Immunology 1979 **38** 577

Effect of stimulation and blockade of mononuclear phagocyte system on the delayed footpad reaction to SRBC in mice

Y. YOSHIKAI, S. MIAKE, T. MATSUMOTO K. NOMOTO & K. TAKEYA *Department of Microbiology and Department of Immunology, in Cancer Research Institute, School of Medicine, Kyushu University, Fukuoka, Japan*

Accepted for publication 14 June 1979

Summary. The delayed footpad reaction to sheep erythrocytes (SRBC) was studied in mice whose mononuclear phagocyte system (MPS) was blocked or stimulated. Colloidal carbon or carrageenan was used for the blockade of MPS and *Corynebacterium parvum* or diethylstilbestrol used for the stimulation. The optimal dose of SRBC for the induction of the delayed footpad reaction was lower in MPS-blocked mice than in non-treated mice, whereas a high dose of SRBC was required for the induction of the strongest delayed footpad reaction in MPS-stimulated mice. These results suggest that non-specific phagocytic function of MPS modulates subsequent immune responses after administration of antigens.

INTRODUCTION

Macrophages have functions not only as scavenger cells in the elimination of particulate substances (Metchnikoff, 1899) but also as accessory cells for antigen presentation in the induction of immune responses (Unanue, 1978). The relationship of these two functions, however, is not clear.

Antigenicity of sheep erythrocytes (SRBC) was reported to increase (Ptak, Pryjma & Moskalewski, 1974) or decrease (Gershon & Feldman, 1968; Perkins & Makinodan, 1965) after ingestion into macrophages in *in vitro* studies. In *in vivo* studies, stimulants or blockers of the mononuclear phagocyte system (MPS) have been used for determination of the role of macrophages in immune responses. It has been shown that MPS stimulation or blockade results in modification of antibody production to SRBC. Individual results, however, were variable according to each experimental model. MPS stimulation resulted in enhancement of antibody production in some experiments (Cutler, 1960; Wooles & Di Luzio, 1963; Watson & Šljivič, 1976) but in depression in other experiments (Franzl & McMaster, 1968; Sljivič & Warr, 1973; Ghaffar & Sigel, 1978). In addition, MPS blockade resulted in depression of antibody production in some experiments (Sabet, Newlin & Friedman, 1969; Rumjanek, Watson & Šljivič, 1977) but in enhancement in other experiments (Souhami, 1972; Turner & Higginbotham, 1977).

In this paper, a study is described of the relationships between phagocytic function of MPS and delayed footpad reaction against varying doses of SRBC in MPS-blocked or -stimulated mice. Non-specific phagocytic function of MPS appears to play an important role in the modification of subsequent immune responses after administration of SRBC. It may be ascribed to different contribution of macrophages as scavenger cells resulting in different doses of retaining SRBC and different periods of antigenic stimulation.

Correspondence: Dr. Yoshikai, Department of Microbiology, School of Medicine, Kyushu University, 3-1-1 Maidashi, Higashiku, Fukuoka City, Japan, 812.

0019-2805/79/1100-0577 $02.00

MATERIALS AND METHODS

Animals
Male mice of an inbred AKR strain were obtained from the Breeding Unit of Kyushu University. Ten-week-old mice were used for the experiments. Each experimental group consisted of six to eight mice and the same experimental protocols were repeated two or three times.

Antigens
SRBC in Alsever's solution were obtained commercially and kept at 4° until use. SRBC were washed three times with phosphate-buffered saline (saline) and suspended at desirable concentrations in saline.

MPS blockade
Pelican carbon particles (C11/1431a, Günther Wagner, Hanover, Germany) were suspended at 25 mg/ml in saline containing 1% gelatin as described elsewhere (Takeya, Shimotori, Taniguchi & Nomoto 1977) and 0·1 mg/10 g body weight was injected intravenously (i.v.) on day −1 and intraperitoneally (i.p) on day 0. Immunization was carried out on day 0.

Carrageenan Type II (Sigma) was dissolved in distilled water (5 mg/ml) and injected i.p. at a dose of 200 mg/kg 1 day before immunization.

