Immunology 1979 **37** 765

Immune activation by T-independent antigens: lack of effect of macrophage depletion on the immune response to TNP-LPS, PVP and dextran

D. M. WONG* & H. B. HERSCOWITZ *Department of Microbiology Georgetown University, Schools of Medicine and Dentistry, Washington, U.S.A.*

Accepted for publication 26 January 1979

Summary. Carrageenan, a sulphated polysaccharide, and rabbit anti-mouse macrophage serum, were used to inhibit macrophage function in BALB/c mice as well as to deplete macrophages from spleen cell cultures in an attempt to determine the requirement for macrophages in the immune response to several thymus-independent antigens. Carrageenan inhibited macrophage function and was cytotoxic at low concentrations. The ability of T and B lymphocytes to undergo mitogen-induced proliferation in the presence of PHA and LPS, respectively, was not affected by *in vitro* exposure of lymphoid cells to carrageenan. BALB/c mice injected with carrageenan demonstrated a suppressed immune response to SRBC, a thymus-dependent antigen, but not to *E. coli* LPS, polyvinylpyrrolidone or dextran B-1355*S*, all of which are known to be thymus independent antigens. The sensitivity of the *in vivo* immune response to SRBC after depletion of macrophages by carrageenan treatment was confirmed *in vitro* using the Marbrook–Diener culture system. The *in vitro* immune response to TNP-LPS was unaffected by either carrageenan treatment or treatment of BALB/c spleen cells with AMS and complement. The results of experiments which utilized the two anti-macrophage reagents, carrageenan and AMS, both *in vivo* and *in vitro* systems, suggest that the immune response to thymus-independent antigens does not require the participation of macrophages.

Abbreviations: PHA, phytohaemagglutinin; SRBC, sheep red blood cells; TNP-LPS, 2,4,6-trinitrophenyl-lipopolysaccharide; TNP-DEAE-dextran, 2,4,6-trinitrophenyl-diethylaminoethylated dextran; NNP-LPS, 4-hydroxyl-3,5-dinitrophenyl acetyl-lipopolysaccharide; AMS, anti-macrophage serum; PVP, polyvinyl pyrrolidone; PEC, peritoneal exudate cells; BSA, bovin serum albumin; MEM, minimal essential medium; FCS, fetal calf serum.

* Present address: Cellular Immunology Division, Clinical and Experimental Immunology Department, Naval Medical Research Institute Bethesda, Maryland 20014, U.S.A.

Correspondence: Dr H. B. Herscowitz, Department of Microbiology, Georgetown University, School of Medicine, School of Dentistry, Washington D.C. 20007, U.S.A.

0019-2805/79/0800-0765$02.00

INTRODUCTION

The generation of a humoral immune response to many complex multideterminant immunogens (thymus-dependent antigens) appears to require the cooperative interaction of several different functional cell types (Claman & Mosier, 1972). While it is well established that macrophages are required (Mosier, 1967; Pierce & Kapp, 1976) for immune responses to thymus-dependent antigens (e.g. SRBC), the involvement of macrophages in immune responses to thymus-independent antigens (e.g. LPS, PVP, levan, dextran) is less clear and somewhat controversial.

Removal of macrophages from spleen-cell populations by passage through glass bead columns signifi-

cantly reduced the *in vitro* primary response to SRBC, while the response to polymerized flagellin (POL), a thymus-independent antigen, was unaffected (Shortman, Diener, Russell & Armstrong, 1970). In contrast, Lee, Shiozawa, Shaw & Diener (1976) showed that removal of macrophages from mouse spleen-cell populations with carbonyl iron resulted in significant suppression of the response to POL. *In vivo* depletion of macrophages by multiple injections of carrageenan, a polysulphated galactan (Smith, Cook & Neal, 1954), had no effect on the response to TNP–Ficoll while the response to TNP-LPS was reduced to 50% of that of control mice (Ishizaka, Otani & Morizawa, 1977). In other studies, depletion of macrophages from mouse spleen populations by use of glass bead columns (Poe & Michael, 1974) or carbonyl iron (Lemke, Coutinho, Opitz & Gronowicz, 1975) had no effect on the magnitude of the *in vitro* immune response to TNP-LPS and NNP-LPS, respectively. It has also been demonstrated that depletion of macrophages by filtration through Sephadex G-10 columns resulted in suppression of the responses to TNP–Ficoll and TNP-LPS (Chused, Kassan & Mosier, 1976). Thus it appears that the requirement for macrophages in the immune response to a given thymus-independent antigen can be correlated with the method used to deplete macrophages from lymphoid cell populations.

In this report two agents were used in an attempt to deplete macrophages from mouse spleen cell populations both *in vivo* and *in vitro*. Carrageenan was used as an anti-macrophage agent, since it has been suggested to be selectively cytotoxic for macrophages *in vitro* (Catanzaro, Schwartz & Graham, 1971) and has been shown to inhibit macrophage function *in vivo* (Bice, Gruwell, Salvaggio & Hoffman, 1972). The efficiency and specificity of the other agent, anti-macrophage serum, for the depletion of macrophages from spleen cell populations has been documented (Loewi, Temple, Nind & Axelrod, 1969; Hirsch, Gary & Murphy, 1969; Stinnett, Kaplan & Morahan, 1976; Herscowitz & DiBlasio, 1976). The major difficulty encountered in obtaining antisera specific for macrophages has been the presence of antibodies directed against lymphocytes (Loewi *et al.*, 1969; Stinnett *et al.*, 1976). Since much of this non-specific activity can be attributed to lymphocyte contamination of the peritoneal exudate cell (PEC) population routinely used for immunization, we attempted to circumvent this problem in a unique manner. We took advantage of the fact that nu/nu mice are deficient in mature T lymphocytes and thereby eliminated one potential contaminating cell type. We first prepared an anti-lymphocyte serum against adherent cell-depleted nu/nu mouse spleen cells and used this to remove lymphocyte contaminants from thioglycollate-stimulated PEC which were then used for the preparation of the anti-macrophage serum. The results obtained support the concept that the immune response to selected thymus-independent antigens is independent of macrophage function.

