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IN VITRO AND *IN VIVO* STUDIES OF THE PROPERTIES AND EFFECTS OF ANTIMACROPHAGE SERA (AMS)

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SUMMARY

Antimacrophage sera (AMS) were prepared in rabbits by injection of mouse peritoneal cells with or without purification by *in vitro* culturing. These AMS showed high cytotoxic activity against macrophages in culture. The antimacrophage sera were not specific for macrophages; they cross-reacted and were cytotoxic to lymphocytes and granulocytes in the *in vitro* assay. Injection of AMS into mice did not affect anti*Shigella* antibody production, nor did it prolong skin allograft survival. Furthermore, AMS-treated macrophages did not lose their ability to induce anti*Shigella* antibody production in irradiated mice. On the other hand, AMS inhibited phagocytosis of *Bacillus subtilis* by macrophages and likewise increased the synthesis of DNA in cultured macrophages.

INTRODUCTION

The experiments of Fishman (1961), Askonas & Rhodes (1965), Gallily & Feldman (1967) and Mosier (1967) have indicated that macrophages are essential for the induction of antibody production. Heterologous antisera against macrophages (AMS) recently described (Argyris & Plotkin, 1969; Dyminski & Argyris, 1969; Hirsch, Gary & Murphy, 1969; Loewi *et al.*, 1969; Panijel & Cayeux, 1968; Unanue, 1968), could provide an important tool for further study and elucidation of the participation of macrophages in the immune response. It was reported that AMS, when injected into mice, suppressed their immunological response to bacteriophage $\Phi \times 174$ (Panijel & Cayeux, 1968) and also hastened morbidity and mortality following injection with vesicular stomatitis virus (Hirsch *et al.*, 1969). Likewise, it impaired the carbon clearance from the blood of the treated animals (Hirsch *et al.*, 1969). In guinea-pigs, application of AMS decreased the response of delayed hypersensitivity and impaired carbon clearance (Loewi *et al.*, 1969). *In vitro* studies showed that AMS was specific for macrophages and that its application diminished the phagocytic activity (Argyris & Plotkin, 1969), and the adherence and viability of macrophages (Hirsch

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et al., 1969; Unanue, 1968). The present investigation has been designed to establish an *in vitro* assay for titring the cytotoxic activity of antimacrophage sera and to evaluate its specificity. Our intention has been to examine and clarify the affects of AMS on phagocytosis, anti*Shigella* antibody production and allograft survival. The influence of AMS on the DNA contents of cultured macrophages has also been studied.

MATERIALS AND METHODS

Preparation of antisera. Antimacrophage sera were prepared by giving adult New Zealand white rabbits intraperitoneal (i.p.) injections of macrophages. Four days after thioglycollate injection (Gallily & Feldman, 1967), peritoneal cells were withdrawn from 10–12 week-old C57BL/6J or BALB/c female mice. The cells were washed 2–3 times with phosphate-buffered saline (PBS) and resuspended in PBS at a concentration of 10^7–10^8 cells/ml. Differential counts of the peritoneal cell populations showed that about 80% of the cells were macrophages, 10% were granulocytes, and 10% lymphocytes. The antimacrophage antisera were prepared in rabbits 1 and 4 by two successive i.p. injections of 10^9 live peritoneal cells 14 days apart. Rabbit 2 received three i.p. injections of 6×10^8 to 9×10^8 peritoneal cells, 14 and 28 days apart. Rabbit 3 received three i.p. injections of 10^8 cultured macrophages 7 and 14 days apart. These macrophages were cultured *in vitro* in a medium consisting of 10% foetal calf serum (FCS) and 90% Hanks's balanced salt solution (BSS). After 48 hr in culture, the cells were washed and removed from the dishes with a rubber policeman. About 98% of the adhering cells were macrophages. Rabbits 5, 6 and 7 were injected with cultured macrophages withdrawn from BALB/c mice. The number of cells and schedule of injection were similar to that used in immunization of rabbit 3. All the rabbits were bled 7 days after the last injection. The sera were inactivated by heating to 56°C for 30 min. Samples of each serum were adsorbed on equal volumes of packed sheep red blood cells (SRBC) to remove the Forssman antibodies; others were first adsorbed on SRBC and then adsorbed either on washed mouse red blood cells (1/2 volume of packed cells) or on lymphocytes from lymph nodes (3×10^8 lymphocytes to 1·0 ml sera) or from spleen (1×10^9 lymphocytes to 1·0 ml sera). The sera were adsorbed at room temperature for 30–60 min, then filtered and stored at −20°C.

