Immunology, 1969, **17**, 535.

Effects of RES 'Blockade' on Antibody Formation

II. CYTOKINETICS OF THE SECONDARY HAEMOLYSIN RESPONSE AND SUPPRESSED IMMUNOLOGICAL 'MEMORY' IN MICE TREATED WITH CARBON PARTICLES

T. Y. SABET* AND H. FRIEDMAN

Department of Microbiology, Albert Einstein Medical Centre, Philadelphia, Pennsylvania, U.S.A.

(*Received* 14*th February* 1969)

Summary. Suppression of the primary immune response by treatment of mice with carbon 1 day before initial immunization markedly interfered with development of immunological 'memory', since such mice responded to a subsequent challenge injection of RBCs by formation of mainly IgM PFCs and serum antibody. Appearance of IgG PFCs and 2-ME resistant antibody was delayed several days in these carbon treated animals, indicating failure of a typical secondary response. The immune response of these animals was similar to that of a primary response of control animals to a single injection of red cells.

Reticulo-endothelial cell blockade with colloidal carbon suspensions interfered with development of a normal secondary type immune response to sheep red blood cells, as assayed on both the cellular and humoral levels. Fewer antibody PFCs, mainly 19S IgM but also 7S IgG, appeared in spleens of antigen primed mice treated with carbon 1–3 days prior to a challenge injection of red cells, as compared to control primed mice injected with erythrocytes alone. However, the peak day of antibody response was the same for both control and carbon treated animals.

Mice treated with carbon 1–3 days before secondary immunization had much lower peak serum titres, mostly susceptible to 2-ME inactivation.

The time of inoculation of carbon in relation to immunization was important since carbon treatment 1–3 days before secondary RBC immunization resulted in maximum suppression. Injection of carbon 5–7 days before resulted in only a slight effect, whereas injection 30 days before had no detectable effect. Injection of carbon simultaneously or after RBC injection had little effect.

The dose of carbon used for immunosuppression, as well as the concentration of sheep erythrocytes used for immunization affected the number of antibody PFCs and the serum titres in control as well as carbon treated animals.

INTRODUCTION

The role of the reticulo-endothelial system (RES) and its constituent cells in immunity has been well documented during the past several decades by numerous studies using a variety of experimental approaches (Wood, 1960; Rowley, 1962; Thorbecke and Benacerraf, 1962; Hirsch, 1965). A variety of morphological studies, especially with immunofluorescent antibody techniques and radioautography, have demonstrated that specific

* Present address: Department of Histology, University of Illinois, College of Dentistry, Chicago, Illinois, U.S.A.

antigen localizes in RE cells and that antibody is synthesized by lymphocytes and plasmocytes in distinct anatomical sites in lymphoid tissue and in the blood (Nossal, 1967).

It is generally accepted that phagocytosis of particulate antigens by RE cells may be an important step in antibody synthesis (Frei, Benacerraf and Thorbecke, 1965; Gallily and Feldman, 1967; Argyris, 1967; Nossal, 1967). Circulating and fixed macrophages are thought to 'digest' and/or 'process' antigens such as foreign red blood cells or bacteria. There is evidence from a number of laboratories that an important product of phagocytic cells which have ingested a particulate or soluble antigen may be an 'informational' nucleic acid, probably associated with the antigenic determinant, which can directly stimulate antibody formation by other cells (Fishman and Adler, 1963; Friedman, 1964; Askonas and Rhodes, 1965; Friedman, Stavitsky and Solomons, 1965). On the other hand, there have been reports that phagocytic cells, such as macrophages from the peritoneal cavity, may interfere with the immunogenicity of an antigen (Perkins and Makinodan, 1965). Under certain circumstances it appears that phagocytosis may result in rapid digestion or removal of an antigen, resulting in little or no stimulation of antibody formation.

Numerous studies concerning the role of the RES in immunity have been concerned with inhibition of normal RE cell function by agents such as ionizing irradiation, antimetabolites or steroids (Schwartz and Damashek, 1963; Berenbaum, 1964; Schwartz, 1965; Gabrielson and Good, 1967). In this regard, a number of studies during the past decade have indicated that non-toxic agents such as carbon suspensions or oil emulsions may also interfere with RE cell function and, consequently, suppress immune responses (Lewis, 1954; Derby and Rogers, 1961; Koenig, Heysell, Milly and Rogers, 1965). Such interference with immunity is considered to be an example of the effect of RES 'blockade'. However, there have been conflicting reports concerning the effects of RES blockade on specific antibody formation. For example, some investigators observed that the small doses of either India or trypan blue dye would result in decreased antibody formation to sheep red blood cells in rabbits (Gay and Clark, 1924; Canon, Baer, Sulivan and Webster, 1929; Drutz, Koenig and Rogers, 1967). On the other hand, Fisher (1966) and Jenkin, Auzin and Reade (1965) indicated that treatment of animals with carbon or thorium dioxide as blockading agents enhanced the immune response of rodents to sheep red blood cell antigens. Stern, Spencer and Farquar (1955), however, observed suppression of the serum antibody response to sheep erythrocytes in mice injected repeatedly with polyvinyl pyrrolidone. They attributed this depression to RES blockade. More recently Cruchaud (1968) observed a decreased serum antibody response to bovine serum albumin in rabbits injected repeatedly with India ink, gelatin or dextran. No suppression occurred when the animals received only a single injection of carbon each day, rather than multiple injections daily.

In previous studies, we have observed that mice injected once with carbon prior to a single immunizing inoculum of sheep red blood cells had a markedly suppressed primary immune response, as assessed both on the cellular and humoral levels (Sabet, Newlin and Friedman, 1968, 1969). Maximum immunodepression occurred when the mice were injected with 10 mg carbon 1–3 days before challenge immunization. A lesser degree of suppression occurred when lower concentrations of carbon or red cells were used. In this paper, the effects of RES blockade by carbon, prior to a primary or a secondary injection of sheep red blood cells, were investigated. It was found that, under appropriate conditions, 19S antibody response to sheep erythrocytes could be markedly suppressed in antigen primed mice treated with carbon particles prior to secondary immunization. The 7S

antibody response was also suppressed, but to a lesser degree. In addition, mice injected with carbon before initial immunization with red blood cells showed a marked suppression of 7S IgG, but not 19S IgM antibody forming cells following the subsequent secondary immunization.