MATERIALS AND METHODS

Animals

Inbred BALB/c mice, 4–5 weeks of age were obtained from Flow Laboratories, Rockville, Md. Athymic (nu/nu) mice bred in this laboratory on a BALB/c genetic background were originally obtained from Dr William P. Weidanz, Hahnemann Medical College, Philadelphia. Female random bred, New Zealand white rabbits weighing 4–5 lbs were obtained from a local dealer. Animals were given food and water *ad libitum*.

Antigens

Sheep erythrocytes in 50% Alsever's solution were obtained from Microbiological Associates, Bethesda, Md. *E. coli* 055:B5 lipopolysaccharide (Westphal preparation) was purchased from Difco Laboratories, Detroit, Michigan. Dextran was extracted from *Leuconostoc mesenteroides* strain B512 essentially as described by Jeanes, Wilham & Miers (1948). Polyvinylpyrrolidone, with an average molecular weight of 360,000 was obtained from Sigma Chemical Company, St Louis, Mo.

Preparation of anti-cellular sera

An anti-lymphocyte serum prepared by hyperimmunization of New Zealand white rabbits with non-adherent nu/nu mouse spleen cells as follows. Spleen cells from nu/nu mice were depleted of adherent cells by successive plating on plastic dishes according to the method of Mosier (1967) followed by treatment with carbonyl iron (Technicon Instruments, Tarrytown, N.Y.) as described by Lee *et al.* (1976). The recovered non-adherent, non-phagocytic cells ($5–10 \times 10^7$) were emulsified in Freund's complete adjuvant and injected subcutaneously. This procedure was repeated three times at weekly intervals using incomplete adjuvant and the animals were bled 1 week after the last injection.

To prepare the anti-macrophage serum (AMS),

PEC were obtained by lavaging the peritoneal cavity of nu/nu mice with 4 ml of Eagle's MEM (Grand Island Biological Co., Grand Island, N.Y.) 4 days after an intraperitoneal injection of 3 ml of thioglycolate (Difco Laboratories). The PEC ($1–2 \times 10^8$) were washed once with Eagle's MEM and treated with the rabbit anti-mouse lymphocyte serum (1 ml) and guinea-pig complement (0·2 ml) for 1 h at 37°. The treated cells were washed twice in Eagle's MEM and resuspended in 4 ml of 33% bovine serum albumin (Sigma Chemical Co., St Louis, Mo.) and placed into 5 ml polycarbonate centrifuge tubes. Successive 1 ml quantities of 29% and 10% solutions of bovine serum albumin (BSA) were layered over the 33% BSA-cell suspension. The separation of viable macrophages layered over the 33% BSA-cell suspension. The separation of viable macrophages from dead cells and cellular debris was effected by centrifugation for 30 min at 20,000 *g* (HB-4 rotor, Ivan Sorvall, Norwalk, Conn.) at 4°. Cells from the 29% and 10% BSA interface were removed with a Pasteur pipette, washed three times in Eagle's MEM and resuspended in 1 ml of phosphate-buffered saline. The resulting cell population ($5–10 \times 10^7$) containing >95% macrophages, as determined by morphological examination of Giemsa-stained preparations, was emulsified in Freund's complete adjuvant and injected subcutaneously into rabbits. Similarly treated PEC suspensions were injected every 2 weeks for a total of four injections. The second injection was by the subcutaneous route using cells emulsified in Freund's incomplete adjuvant. The third and fourth injections were administered by the intravenous route. The AMS was rendered specific for mouse macrophages by extensive absorption with mouse erythrocytes, nu/+ thymocytes and non-adherent nu/nu spleen cells.

Mitogen assays

Two to three million non-immune mouse spleen cells were suspended in 0·9 ml of Eagle's complete MEM (Spinner modification) supplemented with 10% heat-inactivated foetal calf serum (FCS), non-essential amino acids, 2mM glutamine, 100 units/ml penicillin, 50 μg/ml of streptomycin and 1 μg/ml of fungizone, all obtained from Grand Island Biological Co., Grand Island, N.Y. The cell suspensions were placed into 13×100 mm tissue culture tubes (Falcon Plastics Oxnard, Ca). After the addition of 100 μg/ml of LPS or 25 μg/ml of PHA, the tubes were placed on a roller drum at 1 revolution/6 min (New Brunswick Scienfic, New Brunswick, N.J.) and incubated in an atmosphere of 5% CO_2 at 37° for 24 h. At the end of this time 1 μCi of methyl-[^{3}H]-thymidine (56·5 mCi/mM ICN Isotope and Nuclear Division, Irvine, Ca) was added to tubes containing LPS and the tubes reincubated for an additional 16–20 h. Cultures stimulated with PHA were incubated for 48 h before a similar amount of methyl-[^{3}H]-thymidine was added. At the end of the incubation period, the cell samples were harvested and assayed for the incorporation of [^{3}H]-thymidine as described previously (Proctor & Herscowitz, 1975).

Cytotoxicity assays

The procedure used to determine the amount of ^{51}Cr released from labelled cells by cytotoxic antibody was described previously (Proctor & Herscowitz, 1975). Briefly, 1×10^8 cells in 1 ml of Eagle's MEM containing 15% FCS were incubated at 37° for 30 min with 50 μCi of ^{51}Cr (100–300 mCi/mg [^{51}Cr]-$NaCrO_4$, Amersham-Searle, Arlington Heights, Ill.). The cells were washed three times in Eagle's MEM and 5×10^6 labelled cells in 0·1 ml of media were mixed with 0·1 ml of antiserum, 0·1 ml of guinea-pig complement was added, and the mixture incubated at 37° for 45 min. The treated cells were then sedimented and the supernatant assayed for the amount of ^{51}Cr released in a gamma spectrometer.

Passive sensitization of SRBC

Sheep erythrocytes were coated with PVP by the method of Boyden (1951). Coating of SRBC with *E. coli* 055:B5 LPS was accomplished by mixing 0·1 ml of packed cells with 1 ml of LPS solution (1 mg/ml in saline) and incubated at 37° for 1 h. Conjugation of dextran onto SRBC was carried out according to the method of Ghanta, Hamlin, Pretlow & Hiramoto (1972). Conjugation of TNP onto SRBC was accomplished by the method of Rittenberg & Pratt (1969). The method of Jacobs & Morrison (1975) was used to prepare TNP-LPS.

