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The Effect of Colloidal Carbon on the Organ Distribution of Sheep Red Cells and the Immune Response

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Summary. The organ distribution, blood clearance rate, and immune response to intravenous ^{51}Cr-labelled sheep red blood cells (SRBC) have been studied in mice, following administration of a single intravenous dose of colloidal carbon. In normal mice 80–90 per cent of SRBC were found in the liver and 2–6 per cent in the spleen. The blood clearance rate of SRBC was extremely rapid. Colloidal carbon caused a marked depression of hepatic uptake of SRBC and a corresponding increase in splenic uptake. This effect was maximal at 6 hours after carbon administration and recovery of hepatic phagocytosis occurred over 4 days. The rate of clearance of SRBC was greatly reduced while the hepatic uptake of red cells was depressed. At low doses of cells there was an increase in titre of humoral antibody and in numbers of spleen PFCs while, at high doses of cells, a slight depression in immune response occurred.

'Stimulation' of splenic uptake of antigen and of the immune response by colloidal carbon is associated with depression of hepatic phagocytosis.

INTRODUCTION

The activity of the reticuloendothelial system (RES) has been widely studied, using the rates of disappearance of particulate material from the blood as a measure of phagocytic ability (Benacerraf, Halpern, Biozzi and Benos, 1954; Biozzi and Stiffel, 1965; Benacerraf, 1964). These particles have usually been administered in 'blocking' doses, that is, quantities sufficient to cause a sustained blood concentration over a considerable period of time.

The relationship between RES 'blockade' on the one hand, and the immune response on the other, is far from clear. Fisher (1966) has found a stimulation of splenic uptake of red cell stroma and of antibody production after intravenous administration of a variety of particles, including colloidal carbon, and similar results have been reported by Jenkin, Auzin and Reade (1955) after giving thorotrast. In contrast, Stern, Spencer and Farquar (1955) found depressed humoral antibody production after polyvinylpyrrolidone particles. Sabet, Newlin and Friedman (1969) have also observed suppression of antibody production after the intraperitoneal administration of carbon and sheep red cells.

The studies reported in this paper describe the distribution of sheep red blood cells in the reticuloendothelial system of mice after a single intravenous 'blocking' dose of carbon, and the effects of this alteration in distribution on the subsequent immune response.

MATERIALS AND METHODS

Animals

Outbred, male Porton mice, 6–10-weeks old, weighing approximately 30 g were used. Groups of 4–6 mice were used in each experiment.

Antigen

Sheep red blood cells (SRBC) in Alsever's solution (Burroughs Wellcome) were washed three times in isotonic barbitone-buffered saline and suspended to the desired concentration prior to intravenous injection.

^{51}Cr-labelling

For clearance studies, and for studies of organ distribution, 5 ml SRBC in Alsever's solution were incubated with 100 μc ^{51}Cr (Sodium Chromate B.P., Radiochemical Centre, Amersham) for 45 minutes at 37°. The cells were washed three times in barbitone-buffered saline and used within 2 hours. Less than 2 per cent of the radioactivity of the suspension was present in the supernatant at the time of injection.

RES Blockade

Pelikan carbon particles (C11/1431a, Günther Wagner, Hanover, Germany) were suspended at various concentrations in 1 per cent gelatin at pH 7·4 as described by Slivič (1970). For blockade, a single intravenous injection of carbon suspension (16 mg/ml) at a dose of 0·1 ml/10 g body weight was given.

Clearance studies

For studies of the clearance of ^{51}Cr-labelled SRBC from the blood, the animals were anaesthetized lightly with Nembutal prior to the injection of the cells. After the injection they were bled from the retro-orbital plexus every 10–15 seconds during the first minute, and thereafter at 2, 5, 10 and 15 minutes. Each sample was taken directly into a 20 μl break-off haematocrit tube (Harshaw Chemicals Ltd.) and radioactivity measured in a Nuclear Enterprises gamma counter.

Organ distribution of red cells

At various time intervals after the intravenous injection of ^{51}Cr-labelled SRBC, animals were killed and the whole organs removed and radioactivity measured. The interval between injection and death was 2 hours unless otherwise stated.

Measurement of antibody

Haemagglutinating antibody was measured 5 days after immunization, by a haemagglutination technique (Mowbray, 1966). Results are expressed as the reciprocal of the highest dilution causing haemagglutination.