MATERIALS AND METHODS

Animals

Male NIH albino A mice, 6–8 weeks of age, were used as described previously (Sabet *et al.*, 1968, 1969). They were obtained from a local dealer in the Philadelphia area and fed Purina mouse pellets and water *ad libitum.*

Antigens

Freshly washed sheep erythrocytes, in buffered physiological saline, pH 7·2, were used as antigen, as described previously (Sabet *et al.*, 1969).

Carbon particles

Pelikan carbon was used to induce RES blockade, as described previously (Sabet *et al.*, 1968, 1969). For most experiments, mice were injected intraperitoneally (i.p.) with 0·1 ml of a suspension containing 10 mg carbon.

Antibody titrations

Blood was obtained from individual mice by retro-orbital venous puncture prior to immunization and at various time intervals thereafter. The resulting serum specimens were tested for haemolysins and haemagglutinins to sheep erythrocytes as described previously (Sabet *et al.*, 1969).

Detection of antibody plaque forming cells (PFC)

Direct plaques. The direct method of Jerne, Nordin and Henry (1963) as described previously (Friedman, 1964; Sabet *et al.*, 1969) was used to detect haemolysin forming cells considered to be due to release of 19S IgM antibody (Sterzl and Riha, 1965; Dresser and Wortis, 1965; Plotz, Talal and Asofsky, 1968). In brief, a dispersed spleen cell suspension was prepared and 0·1-ml aliquots were added to tubes containing 2·0 ml warm, melted 0·7 per cent Noble agar (Difco Laboratories, Detroit, Michigan) in Hanks's solution, to which had been added 0·1 ml of a 10 per cent suspension of washed sheep erythrocytes and DEAE Dextran. This mixture was carefully and quickly poured into a previously prepared 100 mm diameter Petri plate containing a base layer of solidified 1·4 per cent Noble agar in Hanks's solution. The plates were incubated for 1 hour at 37° and then treated with approximately 5 ml of a 1 : 15 dilution of guinea-pig serum as a source of complement. The plates were further incubated at 37° for 1 hour so that antibody plaque formation, if present, could be detected as discrete zones of haemolysis against the background 'lawn' of unlysed eythrocytes. In all experiments at least two duplicate plates and several cell concentrations were poured for each spleen cell suspension. Control spleen cells were always assayed at the same time as spleens from experimental mice. The number of plaque forming cells was calculated both per million spleen cells plated and per whole spleen.

Indirect or low-efficiency plaques

An enhancement procedure was used to determine the number of antibody forming cells presumably secreting 7S IgG haemolysins (Dresser and Wortis, 1965; Wortis, Taylor and Dresser, 1966; Plotz *et al.*, 1968). Separate aliquots of each spleen cell suspension were tested exactly as for the direct procedure in additional sets of agar plates. Following the first incubation with complement, as above, the plates were washed with Hanks's solution and then treated with approximately 5 ml of a 1 : 50 dilution of hyperimmune rabbit anti-mouse γ-globulin serum, which had been previously absorbed exhaustively with sheep and mouse erythrocytes. The plates were then incubated for 1 hour at 37°, rinsed with Hanks's solution and developed with guinea-pig complement as before. The additional plaques which appeared following this treatment were considered to be due to low-efficiency 7S IgG haemolysin forming cells (Dresser and Wortis, 1965; Wortis *et al.*, 1966). Plates containing approximately 50–500 plaques were counted and the number of PFCs calculated per spleen and per million cells. The difference between the number of PFCs obtained by the direct technique and the number of PFCs obtained with the indirect technique was considered to be the number of 7S IgG PFCs.

Experimental design

The ability of primed mice to respond to a subsequent injection of sheep erythrocytes by producing large numbers of 7S IgG antibody forming cells and 2-ME resistant haemolysins and haemagglutinins was used as the assay to determine immunological 'memory'. For this purpose groups of mice, generally numbering 100–150, were injected i.p. with erythrocytes. Some of the animals had been previously injected with carbon in order to blockade the RES. Four weeks later, a time which had been found optimal for stimulation of large numbers of 7S IgG antibody forming cells in spleens of primed mice, both control and carbon treated animals were again injected with sheep erythrocytes. Some of the control mice, which had not been injected with carbon prior to initial immunization, were injected with carbon at intervals prior to this challenge injection of red blood cells. All mice were bled from the retro-orbital plexus before and at intervals after immunization. The number of splenic IgM and IgG PFCs, as well as the level of 2-ME sensitive and resistant serum antibody, was determined for three to six mice per group at closely spaced intervals before and after challenge RBC injection.

RESULTS

THE EFFECT OF CARBON TREATMENT BEFORE PRIMARY IMMUNIZATION ON DEVELOPMENT OF IMMUNOLOGICAL 'MEMORY'

For these experiments mice were treated with 10 mg carbon 24 hours prior to an initial immunizing inoculum of $2{\cdot}5 \times 10^8$ RBC. Control mice were injected with RBCs alone. As described previously (Sabet *et al.*, 1968, 1969), the immune responses of these groups differed markedly. Carbon pre-treated animals generally had less than 5–10 per cent of the PFC response of control animals. Few, if any 7S PFCs, could be detected during the 1st week after immunization, using the antiglobulin enhancement procedure. In contrast, control animals developed significant numbers of 7S PFCs following primary immunization. These PFCs first appeared about 5–6 days after initial immunization, and reached a peak several days later (Sabet *et al.*, 1969). As a test for development of immuno-

