EFFECTS OF RES-BLOCKADE ON IMMUNE RESPONSE. II. THE EFFECTS OF CARBON INJECTION ON MEMORY FORMATION AND SECONDARY ANTIBODY RESPONSE

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The effects of reticuloendothelial system (RES)-blockade on immune response and immunological memory formation were studied in mice. To examine the effects of carbon on memory formation, mice were first treated with carbon and then stimulated with 10^4 - 10^9 sheep erythrocytes (SRBC) one day later. The maximum secondary indirect plaque-forming cell (IPFC) responses to restimulation with 10^9 SRBC were obtained in control mice primed with 10^6 SRBC and in mice injected with carbon and primed with 10^5 SRBC. The effects of RES-blockade on memory formation were not prominent and only mice primed with 10^5 SRBC showed increased humoral immune responses in comparison with control mice. Both carbon-treated and control mice primed with 10^4 SRBC showed high secondary direct PFC and low IPFC response to 10^9 SRBC restimulation. In the case of mice primed with 10^9 SRBC 30 days before injection with carbon and restimulated with 10^5 - 10^9 SRBC one day later, the mice given the larger dose of booster antigen had larger PFC responses in spleen compared to mice injected with the smaller antigen dose but a difference in PFC response between carbon-treated mice and control mice was not observed.

It was suggested that RES-blockade increased memory formation in mice primed with a low antigen dose but did not affect the development of the secondary antibody response in mice primed with 10^9 SRBC.

At least 3 different types of cells are needed for a humoral immune response to most antigens. The bone marrow-derived lymphocyte (B-cell) produces the humoral antibody and is triggered by a cooperating thymus-derived lymphocyte (T-cell) to do so (see review reference 7). A phagocytic cell, the macrophage, is often needed to process or handle the antigen for this cooperation (5). In addition, macrophage regulates the immune response with its active phagocytosis that non-specifically removes most antigens from extracellular fluids without contributing to the specific immune response (13, 21, 23, 24). A small part of the injected antigen becomes an effective immunogenic stimulus that requires 2 types of cells to meet with antigen molecules (see review ref. 15).

It is generally accepted that phagocytosis of antigen by RES may be an important step in the immune response. Sabet et al. (20) reported that mice injected with carbon prior to a single immunizing inoculum of SRBC had markedly suppressed primary and secondary immune responses. Silica administered i.v. to DBA/2 mice seriously suppressed the humoral immune response to SRBC and the cell-

mediated immune response to allogeneic fibroblasts (12). Mice injected with agents which are cytotoxic for macrophages showed a reduced resistance to tolerance against protein and polysaccharide antigens (2, 14).

In previous studies, it was observed that mice injected with carbon showed an enhanced humoral response to low dose antigen and a decreased humoral response to high dose antigen (see part I of this paper). In this paper, the effects of carbon induced RES-blockade prior to primary or secondary injection of SRBC were investigated. It was found that the humoral immune response was significantly affected in antigen primed mice treated with carbon particles prior to secondary immunization. In addition, mice injected with carbon before initial immunization with SRBC showed a marked enhancement of IPFC responses at low dose SRBC but not at high dose SRBC following the subsequent secondary immunization.

MATERIALS AND METHODS

The animals, antigen, the methods of RES-blockade and determination of the immune responses employed in this research were described in part I of this paper. In brief, RES-blockade was induced in inbred DDD mice by intravenous injection with 0.3 ml Pelican carbon suspension at a concentration of 2.5 mg/ml followed by immunization with sheep erythrocytes (SRBC) washed freshly in phosphate buffered-saline (PBS). To determine the immune response, serum hemagglutinin titer was determined with microtiter for two-fold serial dilution and the number of anti-SRBC plaque-forming cells (PFC) was measured.

The ability of primed-mice to respond to a booster injection of SRBC was investigated as a means to determine immunological memory formation. For this purpose mice of each group were treated with carbon one day before injection of 10^4 – 10^9 SRBC and boosted i.v. with a constant dose of SRBC. The number of splenic direct and indirect PFC (DPFC and IPFC) was determined 4 days after. In order to test the effects of RES-blockade on secondary antibody response, mice primed with 10^9 SRBC were injected with carbon 29 days later and then challenged with 10^5 – 10^{10} SRBC one day after carbon treatment. Splenic PFC responses were determined 4 days after.