Antibody-forming cells in spleen

Five days after immunization, the number of cells in the spleen producing direct haemolytic plaques against sheep red blood cells was measured. The method used was that of Jerne and Nordin (1963). Results are expressed in numbers of plaque-forming cells (PFCs) per spleen.

RESULTS

THE DISTRIBUTION OF ^{51}Cr SRBC INJECTED INTRAVENOUSLY IN NORMAL MICE

Two hours after the i.v. injection of SRBC, the majority of cells were found in the liver and a very much smaller number in the spleen, the proportion depending on the dose of cells injected. Table 1 shows that, at low doses of cells (4×10^6), 91 per cent were recovered from the liver while only 0·9 per cent could be found in the spleen. When very large doses were given (1×10^9), only 62 per cent were present in the liver while 6·8 per cent were found in the spleen. Similar results have been reported by Halpern, Biozzi, Benacerraf and Stiffel (1957). The remainder of the cells was found in small amounts in lung, bone marrow and gut.

THE EFFECT OF PRIOR ADMINISTRATION OF CARBON ON THE DISTRIBUTION OF ^{51}Cr SRBC

The intravenous injection of colloidal carbon prior to the injection of SRBC produced a striking alteration in the organ distribution of the cells. The degree of alteration depended on the interval between the administration of carbon and the red cells.

TABLE 1
LIVER AND SPLEEN DISTRIBUTION OF I.V. ^{51}Cr-LABELLED SHEEP RED BLOOD CELLS IN MICE

Number of cells	Liver Uptake		Spleen Uptake		Liver/spleen ratio
	% (±S.E.)	No. of cells $\times 10^{-6}$	% (±S.E.)	No. of cells $\times 10^{-6}$	
1×10^9	61·6±3·3	616	6·8 ±1·5	68	9·06
2×10^8	79·8±3·4	159·6	2·9 ±0·15	5·8	23·47
5×10^7	88·9±3·12	44·45	1·63±0·21	0·82	54·54
2×10^7	86·9±3·2	17·71	1·2 ±0·24	0·24	72·4
4×10^6	91·4±2·0	3·97	0·9 ±0·24	0·039	101·5

The distribution of i.v. ^{51}Cr-labelled SRBC in liver and spleen. Animals were killed 2 hours after injection. As the dose of SRBC increases the fraction taken up by the liver does not change until the highest dose, but the fraction taken up by the spleen increases significantly.

When SRBC were given 5 minutes after the carbon, a marked reduction in hepatic uptake of red cells was seen and a clear increase in spleen uptake. This effect was greater when the interval between the injections increased (Fig. 1). When 6 hours elapsed after the administration of carbon before the red cells were given, only 6 per cent of the cells were found in the liver while 36 per cent were taken up by the spleen. When the interval was 24 hours, the liver showed a return of phagocytic activity, and at 4 days the hepatic phagocytosis had returned to normal levels while the splenic uptake was greatly diminished, although still higher than control levels. Table 2 shows that this redistribution into the spleen occurred regardless of the dose of red cells which were given. Whereas with increasing doses of red cells in normal animals the ratio of liver to spleen uptake fell, after carbon blockade the ratio remained constant. The effect of the redistribution was such that as many red cells reached the spleen when 4×10^6 cells were given to a blockaded animal as when 5×10^7 cells were given to a normal animal. A similar organ distribution was found if the animal was killed at 2, 24 or 48 hours after injection of the red cells (Table 3). It will be noted that the total amount of radioactivity accounted for in the liver and spleen was approximately 90 per cent in the control animals given a dose of 5×10^7

cells and only 45 per cent in carbon treated animals. Table 4 shows that more radioactivity was found in bone marrow and lung in the carbon-blockaded animals. Other experiments showed that the same redistribution of radioactivity was observed whether 5, 10 or 15 mg of carbon were given. The injection of 1 per cent gelatin without carbon did not affect the distribution of radioactivity compared with normal animals.