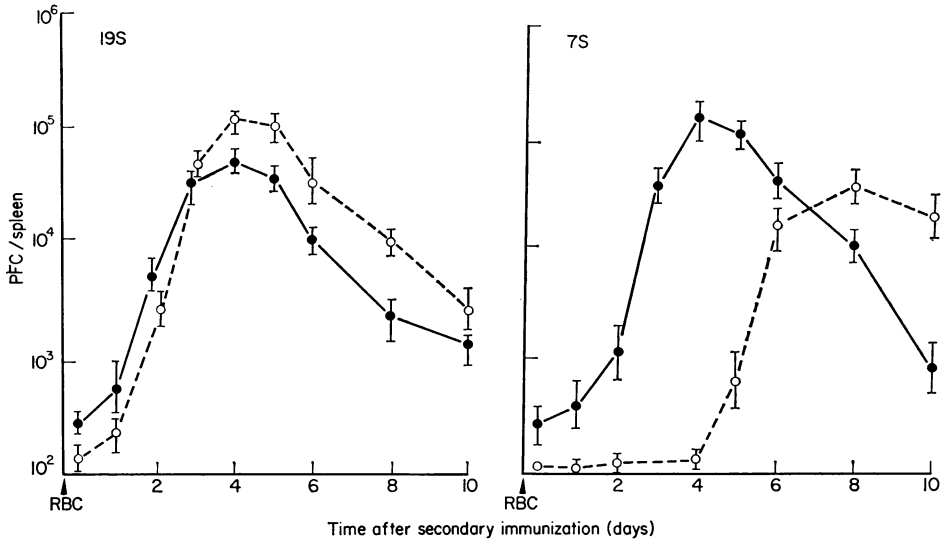

FIG. 1. Effect of Pelikan carbon (10 mg/mouse i.p.) on development of immunological 'memory' in mice treated with carbon 1 day before primary injection of $2{\cdot}5 \times 10^8$ RBCs. All mice challenged with $2{\cdot}5 \times 10^8$ RBCs 4 weeks later and assayed for splenic 19S IgM and 7S IgG PFCs on days indicated. ●, Mice showing normal secondary response, controls; ○, mice treated with carbon 1 day before primary immunization. Each point represents average of three to five mice.

logical 'memory', groups of primed animals, either normal or carbon treated, were challenged with $2{\cdot}5 \times 10^8$ sheep red cells 4 weeks after the primary immunization. As seen in Fig. 1, there was a vigorous 19S PFC response in the spleens of both groups of mice after the secondary immunization. A peak number of approximately 50,000–80,000 PFCs appeared in the spleens of the control mice. The carbon pre-treated animals had fewer 19S PFCs on the day of challenge, but had a peak number of about 100,000 PFCs 4 days later. The major difference between the two groups was the appearance of 7S PFCs in spleens of control mice. Whereas the non-treated animals had a rapid appearance of 7S PFCs, with a peak of 100,000 or more 4 days after secondary immunization, the carbon pre-treated animals had very few or no detectable 7S PFCs until the 4th to 5th day after secondary immunization. The number of 7S PFCs then increased to a peak of about 20,000–30,000 by the 8th to 10th day. This response was similar to that observed routinely in non-primed mice receiving a single injection of red blood cells. The serum antibody response of the carbon pre-treated mice was generally lower than that of control animals. Nearly all of the antibody activity was susceptible to 2-ME treatment until the 5th to 10th day after secondary immunization.

EFFECT OF CARBON INJECTION BEFORE THE SECOND DOSE OF RBCs ON THE SECONDARY IMMUNE RESPONSE

Number of 19S IgM producing cells

Carbon was injected into groups of mice at various times before a second injection of $2{\cdot}5 \times 10^8$ sheep red blood cells. Fig. 2 indicates the number of direct 19S PFCs found at various times after RBC injection in spleens of control primed mice and mice injected with carbon either at the same time as secondary immunization or 1 day

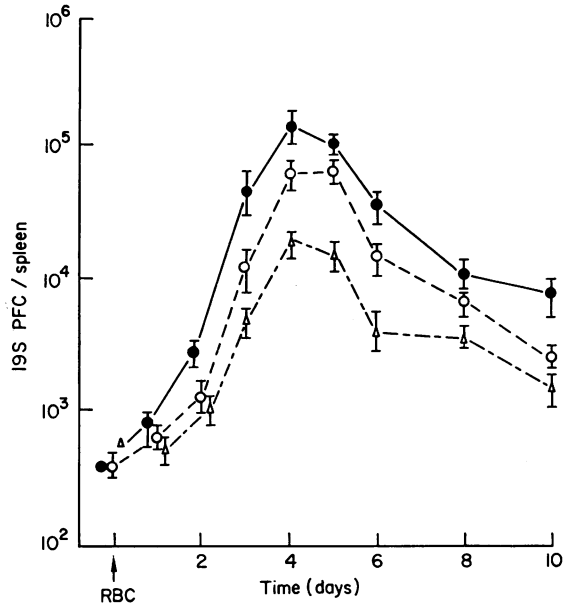

FIG. 2. The effect of Pelikan carbon (10 mg/mouse i.p.) on the number of 19S IgM PFCs in spleens of mice receiving a secondary injection of sheep RBC 4 weeks after primary immunization. Primary and secondary doses were $2{\cdot}5 \times 10^8$ RBCs each. ●, Normal control mice; ○, mice injected with carbon same day as secondary immunization; △, mice injected with carbon 1 day before immunization. Each point represents average of five or more mice. Vertical line indicates standard error.

before. There was a vigorous IgM PFC response in both the control and experimental groups, with a peak four days after immunization. Mice in both groups had between 300 and 600 PFCs/spleen prior to secondary immunization. The peak number in spleens of control mice reached a level of approximately 100,000 PFCs on day 4. The peak number of PFCs in spleens of mice injected with carbon the same day as immunization was about 20–50 per cent less than in controls. Mice injected with carbon 1 day before the red blood cells had approximately 20,000–30,000 PFCs on the peak day after immunization. There was a lag in appearance of such PFCs the first few days after immunization, and a more rapid decline in the number of PFCs between days 6 and 10 for both groups of carbon treated animals, as compared to controls.

Similar results were obtained when the number of 19S PFCs was calculated per 10^6 spleen cells rather than per whole spleen. As can be seen from Fig. 3, there were generally fewer than 10 PFCs/10^6 spleen cells at the time of secondary immunization in all animals tested 4 weeks after primary injection. The control animals had a rapid increase in the number of PFCs after secondary immunization, with a peak of nearly 1000 PFCs/10^6 spleen cells. The carbon treated animals had a slower rise in PFCs. Mice treated with carbon the day of immunization had about half the number of PFCs as compared to controls. Animals treated with carbon 24 hours before immunization had a peak of about 300 PFCs/10^6 spleen cells. By the 10th day after immunization the control and experimental mice had comparable numbers of 19S PFCs.