RESULTS

Effects of carbon injection before primary immunization on the development of immunological memory

Mice stimulated with antigen produced much more vigorous immune responses to the booster injection accompanied by a high level of 2-mercaptoethanol resistant (2ME-R) antibody and a large number of IPFC. The secondary PFC response in mice stimulated with 10^9 SRBC and restimulated with 10^9 SRBC 30 days after is shown in Fig. 1. Mice showed peak DPFC response on the 4th day, as was seen in the primary response. The number of DPFC was slightly suppressed in comparison with the primary response. The peak IPFC response was shown on the 4th day and was about 10 times larger in number the peak primary IPFC response.

Mice treated with carbon were stimulated with intravenous injection of 10^4 –

FIG. 1. Kinetics of secondary PFC response of mice. Mice were primed i.v. with 10^9 SRBC and boosted i.v. with 10^9 SRBC 30 days after initial injection. Data are presented as per 10^6 nucleated spleen cells. DPFC was developed directly and IPFC was developed indirectly by rabbit anti-mouse γ -globulin enhancement technique. —○—: DPFC response, --○--: IPFC response. Vertical bars represent one standard error.

10^9 SRBC one day after carbon treatment. The immune responses of these groups differed markedly from that of control groups (see part I of this paper). The mice were restimulated i.v. with 10^9 SRBC 30 days after priming and the splenic PFC responses were determined 4 days later (Fig. 2), since it was the day of peak secondary PFC response. 10^4 SRBC injection primed mice to produce the suppressed DPFC response as compared with the peak primary DPFC response in mice immunized with 10^9 SRBC.

Carbon-treated and 10^4 SRBC-primed mice produced an enhanced DPFC response similar to that in control 10^4 SRBC-primed mice. The DPFC response of carbon-treated and 10^5 – 10^9 SRBC-primed mice was generally lower than the primary response in mice immunized with 10^9 SRBC and the dose response curve was slightly shifted to the left. Mice stimulated previously with 10^6 – 10^9 SRBC produced a much more vigorous immune response to restimulation with 10^9 SRBC accompanied by a large number of IPFC. At maximum response, IPFC numbers were above one PFC per 100 nucleated spleen cells. When mice were primed with 10^6 – 10^9 SRBC, carbon-treated mice produced secondary IPFC responses similar to those in control mice. 10^4 – 10^5 SRBC-primed mice showed higher response than control mice and the IPFC number were more than 2 times that of control mice. Carbon-treated and unstimulated-mice produced normal primary DPFC and IPFC responses to stimulation with 10^9 SRBC 30 days later. Consequently non-specific memory induction by carbon injection was not observed.

FIG. 2. Effect of carbon on the memory formation in mice. Mice were injected i.v. with carbon one day prior to initial stimulation with 10^4 – 10^9 SRBC. Control mice were injected with saline substitute for carbon. Primed mice were restimulated i.v. with 10^9 SRBC. PFC response was examined 4 days after restimulation. $---\bigcirc---$: DPFC response in control mice, $-\bigcirc-$: IPFC response in control mice, $---\bullet---$: DPFC response in carbon treated mice, $-\bullet-$: IPFC response in carbon treated mice. Vertical bars represent one standard error.

Similar results were obtained in the serum secondary antibody responses (Table 1). RES-blocked mice were primed with 10^5 – 10^{10} SRBC one day after carbon and 29 days later blood was obtained from individual mice. Mice were restimulated with 10^9 SRBC 30 days after initial stimulation and at various intervals thereafter blood was obtained from individual mice. 10^7 – 10^{10} SRBC-primed mice showed high antibody level before restimulation and the formation of far more antibody was elicited by the second antigen. The increase in the amount of antibody was about thirty-fold. Serum hemagglutinin titer showed similar levels among the different groups of priming doses and RES-blocked mice produced the same level of antibody activity in serum as that found in control mice. Before the booster injection RES-blocked and 10^5 SRBC-primed mice showed a low total and 2ME-R antibody activity, 1.8 ± 0.2 and 1.2 ± 0.2 , respectively. When such mice were restimulated with 10^9 SRBC, mice showed a rapid increase in antibody amount and showed a very high total and 2ME-R antibody activity, 8.9 ± 0.4 and 6.0 ± 0.5 respectively, 4 days later. Serum antibody activity in 10^5 –SRBC primed control mice was undetectable before booster injection. A more dramatic increase in antibody amount was observed in such mice after booster injection.