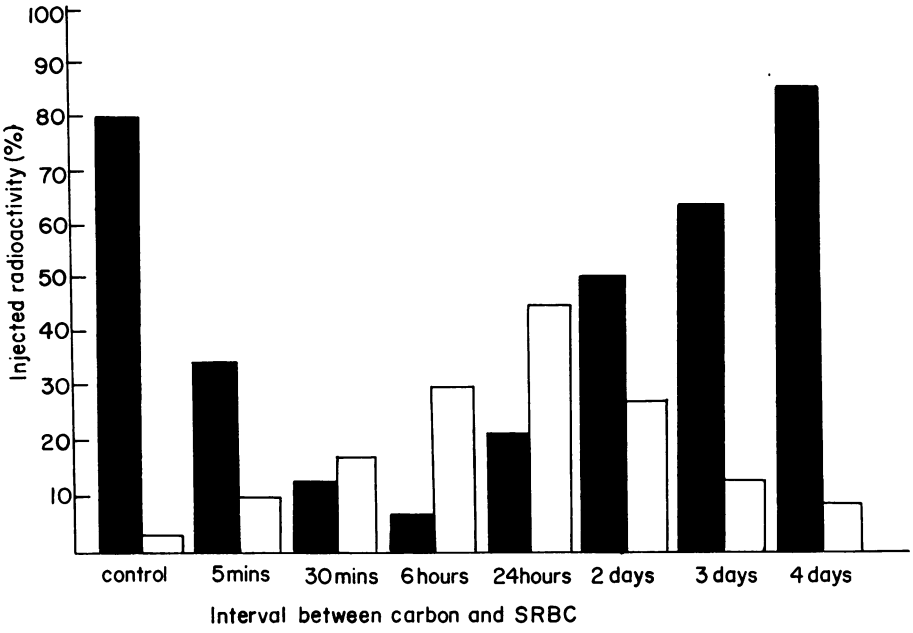

FIG. 1. The effect of 5 mg colloidal carbon given intravenously on the liver and spleen uptake of ^{51}Cr-labelled SRBC given i.v. The reduction in hepatic uptake and increase in splenic uptake begins 5 minutes after carbon injection. Hepatic uptake is at a minimum at 6 hours after carbon injection and splenic uptake is maximal at 24 hours. Solid columns, liver; open columns, spleen.

TABLE 2

LIVER AND SPLEEN DISTRIBUTION OF I.V. ^{51}Cr-LABELLED SHEEP RED BLOOD CELLS IN BLOCKADED MICE

Number of cells	Liver Uptake		Spleen Uptake		Liver/spleen ratio
	% (±S.E.)	No. of cells $\times 10^{-6}$	% (±S.E.)	No. of cells $\times 10^{-6}$	
2×10^8	6·26±1·8	12·5	29·6 ±7·26	59·2	0·21
5×10^7	9·83±1·6	4·92	35·7 ±5·26	17·89	0·28
2×10^7	11·5 ±1·51	2·35	25·14±9·02	5·2	0·46
4×10^6	9·36±1·44	0·42	24·79±3·12	1·08	0·39

The distribution of i.v. ^{51}Cr-labelled SRBC in liver and spleen after 5 mg of colloidal carbon had been given i.v. 6 hours previously. Animals were killed 2 hours after the injection of SRBC. Note that the hepatic uptake of cells is greatly diminished and the splenic uptake increased. The ratio of activity in liver and spleen does not depend on the dose of red cells.

THE CLEARANCE OF ^{51}Cr SRBC FROM THE BLOOD IN NORMAL AND CARBON-TREATED ANIMALS

The normal mice cleared SRBC from the circulation extremely rapidly and prolonged circulation of red cells (for more than 30 minutes) was only seen when very large doses

(1×10^9) were given. The carbon-treated animals showed a very much slower rate of clearance as shown in Fig. 2. The degree of impairment of clearance corresponded with the failure of hepatic uptake of cells. The slowest rates of clearance were found at 30 minutes and 6 hours after the administration of carbon, the clearance was returning towards normal at 24 hours, at a time when there was the beginning of recovery of hepatic uptake of red cells. These findings are in contrast to those reporting that the prior administration one of type of particle does not materially affect the rate of clearance of another type (Murray, 1963).

TABLE 3

PERCENTAGE DISTRIBUTION OF ^{51}Cr-LABELLED SHEEP BLOOD CELLS IN THE ORGANS OF NORMAL AND CARBON-TREATED ANIMALS AT VARIOUS TIMES AFTER INJECTION

Time after giving SRBC (2×10^8 cells)	Spleen		Liver		Lung	
	Normal	Carbon	Normal	Carbon	Normal	Carbon
2 hours	2·5	38·96	84	10·15	0·07	3·12
24 hours	2·95	43·27	79·7	12·38	0·067	0·93
48 hours	2·0	45·6	71·0	7·1	0·04	0·3

The distribution of i.v. ^{51}Cr-labelled SRBC in normal and carbon-treated mice. 5 mg of colloidal carbon was given 6 hours before SRBC. The animals were killed at various times after injection of SRBC. The proportion of cells in the liver, spleen and lungs did not change with time. Results are means of groups of four animals.