Cell cultures

Spleens from BALB/c or nu/+ mice were teased apart, washed twice in complete RPMI 1640 and $2–2·5 \times 10^7$ cells were cultured in Marbrook–Diener tissue culture chambers as described previously (Marbrook, 1967). AMS treatment of mouse spleen cells involved incubating 1×10^8 cells in 1·5 ml of Eagle's MEM containing 2% FCS with 0·2 ml AMS at 4° for 10 min. The cell suspension, after the addition of 0·2 ml guinea-pig complement, was then reincubated at 37° for 45 min.

The treated cells were washed three times in RPMI 1640 and 2–2·5 × 10^7 cells placed in culture. Control spleen cells were treated with normal rabbit serum in an identical manner.

Reconstitution of the macrophage-depleted spleen population after treatment with AMS was effected by the addition of BALB/c peritoneal macrophages. PEC from BALB/c mice were treated with anti-B lymphocyte serum and guinea-pig complement as described above. Spleen cell cultures were incubated at 37° in an atmosphere of 10% CO_2 for 3 days with TNP-LPS and 4 days when SRBC was used as the antigen. At the end of the culture period, the cells were harvested, washed once in RPMI 1640 and assayed for the presence of PFC by the method of Cunningham & Szenberg (1968).

Phagocytosis assay

The ingestion of polystyrene latex particles (1·099 μm in diameter; Coulter Electronics, Inc., Hialeah, Florida) by macrophages was carried out in 13 × 100 mm glass tissue culture tubes. Approximately 2 × 10^6 macrophages in 0·9 ml Eagle's MEM were placed into the tubes containing 0·1 ml of the latex suspension (1–3 × 10^9 particles). The tubes were incubated on a roller drum in an atmosphere of 5% CO_2 at 37° for 24 h. The cells were then harvested from the tubes, washed three times and a smear of the cell suspension was prepared on a glass microscope slide. The ability of splenic macrophages to adhere to glass was tested in 60 × 10 mm plastic petri dishes containing a sterile glass coverslip. Each dish contained approximately 2 × 10^7 spleen cells in 3 ml of complete RPMI-1640. Cultures were incubated for 4 days in an atmosphere of 10% CO_2 at 37°. The glass coverslips were then removed, gently washed in cold 0·15 M NaCl to remove non-adherent cells and air dried.

Non-specific esterase stain

The method of Yam, Li & Crosby (1971) was used for staining macrophages. Cell smears were fixed in a solution of buffered formalin–acetone, pH 6·6 for 30 s at 4–10°. The fixed smears were washed in distilled water and allowed to air dry. The smears were flooded with the esterase-staining reagent, incubated at room temperature for 30–40 min and washed with distilled water. The smears were then counterstained for 1 min with 1% methyl green, rinsed with distilled water, air dried and examined under oil immersion.

Preparation of carrageenan

A stock solution of carrageenan (RE 6918, Seakem 9 kindly provided by Marine Colloids, Inc., Melbourne, N.J.) was prepared by dissolving 40 mg of carrageenan in 20 ml of 0·15 M NaCl maintained in a hot water bath. The carrageenan was dialysed against 4 l of 0·15 M NaCl for 48 h with one complete change of 0·15 M NaCl at 24 h. The non-dialysable material was heated in a boiling water bath for 30 min. The stock solution was diluted in Eagle's MEM for *in vitro* use or in 0·15 M NaCl for *in vivo* use.

RESULTS

Effect of carrageenan on cell function

To determine the effect of carrageenan on cell function, peritoneal macrophages from thioglycollate-stimulated BALB/c mice or normal spleen cells were cultured *in vitro* with varying doses of carrageenan. Table 1 indicates that a large number of PEC were viable during the first 24 h of culture. Phagocytic function, however, as determined by ingestion of latex particles, decreased with increasing doses of carrageenan. Upon further incubation of the cells with carrageenan, cell viability decreased to a low level. This decrease did not appear to be dose-dependent since as little as 50 μg/ml of carrageenan could affect macrophage function. These results indicate that carrageenan was not immediately cytotoxic to macrophages and that the effects of carrageenan on macrophage function preceeded the death of these cells.

The number of adherent cells in the splenic population decreased with increasing doses of carrageenan

Table 1. *In vitro* culture of normal peritoneal macrophages in the presence of carrageenan

Carrageenan* (μg/ml)	Viability† 24 h (%)	Viability† 48 h (%)	Cells ingesting‡ latex (24 h) (%)
0	99	99	99
50	93	30	73
100	95	29	58
200	85	21	46
300	80	21	14
500	70	21	2

* Added at the beginning of culture.

† Determined by trypan blue dye exclusion.

‡ PEC were cultured with carrageenan and latex particles as described in the Materials and Methods section.

until a plateau was reached (Table 2). The adherent cells that remain may represent B lymphocytes, since some of these lymphocytes, especially plasma cells, have been reported to be adherent (Shortman, Williams, Jackson, Russell, Hart & Diener, 1971). The suggestion that the adherent cells were lymphocytes was further supported from observations using the esterase stain of the adherent population. Esterase positive cells are primarily monocytes and macrophages (Yam *et al.*, 1971). As can be seen in Table 2, the esterase positive cells in the adherent cell population sharply decreased with increasing doses of carrageenan.

In order to determine if the inhibitory effects of carrageenan were specific for macrophages, splenic lymphocytes were cultured in the presence of varying doses of carrageenan and were stimulated with optimal doses of PHA and LPS as a measure of T- and B-cell function, respectively. Table 3 indicates that exposure of T and B lymphocytes to carrageenan does not affect the capabilities of these cells to respond to mitogens. A proliferative response was evident in the presence of 100 μg of carrageenan, a concentration which had been shown to adversely affect macrophage phagocytic function and viability. As can be observed in Table 3, the viability of T and B lymphocytes was high and even when exposed to high concentrations of carrageenan. It should be noted that there was no significant difference in the background level of incorporation between control cell cultures containing carrageenan only (e.g. 50 μg carrageenan: 6244 ± 670 c.p.m.) and those with spleen cells only (e.g. 6435 ± 406 c.p.m.), thereby eliminating the argument that carrageenan could exert an inhibitory effect without causing any change in the stimulation index. The possibility of adverse effects on B-lymphocyte function was further investigated by treating plasma cells, which were actively producing antibody, with various concentrations of carrageenan. Splenic lymphocytes obtained from BALB/c mice primed 5 days previously with SRBC were incubated *in vitro* with 10–100 μg of carrageenan for various time intervals up to 24 h. Carrageenan treatment failed to affect ongoing antibody synthesis by previously immunized B lymphocytes since these cells were still able to generate comparable numbers of plaques as did the untreated controls when tested in a haemolytic plaque assay (data not shown).