As can be seen from Fig. 4, an even greater PFC suppression occurred when mice were treated with carbon 2 or 3 days before secondary immunization. In these experiments the PFC response was determined on the 4th day after secondary immunization of groups

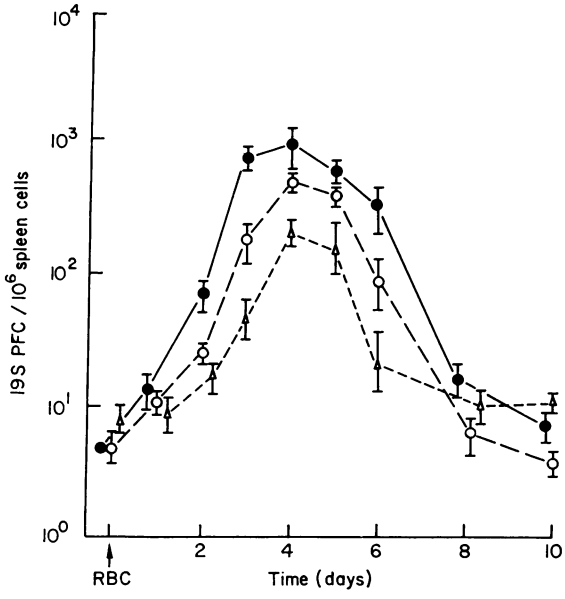

FIG. 3. Effect of Pelikan carbon (10 mg/mouse i.p.) on the number of 19S IgM PFCs/10^6 spleen cells in mice receiving a secondary injection of sheep RBCs 4 weeks after primary immunization. Primary and secondary doses were $2{\cdot}5 \times 10^8$ RBCs each. ●, Normal control mice; ○, mice injected with carbon same time as secondary immunization; △, mice injected with carbon 1 day before immunization. Each point represents average of five or more mice.

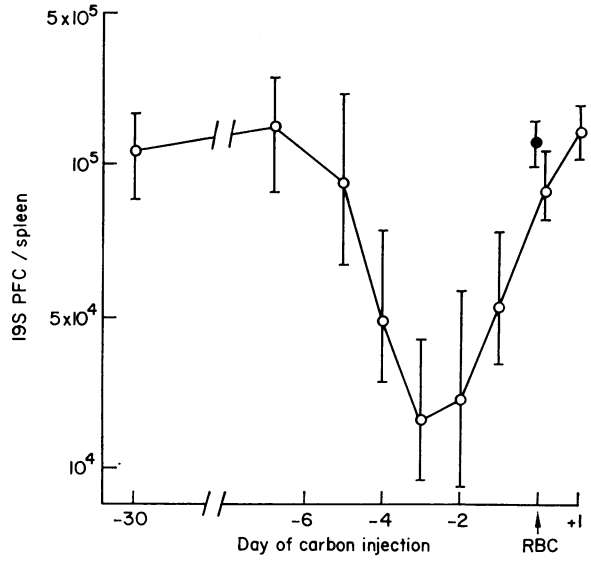

FIG. 4. Effect of time of injection of Pelikan carbon (10 mg/mouse i.p.) relative to day of secondary RBC injection on the number of direct 19S IgM PFCs 4 days after the secondary immunization with $2{\cdot}5 \times 10^8$ RBCs. All mice primed with the same dose of RBCs 30 days earlier. ●, Control mice not injected with carbon; ○, mice treated with carbon. Each point represents average of three to five mice. Vertical lines indicate range.

of mice injected with carbon prior to, simultaneously with, or following secondary immunization. The lowest number of PFCs, generally less than 20 per cent of the control response, occurred in animals treated with 10 mg carbon 3 days before immunization. When carbon was administered 5 days earlier, there was little or no significant suppression. Some mice treated with carbon 7 days before secondary immunization often had more PFCs than did controls. Animals injected with carbon 30 days before secondary immunization (several days after primary RBC injection) had no significant alteration in their response, as compared to controls.

Number of 7S IgG antibody producing cells

The indirect enhancement procedure was used to determine the number of cells secreting low efficiency 7S IgG antibody. Treatment of plates with rabbit anti-mouse immunoglobulin serum resulted in additional antibody plaques in plates containing spleen cells from normal control mice receiving a secondary injection of red blood cells. As seen in Fig. 5, there was a rapid appearance of such additional plaques in spleens of control mice, with a peak occurring 4–5 days after injection of $2{\cdot}5\times10^8$ RBCs. Most control mice had approximately 500 such 7S PFCs at the time of secondary injection. This number increased to several hundred thousand by the 4th day. The rise in the number of PFCs was much more rapid than that which occurred in normal non-primed animals receiving only a single injection of the same concentration of red blood cells (Sabet *et al.*, 1968).

Experimental animals injected with carbon 1 day before secondary immunization also had an increased number of IgG PFCs. These mice often had a more rapid appearance of such PFCs during the first 2 days, as compared to controls, but the peak number, which occurred on the 4th to 6th day, was generally lower (Fig. 5). Animals injected with carbon

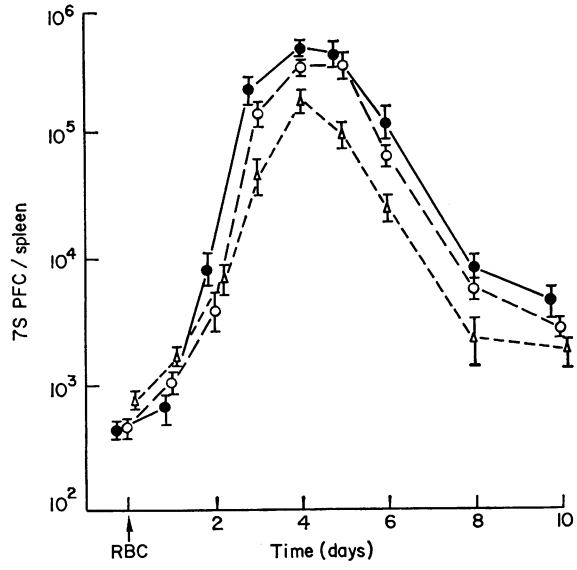

FIG. 5. Effect of Pelikan carbon (10 mg/mouse i.p.) on appearance of 7S IgG PFCs (indirect or enhanced plaques) in spleens of mice receiving a secondary injection of sheep erythrocytes 4 weeks after primary immunization. Primary and secondary doses were $2{\cdot}5\times10^8$ RBCs each. ●, Normal control mice; ○, mice injected with carbon same time as immunization; △, mice injected with carbon 1 day before immunization. Each point represents average results of five or more mice.