Four days after booster injection total and 2ME-R antibody activity were 7.0 ± 0.5 and 2.2 ± 0.4 , respectively. Thirty days after booster injection, serum antibody level did not differ significantly among the experimental groups.

Effects of carbon injection before second stimulation with SRBC on secondary immune response

In order to evaluate the effects of carbon on the immune response of primed-

FIG. 3. Effect of carbon on the secondary PFC response of mice to SRBC. Primed mice with 10^9 SRBC were injected i.v. with carbon at 29 days and challenged with 10^5 – 10^9 SRBC at 30 days after priming. PFC response was determined 4 days after challenge. ---○---: DPFC response in control mice, —○—: IPFC response in control mice, ---●---: DPFC response in carbon treated mice, —●—: IPFC response in carbon treated mice. Vertical bars represent one standard error.

mice to the second antigen injection, mice primed with 10^9 SRBC were restimulated with 10^5 – 10^9 SRBC 30 days later and the DPFC and IPFC number in spleen were determined after 4 days. RES was blocked by the intravenous injection of carbon one day before restimulation (Fig. 2). The DPFC response of carbon-treated mice was essentially the same as that produced in control mice, except in the case of the 10^5 and 10^9 SRBC-challenged groups, where the difference was minimal. The effects of increasing the secondary stimulation dose were prominent in the DPFC and IPFC responses. Mice having the same potential memory capacity showed a larger secondary response when boosted with higher doses than with lower doses. Differences in IPFC response between carbon-treated and control mice were not observed.

DISCUSSION

The primary immune response to SRBC was characterized by the appearance of many DPFC with the peak number reached 4–5 days after immunization and the delayed appearance of IPFC whose number peaked by the 7th–10th days. Serum antibody peaked within 7–10 days after antigen injection (see part I of this paper). If such mice were stimulated with the same antigen 30 days after initial stimulation, a vigorous secondary type immune response characterized by the appearance of large numbers of IPFC in the spleen and enhanced serum antibody production was produced. In the secondary immune response, IPFC appeared

TABLE 1. *Effects of carbon on immunological memory formation^{a)}*

Priming SRBC dose ^{b)}	Carbon injection ^{b)}	Log ₂ anti-SRBC antibody titer at indicated days after the 10 ⁹ SRBC injection ^{c)}				
		-1 ^{d)}	4	8	14	30
10 ⁵	-	- ^{e)} (-)	7.5±0.6 (2.2±0.4)	9.0±0.5 (7.0±0.3)	9.2±0.4 (6.8±0.4)	8.4±0.5 (6.6±0.5)
	+	1.8±0.9 (1.2±0.2)	9.9±0.5 (6.0±0.3)	10.8±0.4 (7.2±0.4)	11.0±0.4 (8.0±0.6)	8.8±0.4 (7.0±0.6)
10 ⁷	-	6.4±0.4 (4.2±0.4)	11.7±0.4 (7.6±0.4)	11.2±1.2 (7.6±0.4)	10.8±0.8 (8.3±0.4)	9.0±0.5 (7.0±0.6)
	+	7.0±0.4 (4.5±0.5)	9.8±0.5 (6.8±0.6)	10.3±0.6 (6.8±0.5)	10.5±0.6 (7.5±0.4)	8.8±0.5 (6.5±0.3)
10 ⁹	-	7.3±0.5 (4.0±0.4)	9.8±0.5 (7.0±0.0)	9.5±0.5 (7.0±0.5)	9.5±0.5 (7.0±0.0)	8.0±0.0 (6.0±0.3)
	+	6.4±0.7 (4.0±0.6)	9.5±0.0 (5.6±0.5)	9.6±0.8 (6.2±0.5)	8.6±0.8 (6.2±0.4)	8.4±0.5 (6.8±0.4)
10 ¹⁰	-	8.0±0.6 (5.4±0.5)	10.1±0.5 (6.8±0.4)	10.8±0.4 (7.6±0.5)	10.2±0.4 (7.4±0.5)	9.2±0.4 (6.6±0.5)
	+	7.0±0.0 (5.0±0.0)	9.5±0.0 (6.0±0.0)	10.0±0.0 (7.3±0.5)	10.0±0.0 (6.7±0.5)	9.2±0.0 (6.3±0.5)

^{a)} : Mice were stimulated i.v. with 10⁵-10¹⁰ SRBC and restimulated with 10⁹ SRBC at 30 days after initial stimulation.