Table 2. Depletion of BALB/c splenic macrophages by treatment with carrageenan *in vitro**

Carrageenan† (μg)	Adherent cells/mm^2	Esterase positive cells (%)
0	384 ± 73‡	96
10	128 ± 42	32
20	64 ± 16	10
30	48 ± 28	3
40	21 ± 9	0·3
50	21 ± 8	0·02
100	16 ± 1	0§

* One ml of BALB/c spleen cells (2×10^7 cells) was added to a 60 × 10 mm plastic petri dish containing 2 ml of cRPMI 1640 and a glass coverslip. The dishes were incubated at 37° in an atmosphere of 10% CO_2 for 4 days. The coverslips were then gently washed in cold 0·15 M NaCl, air dried and stained with a non-specific esterase stain.

† Added at the beginning of the culture.

‡ Mean ± SD of five determinations.

§ 1×10^5 cells were counted.

Table 3. Mitogenic response of BALB/c spleen cells to LPS and PHA when cultured in the presence of carrageenan

Carrageenan* (μg/ml)	LPS† (S.I.)‡	PHA§ (S.I.)	Viability¶ (%)
0	$3·9 \pm 0·9$**	$5·5 \pm 3·3$	91–95
1	$4·3 \pm 1·1$	ND	90
10	$4·4 \pm 1·1$	$5·5 \pm 1·5$	86–89
50	ND	$5·4 \pm 1·7$	88
100	$4·0 \pm 1·6$	$4·4 \pm 2·5$	85

* Added at the beginning of culture.

† Cells were cultured *in vitro* for 24 h with 100 μg *E. coli* 055:B5 LPS, then incubated for 24 h with 1 μCi of [^{3}H]-thymidine.

‡ Stimulation Index:

$$\frac{\text{c.p.m. from mitogen stimulated cells}}{\text{c.p.m. from unstimulated cells}}$$

§ Cells were cultured for 48 h with 25 μg of PHA, then incubated for 24 h with 1 μCi of [^{3}H]-thymidine.

¶ Determined by trypan blue dye exclusion.

** Mean ± SD of five independent experiments

ND, Not done.

Cytotoxic effect of AMS on peritoneal macrophages, thymocytes and B lymphocytes

AMS produced by the injection of lymphocyte-depleted nu/nu peritoneal macrophages into rabbits exhibited high cytotoxic activity against macrophages as shown in Fig. 1. Absorption of the AMS with BALB/c

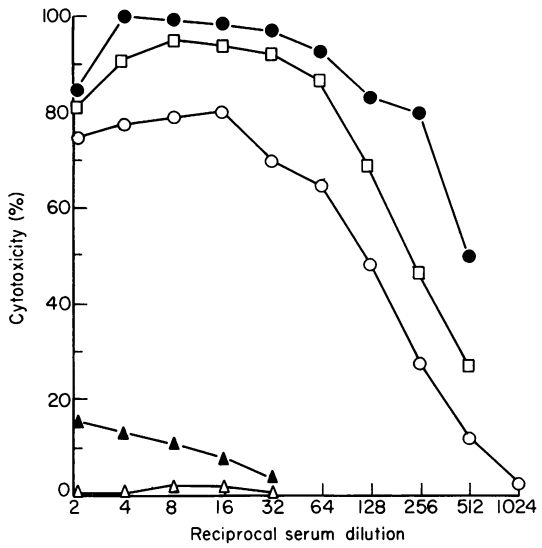

Figure 1. Reactivity of rabbit anti-mouse macrophage serum against nu/nu macrophages. Peritoneal macrophages from thioglycollate-stimulated nu/nu mice were labelled with ^{51}Cr, mixed with serial dilutions of AMS and guinea-pig complement. The mixture was incubated for 45 min at 37° and the reaction was stopped by the addition of cold 0·15 M NaCl. The amount of ^{51}Cr released was determined by measuring the radioactivity of the supernatant fluid in a gamma spectrometer. AMS unabsorbed (●) AMS absorbed with BALB/c erythrocytes and nu/+thymocytes (□), AMS absorbed with nu/nu splenic lymphocytes (○), AMS absorbed with nu/nu peritoneal macrophages (△), normal rabbit serum (▲).

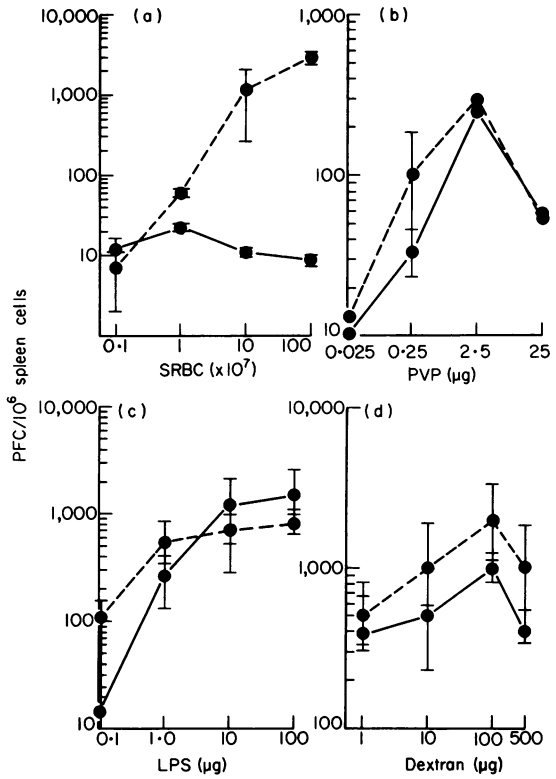

Figure 2. BALB/c mice received a total dose of 10 mg carrageenan through five daily intraperitoneal injections of 2 mg of carrageenan. The antigen was injected through the same route on the fourth day of carrageenan treatment and the haemolytic plaque assay was done 5 days later. The direct plaque-forming response was measured. Each point represents the mean (±SD) of two to three independent experiments employing four to five mice per dose of antigen in each experiment. Untreated mice (--), carrageenan treated mice(—). (a) response to SRBC; (b) response to PVP; (c) response to LPS; (d) response to dextran.