MPS stimulation
A suspension of heat- and formalin-killed *Corynebacterium parvum* (IM1585, Institut Merieux 17 Lyon, France) containing 2 mg/ml dry weight of organisms was obtained commercially. 0·25 ml (0·5 mg) of this suspension was injected i.v. 7 days before immunization.

Diethylstilbestrol (DES, Iwai Kagaku Yakuhin, Japan) was dissolved in peanut oil and administered subcutaneously (s.c.) as a single injection. Mice received 1 mg in oil 3 days before immunization.

Carbon clearance
The clearance rate of colloidal carbon was measured according to Biozzi, Benacerraf & Halpern (1953) and K values were used to express MPS phagocytic function.

Immunization
Varying numbers of SRBC in saline were injected i.p. or into the footpad.

Assay of footpad reaction
For elicitation of delayed footpad reaction, 1×10^8 SRBC in 50 μl of saline were injected into the left hind footpad 4 days after immunization. The right hind footpad was injected with 50 μl of saline as control. Swelling of these footpads was measured 24 h later with a dial-thickness gauge. The degree of reactions was expressed as the difference in thickness between the right and left hind footpad.

Assay of antibody titres
Blood specimens were obtained 4 days after immunization with SRBC from the retro-orbital venous plexus. Haemagglutinin (HA) titres against SRBC were assessed by a microtitration method. HA titres were expressed in $\log_2$ units.

Treatment with cyclophosphamide
Cyclophosphamide (CY, Endoxan, Shionogi) was dissolved in distilled water at a concentration of 10 mg/ml and injected i.p. at a dose of 200 mg/kg 3 days before immunization.

Organ distribution of SRBC
Mice were injected i.p. with [^{51}Cr]-labelled SRBC (2×10^8) and counts of radioactivity in the spleen and liver were measured 24 h after injection (Warr & Šljivič, 1974a).

Statistics
The statistical significance of the data was determined by Student's t test. A P value of less than 0·05 was taken as significant.

RESULTS

Effect of MPS stimulation or blockade on carbon clearance

The phagocytic index K, which is thought to reflect the phagocytic capacity, was determined by carbon clearance test in mice injected with 1 mg of DES s.c. on day −3, 0·5 mg of *C. parvum* i.v. on day −7, 25 mg/100 g of colloidal carbon i.v. on day −1 or 5 mg of carrageenan i.p. on day −1 (Fig. 1). The timing of administration was determined for each reagent in preliminary experiments to obtain the maximal effects (data not shown). These results showed that K values were increased significantly by pre-treatment with DES or *C. parvum* $P < 0{\cdot}001$) and decreased significantly by pre-

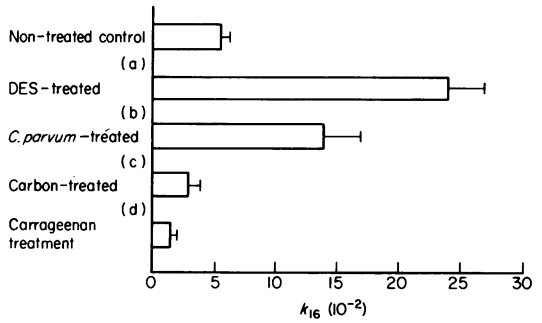

Figure 1. Blockade and stimulation of MPS by 1 mg of DES given s.c. on day −3, 0·5 mg of *C. parvum* given i.v. on day −7, 25 mg/100 g body weight colloidal carbon given i.v. on day −1 or 5 mg of carrageenan given i.p. on day −1. Phagocytic indices (K) were measured by the carbon clearance in different groups consisting of five mice. Horizontal bars represent standard errors. Difference from control was significant in (a) $P<0·001$, (b) $P<0·001$, (c) $P<0·01$, (d) $P<0·001$.

treatment with colloidal carbon ($P<0·01$) or carrageenan ($P<0·001$).

Effect of MPS blockade on the delayed footpad reaction

Colloidal carbon-treated and non-treated mice were immunized i.p. with 10^5, 10^6, 10^7 or 10^8 SRBC on day 0 and delayed footpad reaction was elicited on day 4. 10^7 SRBC induced maximal delayed footpad reaction in non-treated mice, but a lower dose, namely 10^6 SRBC, induced maximal delayed footpad reaction in carbon-treated mice ($P<0·005$; Fig. 2).