^{b)} : Mice were stimulated with 10⁵-10¹⁰ SRBC one day after carbon i.v. injection.

^{c)} : Serum hemagglutinin titer was determined in individual mice. Data are presented as geometric mean of the number of animals examined. Values in parentheses represent the 2-ME-R hemagglutinin titer.

^{d)} : Blood was collected from individual mice one day before the restimulation with 10⁹ SRBC.

^{e)} : -represent that antibody activity could not be detected.

immediately after booster injection without latent phase, increased rapidly to its peak 4 days later and thereafter decreased quickly. In addition, serum antibody responses characterized by a large amount of 2ME-R antibody increased sharply and reached their peak within 4 to 8 days, thereafter remaining stable for over 30 days.

The optimum conditions for the production of a high secondary antibody response were discussed by Nakashima et al. (17) but not determined in detail. Sterzl et al. (22) found that neonatal rabbits and piglets primed with a suboptimal dose of SRBC produced higher DPFC and IPFC responses to second antigen injection than did animals primed with an optimal dose for maximum primary response. Similar results were reported in adult mice immunized with SRBC (8, 9, 10). Nossal et al. (18) obtained the maximum anamnestic response in mice challenged with an antigen dose larger than that used in primary stimulation. In our present experiments, the maximum secondary response was obtained in mice stimulated with 10⁶ SRBC and restimulated with 10⁹ SRBC 30 days later. However the

difference in IPFC response between 10^6 SRBC-primed mice and 10^7 – 10^9 SRBC-primed mice was not that large. The number of IPFC found in 10^6 SRBC-primed mice did not exceed twice the amounts found in other groups.

Mice stimulated with 10^4 – 10^5 SRBC produced an undetectable primary response or minimal primary response unaccompanied by IPFC production (see part I of this paper). These mice produced a smaller secondary response than that of 10^6 – 10^9 SRBC-stimulated mice which had produced the primary IPFC response to initial stimulation. Carbon-treated and 10^5 SRBC-stimulated mice initially showed a weak primary response accompanied by small IPFC and 2ME-R antibody responses. These mice produced the highest anamnestic response among the carbon-treated groups after restimulation with 10^9 SRBC. These results suggested that the induction of minimal IPFC response with initial antigen stimulation was essential for maximum secondary IPFC response.

DPFC responses were slightly suppressed in mice primed with 10^6 – 10^9 SRBC in comparison with the primary response in 10^9 SRBC-stimulated mice. However, mice primed with 10^4 SRBC showed anamnestic response to the challenge. This may be the effect of feedback inhibition by the specific 7 S antibody, which is known to suppress the immune response in small concentrations and to have more marked effects on DPFC than on IPFC production (8, 11). Enhanced DPFC and IPFC response in 10^4 SRBC-primed mice may be the result of production of extremely low or entirely absent levels of 19 S antibody in the mice, since it is known that 19 S antibody enhances antigen localization in the spleen and antibody formation (3). On the other hand, it may be the result of selective sensitization of the helper T-cells (4, 16).

Several studies concerning the function of macrophages in the secondary immune response showed that antigen handling by macrophage was essential for *in vivo* and *in vitro* secondary immune response (1, 5). Sabet et al. (20) reported that in primed-mice, the secondary IgM and IgG antibody formation were markedly suppressed by carbon injection 1–3 days before second immunization. In the present investigation, however, RES-blockade with carbon injection one day before the second immunization did not affect secondary immune response in mice primed with 10^9 SRBC and restimulated with 10^5 – 10^9 SRBC.

Previously, it was reported that RES-blockade increased antigen distribution to spleen (6, 21). In present experiments, however, increased antigen distribution in the spleen did not affect the magnitude of the secondary response. There may be the possibility of feedback inhibition by specific antibody, since mice had a large amount of 2 ME-R antibody at the time of booster injection.

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