TABLE 4

PERCENTAGE DISTRIBUTION OF ^{51}Cr-LABELLED SHEEP RED BLOOD CELLS IN THE ORGANS OF NORMAL AND CARBON-TREATED ANIMALS*

Organ	Normal	Carbon-treated
Liver	71	7·1
Spleen	2·0	45·6
Lung	0·04	0·3
Lymph node	0	0
Gut	0·43	0·48
Bone marrow	0·17	4·0

* The distribution of i.v. ^{51}Cr-labelled SRBC (2×10^8) in the organs of normal and carbon-treated mice. 5 mg of colloidal carbon given 6 hours before SRBC, and animals were killed 48 hours after the injection of labelled red cells. Bone marrow was obtained from two femora. Note that both spleen and bone marrow uptake was increased in the carbon-treated animals.

THE EFFECT OF CARBON TREATMENT ON FORMATION OF SPLEEN PFCs AFTER IMMUNIZATION WITH SRBC

Fig. 3 shows the numbers of direct PFC/spleen in normal mice given various doses of red cells 5 days previously, and the effect of treatment with intravenous carbon 6 hours before the immunizing dose of cells.

The normal mice show an increasing number of PFCS in the spleen with doses of red cells up to 2×10^7, but at higher doses there is no increase, or even a slight reduction in number. As previously shown, the number of red cells taken up by the spleen increases with dose.

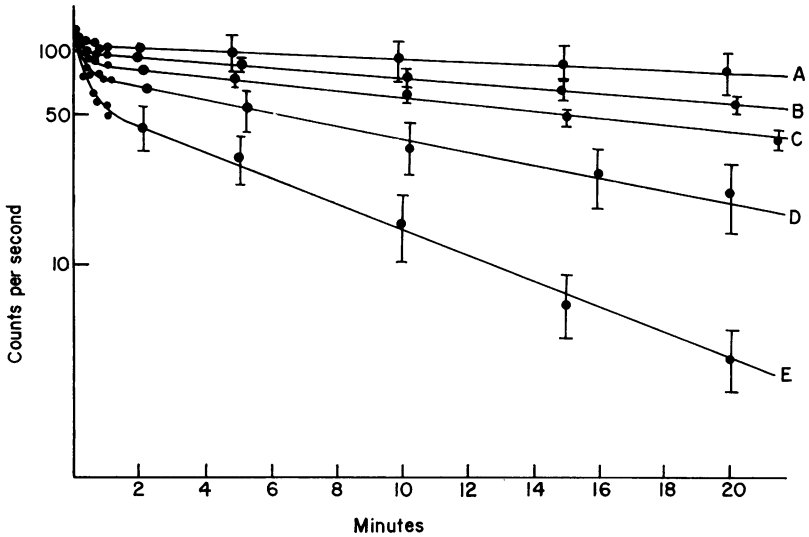

FIG. 2. The rate of clearance of ^{51}Cr-labelled SRBC from the blood in normal mice and mice given 5 mg colloidal carbon intravenously at various times before the injection of the cells. Clearance is significantly slower than normal 5 minutes after the carbon injection and maximal slowing is seen at 30 minutes. The clearance rate is returning towards normal at 24 hours. A, 30 minutes; B, 6 hours; C, 24 hours; D, 5 minutes; E, control.