Antithymocytic sera (ATS) were prepared by giving rabbits two successive i.p. injections of 10^9 thymocytes, 14 days apart. The rabbits were bled 7 days after the last injection.

Cytotoxicity assay for macrophages. Peritoneal cells were withdrawn from C57BL/6J female mice 4 days after thioglycollate injection. The cells were sedimented and resuspended in a medium consisting of 10% FCS and 90% Hanks's solution. Then, 1 ml of the cell suspension containing $2{\cdot}5 \times 10^6$ cells/ml was transferred to a disposable plastic petri dish (35 × 10 mm). The cells were incubated at 37°C in an atmosphere of 5% CO_2 in air for 24 hr. The medium was removed and replaced by 10% of a varying dilution of rabbit antimacrophage serum (0·1 ml/dish) and 90% Hanks's solution (0·9 ml/dish). All samples of AMS were inactivated for 30 min at 56°C and adsorbed on SRBC prior to the assay. The cells were incubated for 1 hr, and then a commercial guinea-pig complement (dried complement List No. 070-432; Hyland Laboratories, Los Angeles, California) was added so that its final concentration was 5% (V/total volume). After further incubation for an additional 3–4 hr, the viability of the cells was determined by exposing them to 0·4% erythrosin B (Phillips & Andrews, 1959) (National Aniline Division, Allied Chemical

Corp.) in Tyrode solution. Only the dead cells were stained red by this solution, which was not toxic at this concentration to viable cells. 200 cells were counted in each sample. The cytotoxicity of the antisera was expressed either as the dilution which caused the death of 50% of the cells or as the cytotoxic titre, which is the reciprocal of this dilution. Two samples of each serum dilution were titred at least twice.

Cytotoxicity assay for lymphocytes. Cell suspensions of $2{\cdot}5 \times 10^7$ cells/ml in Eagle's medium were prepared from the spleen or lymph nodes of C57BL/6J mice. 0·1 ml amounts of this suspension were transferred to 13 × 100 mm tubes that contained 0·1 ml of varying dilutions of inactivated and SRBC adsorbed antimacrophage antiserum and 0·9 ml of Eagle's Basal Medium. The cells were incubated in a water bath for 1 hr at 37°C, and then commercial guinea-pig complement was added to make a final concentration of 5%. After additional incubation for 3 hr, the viability of the cells was determined by exposing them to 0·4% erythrosin B. 200 cells per sample were scored in a haemocytometer.

Cytotoxicity assay for granulocytes. Granulocytes were withdrawn from the peritoneal cavities of C57BL/6J female mice 14 hr after i.p. injection of 2·0 ml thioglycollate. A differential count showed that 89% of the cells were granulocytes, 4% macrophages and 7% lymphocytes. The cytotoxic assay of antimacrophage sera was done by a technique similar to that used for lymphocytes. The cells ($2{\cdot}5 \times 10^6$ per tube) were incubated with 0·1 ml of diluted AMS and 0·9 ml Hanks's solution at 37°C for 1 hr. 5% guinea-pig complement was added, and the cells were incubated for 3 hr more. The viability of the cells was determined after exposing them to 0·4% erythrosin B.