erythrocytes and nu/+ thymocytes did not appreciably decrease its cytotoxic activity against macrophages. Absorption of the AMS with nu/nu splenic lymphocytes, however, reduced the anti-macrophages activity by 25%. BALB/c peritoneal macrophages treated with dilutions of absorbed AMS as high as 1:1000 and guinea-pig complement adversely affected the ability of these cells to adhere to glass and to phagocytose latex particles (data not shown). Similar treatment of BALB/c spleen cells with the absorbed AMS preparation did not affect the ability of T or B lymphocytes to respond to the mitogens, PHA and LPS, respectively (data not shown).

In vivo depletion of macrophages by carrageenan

As shown in Fig. 2a the plaque-forming response to SRBC was suppressed in BALB/c mice treated with 10 mg of carrageenan. There was a 300-fold decrease in the number of plaque-forming cells in carrageenan-treated mice when immunized with the highest dose of SRBC (1×10^9), as compared to untreated control animals. In contrast, the immune response to PVP was unaffected in mice treated with carrageenan (Fig. 2b). The plaque-forming response increased with increasing doses of the antigen in both carrageenan-treated and untreated mice until a maximum response was obtained with 2·5 μg of PVP. The response to LPS (Fig. 2c) in carrageenan-treated mice was slightly depressed at low concentrations of LPS (0·1–1·0 μg) and slightly enhanced at optimal doses of LPS (10–100 μg). Both carrageenan-treated and untreated mice, however gave comparable immune responses at all doses tested. The plaque-forming response to dextran

B-1355S was slightly suppressed in carrageenan-treated mice (Fig. 2d). However, the plaque-forming response of both carrageenan-treated and untreated mice peaked at 100 μg of dextran. Variability in the plaque-forming response was evident at all doses of dextran tested. Similar results with all three TI antigens were consistently observed in two to three independent experiments.

In vitro immune responses of spleen cells depleted of macrophages by carrageenan or AMS

When macrophages were depleted by culturing BALB/c spleen cells in the presence of carrageenan, the immune response to SRBC was suppressed as shown in Table 4. There was a four-fold decrease in the number of plaque-forming cells obtained from cultures which contained the lowest dose of carrageenan. This suppression increased with increasing doses of carrageenan until a background level of PFC was obtained from cultures containing the highest dose of carrageenan. In contrast, the immune response to TNP-LPS, a thymus-independent antigen (Jacobs & Morrison, 1975), was unaffected by similar carrageenan treatment. A significant response to TNP-LPS was obtained at all concentrations of carrageenan tested. The optimal dose of TNP-LPS was found to be 5 μg/ml since lower doses (e.g. 0·001–1 μg/ml) consistently failed to generate an observable immune response whereas higher doses (e.g. 5–10 μg/ml) elicited strong polyclonal responses which resulted in a high background level. Haptenated LPS was used in preference to LPS since the *in vitro* immune response to the latter was observed to be inconsistent and highly variable (unpublished observations).

To corroborate observations obtained from the carrageenan-treated *in vitro* cell cultures, spleen cells were depleted of macrophages by treatment with AMS and then the cells were cultured with SRBC or TNP-LPS. Table 5 shows that treatment with AMS suppressed the immune response to SRBC to background levels. The immune response to SRBC in the macrophage-depleted cell cultures was restored by the addition of BALB/c peritoneal exudate cells depleted of B lymphocytes by treatment with anti-lymphocyte serum. Spleen cell cultures depleted of macrophages by treatment with AMS were still able to generate a plaque-forming response to TNP-LPS.

Table 4. The *in vitro* immune response to SRBC and TNP-LPS of BALB/c spleen cells depleted of macrophages by treatment with carrageenan*

Antigen	Carrageenan† (μg/ml)	Direct PFC/culture‡
None	0	17 ± 9
SRBC	0	263 ± 184
	20	65 ± 35
	30	77 ± 13
	40	39 ± 16
	50	18 ± 12
None	0	76 ± 18
TNP-LPS	0	397 ± 115
	20	479 ± 107
	30	477 ± 114
	40	426 ± 104
	50	422 ± 125

* Spleen cells were cultured with 5 μg/ml of TNP-LPS for 3 days, or 6×10^7 SRBC for 4 days and then assayed for a plaque-forming response.

† Carrageenan was added at the beginning of culture.

‡ Mean (±SD) of three to five independent experiments with duplicate cell cultures.

Table 5. *In vitro* immune response to SRBC and TNP-LPS of BALB/c spleen cells depleted of macrophages by treatment with AMS*

Antigen	Direct PFC/cell culture†
None	35 ± 21
SRBC‡	329 ± 152
SRBC + AMS§	44 ± 23
SRBC + AMS + macrophages¶	325 ± 149
Macrophage control**	0
None	25 ± 14
TNP-LPS	250 ± 111
TNP-LPS + AMS§	302 ± 220

* See Table 4 for culture conditions.

† Mean (±SD) of three to four independent experiments with duplicate cell cultures.

‡ Antigen added to cultures containing spleen cells which had not been treated with AMS.

§ Spleen cells were treated with AMS for 10 min at 4° with a 45 min incubation at 37° following the addition of guinea-pig complement, washed twice in Eagle's MEM and placed into culture.

¶ Reconstitution of macrophage-depleted spleen cells was effected by the addition of BALB/c peritoneal exudate cells depleted of lymphocytes by a one hour incubation at 37° with anti-B lymphocyte serum and guinea-pig complement. The peritoneal exudate cells were washed twice in Eagle's MEM and 4×10^5 cells were added to each culture.

** BALB/c peritoneal exudate cells (2×10^7 cells), used to reconstitute the macrophage-depleted cell cultures were cultured with 6×10^7 SRBC.