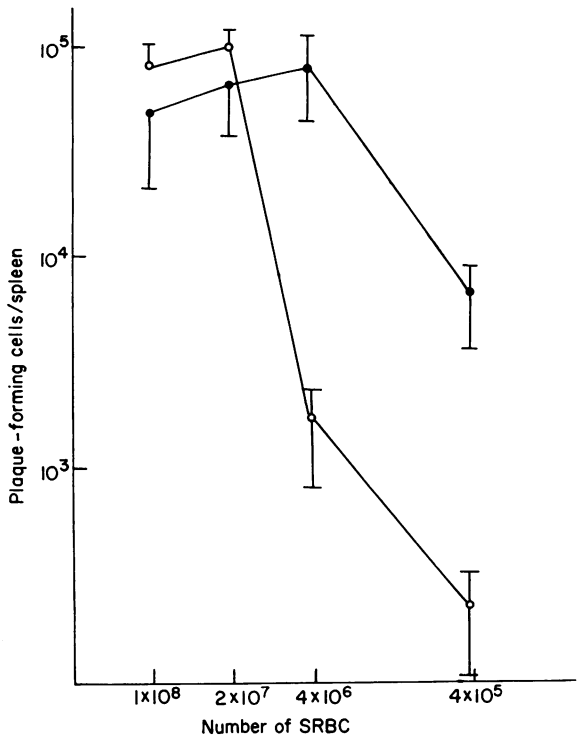

FIG. 3. The number of direct plaque-forming cells, 5 days after the injection of SRBC, in the spleens of normal mice, and in mice which had received 5 mg of colloidal carbon 6 hours before injection of red cells. There is an increase in the number of PFCs in the carbon-blockaded animals at low doses of SRBC, but not at high doses. ●, Carbon; ○, control.

The carbon-treated animals showed a marked increase in number of PFCs at low doses of cells, reaching a maximum when 4×10^6 cells were given, and with a tendency to fall at higher doses. The number of PFCs were the same in normal animals receiving 1×10^8 cells. The number of cells reaching the spleen under these two circumstances were approximately the same. Thus, the effect of carbon had been to cause a redistribution of SRBC into the spleen, which, when low doses of cells were given, resulted in a great increase in the number of PFC. At higher doses the numbers of PFCs fell to 60 per cent of control although this is associated with a greatly increased splenic uptake of cells.

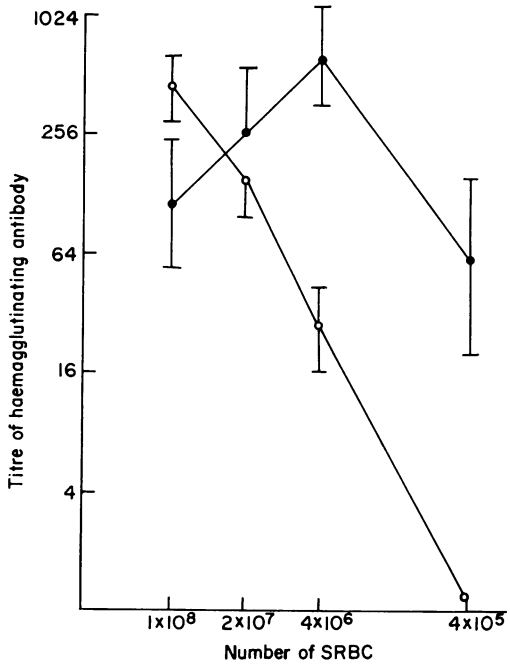

FIG. 4. The titre of haemagglutinating antibody 5 days after injection of SRBC in normal mice, and in mice which had received 5 mg of colloidal carbon 6 hours before the injection of red cells. At low dose of cells there is a higher titre of antibody in the carbon-treated group than in the controls, while at high doses of cells the carbon-blockaded animals show a slight reduction in antibody titre. ●, Carbon; ○, control.

THE EFFECT OF CARBON TREATMENT ON HUMORAL ANTIBODY PRODUCTION AFTER IMMUNIZATION WITH SRBC

Fig. 4 shows the titre of haemagglutinating antibody measured 5 days after immunization with varying doses of SRBC. Normal animals show a steady increase in amount of antibody produced as the dose increases. Animals given carbon 6 hours previously on the other hand showed a 'stimulation' of antibody production at lower doses of cells, reaching a maximum when 4×10^6 cells are given, and thereafter a slight diminution in response compared with controls. Thus, either a greatly increased or slightly decreased production of antibody could be seen, depending on the immunizing dose of red cells.

DISCUSSION

Studies of the immune response after the administration of particles to induce reticulo-endothelial blockade have given conflicting results. An increased splenic uptake and antibody production has been shown by Fisher (1966), who injected ^{131}I-labelled sheep red cell stroma intravenously after a single intravenous dose of carbon particles. He also observed maximal splenic uptake if the stroma was given 21–26 hours after the carbon. A similar increase in splenic uptake occurred after thorotrast, saccharated iron oxide and polystyrene particles. He interpreted his findings as showing a direct 'stimulation' of uptake of antigen into the spleen as a result of the blockading particles, but did not record hepatic uptake of antigen. Although there is an increase in spleen weight after treatment with carbon, this occurs over a period of several days. In the results reported here, the increased uptake of red cells occurs 5 minutes after the administration of carbon and is maximal at 6–24 hours. The reason for the increased uptake of antigen is probably that carbon has preferentially impaired hepatic phagocytosis, so that more antigen is available to the spleen. A similar increase in spleen uptake is observed when very high doses of red cells are given to normal animals. Associated with the depression of hepatic phagocytosis is a failure of rapid clearance of red cells from the blood stream, confirming that the liver has the major role in eliminating red cells injected intravenously. A similar increase in antibody production to a bacterial antigen and to sheep red cells has been reported by Jenkin *et al.* (1965) after thorotrast blockade.