Phagocytosis. Thioglycollate stimulated macrophages were withdrawn from C57BL mice and transferred into disposable Petri dishes. 2×10^6 macrophages per plate were incubated in media consisting of 10% newborn calf serum (NBCS) and 90% Hanks's BSS. After 24 hr *in vitro* the media were replaced by different samples of 10% AMS and 90% Hanks's. The cells were incubated for 1 hr with these antisera and then *Bacillus subtilis* was added. The ratio of bacilli to macrophages was 10 : 1. One hour later, the cultures were washed, fixed in methanol and stained with Giemsa stain. The percentage of bacilli containing macrophages was determined by counting 600–1000 cells in each test.

Shigella, antiShigella agglutinins. The antigen used was *Shigella paradysenteria* obtained by courtesy of Dr T. N. Harris. The agglutination procedure for titring anti*Shigella* antibody was described previously (Gallily & Feldman, 1967).

Skin allograft. The technique used for skin grafting was basically described by Billingham & Medawar (1951). After transferring the grafts to their beds, Nobecutane spray (Bofors, Novel-Pharma, Sweden) was applied instead of silk sutures.

DNA determination. The DNA content of macrophages in culture was determined by the indole colorimetric method (Bonting & Jones, 1957).

RESULTS

Normal rabbit sera. Thirty samples of inactivated normal rabbit sera (NRS) were assayed for their cytotoxic effect on macrophages growing in culture. The replacement medium consisted of 10% normal rabbit serum, 85% Hanks's solution, and 5% guinea-pig complement. After incubation for 3–4 hr in this medium, many macrophages detached from the dishes; four sera out of thirty were cytotoxic to 20–30% of the macrophages and one (No. 2) to 73% of the cells after this period of time.

Cytotoxicity of AMS to macrophages. The cytotoxicity of seven preimmunization sera and seven immune antimacrophage sera was assayed against macrophages growing in culture. After incubation with the antisera, agglutination of the macrophages occurred. When 5% complement was added, the seven antisera killed 52–88% of the macrophages in 1: 1000–1: 5000 dilutions (Table 1). Only one of the seven normal rabbit sera tested, No. 2, was cytotoxic in a 1: 10 dilution (73% dead cells); but in the 1: 100 dilution it was not cytotoxic to the macrophages. The remaining six normal sera did not kill any of the cells during the assay.

TABLE 1. Cytotoxicity of pre-immunization sera and antimacrophage sera (AMS) against macrophages

	Pre-immunization sera		AMS	
Rabbit no.	Dilution	% Dead cells	Dilution	% Dead cells
1	1:10	0	1:5000	66
2	1:100	0	1:5000	73
3	1:10	0	1:1000	72
4	1:10	0	1:1000	73
5	1:10	0	1:1000	52
6	1:10	0	1:1000	88
7	1:10	0	1:1000	55

TABLE 2. Cytotoxicity of AMS against lymphocytes and granulocytes

	Cytotoxicity against lymphocytes		Cytotoxicity against granulocytes	
Serum	Dilution	% Dead cells	Dilution	% Dead cells
AMS 1	1:1000	96	1:100	96
AMS 2	1:1000	97	1:1000	50
AMS 3	1:1000	82	1:1000	60
AMS 4	1:1000	55	1:100	82

Cross-reactivity of AMS. All four AMS tested were found to be cytotoxic to lymphocytes (Table 2). Two of the antisera had the same cytotoxic titres against lymphocytes as against macrophages. One of them was obtained after immunizing rabbit 3 with a pure population of macrophages. The other two antisera had somewhat lower titres, 1000 for lymphocytes as compared to 5000 for macrophages.

