Secondary Immunization with Sheep Erythrocytes.

Mouse treatment prior to first immunization[a]	Primary immunization[b]				Secondary immunization[b]			
	PFC/spleen[a]		Serum titers[d]		PFC/spleen		Serum titers[d]	
	19S	19S & 7S	No ME	With ME	19S	19S & 7S	No ME	With ME
Sheep RBC-immunized								
No carbon	60,633	60,670	1:181	1:13	58,450	98,465	1:642	1:518
Carbon-injected[c]								
Same day	11,740	11,865	1:26	1:4	48,465	49,950	1:212	1:8
24 hr before	4,250	4,340	1:8	1:4	68,730	69,105	1:129	1:4
48 hr before	4,790	4,815	1:8	1:4	62,480	62,650	1:210	1:4
96 hr before	5,876	5,869	1:12	1:4	57,730	56,950	1:263	1:8
35 days before	61,384	61,565	1:190	1:11				

[a] Average results of at least four mice per group on Day 4 after immunization.

[b] Immunized mice injected ip with 2×10^8 sheep erythrocytes for primary inoculation and a similar dose 35 days later for secondary immunization.

[c] Carbon-treated mice treated ip with 10.0 mg Pelikan carbon at time interval indicated relative to primary immunization with sheep erythrocytes.

[d] Mean hemagglutinin titers 4 days after primary or secondary immunization: titration without 2-ME treatment of sera reflects total antibody activity; titer after 2-ME treatment reflects mainly 7S IgG antibody activity.

carbon-treated mice first appeared about Day 4 after second immunization, and reached titers similar to that of controls only by Day 7 to 8. Most of the antibody activity in carbon-pretreated mice during the first week after secondary immunization was sensitive to 2-ME treatment. The mercaptoethenol-resistant antibody did not appear until the second week.

Discussion. The results presented here indicate that mice injected for a second time with sheep RBC, but treated with colloidal carbon prior to the first immunization with the same antigen, had a "primary" type immunologic response similar to that which occurred normally in mice injected only once with sheep erythrocytes. The absence of a typical anamnestic response suggests that "immunologic memory" did not develop when mice were treated before primary immunization with an RES-blockading agent such as carbon. Thus, RES blockade interfered not only with the initial immune response, as observed previously (11, 12), but also markedly affected development of cells capable of "immunologic memory."

A direct effect of carbon on secondary responses did not appear to be involved, since correlary studies, not reported here, have shown that carbon injection into RBC-primed mice shortly before a second injection of red cells results in a marked suppression of appearance of both 19S and 7S PFC. Such mice generally exhibited a greater suppression of 19S plaque-forming cells than 7S PFC. Treatment of mice with carbon alone 35 days prior to a single injection of sheep RBC had no detectable effect on the immune response (Table I). In addition, there was no evidence that carbon particles had any toxic effect on lymphoid cells *per se*, or that there was a prolonged depression of phagocytic activity.

A number of investigators have suggested that certain cells develop after initial antigenic stimulation which can "remember" the antigen at a subsequent time (2). It is thought that such "memory" cells, if they exist, would probably be small lymphocytes. Initial contact with antigen is thought to stimulate proliferation of specific stem cells into both antibody-producing cells and into "memory"

cells. In this regard, it seems plausible that interference with the initial steps of the immune response by RES blockade may result in alteration or suppression of antigen "processing" by phagocytes so that "antigen-sensitive" lymphoid cells do not become stimulated. Absence of such stimulation should lead to an absence of an anamnestic response after subsequent challenge immunization with the same antigen.

It should be pointed out that in relatively similar situations treatment of experimental animals with certain immunosuppressive agents, such as inoizing irradiation, nucleic acid analogs, or other cytotoxic agents, readily induces a well described nonspecific immunologic unresponsiveness. Recovery from such unresponsiveness, however, is not specific or selective in regard to IgM or IgG antibody-producing cells. Furthermore, it has been recently observed that depression of a primary immune response by an immunosuppressant derived from group A streptococci had no effect on "memory" (17).

In the present study essentially normal numbers of 19S IgM antibody forming cells, as well as a normal level of 2-ME–sensitive serum antibody, appeared after the second injection of RBC into mice treated with carbon particles before the initial priming injection of antigen. Such results indicate that the mice were fully capable of a "primary" type 19S PFC response to sheep RBC, but did not have the "memory" component necessary for 7S PFC, as occurs in normal primed mice. It is possible, however, that absence of the secondary type response in this system was not due only to a defect in the cellular mechanism. A number of recent studies have indicated that a critical level of serum antibody may be necessary for optimum opsonization and processing of particulate antigen (16). Development of 7S PFC may depend wholly or in part on the presence of an optimum level of serum opsonins, or a required number of antibody-producing cells, or their progenitors, rather than a separate cell compartment presumably containing nonantibody-forming "memory" cells.

Although the actual mechanism involved in induction of immunologic "amnesia" in an RES-blockaded animal is still unclear, it seems apparent that further evaluation of the relationship between cellular formation of antibody and the RES in blockaded animals, as well as the relationship between phagocytes and immunocytes, may be of value in understanding the mechanism of immunity.

Summary. Cellular and humoral antibody formation was markedly inhibited in mice treated with colloidal carbon particles to blockade the reticuloendothelial system. Appearance of 19S IgM and 7S IgG hemolysin-forming cells was prevented when adult mice were treated with carbon prior to a single inoculation of sheep erythrocytes. Carbon treatment also reduced markedly the expected secondary immune response in mice primed several weeks previously with sheep red blood cells. Few antibody-forming cells, either IgG or IgM, and only low levels of serum hemolysins appeared in primed mice injected with carbon 1 to 4 days before a secondary immunization. Mice injected with carbon before the primary inoculation of antigen had normal 19S IgM antibody-plaque responses after a second injection of red blood cells 1 month later. However, there was a marked absence of 7S IgG antibody-forming cells. Also, there was little, if any, 2-mercaptoethenol–resistant antibody in the sera of these mice after booster immunization. The immune response of these animals was similar to that of a primary-type response of normal control mice injected only once with erythrocytes. Specific immunologic "amnesia," as distinct from immunological tolerance, had developed as a consequence of RES blockade by carbon injection prior to initial immunization.

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