The results reported here show that the change of proportions of red cell phagocytosis in the liver and spleen occurs whatever dose of red cells is given. The arrival of excess antigen in the spleen is accompanied by an increased number of plaque-forming cells in the spleen compared with controls when low doses of cells are given, and a corresponding increase in the titre of antibody. This can probably be accounted for by the increased number of cells which have been diverted to the spleen and it does not seem necessary to postulate a direct stimulating effect of carbon on splenic uptake.

At high doses of cells, on the other hand, there can be no increase in numbers of plaque-forming cells in the spleen, or in the titre of circulating antibody, since the spleen is maximally stimulated in normal mice without carbon treatment. On the contrary, there is a reduction both in numbers of plaque-forming cells and in humoral antibody titre in the carbon-treated animals given high doses of cells, although this is slight. It is suggested that the depression of the immune response seen in these circumstances is a consequence of the greatly increased amounts of antigen arriving at the site of antibody production, and is not due to interference of macrophage processing of antigen as a result of the carbon.

The clearest demonstration of a depressed immune response after carbon treatment comes from the work of Sabet *et al.* (1969). A very important difference from the present studies is that they gave both carbon and red cells intraperitoneally. Further, they found that labelled red cells injected under these circumstances were distributed to the liver and spleen in the same way whether or not carbon was given. However, only 30–40 per cent of the red cells were found in the liver and 1·5–3·0 per cent in the spleen, 55 per cent of the labelled antigen being unaccounted for. The results are quite unlike those described here where the carbon and red cells are given intravenously. It is possible that intraperitoneal carbon may alter both the organ distribution and the immunogenicity of red cells injected subsequently, and that the depression of the immune response seen in these circumstances is not due to a defect of antigen 'processing' in the spleen or lymph nodes. Support for

this view comes from the further finding of Chakrabarty and Friedman (1971) who showed that Freund's incomplete adjuvant given intraperitoneally also lowered the numbers of antibody-forming cells in the spleen after i.p. injection of sheep red cells.

Other reports of depressed immune responses after blockade of the reticuloendothelial system have come from Stern *et al.* (1955) but in these experiments both polyvinyl pyrrolidone used as a blocking agent and sheep red cells used as an antigen were given intraperitoneally. Only a slight reduction in antibody production was observed. Cruchaud (1968) gave repeated large doses of either carbon, dextran or saccharated iron oxide to rabbits. The blockading substances were given once or twice daily for 5 days before immunization and for 10–15 days afterwards. He observed a failure of antibody production to a first and second challenge with intravenous bovine serum albumin (BSA) and some animals showed evidence of induction of tolerance to BSA. It is possible that tolerance to a protein antigen such as BSA might have been induced in his experiments in a manner similar to the reduction in immune responses with high doses of sheep red cells reported in the present paper.

Lewis (1954) also observed a small reduction in antibody formation to intravenous TAB vaccine in rabbits treated with repeated intravenous doses of thorotrast. The animals showed widespread histological evidence of thorotrast toxicity and the depression of the immune response was observed only in the first 6 weeks of blockade. Gay and Clark (1924) observed a reduction in antibody response to intraperitoneal and intravenously administered sheep red cells after repeated intraperitoneal injections of trypan blue.

Reports to date, therefore, do not show that reticuloendothelial blockade leads to a reduction in the immune response unless the blockading agent is given frequently and in high dosage, or unless the blockading agent and antigen are given intraperitoneally. Stimulation of the immune response, on the other hand, can be accounted for by a diversion of antigen to the spleen as a result of blockade.

It is not clear why carbon preferentially depresses hepatic phagocytosis. Slivič (1969) has shown that the spleen takes up four times as much carbon as the liver per gram wet weight. The number of macrophages in the spleen per gram is far greater than in the liver, but the blood flow through the liver is much greater than that through the spleen. Hepatic phagocytes may be exposed to a greater local concentration of carbon and this may result in their inability to remove sheep red cells subsequently injected into the circulation.

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