Likewise, the four AMS were found to be cytotoxic to another cell type—the granulocytes in the *in vitro* assay (Table 2). However, their cytotoxic titres against granulocytes were lower than against macrophages in three out of four antisera tested. Two of the four antisera were cytotoxic to granulocytes in a 1: 100 dilution and the remaining two were cytotoxic in 1: 1000 dilution. Only one of the four normal rabbit sera (No. 2) in a 1: 100 dilution was cytotoxic to granulocytes as well as to lymphocytes when 5% guinea-pig complement was

added. During the assay, the other three normal sera (in 1: 10 dilution) killed 15–22% of the cells, probably due to unfavourable *in vitro* conditions. Incubation of granulocytes and lymphocytes with 10% foetal calf serum in Eagle's Basal Medium also killed 15–25% of these cells when 5% guinea-pig complement was added.

Adsorption of AMS. Samples of four AMS were adsorbed on different cell types and their cytotoxicity to macrophages was scored. It is clear (Table 3) that adsorption of sheep red blood cells, which removed the Forssman antibodies, did not lower the cytotoxicity of AMS to macrophages in three out of four sera. Similarly, adsorption on isologous red blood cells did not change grossly the cytotoxicity of these antisera. On the other hand, the

TABLE 3. Cytotoxicity of antimacrophage sera (AMS) against macrophages following adsorption with different cell types

	AMS titre*			
Adsorbing cells	Rabbit 1	Rabbit 2	Rabbit 3	Rabbit 4
None	5000	5000	1000	5000
Sheep red blood cells	5000	5000	1000	1000
Mouse red blood cells	5000	2000	1000	1000
Mouse spleen cells	100	500	100	100
Mouse lymph node cells	1000	1000	1000	1000

* The reciprocal of the dilution which killed at least 50% of the cells.

TABLE 4. Effect of AMS, ATS and NRS on phagocytosis of *Bacillus subtilis* by macrophages *in vitro*

Medium	No. of serum samples	Percentage of phagocytosis
Hanks's BSS		49
Hanks's BSS+10% NBCS	2	67
Hanks's BSS+10% NRS	2	33
Hanks's BSS+10% ATS	2	20
Hanks's BSS+10% AMS	5	3

cytotoxic effect against macrophages was markedly reduced by prior adsorption on mouse spleen cells, and was effected to a lesser degree after adsorption on lymph node lymphocytes.

Effect of AMS on phagocytosis. The phagocytosis of *Bacillus subtilis* by macrophages growing in culture and treated with different samples of normal sera and antisera is presented in Table 4. It was found that while 67% of the macrophages maintained in newborn calf serum (NBCS) phagocytized the bacilli, this ability of the macrophages was almost totally abrogated (1–3%) after treatment with AMS. It is interesting to note that although ATS significantly decreased the phagocytotic ability of macrophages, it did not completely abolish it, and that 30% of the macrophages showed phagocytosis.

In order to find out whether the ability of macrophages to phagocytize could recover from the AMS effect, the macrophages were washed four times (with Hanks's BSS) after

incubation for 2 hr in AMS containing media, and then 10% NBCS and 90% Hanks's BSS was added. Twenty-four hours later, the phagocytosis of *Bacillus subtilis* by macrophages was scored. Recovery of the phagocytic activity of macrophages was found (Table 5).

Effect of AMS on antiShigella antibody production. C57BL mice were injected intraperitoneally with AMS 2 hr before *Shigella* inoculation and three or four additional injections of this antisera were given thereafter. No decrease of anti*Shigella* antibody

TABLE 5. Recovery of the phagocytic activity of AMS-treated macrophages after incubation with new born calf serum (NBCS)

Medium	Phagocytosis	
	AMS treated macrophages	AMS treated macrophages maintained in NBCS
	%	%
Hanks's BSS+AMS 5	3	19
Hanks's BSS+AMS 6	2	12
Hanks's BSS+AMS 7	1	14
Hanks's BSS+NBCS*	38	18

* A new batch of NBCS was used in this experiment.