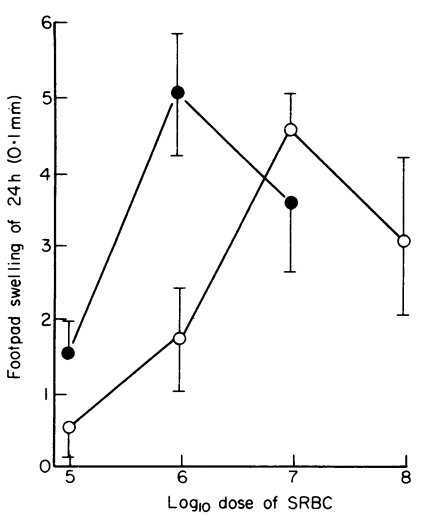

Figure 2. Effect of colloidal carbon on the delayed footpad reaction in mice immunized i.p. with varying doses of SRBC on day 0. Colloidal carbon was given i.v. on day −1 and i.p. on day 0. Footpad test was carried out on day 4. o, Non-treated control; •, carbon-treated. Vertical bars represent standard errors.

The effect of MPS blockade on the delayed footpad reaction was studied in carbon-treated, carrageenan-treated and non-treated mice after immunization via the footpad with 10^7, 10^8 or 10^9 SRBC. 10^8 SRBC induced maximal delayed footpad reaction in non-treated mice, but a lower dose of SRBC, namely 10^7 SRBC, induced a higher degree of delayed footpad reaction in carbon- or carrageenan-treated mice than in non-treated mice ($P<0·001$; Fig. 3a and b).

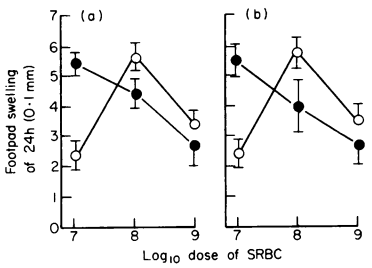

Figure 3. Effect of colloidal carbon or carrageenan on the delayed footpad reaction in mice immunized via the footpad with varying doses of SRBC on day 0. Colloidal carbon (25 mg/100 g) was given i.v. on day −1 and given i.p. on day 0. Carrageenan (5 mg) was given i.p. on day −1. The footpad test was carried out on day 4. (a) o, Non-treated control; •, carbon-treated (b) o, Non-treated control; •, carrageenan-treated. Vertical bars represent standard errors.

Effect of CY and colloidal carbon on the delayed footpad reaction

Suppression of the delayed footpad reaction occurs in non-treated mice when the immunizing dose of SRBC is increased and this suppression is reported to be CY-sensitive (Lagrange, Mackaness & Miller, 1974). When mice are pre-treated with CY to remove suppressor cells, the delayed footpad reaction may reflect the net generation of effector T cells responsible for the delayed footpad reaction. The relationship between various immunizing doses of SRBC and induction of the delayed footpad reaction was studied in CY-pre-treated or non-treated mice. Results showed that the delayed footpad reaction was augmented in an SRBC-dose dependent fashion in mice pre-treated with CY (Fig. 4). This result suggests that the net generation of effector T cells for the delayed footpad reaction can be assessed in CY-pre-treated mice. Therefore, the effect of MPS blockade with colloidal carbon on delayed footpad reaction was observed in CY-pre-treated mice (Fig. 5). Immunization with 10^7 SRBC induced a sig-