DISCUSSION

Various functions have been ascribed to macrophages to account for their apparent obligatory role in the generation of an immune response to thymus-dependent antigens; these include antigen processing, antigen presentation, generation of helper T cells, lymphocyte viability promotion and the production of immunoregulatory factors (Pierce & Kapp, 1976). Studies of immunological function in mice treated with carrageenan have suggested that inhibition of macrophage function may be responsible for the observed depression of the immune response to thymus-dependent antigens (Bice *et al.*, 1972; Thomson, Wilson, Cruickshank & Jeffries, 1976; Rumjanek, Watson & Sljivic, 1977). Although phagocytosis has not been demonstrated to be an absolute requirement for the generation of an immune response, this physiological function appears to be a normal activity of macrophages which occurs during the early stages of immune co-operation with immunocompetent cells since agents such as silica and anti-macrophage serum, which block reticuloendothelial (RE) function also depress immune responses (Argyris, 1974; Unanue, 1972). While it has been suggested that carrageenan may interfere with immune responsiveness by inhibiting phagocytosis, this has not been unequivocally proven. Sawicki & Catanzaro (1975) observed that carrageenan could only be detected in a small proportion of phagocytic cells after *in vivo* administration of doses which were previously shown to be immunosuppressive, suggesting that such effects could not be attributed to macrophages. On the other hand, studying organ uptake of isotopically labelled erythrocytes as an indicator of *in vivo* phagocytosis, Rumjanek *et al.* (1977) observed a marked depression in hepatic uptake concomittant with an increase in uptake by lung and spleen in animals treated with carrageenan. They also observed that the antibody response to SRBC was markedly suppressed in carrageenan-treated mice at a time coincident to the decrease in hepatic uptake. Similarly, Chaouat & Howard (1976) reported that treatment of mice with carrageenan to achieve RE blockade resulted in a marked depression of carbon clearance after 1 day and that phagocytic indices remained subnormal for 5 days. They also showed that mice treated with carrageenan 24 h previously demonstrated an impaired clearance of levan which was less striking than that observed with carbon particles. Table 1 indicates that phagocytosis by macrophages was inhibited by treatment with carrageenan. Thus there appears to be some correlation between the ability of macrophages to phagocytose and their ability to provide accessory function for the generation of an immune response.

In the present study, large doses of carrageenan (e.g. 10 mg) were injected intraperitoneally into BALB/c mice in an attempt to inhibit macrophage activity both in the peritoneal cavity, the site of antigen deposition and in the spleen, the site of antibody formation. While the immune response to SRBC, a thymus-dependent antigen was markedly reduced at all immunogenic doses tested, the responses to *E. coli* LPS, PVP and dextran (Fig. 2) were unaffected by such treatment suggesting that severe depletion of macrophages *in vivo* did not affect the response to these thymus-independent antigens. Similar results were obtained by Rumjanek *et al.* (1977) who observed that *in vivo* administration of carrageenan resulted in suppression of the PFC response to suboptimal and optimal doses of SRBC. Using adherent and non-adherent spleen cell populations they also demonstrated that *in vivo* treatment with carrageenan resulted in a suppressed ability to generate an *in vitro* PFC response to SRBC but was without effect on the response to DNP-Ficoll, a thymus-independent antigen. They provided convincing evidence that carrageenan manifested its effect on macrophages. On the other hand, while demonstrating that carrageenan treatment resulted in suppression of the response to donkey erythrocytes, Chaouat & Howard (1976) also observed that the response to levan, a thymus-independent antigen, was markedly suppressed. They provided evidence that B-cell reactivity was not impaired since spleen cells from carrageenan-treated mice responded normally to levan after transfer into irradiated recipients. These authors speculated that the immunosuppressive effect of carrageenan could possibly be explained by the release of inhibitory factors from macrophages which exerted regulatory effects on the responding lymphocytes. A similar mechanism was suggested by Rumjanek *et al.* (1977).

Ishizaka *et al.* (1977) reported that carrageenan treatment of mice had no effect on the immune response to TNP-PVP or TNP-DEAE- dextran but did suppress the response to TNP-LPS. The difference in results obtained in their study and those reported herein, employing *E. coli* as the antigen, may be explained in two ways. One is that possibly a suboptimal dose of LPS (1 μg) was used in the former study since in the present study the number of PFC was maximal in mice injected with 100 μg LPS. A second

explanation may be associated with the strains of mice used. Ishizaka *et al.* (1977) noted a 90% decrease in the response to TNP-LPS in C3H/HeMs mice treated with carrageenan, while AKR/J mice exhibited only a 60% decrease in response under identical treatment conditions. Glode & Rosenstreich (1976) have shown that the magnitude of the proliferative response of B lymphocytes to *E. coli* LPS is highly strain-dependent. In the present study BALB/c mice, when treated with carrageenan and immunized with 1 μg LPS, showed a slightly depressed plaque-forming response (Fig. 2).

Employing Sephadex G-10 columns to deplete macrophages from spleen-cell populations, Chused *et al.* (1976) found that the *in vitro* response to TNP-Ficoll and SRBC were reduced by 90% while the response to TNP-LPS was reduced by 60% compared to the unseparated populations. These investigators proposed that there is a hierarchy of macrophage dependence of the immune response such that the response to TNP-LPS is less dependent on macrophage function than is the response of TNP-Ficoll or SRBC. When macrophages were depleted by carrageenan treatment *in vivo* (Fig. 2) and *in vitro* (Table 4), the immune response to TNP-LPS was unaffected, suggesting that the response did not require macrophage function. Treatment of the spleen-cell population with carrageenan, as shown in Table 2, reduced the proportion of macrophages to very low levels. One cannot rule out the possibility that a sufficient number of macrophages may have survived the depletion treatment and were still capable of providing accessory function although we find this argument unlikely. The difference in results obtained in this study and that of Chused *et al.* (1976) may also be related to the type of cells removed by the different depletion methods. Culture of spleen cells in the presence of varying doses of carrageenan did not significantly affect the viability or function of T or B lymphocytes (Table 3). Depletion of splenic adherent cells by Sephadex G-10 columns results in the loss of a large number of spleen cells (e.g. 50% of the cells recovered in the effluent; (Ly & Mishell, 1974). Studies of the adherence properties of lymphocytes by Shortman, Byrd, Williams, Brunner & Cerottini (1972) using glass bead columns, revealed that B lymphocytes exhibited a greater degree of adherence than T lymphocytes. In addition, passage of spleen-cell suspensions through these columns resulted in a selective loss of antigen-binding lymphocytes (Shortman *et al.*, 1971). Thus, the use of physical adherence techniques can also deplete certain T and B lymphocyte subpopulations (e.g. blasts, plasma cells and antigen-binding cells).