TABLE 6. Effect of AMS injections on anti*Shigella* antibody production by C57BL mice

Treatment of recipients	No. of mice	Agglutinin titre	
		5 days $\log_2$ of titre (mean)	8 days $\log_2$ of titre (mean)
Shigella	5	5·6	9·4
AMS* + *Shigella*	5	7·4	9·8
AMS† + *Shigella*	5	—	10·0

* Recipients were treated with a total dose of 0·1 ml AMS given on days 0, 1, 2 and 3.

† Recipients were treated with a total dose of 0·8 ml AMS given on days 0, 1, 2, 4 and 6.

production was detected 5 and 8 days after the injection of the bacteria (Table 6). As the phagocytic activity of macrophages was abolished almost completely after AMS treatment (see Table 5), the peritoneal macrophages were treated *in vitro* for 1 hr with AMS before incubation with the *Shigella* (Gallily & Feldman, 1967). The washed macrophages were then inoculated into 550 r X-irradiated mice. No decrease of anti*Shigella* agglutinin titres was observed in the irradiated mice injected with AMS-treated macrophages (Table 7).

Effect of AMS on allograft survival. Skin allografts were transplanted reciprocally between C57BL and BALB/c mice. The recipients were injected intraperitoneally with

AMS, NRS or ATS, 1 day before allograft transfer, as well as on the day of transfer. Four or five additional injections of these sera were given subsequently every 3 or 4 days. As may be seen from Table 8, no significant increase of allograft survival in AMS treated mice was detected above the survival times found after treatment with normal rabbit sera (NRS). On the other hand, injections of ATS prolonged the survival time of skin allografts in both strains of mice.

TABLE 7. The effect of macrophages treated *in vitro* with AMS on the induction of antibody by irradiated mice*

Treatment of recipients	No. of mice	Agglutinin titre on day 7 (log_2)
15×10^6 macrophages incubated with *Shigella*	13	6·7
15×10^6 macrophages incubated with AMS and *Shigella*	11	6·8
Shigella	15	1·8

* Mice were irradiated with 550 r 2 days before treatment.

Table 8. Effect of NRS, AMS and ATS injections on skin allograft survival

	BALB/c graft on C57BL mice		C57BL graft on BALB/c mice	
Serum*	No. of mice	Mean survival ±SD (days)	No. of mice	Mean survival ±SD (days)
—	(5)	13·5±1·3	(8)	12·6±0·4
NRS	(4)	17·3±1·7(a)	(6)	19·0±2·1(a)
AMS	(9)	16·8±2·1(b)	(7)	21·0±2·7(b)
ATS	(6)	23·1±5·8(c)	(5)	23·2±3·1(c)

* Recipients were treated with a total dose of 0·25 ml given in six or seven injections.

The differences between the values of (a) and (b) were checked according to the rank sum test and found to be insignificant. The differences between the values of (a) and (c) were found to be significant at a level between 95 and 99%.

Effect of AMS on DNA synthesis in macrophages. Macrophages from unstimulated and thioglycollate stimulated mice were incubated *in vitro* with 10% AMS and 90% Hanks's BSS for 24 hr. It was found (in collaboration with Dr Wiener) that the DNA content of the macrophages per plate increased 50–100% (Fig. 1). DNA content of similarly cultured macrophages was not affected when normal rabbit serum (NRS) was added.

AMS effect on the morphology of cultured macrophages. 24 hr after 10% AMS was added to cultured macrophages, marked morphological changes were noted. Long cytoplasmic protrusions extended from single or aggregated macrophages (Fig. 2). Sometimes, these protrusions seemed to fuse together. Macrophages maintained in 10% NBCS and 90% Hanks's BSS remained round.