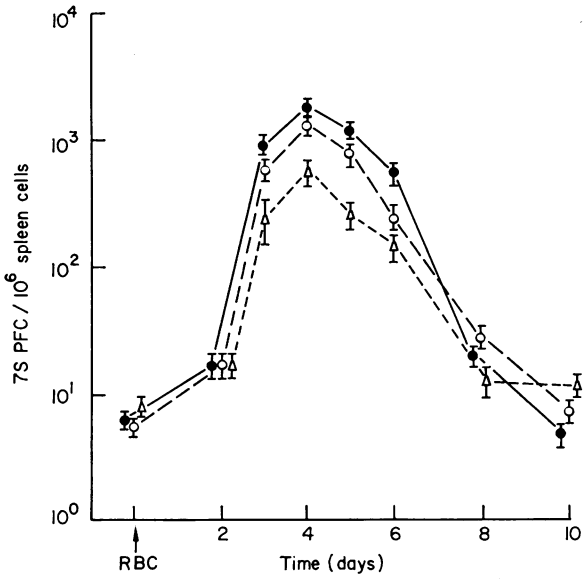

FIG. 6. Effect of Pelikan carbon (10 mg/mouse i.p.) on the number of 7S IgG PFCs/10^6 spleen cells in mice receiving a secondary injection of sheep RBCs 4 weeks after primary immunization. Primary and secondary dose $2{\cdot}5 \times 10^8$ RBCs. ●, Normal control mice; ○, mice injected with carbon same day as secondary immunization; △, mice injected with carbon 1 day before immunization. Each point represents average results of five or more mice.

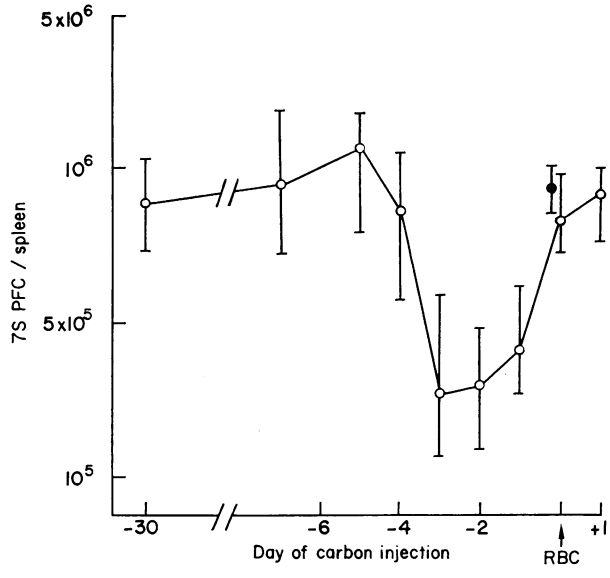

FIG. 7. Effect of time of injection of Pelikan carbon (10 mg/mouse i.p.) relative to day of secondary RBC injection on the number of enhanced 7S IgG PFCs 4 days after immunization with $2{\cdot}5 \times 10^8$ RBCs. All mice primed with the same dose of RBCs 30 days earlier. ●, Control mice not injected with carbon. Each point represents average of three to five mice.

the same day as RBC immunization responded essentially the same as control animals. There was only a slight depression of the number of IgG PFCs per spleen the 4th day after immunization. The number of such PFCs often decreased more rapidly than in control animals by the 6th to 10th day.

Calculation of the number of IgG PFCs/10^6 spleen cells revealed little difference between control and experimental mice injected with carbon the same day as immunization (Fig. 6). Mice injected with carbon 1 day before red blood cells responded essentially the same as controls during the first 2 days, but then had consistently fewer PFCs per million spleen cells tested during the following 6 days.

The greatest suppression of IgG PFCs, as calculated per spleen, occurred in mice treated with carbon 2–3 days prior to secondary immunization (Fig. 7). Animals injected 4–5 days before immunization had little or no significant decrease in the number of IgG PFCs/10^6 spleen cells, as compared to controls. Injection of carbon 7 days or longer before secondary immunization also had no effect.

Serum antibody responses

Serum titres to sheep erythrocytes were determined for all mice tested for antibody plaque formation. As can be seen from Table 1, most of the antibody activity in these sera was resistant to 2-ME treatment. However, there was a marked variation in the mean titres between the various groups of primed mice which were either untreated or injected with carbon at various times before secondary immunization. Whereas control mice had

TABLE 1

EFFECT OF PELIKAN CARBON (10 mg/MOUSE) ON PEAK SERUM HAEMAGGLUTININ AND HAEMOLYSIN TITRES OF MICE AFTER SECONDARY IMMUNIZATION WITH SHEEP ERYTHROCYTES

Day of carbon injection*	Peak serum titre ($\log_2$)†			
	Haemagglutinin		Haemolysin	
	No 2-ME	Plus 2-ME	No 2-ME	Plus 2-ME
+1	10·3	10·1	12·9	12·2
0	9·5	8·1	10·3	9·1
−1	8·9	6·5	8·3	7·8
−2	8·3	5·1	7·1	6·3
−3	7·9	6·2	7·3	5·9
−4	8·9	5·9	9·8	6·8
−6	10·5	8·6	12·3	10·2
−8	12·8	11·9	13·9	11·5
None	11·9	11·4	13·5	12·6

* Mice injected with carbon i.p. on day indicated relative to day of immunization with $2{\cdot}5 \times 10^8$ RBCs 4 weeks after primary immunization with the same dose of red cells.

† Mean titre of four to six mice per group 5–8 days after secondary immunization; No 2-ME indicates total 19S and 7S antibody titres; plus 2-ME indicates 7S antibody activity only.

relatively high titres, generally between 1 : 1024 to 1 : 4096, many of the carbon treated animals had lower titres. For example, mice treated with carbon 1–3 days before secondary immunization generally had titres less than 1 : 256. The lowest titres occurred in mice treated with carbon 3 days before secondary immunization. There was a lesser suppression when carbon was injected 4 or 6 days before red blood cells. Mice treated 1 week or longer before immunization had no significant suppression. Although haemolysin titres were generally higher than the haemagglutinin titres, the results obtained with either serological procedure indicated similar differences. The haemolysin activity of sera from carbon

treated mice, generally was more susceptible to 2-ME treatment, as compared to controls. Sera from animals treated with carbon 1–4 days before immunization had the least 2-ME resistant antibody.

The effect of carbon treatment after secondary immunization

As can be seen in Figs. 4 and 7 and Table 1, injection of carbon 1 day after secondary immunization had little or no effect on either the 7S or 19S PFC response. There was also little effect on the serum haemolysin or haemagglutinin response. In general, the level of the immune response was similar for control and experimental groups. On occasion some experimental animals had higher plaque counts, but this was not consistent. When carbon was injected 2 or 3 days after secondary immunization, the number of PFCs, either 7S or 19S, determined on the 4th day was occasionally depressed. However, this appeared to be due to technical artifacts.