In an attempt to confirm the results obtained by *in vitro* depletion of macrophages with carrageenan, another anti-macrophage agent, AMS, was used in similar experiments. The AMS was prepared by a novel method. Previous attempts at preparation of specific anti-macrophage sera have utilized physical techniques to obtain an enriched population of macrophages. The production of an anti-nu/nu mouse lymphocyte serum with predominant cytotoxic activity against B cells, facilitated the isolation of peritoneal macrophages depleted of lymphocytes. This not only minimized lymphocyte contamination of the peritoneal exudate cell population, but also decreased the likelihood of depleting functional subpopulations of macrophages which may differ in adherence, phagocytosis, size or density (Walker, 1974; Walker, 1976).

The specificity of the AMS was directed against peritoneal macrophages and not lymphocytes (Fig. 1). AMS absorbed with mouse erythrocytes, thymocytes and B lymphocytes had significant cytotoxic activity against nu/nu mouse peritoneal macrophages (75–80%), while it exhibited very low cytotoxic activity against BALB/c T lymphocytes (2–3%) and nu/nu B lymphocytes (10–12%) at low serum dilutions (1:8 to 1:10). This low level of cytotoxic activity could possibly be attributed to the presence of macrophages or monocytic precursors in the lymphocyte populations used to evaluate the absorbed AMS. The inhibitory effect of AMS treatment on macrophage function was demonstrated by its ability to interfere with the adherence of macrophages to glass and by its ability to depress the phagocytosis of latex particles by macrophages treated with high AMS dilutions (1:1000 to 1:10,000). Although the AMS was not cytotoxic for 100% of the peritoneal macrophage population, the treated macrophages were adversely affected in their ability to carry out normal functions.

Depletion of macrophages in the spleen-cell population by AMS treatment resulted in the abrogation of the *in vitro* immune response to SRBC, but not to TNP-LPS (Table 5). These observations are in agreement with our studies employing carrageenan as the anti-macrophage reagent (Table 4). The advantage of AMS as a macrophage-depleting agent is its specificity for macrophages. Further, in our hands, treatment with AMS was more effective for macrophage depletion than were carrageenan, adherence or phagocytosis of carbonyl iron followed by magnetic separation (unpublished observations).

The major emphasis of this study was to determine the requirement for macrophages in one type of B-lymphocyte activation, that is, the triggering of specific B cells by T-independent antigens leading to antibody production. The finding that macrophages do not appear to be required suggests a direct antigen–B cell interaction in the activation process. Previous studies have shown that B cells can be activated non-specifically by LPS without the need for accessory cells, e.g. macrophages (Lemke *et al.*, 1975; Yoshinaga, Yoshinaga & Waksman, 1972; Nelson, 1973; Wahl, Wilton, Rosenstreich & Oppenheim, 1975). The results of this study also suggest that this may be the process by which specific activation of B cells by thymus-independent antigens occurs for the initiation of an immune response. An alternative conclusion that must not be overlooked is that the immune response to thymus-independent antigens may be less macrophage-dependent. Therefore fewer macrophages are required for the initiation of an immune response to these antigens than are required for the response to thymus-dependent antigens. This interpretation is based on the realization that it is virtually impossible to obtain a splenic B-cell population completely devoid of macrophages. Finally, the macrophage dependence or independence of an immune response to a given antigen could be related to a functional subpopulation of macrophages that may be either selected or depleted by the method used to separate macrophages from lymphoid cell populations.

ACKNOWLEDGMENTS

We thank Ms Janet Phoenix Dixon for her excellent secretarial assistance and Dr K. J. Pennline for his critical review of this manuscript. This study was supported in part by USPHS grant HL-16748, The Council for Tobacco Research–U.S.A., Inc. and USPHS Training Grant TI-AI 298.

REFERENCES

ARGYRIS B.F. (1974) Role of macrophages in the immune response. In: *Developments in Lymphoid Cell Biology* (Ed. by A. A. Gottlieb), p. 28. CRC Press, Cleveland, Ohio.

BICE D.E., GRUWELL D.G. SALVAGGIO J.E. & HOFFMAN E.O. (1972) Suppression of primary immunization by carrageenan: a macrophage toxic agent. *Immunol. Commun.* **1,** 615.

BOYDEN S.V. (1951) The absorption of proteins on erythrocytes treated with tannic acid and subsequent hemagglutination by anti-protein sera. *J. exp. Med.* **93,** 107.

CATANZARO P.J., SCHWARTZ H.J. & GRAHAM R.C. (1971) Spectrum and possible mechanism of carrageenan cytotoxicity. *Am. J. Pathol.* **64,** 387.

CHAOUAT G. & HOWARD J.G. (1976) Influence of reticuloendothelial blockade on the induction of tolerance and immunity by polysaccharides. *Immunology,* **30,** 221.

CHUSED T.M., KASSAN S.S. & MOSIER D.E. (1976) Macrophage requirement for the *in vitro* response to TNP-Ficoll: a thymic independent antigen. *J. Immunol.* **116,** 1579.

CLAMAN H.N. & MOSIER D.E. (1972) Cell–cell interactions in antibody production. *Progr. Allergy,* **16,** 40.

CUNNINGHAM A.J. & SZENBERG A. (1968) Further improvements in the plaque technique for detecting single antibody-forming cells. *Immunology,* **14,** 599.

GHANTA V.K., HAMLIN N.M., PRETLOW T.G. & HIRAMOTO R.N. (1972) Use of dextran-conjugated SRBC for the detection of MOPC-104E tumor cells. *J. Immunol.* **109,** 810.

GLODE L.M. & ROSENSTREICH D.L. (1976) Genetic control of B cell activation by bacterial lipopolysaccharide is mediated by multiple distinct genes or alleles. *J. Immunol.* **117,** 2061.