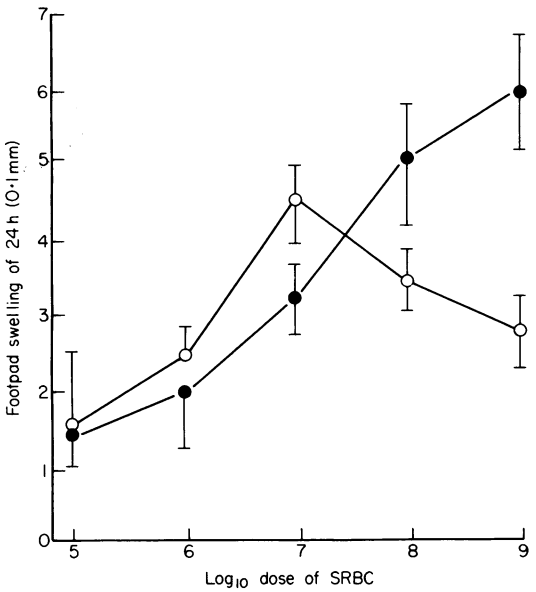

Figure 4. Effect of CY on the delayed footpad reaction in mice immunized i.p. with varying doses of SRBC on day 0. CY (200 mg/kg) was given i.p. on day −3. The footpad test was carried out on day 4. ○, Non-treated control; ●, CY-treated. Vertical bars represent standard errors.

nificantly higher degree of delayed footpad reaction in mice pre-treated with CY and colloidal carbon than in mice pre-treated with CY alone ($P < 0.02$).

Effect of MPS stimulation on the delayed footpad reaction

C. parvum-treated, DES-treated or non-treated mice were immunized i.p. with 10^6, 10^7, 10^8, 10^9 or 10^{10} SRBC on day 0 and the delayed footpad reaction was elicited on day 4. 10^7 SRBC induced maximal delayed footpad reaction in non-treated mice. Maximal reaction was induced with a higher dose of SRBC, namely 10^9 SRBC, in *C. parvum*- or DES-treated mice ($P < 0.005$; Fig. 6a and b).

Effect of colloidal carbon or DES on antibody production

Blood specimens were obtained from mice used in the former experiments just before footpad elicitation on day 4. In carbon-treated mice, increased antibody production was detected at low doses of SRBC ($P < 0.05$), whereas a slight depression was observed at high doses of SRBC ($P < 0.05$; Fig. 7a).

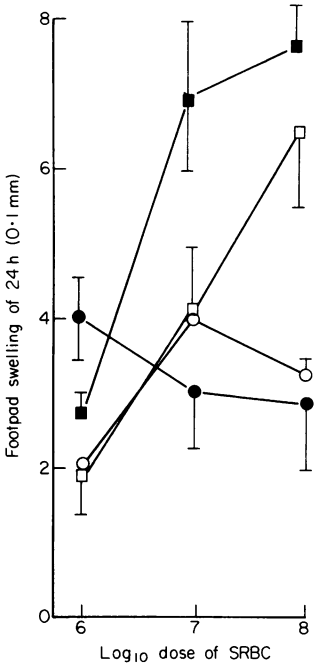

Figure 5. Additive effect of colloidal carbon and CY on the delayed footpad reaction in mice immunized i.p. with varying doses of SRBC on day 0. CY (200 mg/kg) was given i.p. on day −3. Colloidal carbon (25 mg/100 g) was given i.v. on day −1 and i.p. on day 0. Footpad test was carried out on day 4. ○, Non-treated control; ●, carbon-treated; □, CY-treated; ■, CY- and carbon-treated. Vertical bars represent standard errors.

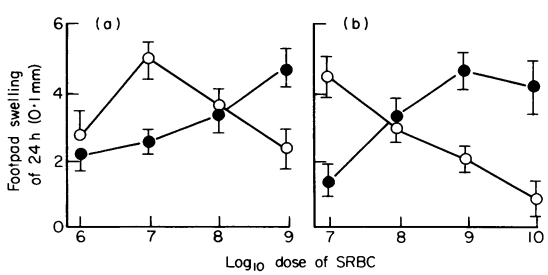

Figure 6. Effect of *C. parvum* or DES on the delayed footpad reaction in mice immunized i.p. with varying doses of SRBC on day 0. *C. parvum* (0·5 mg dry weight) was given i.v. on day −7. DES (0·1 mg) was given s.c. on day −3. The footpad test was carried out on day 4. (a) ○, Non-treated control; ●, *C. parvum*-treated. (b) ○, Non-treated control; ●, DES-treated. Vertical bars represent standard errors.