Effect of antigen dose on the secondary immune response in carbon treated animals

In further experiments, groups of primed mice were injected with 10 mg carbon 24 hours

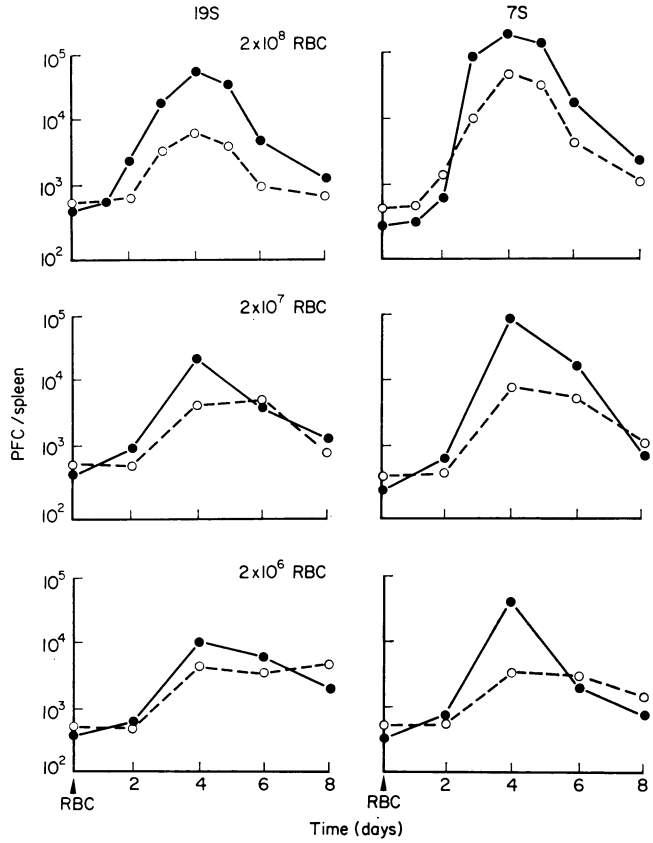

FIG. 8. Effect of Pelikan carbon (10 mg/mouse i.p.) on the 19S and 7S PFC response in spleens of mice receiving a secondary injection of either 2×10^6, 2×10^7 or 2×10^8 RBCs 4 weeks after primary immunization with $2{\cdot}5 \times 10^8$ RBCs. ●, Normal control mice; ○, mice treated with carbon 1–2 days before secondary immunization. Each point represents PFC response of spleens pooled from three to four mice.

prior to secondary immunization with 2×10^6, 2×10^7 or 2×10^8 red blood cells. All of the mice had been injected with $2{\cdot}5\times10^8$ RBCs 1 month previously. The control animals responded vigorously with both 19S and 7S PFCs (Fig. 8). Mice injected with the largest concentration of RBCs had, in general, the largest 7S and 19S PFC response, as compared to animals injected with the smaller antigen doses. Carbon treatment had similar effects on the immune responses of all three groups. In general, the 19S PFC response was less suppressed than the 7S response in the mice injected with the two lower RBC doses. However, it should be noted that the total peak PFC response was generally less than 10,000 in spleens of control animals receiving the lowest dose of red cells.

Effect of carbon concentration on the secondary PFC response

In additional experiments groups of primed mice were injected with varying concentrations of carbon 24 hours prior to a secondary injection of $2{\cdot}5\times10^8$ RBCs. As can be seen from Table 2, maximum suppression of both 19S and 7S PFCs occurred in animals receiving 10–30 mg carbon. Five milligrams of carbon had little effect. Serum titres were most suppressed when mice were injected with the highest concentration of carbon.

TABLE 2

EFFECT OF CARBON CONCENTRATION ON PEAK NUMBER OF SPLENIC PFCs AND SERUM ANTIBODY RESPONSE IN MICE RECEIVING A SECOND INJECTION OF RBCs

Carbon concentration* (mg)	Peak PFC response ($\times10^5$)				Peak serum titre ($\log_2$)	
	19S	Per cent of control	7S	Per cent of control	No 2-ME	+ 2-ME
None (saline)	1·62	–	4·65	–	12·8	12·3
5·0	1·15	71·0	4·68	100·6	11·9	10·1
10·0	0·46	28·4	2·56	55·1	8·5	4·5
20·0	0·32	19·8	1·31	28·2	7·9	4·8
30·0	0·52	32·1	1·28	27·5	8·8	5·3

* Mice injected i.p. with indicated concentration of carbon 1 day prior to secondary injection of $2{\cdot}5\times10^8$ RBC 4 weeks after primary injection of same dose of RBCs. Groups of three to five mice assayed per point.

Response of carbon treated mice to unrelated antigens

In order to test the specificity of 'memory' suppression, mice were treated with carbon 1 day before a primary injection of sheep erythrocytes, and then challenged with both sheep and chicken erythrocytes 4 weeks later. The peak PFC response to both red blood cells occurred 4 days later, with essentially no enhanced PFCs detected during the first 5 days. The response to both red blood cells was essentially similar, indicating that the mice had reacted to both antigens in a similar manner. In an additional experiment, mice were primed with both sheep and chicken erythrocytes and challenged 4 weeks later either with: (1) chicken RBCs 2 days before carbon treatment, or (2) with sheep RBCs 1 day after carbon treatment. Immunosuppression occurred only to the sheep RBCs. There was a vigorous 7S and 19S PFC response to the chicken RBCs. The carbon treated animals had 20–60 per cent fewer PFCs to sheep red cells than did the controls.

DISCUSSION

The primary immune response to sheep erythrocytes is characterized by the appearance of many 19S IgM PFCs with peak numbers in the spleen 4–5 days after immunization. Few, if any, 7S IgG PFCs appear until the 6th to the 10th day. Humoral antibodies

(haemagglutinins and haemolysins) appear in the serum with a peak at 7 days following immunization. These antibodies are 2-ME sensitive. Such mice, if challenged 4 weeks later with the same antigen, would respond with a vigorous secondary type immune response characterized by the appearance of large numbers of both 19S IgM and 7S IgG PFCs in their spleens. There is also a rapid increase in the level of serum haemagglutinins and haemolysins, most of which is resistant to 2-ME treatment. In contrast, when mice were primed with sheep red cells after a single injection of carbon, there was an apparent failure of development of immunological memory, as assessed after challenge immunization. Injection of such mice with sheep red cells 4 weeks after primary immunization resulted in a relatively normal IgM PFCs response, but a marked delay in appearance of IgG PFCs. Such a response was similar to that which occurred normally in non-primed mice receiving a single inoculum of red blood cells. These results indicate that 'memory' cells, if they exist, do not develop in mice primed with RBCs after RES blockade with carbon. It seems plausible that inhibition of the initial steps of the immune response by RES blockade not only suppressed the primary response, but also suppressed development of immunological 'memory' characterized by 7S antibody formation. Since essentially normal numbers of 19S PFCs, as well as 2-ME sensitive serum antibody, appeared after challenge injection of the mice treated with carbon before primary immunization, it is likely that the animals were fully capable of a primary type response, but did not have the necessary 'memory' component for formation of IgG PFCs, as occurs in normal primed animals.