HERSCOWITZ H.B. & DIBLASIO R.C. (1976) Suppression of the primary immune response to Keyhole limpet haemocyanin by anti-macrophage globulin. *Immunology,* **30,** 35.

HIRSCH M.S., GARY G.W. & MURPHY F.A. (1969) *In vitro* and *in vivo* properties of antimacrophage serum. *J. Immunol.* **102,** 656.

ISHIZAKA S., OTANI S. & MORISAWA S. (1977) Effects of carrageenan on immune responses. I. Studies on the macrophage dependency of various antigens after treatment with carrageenan. *J. Immunol.* **118,** 1213.

JACOBS D.M. & MORRISON D.C. (1975) Stimulation of a T-independent primary anti-hapten response *in vitro* by TNP-lipopolysaccharide (TNP-LPS). *J. Immunol.* **114,** 360.

JEANES A., WILHAM C.A. & MIERS J.C. (1948) Preparation and characterization of dextran from *Leuconostoc mesenteroides. J. biol. Chem.* **176,** 603.

LEE K.C., SHIOZAWA C. SHAW, A. & DIENER E. (1976) Requirement for accessory cells in the antibody response to T cell-independent antigens *in vitro. Europ. J. Immunol.* **6,** 63.

LEMKE H., COUTINHO, A. OPITZ, H.G. & GRONOWICZ. E. (1975) Macrophages suppress direct B-cell activation by lipopolysaccharide. *Scand. J. Immunol.* **4,** 707.

LOEWI G., TEMPLE, A. NIND, A.P.P. & AXELROD M. (1969) A study of the effects of anti-macrophage sera. *Immunology,* **16,** 99.

LY I.A. & MISHELL R.I. (1974) Separation of mouse spleen cells by passage through columns of Sephadex G-10. *J. immunol. Meth.* **5,** 239.

MARBROOK J. (1967) Primary immune response in cultures of spleen cells. *Lancet,* **i,** 1279.

MOSIER D.E. (1967) A requirement for two cell types for antibody formation *in vitro. Science,* **158,** 1573.

NELSON D.S. (1973) Production by stimulated macrophages of factors depressing lymphocyte transformation. *Nature (Lond.),* **246,** 306.

PIERCE C.W. & KAPP J.A. (1976) The role of macrophages in antibody responses *in vitro*. In: *Immunobiology of the Macrophage* (Ed. by D. S. Nelson), p. 2. Academic Press, New York.

POE W.J. & MICHAEL J.G. (1974) The lack of cellular cooperation in the immune response to *E. coli* 0127. *J. Immunol.* **113,** 1033.

PROCTER M.L. & HERSCOWITZ H.B. (1975) Preparation and properties of an anti-plasma cell serum directed against a defined plasmacytoma cell population. *J. Immunol.* **115,** 1642.

RITTENBERG M.B. & PRATT K.L. (1969) Antitrinitrophenyl (TNP) plaque assay. Primary response of BALB/c mice to soluble and particulate immunogen. *Proc. Soc. exp. Biol. Med.* **132,** 575.

RUMJANEK, V.M., WATSON S.R. & SLJIVIC V.S. (1977) A re-evaluation of the role of macrophages in carrageenan-induced immunosuppression. *Immunology,* **33,** 423.

SAWICKI J.E. & CATANZARO P.J. (1975) Selective macrophage cytotoxicity of carrageenan *in vivo*. *Int. Archs Allergy appl. Immun.* **49,** 709.

SHORTMAN K., DIENER E. RUSSELL P. & ARMSTRONG W.D. (1970) The role of non-lymphoid accessory cells in the immune response to different antigens. *J. exp. Med.* **131,** 461.

SHORTMAN K., WILLIAMS, N. JACKSON, H. RUSSELL, P. HART P. & DIENER E. (1971) The separation of different cell classes from lymphoid organs. IV. The separation of lymphocytes from phagocytes on glass bead columns and its effect on subpopulations of lymphocytes and antibody-forming cells. *J. cell. Biol.* **48,** 566.

SHORTMAN K., BYRD, W. WILLIAMS, N. BRUNNER K.T. & CEROTTINI J.C. (1972) The separation of different cell classes from lymphoid organs. The relationship between the adherence properties and the buoyant density of subpopulations of 'B' and 'T' lymphocytes. *Aust. J. exp. Biol. Med. Sci.* **50,** 323.

SMITH D.B., COOK W.H. & NEAL J.L. (1954) Physical studies on carrageenan and carrageenan fractions. *Arch. Biochem. Biophys.* **53,** 192.

STINNETT J.D., KAPLAN A.M. & MORAHAN P.S. (1976) Identification of a macrophage-specific cell surface antigen. *J. Immunol.* **116,** 273.

THOMSON A.W., WILSON, A.R. CRUICKSHANK W.J. & JEFFRIES A.H. (1976) Evidence for a selective cytotoxic effect of carrageenan on cells of the immune system *in vivo* and *in vitro*. *Experientia,* **32,** 525.

UNANUE E.R. (1972) The regulatory role of macrophages in antigenic stimulation. *Adv. Immunol.* **15,** 95.

WAHL S.M. WILTON J.M. ROSENSTREICH D.L. & OPPENHEIM J.J. (1975) The role of macrophages in the production of lymphokines by T and B lymphocytes. *J. Immunol.* **114,** 1296.

WALKER W.S. (1974) Functional heterogeneity of macrophages: subclasses of peritoneal macrophages with different antigen-binding activities and immune complex receptors. *Immunology,* **26,** 1025.

WALKER W.S. (1976) Functional heterogeneity of macrophages in the induction and expression of acquired immunity. *J. Reticuloendothel. Soc.* **20,** 57.

YAM L.T., LI C.Y. & CROSBY W.H. (1971) Cytochemical identification of monocytes and granulocytes. *Am. J. Clin. Pathol.* **55,** 283.

YOSHINAGA M., YOSHINAGA A. & WAKSMAN B. (1972) Regulation of lymphocyte responses *in vitro*. I. Regulatory effect of macrophages and thymus-dependent (T) cells on the response of thymus-independent (B) lymphocytes to endotoxin. *J. exp. Med.* **136,** 956.