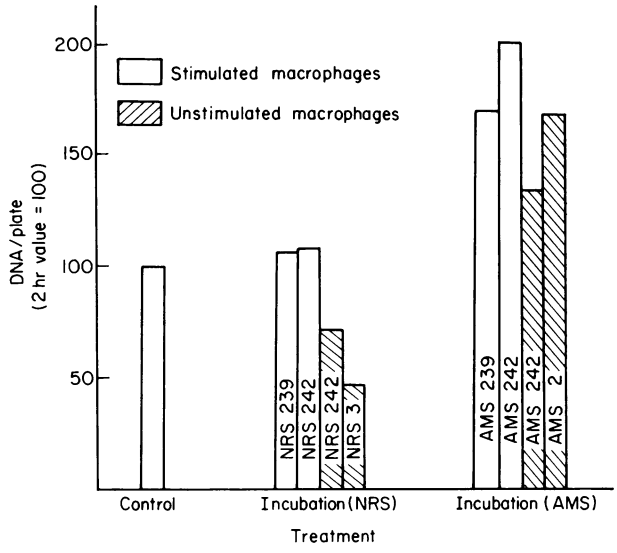

FIG. 1. The effect of AMS on DNA content of macrophage cultures after 24 hr of incubation.

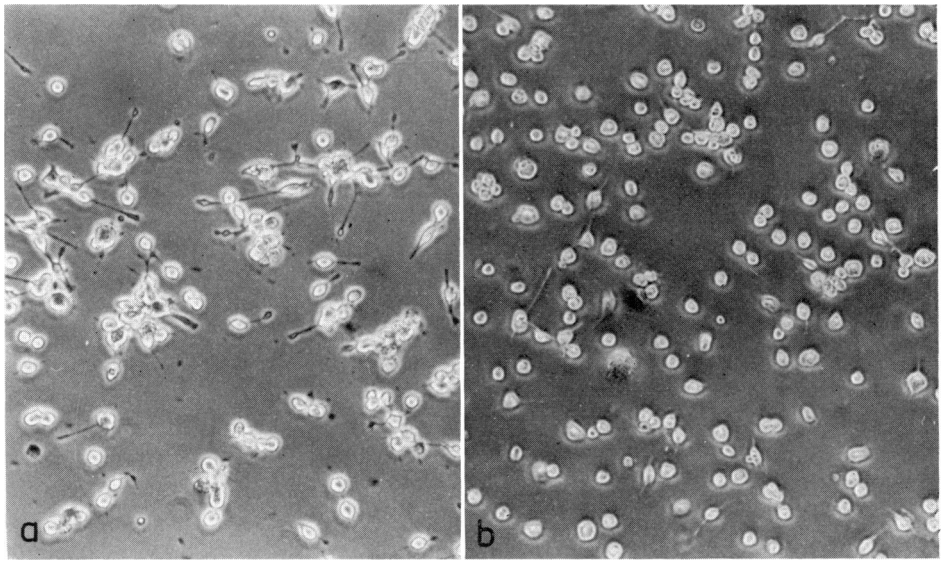

FIG. 2. Monolayer of macrophages in culture. Phase contrast, ×378. (a) Macrophages maintained for 24 hr in media containing 10% AMS and 90% Hanks's BSS. Macrophages are aggregated and many show numerous cytoplasmic protrusions. (b) Macrophages maintained for 24 hr in media containing 10% NBCS and 90% Hanks's BSS. Most macrophages are round.

DISCUSSION

The present report describes an *in vitro* assay for titring antimacrophage serum (AMS). This assay may help to define the activity of different preparations of AMS before evaluating their other *in vitro* and *in vivo* effects.