It should be pointed out that absence of a secondary type response in this system could be due to lack of a humoral rather than a cellular factor. Various studies have indicated that a critical level of serum antibody may be necessary for opsonization or 'processing' of particulate antigens (Rowley, 1962; Nossal, 1967). Development of IgG antibody forming capacity in experimental animals may depend wholly or in part on the presence of an optimum level of serum opsonins, rather than specific numbers of antibody producing cells or their precursors. It seems probable that injection of carbon prior to primary immunization suppressed the immune response so that sufficient antibody was not present at the time of secondary immunization to permit adequate opsonization of the RBCs, possibly a necessary step for stimulation of IgG PFC formation. The effect of RES blockade with carbon treatment on the secondary response was studied in mice primed with sheep red blood cells and then challenged 4 weeks later with the same antigen. In contrast to controls, primed mice injected with carbon the same time or a few days before secondary immunization had a suppressed immune response to the red blood cells, both on the cellular and humoral levels. The number of 19S IgM and 7S PFCs was significantly reduced in spleens of animals treated with carbon 1–2 days before secondary immunization. The time of carbon administration is very important since less suppression occurred when carbon was injected the same day as the red blood cells. There was no significant effect when carbon was administered either 1 day after, or 5–7 days or longer before secondary immunization.

It seems of interest that a secondary type PFC response occurred in all carbon pretreated animals. The major difference between treated and control mice was the lower degree of responsiveness.

The mechanism whereby carbon blockade affects the cytokinetics of the secondary response, as well as the primary response, is not clear. It should be noted, however, that other investigators have previously indicated that multiple injections of substances such as

polyvinyl pyrrolidone, oil emulsions or thorotrast, as well as carbon, can suppress the humoral immune response of animals to single or multiple injections of antigen. Recent experiments by Cruchaud (1968) have indicated that 'overloading' of the RES with India ink, dextran 200, gelatin or saccharated iron oxide can also suppress the immune response of rabbits to bovine serum albumin. It was found that carbon or colloidal iron readily caused a decrease in the secondary serum antibody response in some of the tested rabbits. Specific immunological tolerance, as indicated by a lack of detectable antibody response, was also observed in a few rabbits. Although the blockading agents had no detectable effect on localization of antigen, Cruchaud postulated that antigen administered to RES suppressed animals may react directly with potential antibody forming cells, rather than intermediary phagocytic cells. As suggested by Nossal (1967), such contact may result in immunological tolerance, rather than antibody formation.

Experiments concerning immunological 'memory' suggest that classic immunological tolerance does not occur in mice treated with carbon prior to initial immunization. If immunological tolerance had occurred, the animals should have been completely incapable of responding to sheep red cells following challenge immunization. If only partial tolerance had been induced, it would be expected that small numbers of both 7S and 19S antibody forming cells would appear. However, the results of those experiments indicate that only 'memory' did not develop in carbon treated animals, since such mice responded relatively similarly to normal animals receiving a single primary injection of red cells.

It is of interest to note that a few investigators have reported that injection of blockading agents into experimental animals stimulates, rather than suppresses antibody responses. Such conflicting results may be due to differences in the method of administration of blockading agents, in relation to antigen, as well as the nature of the antigen and the procedures used for detecting serum antibody. For example, Fisher (1966) immunized animals with sheep red cell stroma, rather than whole red blood cells. Control animals injected with stroma alone had very low haemagglutinin titres. When such animals were pre-treated with multiple inoculations of carbon, the serum titres were often twice as high as for the controls. However, all of the titres were quite low as compared to the much higher titres generally observed in animals injected with red blood cells suspensions. Similarly, experiments with thorotrast treated rabbits indicated that the agglutinins to *Salmonella* vaccine were suppressed during the first few weeks after multiple injections of the antigen, but then became enhanced after further treatment.

A number of theories, as well as experiments, concerning antibody formation and secondary responses have suggested that antibody stem cells in primed animals either differentiate or proliferate into additional antibody producing cells and into 'memory' cells (Nossal, 1967). Since treatment of RBC primed mice with carbon shortly before a second inoculation of erythrocytes resulted in only a moderate suppression of the secondary antibody response, both on the cellular and humoral levels, it appears that only a portion of the expected secondary response can be suppressed by RBC blockade. This may happen because less antigen is 'processed' and thus fewer antigenic determinants become available to stimulate either committed or uncommitted cells. It seems plausible that RES blockade could result in an inhibition of adequate uptake or processing of antigen. Thus inoculation of $2{\cdot}5 \times 10^8$ into primed mice treated with carbon may actually be similar to injection of a much lower dose of red cells. As shown in experiments concerning antigen dose, a significantly lower immune response occurred when one-hundredth the optimum immunizing dose was used. If carbon treatment impairs antigen processing, it seems plausible that

there may be a competition for a limited number of phagocytic cells between the erythrocytes and carbon particles. If most of the available macrophages had already ingested carbon particles, or if postulated antigen-receptor sites on the macrophage surface were occupied non-specifically by carbon particles, there would be less than optimum uptake of antigen, leading to suppressed response. It is also possible that some of the antigen could bypass the 'blockaded' macrophages and react directly with lymphocytes. If this occurred, immunological tolerance might occur, as indicated earlier.

Additional experiments in progress concerning tissue culture systems, as well as cell transfer models, may reveal further information concerning the possible mechanism involved in depression of both the primary and secondary immune response of mice treated with carbon as an RES blockading agent.

ACKNOWLEDGMENTS

The capable technical assistance of Mrs Carol Newlin, Mrs Leoney Mills and Miss Joyce Jenderowski during various portions of this study is acknowledged.

This work was supported in part by research grants from the National Science Foundation (GB3278), The American Cancer Society (T328) and United States Public Health Service Special Fellowship No. 1-F3-AM-21, 827-02 to T.Y.S.

REFERENCES

ARGYRIS, B. F. (1967). 'Role of macrophages in antibody production. Immune response to sheep erythrocytes.' *J. Immunol.*, **99**, 744.