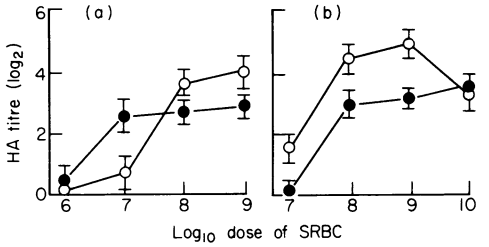

Figure 7. Effect of colloidal carbon or DES on total haemagglutinin titres on day 4 in mice immunized i.p. with varying doses of SRBC on day 0. Colloidal carbon (25 mg/100 g) was given i.v. on day −1 and i.p. on day 0. DES (0·1 mg) was given s.c. on day −3. (a) ○, Non-treated control; ●, carbon-treated. (b) ○, Non-treated control; ●, DES-treated. Vertical bars represent standard errors.

In DES-treated mice, on the contrary, depression in antibody production was detected at doses from 10^7 to 10^9 of SRBC ($P < 0{\cdot}05$; Fig. 7b).

Effect of colloidal carbon or DES on organ uptake of [^{51}Cr]-labelled SRBC

The uptake of labelled SRBC injected i.p. was studied in non-treated, carbon-treated or DES-treated mice. The results summarized in Table 1 indicated that the uptake by the liver was reduced significantly in carbon-treated mice ($P < 0{\cdot}001$), but not modified significantly in DES-treated mice as compared with non-treated mice ($P > 0{\cdot}05$). There was no significant difference in the splenic uptake of SRBC in carbon-treated, DES-treated and non-treated mice ($P > 0{\cdot}6$).

Table 1. Effect of colloidal carbon or DES on 24 h uptake of [^{51}Cr]-labelled SRBC in the liver and spleen. Values are expressed as mean ± standard error

Mouse group*	Liver Percentage c.p.m. whole organ	Spleen Percentage c.p.m. whole organ
Control	38·5 ± 8·2	1·06 ± 0·55
Carbon-treated†	10·5 ± 4·5[(a)]	1·58 ± 0·65[(b)]
DES-treated‡	32·7 ± 8·1[(c)]	1·17 ± 020[(d)]

* Ten mice of each group were injected i.p. with 2×10^8 [^{51}Cr]-labelled SRBC on day 0.

† Colloidal carbon (25 mg/100 g) was given i.v. on day −1 and i.p. on day 0.

‡ DES (0·1 mg) was given s.c. on day −3.

Difference from control was significant in (a) $P < 0{\cdot}001$, but not significant in (b) $P > 0{\cdot}6$, (c) $P > 0{\cdot}05$, (d) $P > 0{\cdot}8$.

DISCUSSION

Our results showed that optimal doses of SRBC for the induction of the delayed footpad reaction varied according to the phagocytic activity of MPS. Low doses of SRBC could induce high degrees of delayed footpad reaction when mice were pre-treated with MPS-blockers such as colloidal carbon or carrageenan (Figs 2 and 3). On the other hand, high doses of SRBC were required for the induction of the delayed footpad reaction when mice were pre-treated with MPS-stimulants such as *C. parvum* or DES (Fig. 6). Similar patterns of modification were observed in the induction of the delayed footpad reaction when the immunizing route was changed from the peritoneal cavity to the footpad (Fig. 3). SRBC have been known to be ingested by macrophages as scavenger cells and to lose their immunogenicity after digestion (Perkins & Makinodan, 1965). In an MPS-blocked state where SRBC could not be eliminated efficiently by scavenger cells, low doses of SRBC may give rise to enough antigen stimulation for the induction of immune responses. On the other hand, in an MPS-stimulated state where SRBC were eliminated very effectively by stimulated macrophages, enough antigen stimulation for the induction of immune responses may be raised by large doses, but not by low doses.

Suppression of the delayed footpad reaction appears to occur readily with an increase in immunizing dose in MPS-blocked mice, although low doses of SRBC can induce high degrees of the reaction in such mice (Figs 2 and 3). Additional treatment with CY augmented the delayed footpad reaction in MPS-blocked mice even after immunization with large doses of SRBC (Fig. 5). The degree of the delayed footpad reaction was higher in MPS-blocked, CY-treated mice than in MPS-blocked, CY-non-treated mice. In MPS-blocked mice, stimulation with SRBC may have a potential to generate larger numbers of effector cells and suppressor cells than in non-treated mice. In the presence of CY-sensitive suppressor cells, suppressor activity may over-ride the activity of effector cell generation in the range of large doses of SRBC. In the absence of CY-sensitive suppressor cells, the degree of the delayed footpad reaction may increase with an increase in immunizing dose followed by augmented generation of effector cells.