Prior to immunizing rabbits with macrophages, it is advisable to assay their sera for cytotoxicity to macrophages and to choose animals that do not possess this property. Also, in order to evaluate the activity of AMS in *in vitro* and *in vivo* studies the Forssman antibody should be eliminated. This antibody may be removed from rabbit antimouse macrophage antisera by adsorption on sheep erythrocytes or guinea-pig kidney, either of which possesses Forssman antigen (Hawes & Coombs, 1960; Tanaka & Leduc, 1956). In the recently published studies on the properties of anti-macrophage sera (Hirsch *et al.*, 1969; Loewi *et al.*, 1969; Panijel & Cayeux, 1968; Unanue, 1968), the macrophage has been considered to be the cell primarily affected. However, the present study shows that other cell types were also damaged. Three out of the four antimacrophage sera used in the present investigation were prepared in rabbits by injection of a mixed population of C57BL peritoneal cells consisting chiefly of macrophages but also including approximately 10% granulocytes and 10% lymphocytes. It was not unexpected to find that these three AMS cross-reacted and caused cytotoxic damage to granulocytes and lymphocytes as well as to macrophages. However, the same cytotoxic cross-reactivity was observed with AMS No. 3, obtained after injection of cultured macrophages. This antiserum was cytotoxic to all three types of cells. Loewi *et al.* (1969) showed partial cross-reactivity of AMS with lymphocytes. Their AMS, prepared by injecting rabbits with cultured macrophages, released ^{51}Cr from lymphocytes as well as from macrophages, though to a lesser degree from the former. On the other hand, Penijel & Cayeux (1968) and Unanue (1968) maintained that their AMS was specific to macrophages and did not damage the lymphocytes. Though their site and method of immunization differed from the present one, it is difficult to explain why, having injected a mixed population of peritoneal cells into rabbits, Panijel & Cayeux did not obtain cytotoxic antisera against lymphocytes as well as macrophages. Possibly the 'heavy' immunization schedule (Panijel & Cayeux, 1968)—three times weekly for 4 weeks—resulted in their obtaining relatively ineffective antisera against lymphocytes (Levey & Medawar, 1966). The AMS prepared by Unanue (1968), who injected cultured macrophages into rabbits, had a low cytotoxic titre against macrophages (maximum 1: 80), and no cytotoxic activity against lymphocytes. This specificity of AMS should be studied further by means of antisera with higher cytotoxic titres and a more sensitive *in vitro* assay. In the present study, the adsorption of antisera on different cell types also revealed the cross-reactivity of AMS with lymphocytes. While the cytotoxicity of AMS was not grossly affected by adsorption on mouse erythrocytes, it was markedly reduced by prior adsorption on mouse-spleen cells.

In view of the present results, it will be of interest to study more thoroughly the cross-reactivity of antilymphocyte serum (ALS) with macrophages, especially as there are indications that ALS impairs the uptake of antigen by dendritic macrophages (Barth *et al.*, 1969) and affects the phagocytic activity of the reticuloendothelial system (RES) of mice (Marshall & Knight, 1969) and rats (Grogan, 1969). It seems very likely that macrophages possess antigens in common with lymphocytes and granulocytes (Bodmer *et al.*, 1966). Indeed, recent publications have shown that antilymphocyte serum does interact with macrophages

(Huber, Michlmaayr & Fudenberg, 1969; Maclaurin & Humm, 1970; Marsman, Van der Hart & Van Loghem, 1970; Caspary, Hughes & Field, 1970). Further studies are needed to determine whether macrophage-specific antigen or antigens can be detected.

The damage to the phagocytic activity of macrophages proved to be reversible and temporary. After incubation in media containing NBCS the macrophages regained the ability to phagocytize bacilli. This recovery might explain the lack of immunosuppressive activity of AMS found in our experiments. AMS-treated macrophages which were incubated with *Shigella* were able to trigger anti*Shigella* antibody production in irradiated mice. Apparently the small amount of *Shigella* phagocytized or stuck to the macrophages was sufficient to trigger the antibody response. Likewise, this recovery of macrophages from the AMS effect might explain the unmodified immune response of AMS-treated mice after *Shigella* injection and allograft transfer which was found in the present study. On the other hand, one cannot exclude the possibility that the amount and schedules used in our AMS treatment were not effective *in vivo* at the macrophage level, especially, as other investigators have claimed that their AMS preparations suppressed humoral antibody formation (Panijel & Cayeux, 1968; Argyris & Plotkin, 1969) or prolonged allograft survival (Dyminski & Argyris, 1969).

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