ASKONAS, B. A. and RHODES, I. (1965). 'Immunogenicity of antigen-containing RNA preparation from macrophages.' *Nature (Lond.)*, **205**, 470.

BERENBAUM, M. C. (1964). 'Effects of carcinogens on immune process.' *Brit. med. Bull.*, **20**, 159.

CANON, P. R., BAER, R. B., SULIVAN, F. L. and WEBSTER, J. R. (1929). 'The influence of blockade and the reticuloendothelial system on the formation of antibodies.' *J. Immunol.*, **17**, 441.

CRUCHAUD, A. (1968). 'The effect of reticuloendothelial blockade on antibody formation and immunologic tolerance.' *Lab. Invest.*, **19**, 15.

DERBY, B. N. and ROGERS, D. E. J. (1961). 'Studies on bacterimia. The effect of simultaneous leukopenia and RE blockade on the early blood stream clearance of staphylococci and *E. coli*.' *J. exp. Med.*, **113**, 1953.

DRESSER, D. W. and WORTIS, H. H. (1965). 'A localized hemolysis in gel method for detection of cells producing 7S antibody.' *Nature (Lond.)*, **208**, 859.

DRUTZ, D. J., KOENIG, M. C. and ROGERS, D. E. (1967). 'Further observations on the mechanism of reticuloendothelial blockade.' *J. exp. Med.*, **126**, 1087.

FISHER, S. (1966). 'Stimulation of splenic antigen uptake and of antibody response in mice by India ink or other "blockading" agents.' *Immunology*, **11**, 127.

FISHMAN, M. and ADLER, F. L. (1963). 'Antibody formation initiated *in vitro*. III. Antibody formation in X-irradiated recipients of diffusion chambers containing nucleic acid derived from macrophages incubated with antigen.' *J. exp. Med.*, **117**, 595.

FREI, P. C., BENACERRAF, B. and THORBECKE, C. J. (1965). 'Phagocytosis of the antigen, a critical step in the induction of the primary response.' *Proc. nat. Acad. Sci. (Wash.)*, **93**, 20.

FRIEDMAN, H. (1964). 'Distribution of antibody plaque forming cells in various tissues of several strains of mice injected with sheep erythrocytes.' *Proc. Soc. exp. Biol. (N.Y.)*, **117**, 526.

FRIEDMAN, H. P., STAVITSKY, A. B. and SOLOMONS, J. M. (1965). 'Induction *in vitro* of antibody to phage T_2-antigen in the RNA extracts employed.' *Science*, **149**, 1106.

GABRIELSON, A. E. and GOOD, R. A. (1967). 'Chemical suppression of adoptive immunity.' *Advanc. Immunol.*, **6**, 92.

GAY, F. P. and CLARK, A. R. (1924). 'The reticuloendothelial spleens in relation to antibody formation.' *J. Amer. med. Ass.*, **83**, 1296.

GALLILY, R. and FELDMAN, M. (1967). 'The role of macrophages in the induction of antibody formation in X-irradiated animals.' *Immunology*, **12**, 197.

HIRSCH, J. G. (1965). 'Phagocytosis.' *Ann Rev. Microbiol.*, **19**, 335.

JENKIN, C. R., AUZIN, I. and READE, P. P. (1965). 'The synthesis of macroglobulin antibody to a bacterial antigen in mice after treatment with thorotrast.' *Aust. J. exp. biol. Med. Sci.*, **43**, 607.

JERNE, N. K., NORDIN, A. A. and HENRY, C. (1963). 'The agar plaque technique for recognizing antibody-producing cells.' *Cell-Bound Antibodies* (Ed. by H. Koprowski and B. Amos), p. 109. Wistar Institute Press, Philadelphia.

KOENIG, M. G., HEYSELL, R. M., MILLY, M. A. and ROGERS, D. E. (1965). 'The dynamics of reticuloendothelial blockade.' *J. exp. Med.*, **122**, 117.

LEWIS, L. A. (1954). 'Effect of repeated injections of thorotrast on antibody formation.' *Amer. J. Physiol.*, **179**, 285.

NOSSAL, G. J. V. (1967). 'Mechanisms of antibody production.' *Ann. Rev. Med.*, **18**, 81.

PERKINS, E. H. and MAKINODAN, T. (1965). 'The suppressive role of mouse peritoneal exudate phagocytes on immune responses.' *J. Immunol.*, **94**, 765.

PLOTZ, P. H., TALAL, N. and ASOFSKY, R. (1968). 'Assignment of direct and facilitated hemolytic plaques in mice to specific immunoglobulin doses.' *J. Immunol.*, **100**, 744.

ROWLEY, D. (1962). 'Phagocytosis.' *Advanc. Immunol.*, **2**, 241.

SABET, T., NEWLIN, C. and FRIEDMAN, H. (1968). 'The effect of RES blockade on cellular antibody formation to sheep erythrocytes.' *Proc. Soc. exp. Biol. (N.Y.)*, **128**, 274.

SABET, T., NEWLIN, C. and FRIEDMAN, H. (1969). 'Effects of RES "blockade" on antibody formation. I. Suppressed cellular and humoral hemolysin responses in mice injected with carbon particles.' *Immunology*, **16**, 433.

SCHWARTZ, R. S. (1965). 'Immunosuppressive drugs.' *Progr. Allergy*, **9**, 246.

SCHWARTZ, R. S. and DAMASHEK, W. (1963). 'The role of antigen dose in drug-induced immunologic tolerance.' *J. Immunol.*, **90**, 703.

STERN, K., SPENCER, K. and FARQUAR, M. (1955). 'Effect of polyvinyl pyrrolidine on immune responses in mice.' *Proc. Soc. exp. Biol. (N.Y.)*, **89**, 126.

STERZL, J. and RIHA, J. (1965). 'A localized hemolysis in gel method for the detection of cells producing 7S antibody.' *Nature (Lond.)*, **208**, 859.

THORBECKE, G. J. and BENACERRAF, B. (1962). 'The RES in immunological phenomenon.' *Progr. Allergy*, **6**, 559.

WOOD, W. E. (1960). 'Phagocytosis with particular reference to encapsulated bacteria.' *Bact. Rev.*, **24**, 41.

WORTIS, H. H., TAYLOR, R. B. and DRESSER, D. W. (1966). 'Antibody production studied by means of LHG Assay. I. The splenic response of CBA mice to sheep erythrocytes.' *Immunology*, **10**, 603.