There are few reports concerning the relationship between the delayed footpad reaction and the activity of MPS. Scott (1974) described a suppressive effect of *C. parvum* on the delayed footpad reaction to SRBC

and explained it as a result of trapping of sensitized lymphocyres in *C. parvum*-stimulated spleens. McCarthy, Arnold & Babcock (1977) reported that the delayed footpad reaction to SRBC was augmented by treatment with dextran sulphate. They suggested that dextran sulphate may be a potent adjuvant for cell-mediated delayed hypersensitivity. The delayed footpad reaction was reported to be modulated by treatment with BCG (*Bacillus* Calmette-Guérin) (Mackaness, Lagrange & Ishibashi, 1974). In BCG-treated mice, footpad reaction was shown to convert from a labile form to a stable form and suppression of the reaction was not observed even with a large dose of immunizing SRBC. In such mice, the delayed footpad reaction was reduced at the dose of SRBC which was optimal for the induction of maximal reaction in non-treated mice. Such a phenomenon observed in BCG-treated mice may be explained on common basis of MPS activity with out results, since BCG was known to be a potent non-specific stimulant of MPS (Old, Benacerraf, Clarke, Carswell & Stockert, 1961).

There are several reports of the relationship between antibody production and MPS activity. It was reported that antibody production against a low dose of SRBC was increased by MPS-blockade with colloidal carbon, and splenic uptake of SRBC was increased concurrently (Fisher, 1966; Souhami, 1972). Bradfield, Souhami & Addison (1974) reported the results with dextran sulphate similar to those with colloidal carbon. According to Šljivić & Warr (1973), antibody production and splenic uptake of SRBC was decreased by pre-treatment with DES. In some other papers, the degrees of splenic uptake of SRBC and antibody production were dissociated (Cutler, 1961; Šljivić & Warr, 1974; Warr & Šljivić, 1974b). In our experiments, MPS-blockade with colloidal carbon augmented antibody production against a low dose of SRBC, but reduced antibody production against large doses (Fig. 7a). MPS stimulation with DES reduced antibody production against a wide range of immunizing dose (Fig. 7b). Such effects of MPS activity may not be explained by the difference in splenic uptake of SRBC, since almost the same amount of labelled SRBC was uptaken by spleens in non-treated, MPS-blocked or MPS-stimulated mice (Table 1). Modulation of antibody production by MPS activity may be explained by different functions of macrophages as scavenger cells.

ACKNOWLEDGMENT

This work was supported by the grants from the Ministry of Eduction, Science and Culture and from the Ministry of Health and Welfare, Japan.

REFERENCES

Biozzi, G., Benacerraf B. & Halpern B.N. (1953) Quantitative study of the granulopectic activity of the reticulo-endothelial system. II. A study of the kinetics of the granulopectic activity of the R.E.S. in relation to the dose of carbon injected. Relationship between the weight of the organs and their activity. *Br. J. exp. Path.* **34,** 441.

Bradfield J.W.B., Souhami R.L. & Addison I.E. (1974) The mechanism of the adjuvant effect of dextran sulphate. *Immunology,* **26,** 383.

Cutler J.L. (1960) The enhancement of hemolysin production in the rat by Zymozan. *J. Immunol.* **84,** 416.

Cutler J.L. (1961) The effect of Zymozan on the phagocytosis of foreign erythrocytes. *J. Immunol.* **86,** 73.

Fisher S. (1966) Stimulation of splenic antigen uptake and of antíbody response in mice by India Ink or other 'blocking' agents. *Immunology,* **11,** 127.

Franzl R.E. & McMaster P.D. (1968) The primary immune response in mice. I. The enhancement and suppression of hemolysin production by a bacterial endotoxin. *J. exp. Med.* **127,** 187.

Gershon H. & Feldman M. (1968) Studies on the immune reconstitution of sublethally irradiated mice by peritoneal macrophages. *Immunology,* **15,** 827.

Ghaffar A. & Sigel M.M. (1978) Immunomodulation by *Corynebacterium parvum*. I. Variable effects on anti-sheep erythrocyte antibody responses. *Immunology,* **35,** 685.

Lagrange P.H., Mackaness G.B. & Miller T.E. (1974) Potentiation of T-cell-mediated immunity by selective suppression of antibody formation with cyclophosphamide. *J. Exp. Med.* **139,** 1529.

Mackaness G.B., Lagrange P.H. & Ishibashi T. (1974) The modifying effect of BCG on the immunological induction of T cells. *J. exp. Med.* **139,** 1540.

McCarthy R.E., Arnold L.W. & Babcock G.F. (1977) Dextran sulphate: an adjuvant for cell-mediated immune responses. *Immunology,* **32,** 963.

Metchnikoff E. (1899) Études sur la résorption des cellules. *Ann. Rev. Inst. Pasteur,* **13,** 47.

Old L.J., Benacerraf B., Clarke D.A., Carswell E.A. & Stockert E. (1961) The role of the reticuloendothelial system in the host reaction to neoplasia. *Cancer Res.* **21,** 1281.

Perkins E.H. & Makinodan T. (1965) The suppressive role of mouse peritoneal phagocytes in agglutinin response. *J. Immunol.* **94,** 765.

Ptak W., Pryjma J. & Moskalewski S. (1974) Macrophages in mouse placenta: immunogenicity of macrophage-assocated antigen. *J. reticuloendothel. Soc.* **16,** 15.

Rumjanek V.M., Watson S. & Šljivić V.S. (1977) A re-evaluation of the role of macrophages in carrageenan-induced immunosuppression. *Immunology,* **33,** 423.

Sabet T., Newlin C. & Friedman H. (1969) Effects of RES 'blockade' on antibody formation. I. Suppressed cellular and humoral haemolysin responses in mice injected with carbon particles. *Immunology,* **16,** 433.

SCOTT M.T. (1974) Depression of delayed-type hypersensitivity by *Corynebacterium parvum:* mandatory role of the spleen. *Cell. Immunol.* **13,** 251.

ŠLJIVIČ V.S. & WARR G.W. (1973) Activity of the reticuloendothelial system and the antibody response. II. Effect of stilbestrol on the immune response to sheep erythrocytes in the mouse. *Br. J. exp. Path.* **54,** 69.

ŠLJIVIČ V.S. & WARR G.W. (1974) Activity of the reticuloendothelial system and the antidy response. III. The fate of type III pneumoccocal polysaccharide and the antibody response. *Immunology,* **27,** 1009.

SOUHAMI R.L. (1972) The effect of colloidal carbon on the organ distribution of sheep red cells and the immune response. *Immunology,* **22,** 685.

TAKEYA K., SHIMOTORI S. TANIGUCHI T. & MOMOTO K. (1977) Cellular mechanisms in the protection against infection by *Listeria monocytogenes* in mice. *J. Gen. Microbiol.* **100,** 373.

TURNER E.V. & HIGGINBOTHAM R.D. (1977) Carrageenan induced alterations of antigen distribution and immune responses in mice. *J. reticuloendothel. Soc.* **22,** 545.

UNANUE E.R. (1978) The regulation of lymphocyte functions by the macrophage. *Immunol. Rev.* **40,** 227.

WARR G.W. & ŠLJIVIČ V.S. (1974a) Studies on the organ uptake of ^{51}Cr-labelled sheep erythrocytes in the evaluation of stimulation of RES phagocytic function in the mouse. *J. reticuloendothel. Soc.* **16,** 193.

WARR G.W. & ŠLJIVIČ V.S. (1974b) Enhancement and depression of the antibody response in mice caused by *Corynebacterium parvum. Clin. exp. Immunol.* **17,** 519.

WATSON R.S. & ŠLJIVIČ V.S. (1976) The role of macrophages in the adjuvant effect on antibody production of *Corynebacterium parvum. Clin. exp. Immunol.* **23,** 149.

WOOLES W.R. & DI LUZIO N.R. (1963) Reticuloendothelial function and the immune responses. *Science,* **142,** 1078.